REVIEW ARTICLE

Role of lipids in the translocation of proteins across membranes

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The architecture of cells, with various membrane-bound compartments and with the protein synthesizing machinery confined to one location, dictates that many proteins have to be transported through one or more membranes during their biogenesis. A lot of progress has been made on the identification of protein translocation machineries and their sorting signals in various organelles and organisms. Biochemical characterization has revealed the functions of several individual protein components. Interestingly, lipid components were also found to be essential for the correct functioning of these translocases. This led to the idea that there is a very intimate relationship between the lipid and protein components that enables them to fulfil their intriguing task of transporting large biopolymers through a lipid bilayer without leaking their contents. In this review we focus on the Sec translocases in the endoplasmic reticulum and the bacterial inner membrane. We also highlight the interactions of lipids and proteins during the process of translocation and integrate this into a model that enables us to understand the role of membrane lipid composition in translocase function.

Key words: lipid–protein interactions, secretion, SecY/Sec61, targeting signal.

PREPROTEIN TRANSLOCASES

Preprotein translocases are multimeric protein complexes that catalyse the transport of newly synthesized proteins across membranes towards their functional location in the cell [1]. Therein, these protein complexes fulfil an essential role in the biogenesis of any cell. Preprotein translocases are found in archaea, Gram-negative and Gram-positive bacteria [2–4], and in the membrane of the endoplasmic reticulum (ER) of yeast and higher eukaryotes [2,3]. Further, chloroplast thylakoid membranes contain preprotein translocases that resemble those from their presumed prokaryotic ancestors [4]. As well as the classical Sec systems that transport proteins in an unfolded conformation, translocases for fully folded proteins, such as the peroxisomal import machinery [5], the chloroplast ΔpH pathway, the similar prokaryotic twin arginine system [6], and the pullulanase system in bacterial outer membranes [7], have been described. Furthermore, translocases that transport proteins across two membranes simultaneously have been described, such as the mitochondrial import machinery [8–10], the chloroplast import machinery [11] and the nuclear import pore [12].

In addition to protein components, several classes of lipids have been demonstrated to assist in the transport process. Well established is the need for acidic lipids and non-bilayer-preferring lipids in *Escherichia coli* [13,14]. Acidic lipids have also been suggested to play a role in import in mitochondria [15]. Chloroplast-specific glycolipids have been suggested to play a role in the sorting of precursor proteins to the membrane of this organelle [16]. In this review, we will focus on the role of lipids in the bacterial Sec system and in the Sec system in the ER of yeast and higher eukaryotes.

THE BACTERIAL Sec SYSTEM

E. coli preprotein translocase (Figure 1a) consists of the integral membrane protein complexes SecYEG and SecDFYajC, and the peripheral ATPase SecA [17]. The SecYEG complex forms the actual channel through which precursors are guided to the periplasm and provides the high-affinity binding site for SecA at the membrane [18]. Proteins destined for translocation are synthesized with an N-terminal extension: the signal peptide. Signal peptides consist of an N-terminal domain containing between one and three positive charges, a more hydrophobic central core often containing a helix-breaking residue, and a more polar cleavage site [19,20]. The signal peptide functions as the address for targeting to the translocase. The signal recognition particle (SRP) or SecB may assist precursor proteins in their transport to the membrane [21]. Once at the membrane, SecB or SRP hands the precursor over to SecA, which by cycling through different conformational states, at the expense of ATP, pushes the precursor forward through the channel [22,23]. The SecA conformational change is accompanied by a reverse in the membrane topology of SecG [24] and a change in the interaction between the SecY and SecE subunits [25]. Furthermore, SecA membrane cycling is regulated by the SecDFYajC complex [26,27]. The proton motive force (pmf) acts as an additional energy source for forward movement of the precursor protein [28]. During or shortly after translocation, the signal sequence is cleaved off by the signal peptidase [29].

THE EUKARYOTIC Sec SYSTEM

The Sec systems of yeast and mammals contain heterotrimeric complexes similar to the prokaryotic SecYEG complex (Figures 1b and 1c). Sec61p in *Saccharomyces cerevisiae* and Sec61α in mammals are homologous to SecY. Sss1p in yeast and Sec61γ in mammals are single-transmembrane-spanning proteins with homology to *E. coli* SecE. The third subunits, Sbh1 in yeast and Sec61β in mammals, show similarity to each other, but are not similar to *E. coli* SecG [30]. Cross-linking experiments revealed that, during early translocation in the mammalian ER membrane, nascent proteins interact with the translocating-chain-associating

Abbreviations used: ER, endoplasmic reticulum; NBD, 7-nitrobenz-2-oxa-1,3-diazole; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; pmf, proton motive force; PMME, phosphatidylmonomethylethanolamine; PS, phosphatidylserine; SRP, signal recognition particle; TRAM, translocating-chain-associating membrane protein.

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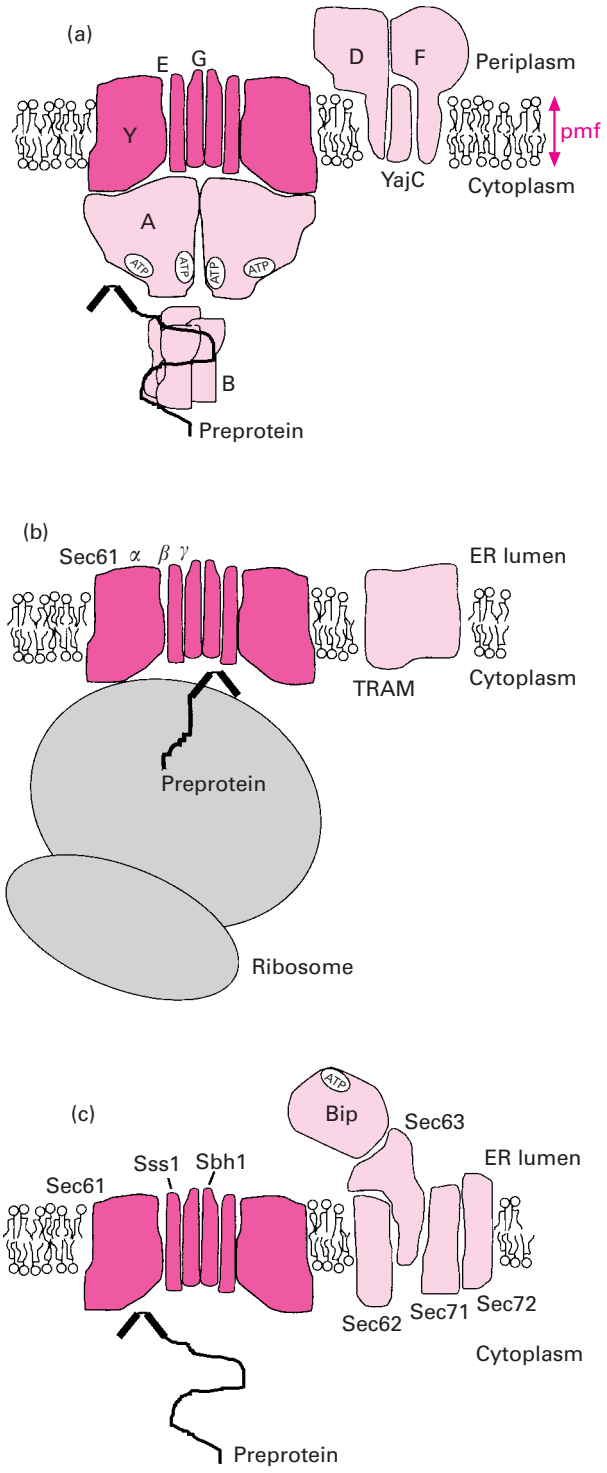

Figure 1 Preprotein translocases

Schematic representations, not drawn to scale, are shown of the *E. coli* translocation system (**a**), the co-translational mammalian system (**b**) and the post-translational *S. cerevisiae* system (**c**). Corresponding components among the three systems are coloured dark pink; those that do not correspond are coloured light pink. The ribosome in (**b**) is shaded grey.

membrane protein (TRAM) [31]. The function of TRAM is required for most, but not all, secretory proteins [32]. Targeting to the translocon is mediated by the SRP pathway. SRP binds to signal sequences of nascent proteins as soon as they emerge from the ribosome and subsequently targets the ribosome nascent chain complex to the SRP receptor on the membrane. Some precursors, such as prepro-α-factor, however, follow a SRP-independent post-translational pathway. In this case, the energy needed to drive translocation is derived from ATP hydrolysis by the lumenal Hsp70 family member Bip. A tetrameric complex consisting of Sec62p, Sec63p, Sec71 and Sec72 is involved in post-translational translocation. Sec63p interacts via its lumenal DnaJ domain with Bip, thereby activating Bip for polypeptide binding. Bip may act via two mechanisms. First, it might actively pull precursor proteins through the translocation channel, similar to the pushing model for SecA. Alternatively, it may exploit Brownian motion of the precursor in the channel by allowing forward movement of the precursor and preventing movement in the direction of the cytosol. Recently it was demonstrated that the Brownian ratchet model is sufficient to explain the translocation of prepro-α-factor. Other substrates than prepro-α-factor may require pulling activity of Bip for translocation, although this has not been tested experimentally [3]. During or directly after translocation, the signal sequence is removed by the signal peptidase.

MEMBRANE PHOSPHOLIPIDS

Lipids form the building blocks of all biomembranes. Variation in headgroup and in acyl chain composition results in the presence of hundreds of different lipid species. This diversity suggests that lipids do more than just form a hydrophobic barrier between the inside and outside [33]. We will focus on the properties of phospholipids that we believe to be relevant for their role in protein translocation.

Obviously, the nature of the headgroup is an important feature for the interaction with proteins. The acidic headgroups of phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylserine (PS) and phosphatidylinositol (PI) may give rise to electrostatic interactions with positively charged residues. The locally lowered pH of a membrane containing acidic lipids may also give rise to the protonation of negatively charged side chains, facilitating their insertion into the membrane [34]. Furthermore, the size of the headgroup is of importance, i.e. bulky headgroups leave less space for proteins to insert.

Regulation of acyl chain composition is important to keep the bilayer in the liquid crystalline phase [35]. The length of the acyl chain has to match with the hydrophobic length of the transmembrane segment that it needs to accommodate [36]. The average length of phospholipid acyl chains varies through different organelles. The hydrophobic match, between the transmembrane helices of Golgi resident proteins and the acyl chains in that membrane, determines retention at this location [37]. Interestingly, the combination of acyl chains and headgroup size determines the shape of the lipid. Lipids such as dioleylphosphatidylcholine (dioleyl-PC) and dioleyl-PG tend to organize into bilayers, because the surface area occupied by the headgroups is similar to the area occupied by the acyl chains. Detergents and lysophospholipids tend to organize in micelles with their acyl chains pointing inwards, because the area occupied by the headgroup exceeds that of the acyl chains. Lipids with small headgroups tend to organize in non-bilayer tubular structures, termed inverted hexagonal (H_{II}) phases. Cardiolipin normally behaves like a bilayer-preferring lipid; however, complex-formation between the headgroups and bivalent cations, such as Ca^{2+} and Mg^{2+}, results in a hexagonal (H_{II}) phase preference. A biomembrane is composed of lipids with all these shapes, along with membrane proteins, and forms a bilayer. The

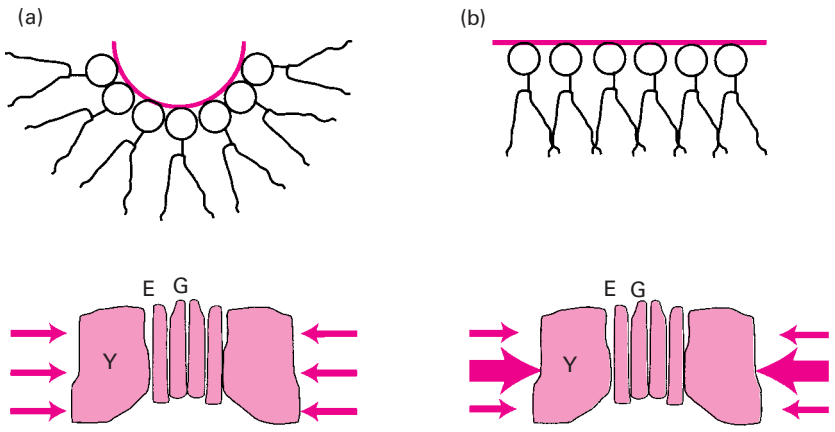

Figure 2 The 'frustrated bilayer'

(**a**) Non-bilayer lipids prefer to adopt curved monolayer structures. (**b**) Constriction in a flat layer results in compression of the acyl chains and spacing of the headgroups.

transition point from bilayer to non-bilayer structure is kept close to the physiological temperature by regulation of the acyl chain composition [35].

Lipids assemble spontaneously into bilayers. This spontaneous process is driven by the shielding of the hydrophobic acyl chains from the water phase. Since a lipid bilayer is free to expand in all directions, the net internal pressure must be zero. The hydrophobic force, which acts at the polar–apolar interface, is counterbalanced by repulsive forces between headgroups and acyl chains. The above implies that the pressure at a certain depth in the membrane does not necessarily need to be zero. The attractive hydrophobic force in the polar–apolar interface is compensated by repulsion close to the centre of the membrane. Theoretical models have been proposed to calculate the internal pressure distribution in a membrane [38]. Using these models it can be predicted that, for example, the level of unsaturation of the acyl chains influences the lateral pressure profile of these membranes. Unsaturated bonds tend to shift the region with the highest lateral surface pressure towards the polar–apolar interface.

Non-bilayer lipids tend to organize into aggregates with a concave interface because of their relatively small headgroups (Figure 2a). When these lipids are constrained in a flat bilayer, this results in a 'frustrated bilayer' with a high internal surface pressure, in which the acyl chains are compressed and the headgroups are spaced apart (Figure 2b). This suggests that non-bilayer lipids also strongly influence the lateral pressure profile of the membrane.

The lateral pressure profile may have great implications for the functioning of membrane proteins. Consider a membrane protein that, in order to perform its function, needs to undergo a conformational change within the membrane. This protein will, at certain depths, increase its cross-sectional area, whereas at other depths it will decrease its cross-sectional area. The energy required to go from one conformation to the other will depend on the surface pressure at the specific depths of the membrane where the protein needs to expand or decrease its cross-sectional area. Therefore it is likely that a membrane protein is adapted to the lateral surface pressure profile present in its native membrane. A change in the lateral surface pressure profile would potentially interfere with conformational changes and therefore interfere with membrane protein function [39]. Local anaesthetics are believed to function by changing the lateral surface pressure profile [40]. Interestingly, the local anaesthetic procain inhibits processing of precursor proteins in *E. coli*, suggesting that it interferes with translocase function [41]. The importance of the above-mentioned properties of lipids for protein translocation will be discussed in the following paragraphs.

MANIPULATION OF LIPID COMPOSITION

The relative simplicity of the *E. coli* membrane system, with only two membranes, has enabled geneticists to create strains with drastically changed phospholipid compositions. These mutant strains have provided direct evidence for the importance of phospholipid properties for protein translocation. Although in yeast the lipid composition can be manipulated genetically, this has not as yet resulted in direct demonstration of an essential role of lipids for protein translocation. Due to the complex membrane organization of a eukaryotic cell, it is not easy to drastically modify the lipid composition of one membrane without killing the cell.

The inner membrane of *E. coli* consists of 75–80 % phosphatidylethanolamine (PE), which contributes to the non-bilayer-forming property of the membrane [42]. The negatively charged lipids PG and cardiolipin form 20 % and 1–5 % respectively of the total phospholipids. Cardiolipin in complex with bivalent cations also contributes to the non-bilayer properties of the membrane. The predominant membrane phospholipids of yeast are PC, PE, PS, PI and sphingolipids [43,44]. Mitochondrial membranes contain, in addition, cardiolipin and PG. Strikingly, all of these membranes contain negatively charged lipids and non-bilayer-preferring lipids.

In *E. coli* the biosynthesis of all phospholipids starts with the acylation of *sn*-glycerol 3-phosphate to form PA. Subsequently, PA is converted into CDP-diacylglycerol. This intermediate forms a branch point in phospholipid biosynthesis. PS synthetase converts CDP-diacylglycerol into PS, which is rapidly decarboxylated to form PE by PS decarboxylase. PG phosphate synthase produces PG phosphate, from which the phosphate is removed by PG phosphate phosphatase to produce PG. Cardiolipin synthetase produces cardiolipin by condensation of two PG molecules. The importance of lipid composition for membrane function is demonstrated by several *E. coli* strains that contain mutations in the lipid biosynthetic enzymes.

Disruption of the *pss* gene is lethal under normal growth conditions; however, the presence of high concentrations of the

bivalent cations Ca^{2+}, Mg^{2+} and Sr^{2+} restores the growth of the mutant [45]. PG and cardiolipin form the main lipids of this mutant. The ratio between these lipids is dependent on the type of cation present during growth. Apparently, restoration of growth requires a specific lipid composition in combination with a specific cation. Analysis of the phase behaviour of these lipids in the presence of the corresponding cation demonstrates that the bilayer to non-bilayer transition temperature is conserved. Addition of Ba^{2+}, which has similar effects on acyl-chain packing and surface charge screening, but does not promote formation of non-bilayer structures, does not restore growth of this mutant. This demonstrates that the non-bilayer-forming property of the membrane is essential for *E. coli* [46,47].

Protein translocation functions normally in PE-depleted cells. Clearly, the specific chemical structure of PE is not required for correct assembly and function of the translocase. However, *in vitro* translocation in inverted inner membrane vesicles, devoid of growth-promoting cations, is severely hampered. Inclusion of bivalent cations which restore the growth and the non-bilayer-forming property of the lipids results in active protein translocation. Re-introduction of the non-bilayer lipid dioleyl-PE, but not the bilayer-preferring lipid dimeristoyl-PE, into these membranes restored translocation to the same extent. This shows that the non-bilayer property of the membrane is essential for the function of the translocase [14]. Moreover, bivalent cations need to be present in the lumen of the vesicles, demonstrating that non-bilayer-preferring lipids are essential at the periplasmic side of the membrane. Possibly, the altered physical properties of PE-depleted membranes disable an essential conformational change in the SecYEG complex, rendering it inactive.

Active import of β-galactosides by lactose permease (lacY) is inhibited in PE-deficient cells, although passive transport is still present [48]. This defect is not related to the phase behaviour of the lipids, since the bilayer to non-bilayer transition temperature is regulated such that SecYEG is fully active [14]. A domain-specific monoclonal antibody was used to demonstrate that the lack of PE results in the absence of a properly folded periplasmic domain between transmembrane helices VII and VIII. Correct folding of this domain could be induced by blotting to nitrocellulose sheets containing PE, but not PG or cardiolipin [49]. *In vitro* transcription/translation reactions in the presence of inner-membrane vesicles devoid of PE demonstrated that PE is not needed for the membrane insertion reaction, but for a late step in folding. Synthesis of PE in these membranes, after lacY insertion, resulted in completion of this final folding step [50]. Therefore it was concluded that PE fulfils a role as a molecular chaperone in the folding of lactose permease. The only requirement for its function in folding is the non-methylated amino headgroup. PS catalyses folding as well. Strikingly, only the natural stereoisomer of PS appears to be functional [51]. This strongly suggests a very specific role of a phospholipid headgroup in membrane protein folding.

Disruption of the *PgsA* gene, which is essential for the synthesis of PG, appears to be lethal [52]. Lipoprotein biosynthesis requires PG as a precursor, and would use all the remaining PG in strains with low PG synthesis. Therefore a lipoprotein-deficient strain was used to bring the *PgsA* gene under regulation of the lactose operator promotor. This resulted in a strain in which the PG level could be varied from a limiting 3 % to wild-type levels [53]. Using this strain it was unambiguously demonstrated that PG is essential for translocation of prePhoE and proOmpA *in vivo* [13]. Translocation *in vitro* was also strongly inhibited in the absence of PG; however, re-introduction of acidic lipids, irrespective of headgroup structure, restored translocation [54]. This demonstrated that it is the negative charge that is needed for proper translocation. *In vitro* membrane insertion of leader peptidase and Sec-independent insertion of M13 coat protein also depends on the presence of acidic phospholipids [55,56].

The purification of delipidated SecYEG and its functional reconstitution into liposomes of defined lipid composition resulted in the precise definition of the lipid requirement for translocase function [57]. PG appeared to be absolutely essential. Translocation activity was proportional to the amount of PG in the membrane, and optimal translocation was observed at levels similar to the amount in wild-type membranes. PE is not essential, but stimulates translocation activity up to 3-fold. The reconstituted SecYE complex showed the same lipid requirements, demonstrating that lipids do not exert their effect directly through SecG. Furthermore, the *Bacillus subtilis* translocase showed an absolute requirement for both acidic and non-bilayer lipids. Interestingly, optimal translocation required 70 % PG, consistent with the high amount of PG in *B. subtilis* membranes. Both anionic and non-bilayer lipids are needed at high concentrations, close to their physiological levels, suggesting a more global role of these lipids in translocation.

The orientation of proteins in the membrane is often determined by the 'positive-inside' rule [58]. Domains of inner membrane proteins flanking transmembrane helices contain more positively charged residues on the inside than on the outside of the membrane. *E. coli* leader peptidase contains two transmembrane helices connected by a positively charged cytosolic loop, and a large catalytic C-terminal periplasmic domain. The positively charged loop is normally localized at the cytosolic side of the membrane (Figure 3). Leader peptidase serves as a model for demonstrating the positive-inside rule [59]. Introduction of positively charged residues in the N-terminal domain results in an inversion of the orientation, such that the newly introduced positive residues stay inside. Depletion of negatively charged lipids results in insertion of these constructs in the wild-type orientation. Depletion of acidic lipids allows the positively charged residues to translocate, resulting in the original orientation. Depletion of the zwitterionic lipid PE results in a membrane composed entirely of negatively charged lipids. Expression of a Lep–PhoA fusion protein in this strain resulted in membrane integration in the wild-type orientation. Introduction of one single arginine residue flanking C-terminal to transmembrane helix 2 abolished translocation of the C-terminal domain completely. Apparently, the acidic lipids keep the positively charged residues on the *cis*-side of the membrane and thereby function as a determinant of membrane protein orientation [60].

The catalytic domain of leader peptidase binds strongly to both the inner and outer membranes of *E. coli*. A deletion mutant, Δ2–75, which lacks the two transmembrane segments, does bind to *E. coli* membranes and phospholipid vesicles, and is inserted in phospholipid monolayers [61]. A shorter truncate, Δ2–98, which lacks the hydrophobic domain H3 surrounding the active site, did not show any affinity for *E. coli* or model membranes. Interestingly, insertion in membranes containing the non-bilayer-preferring lipid PE was more efficient than insertion in membranes composed of PC or PG. This suggests that the larger headgroup spacing in a PE-containing bilayer facilitates membrane insertion of the catalytic domain. The crystal structure of the catalytic domain revealed the presence of a hydrophobic surface surrounding the active site [62]. This suggests that, indeed, insertion of the catalytic domain into the membrane functions to bring the active site closer to the substrate (Figure 3). Most signal sequences have a hydrophobic length that is insufficient to span the bilayer, explaining the need to bring Lep closer to its substrate.

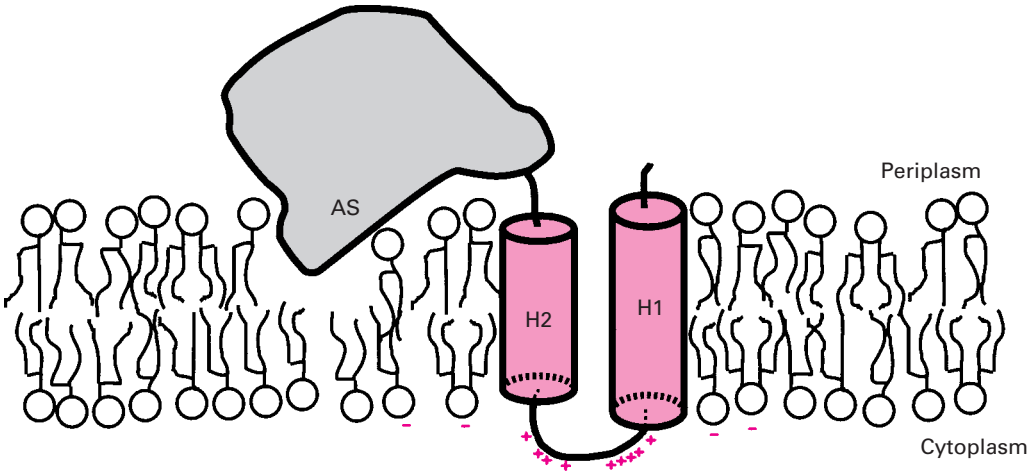

Figure 3 Orientation of leader peptidase in the membrane

Leader peptidase inserts with its active site (AS) in the bilayer. The positively charged residues in the cytosolic loop interact with the acidic lipids of the membrane.

Microsomes isolated from livers of rats fed on a diet enriched in monomethylethanolamine are enriched in phosphatidylmonomethylethanolamine (PMME). The PMME content increases from 0.1 % for control microsomes to 6.4 % in enriched microsomes. These microsomes are fully functional in the translocation of β-lactamase compared with the control microsomes. However, translocation of apolipoprotein B is impaired. Furthermore, translocationally paused intermediates accumulate in PMME-enriched vesicles. This indicates that, in mammalian systems also, the membrane lipid composition is crucial for optimal translocation of some precursor proteins [63].

LIPID–SIGNAL-SEQUENCE INTERACTIONS

The work described above clearly established the important role of lipids in protein translocation across membranes and insertion into membranes *in vivo* and *in vitro*. However, it does not explain the underlying molecular basis for this lipid dependency. *In vitro* approaches appeared to be successful in demonstrating interactions between purified components and phospholipid model membranes. The use of photoactivatable cross-linking reagents and fluorescent probes shed light on the occurrence of lipid–protein interactions during the membrane transit of precursors. These approaches are not only limited to the bacterial process, but also have been rather successful for the yeast and mammalian systems.

The signal sequence is one of the most obvious components to interact with phospholipids, as already proposed in the helical hairpin hypothesis [64]. The positively charged N-terminus enables the signal sequence to interact with anionic phospholipid headgroups, and the hydrophobic core enables it to insert into a mono- or bi-layer in a negatively charged lipid-dependent way [65–68]. Membrane insertion is accompanied by formation of a helix–break–helix motif [69,70]. The hydrophobic length of a signal sequence is shorter than the thickness of the hydrophobic core of the bilayer. Hydrophobic mismatch may result in formation of non-bilayer structures [36]. Indeed, insertion of signal sequences in lipid bilayers induces the formation of inverted hexagonal (H_{II}) phases [66]. These model membrane studies led to a model in which the signal peptide initially binds to the membrane via an interaction between its positively charged N-terminus and the acidic lipids. Subsequently, the peptide inserts in a helix–break–helix structure into the hydrophobic phase and then stretches, resulting in a transmembrane configuration with the cleavage side on the *trans* side of the membrane [71]. Model membrane studies only lead to a hypothetical model based on the physical properties of the components, but do not prove that this route for insertion is the one followed in the biological system. However, a striking amount of evidence is accumulating in both prokaryotic and eukaryotic translocation systems that signal-sequence–lipid interactions indeed play an important role in the biological process.

The importance of the helix–break–helix structure was demonstrated by engineering two cysteine residues into the PhoE signal sequence [72], one close to the N-terminus and the other close to the cleavage site. Oxidation of the cysteines resulted in a precursor with a looped signal sequence that was still targeted to the translocase. Targeting in this case most probably occurred through the SecA–SecB interaction. Translocation was blocked in an early phase and only resumed after reduction of the disulphide bond, demonstrating the importance of unlooping.

Cross-linking experiments performed with prepro-α-factor in the presence of proteoliposomes containing the functional yeast Sec61 system provided a detailed view of the molecular environment of the signal sequence in the membrane directly after targeting. Targeting of prepro-α-factor containing photoreactive lysine derivatives to the translocase and subsequent photoactivation resulted in cross-link products containing Sec61, Sec62, Sec71 and Sec72 [73]. Systematic cross-linking from a series of positions starting at position 5 in the signal sequence through to position 59 in the mature part of the protein revealed a periodicity in cross-linking, from amino acid residues in the signal sequence, with these components. For all positions in the signal sequence cross-linking was shown with phospholipid molecules, whereas lipid cross-links were not found for positions in the mature part of the protein. This result is consistent with an α-helical conformation of the signal sequence bound at the lipid–protein interface. The cross-linking sites in Sec61 were localized to transmembrane segments TM2 and TM7. Experiments with the mammalian translocase revealed that, during co-translational translocation, the signal sequence is localized in a similar way. The signal sequence appears to be

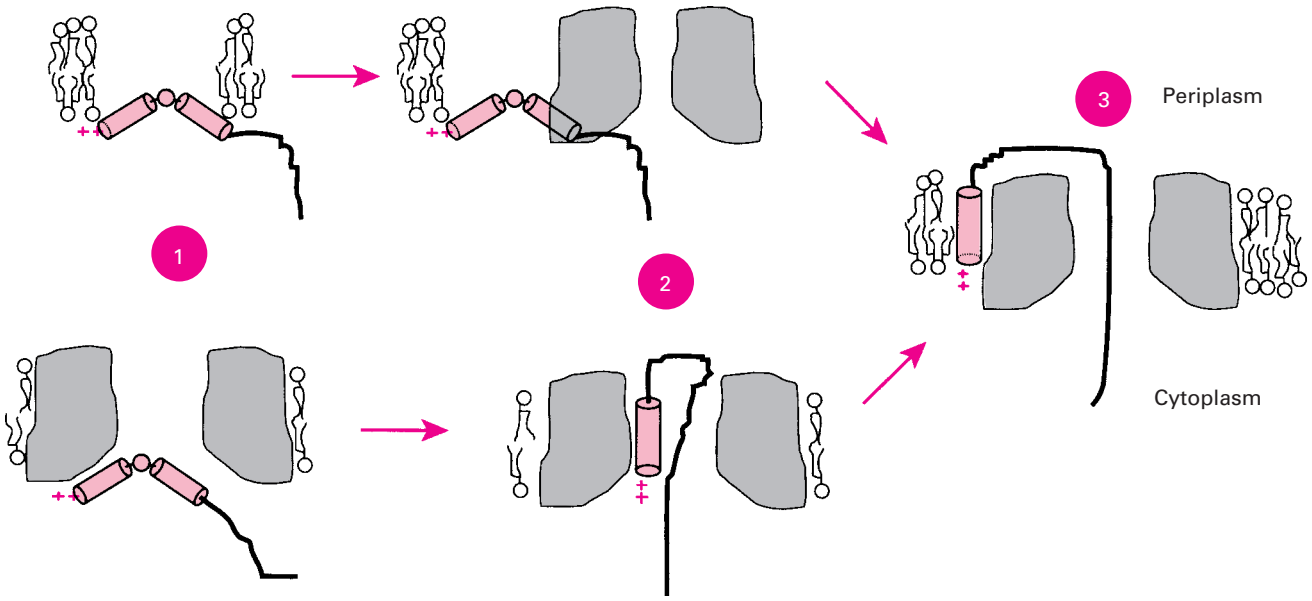

Figure 4 Two possible routes for membrane insertion of the signal sequence

The signal inserts in the lipid bilayer in a looped conformation, unloops at the lipid–protein interface of the translocase and drags the mature part of the protein into the channel (upper route). Alternatively, the signal sequence inserts directly into the channel and moves laterally towards the interface with the bilayer. For reasons of clarity, not all the bilayer lipids are depicted.

oriented at the lipid–protein interface of the translocase in an early translocation intermediate. An intriguing question still is: how did it get there? Possibilities are through the lipids or via insertion into the aqueous channel.

The ability of the signal sequence to translocate through the hydrophobic phase of the bilayer was demonstrated by reconstitution of leader peptidase into proteoliposomes, such that the catalytic site resides in the lumen of the vesicle [74]. Addition of synthetic prePhoE signal peptides containing seven residues of the mature part of PhoE led to processing by leader peptidase. This processing was markedly enhanced by generation of a pmf in these vesicles. Replacing the helix-breaking glycine with a leucine residue resulted in efficient processing, independent of the pmf. These observations are strikingly similar to what was observed for prePhoE translocation in inverted inner membrane vesicles [75]. This demonstrates that the PhoE signal, in the absence of most of its mature moiety, is able to translocate in a SecYEG-independent and pmf-dependent manner. The observation that the signal does not need a transmembrane channel means that there is no obstruction to the signal sequence reaching its location at the lipid–protein interface through the hydrophobic phase of the bilayer.

This leads to the following model. The signal sequence inserts in a looped conformation into the lipid bilayer. It is not able to insert the hydrophilic mature region of the protein into the bilayer. At the interface of the lipid bilayer and translocase, the signal unloops and thereby drags the mature part into the hydrophilic channel. The signal stays at the border, ready to be rejected after cleavage by leader peptidase (Figure 4).

INTEGRATION OF MEMBRANE PROTEINS

The integration of hydrophobic transmembrane helices into the bilayer can also be catalysed by the translocase. In *E. coli* the hydrophobicity of a precursor domain largely determines whether it is inserted into the membrane or fully translocated. Translocation of precursors occurs in discrete steps of approximately 30 amino acid residues [23]. This is thought to correspond to one SecA insertion/de-insertion cycle. Small hydrophobic domains, four amino acid residues in size, slow down the translocation of proOmpA and thereby give rise to abundant translocation intermediates [76]. Duplicating one of these hydrophobic domains resulted in the formation of an artificial transmembrane protein [76]. Apparently, translocation stalls at hydrophobic segments and allows stop-transfer sequences to leave the translocon. A proOmpA mutant (MSI) in which the hydrophobic domain was enlarged by five hydrophobic residues resulted in a protein that was partially integrated into the membrane and partially translocated across the membrane [77]. Increasing the hydrophobic domain by another four residues resulted in a protein (MSII) that was fully integrated into the membrane. This shows that there are two paths that may be followed after insertion into the translocon, either leaving the translocase on the other side of the membrane or leaving laterally into the lipid bilayer. Sorting into one of these pathways is determined by the rate of translocation. The partitioning of MSI into the membrane was increased by slowing down translocation, either by removing the pmf or by using limiting ATP concentrations. MSI partitioned almost completely into the bilayer when further translocation was blocked by the attachment of bovine pancreatic trypsin inhibitor. Apparently, translocation of the precursor pauses upon the insertion of a hydrophobic segment, which then leads to insertion into the bilayer. The hydrophobicity needed for stable membrane integration appears to be lower than that needed for translocational pausing. This suggests that additional signals may function in translocational pausing to facilitate the insertion of proteins with less hydrophobic transmembrane anchors. Indeed, the membrane integration of prion protein is promoted by a short hydrophilic domain flanking the transmembrane segment on the extracytoplasmic site [78]. Removal of this domain results in translocation instead of membrane in-

tegration. When adjacent to a normally translocated hydrophobic domain, it promotes insertion.

Photocross-linking demonstrated that the eukaryotic translocation pore is open laterally towards the phospholipid bilayer during the early stages of co-translational translocation [79]. Both the signal sequence of preprolactin and the signal anchor sequence of the type II membrane protein invariant chain were found to be in contact with phospholipids. This resembles the situation in post-translational translocation where the signal sequence of prepro-α-factor was found at the lipid–protein interface. Although hydrophobic parts are able to contact lipids, hydrophilic domains were found to translocate through an aqueous milieu across the membrane [80]. This again raises the question: do signal sequences and signal-anchor sequences insert into the lipid bilayer and then deliver the hydrophilic precursor to the aqueous channel, or are they inserted into the channel, possibly via hydrophobic protein–protein interactions, which subsequently results in opening the channel towards the lipid bilayer (Figure 4)? The experiments with the *E. coli* translocase indicate that a transmembrane segment follows the same route as a hydrophilic domain into the translocase. Next, possibly due to a hydrophobic interaction between the channel wall and the precursor, translocation stalls; after channel opening, the transmembrane segment is released.

Cross-linking analysis of the insertion of the multispanning membrane protein opsin demonstrated an initial interaction with Sec61β [81]. Cross-linking occurred from a cysteine residue that was still present within the ribosome translocation intermediate, suggesting that Sec61β extends into the ribosome. Increasing the chain length of the intermediates resulted in cross-linking of both Sec61α and Sec61β.

To investigate the insertion of a transmembrane anchor in the mammalian system, a photoactivatable amino acid analogue was incorporated into the transmembrane anchor of an artificial membrane protein [82]. By using truncated mRNAs, translocation intermediates were generated and the cross-linker was photoactivated. In early stages cross-linking was found exclusively to the channel component Sec61α. Further elongation of the nascent chain resulted in the disappearance of this cross-link. Cross-linking to TRAM was found early in integration; however, after the chain moved away from Sec61α, TRAM cross-links were still found. Puromycin treatment of the nascent chains, to dissociate the ribosomes prior to photolysis, prevented cross-linking to Sec61α, but not to TRAM. Further elongation of the nascent chains resulted in puromycin-sensitive cross-linking to TRAM. This demonstrates that a transmembrane anchor travels via at least three different translocase-bound topologies to the phospholipid bilayer.

Taken together, these results indicate that transmembrane helices and signal anchor sequences are inserted into the translocon and leave through a lateral exit to the lipid bilayer. This mechanism leaves two possibilities by which the signal sequence may insert into the membrane. It may insert in the hydrophilic pore, is recognized via a protein–protein interaction, and subsequently follows a route to the lipid–protein interface, where it can be cross-linked to Sec61 and lipid [73]. Alternatively, the signal inserts into the lipid bilayer; subsequently the lipid-inserted signal is recognized via a protein–protein interaction and the hydrophilic part of the precursor is inserted into the hydrophilic channel (Figure 4).

TRANSLOCASE DYNAMICS

The increasing detail of our knowledge of translocation results in a picture whereby the translocase is not just a static pore, but a machine with many moving parts. Electron microscopy of purified Sec61 complexes from mammals and yeast has revealed that these form channels with a quasi-pentagonal shape, a 20 Å (2 nm) pore and an overall diameter of 85 Å. The size of the particles suggests that they consist of three to four Sec61 heterotrimers [83]. Reconstitution of the Sec61 complex in proteoliposomes resulted in an almost complete absence of these complexes. However, upon addition of ribosomes, pores were visible. Apparently, the pores assemble upon interaction with the ribosome. Also, reconstitution in the presence of the Sec62/63 complex resulted in the formation of channels. The assembly of the channels upon addition of ribosomes may be of functional importance; alternatively, the disassembly may be an artefact of the reconstitution technique. The ring structures were also seen in freeze-fractured ER membranes. In this case removal of the ribosomes by puromycin treatment did not result in a decrease in the number of pores. This suggests that the pore does not disassemble after dissociation of the ribosome, or that additional factors function in disassembly.

Investigation of the size of the translocation pore revealed that the assembled channel is highly dynamic. Translocation intermediates were created by using *in vitro* translation of truncated mRNAs in the presence of fluorescent 7-nitrobenz-2-oxa-1,3-diazole (NBD)-Lys-tRNA analogues, resulting in the incorporation of fluorescent probes at desired positions in the nascent chain. These probes are fluorescent when present in the channel and in the cytosol, indicating that they are present in an aqueous environment [84]. The fluorescence of these probes present in the translocation channel can be quenched by iodide ions, but only when these get access to the lumenal site of the membrane via pore formation by perfringolysin O. This demonstrates that the cytoplasmic site of the translocase is sealed by the ribosome, while the other site is open towards the lumen of the ER. Using quenchers of increasing size it was possible to demonstrate that NAD^+ ions had full access to the probes in the translocon in a perfringolysin O-dependent way, but an antigen-binding (Fab) fragment generated against the NBD fluorescent moiety was not able to quench the fluorescence of the probe in the channel. Consequently, the pore size was calculated to be 40–60 Å, i.e. larger than NAD^+ and a nascent chain, and smaller than the Fab fragment [85]. This calculated pore size of the active translocon is substantially larger than the pore observed on electron microscopy of an inactive translocon. Does the pore size change during translocation?

Fluorescent probes incorporated into nascent chains shorter than ~ 70 amino acid residues are shielded from quenching by NAD^+ from both sides of the membrane, indicating that the translocon is also closed on the cytosolic side of the membrane [80]. Extraction and reconstitution of lumenal proteins was used to demonstrate that Bip closes the channel on the lumenal side [86]. After removal of Bip from the channel, NAD^+ has access to probes located in the channel from the lumenal side of the membrane. The cytosolic side is still sealed by the ribosome. Interestingly, in this experiment a substantial fraction of the translocon was not bound to a ribosome. Since NAD^+ does not diffuse through the membrane even after removal of Bip, this suggests that these ribosome-free translocons are not assembled or have a smaller pore than active translocons. Using iodide ions as a collisional quencher, it was demonstrated that these pores were permeable to iodide after the removal of Bip. This demonstrates the presence of inactive translocons gated by Bip. The pore size of these inactive translocation channels was calculated to be 9–15 Å, consistent with the pore size observed in electron microscopy. The large pores containing a ribosome are converted into the smaller pores after dissociation of the ribosome. This

demonstrates that the translocation pore is able to dramatically alter its conformation in the bilayer. With this knowledge, it is not hard to imagine that changing the lateral pressure profile of the membrane by depleting the non-bilayer-preferring lipids would dramatically affect translocation [14].

The above experiments demonstrate the possible role of assembly and disassembly versus a model in which the pore size is regulated and gated. In the bacterial system more is known about the dynamics of the individual components. SecA, the peripheral ATPase, binds with high affinity to the SecYEG translocon [18]. Upon binding of ATP and the precursor protein, SecA inserts into the membrane. This results in protease resistance of two domains of 30 and 65 kDa [22,87]. Protease resistance may be conferred by protection from proteolysis through insertion in the translocase, or may be a result of a protease-resistant conformation of SecA [88,89]. In the inserted state SecA is accessible to proteases and modifying reagents from the periplasmic side of the membrane [90–92]. This might indicate that SecA protrudes through the membrane; alternatively, reagents may permeate through the pore, as was observed for the mammalian translocase [86]. Upon hydrolysis of ATP this inserted state reverts [27]. Translocation is believed to be driven by repeated cycles of insertion and de-insertion of SecA, pushing ~ 30 amino acid residues through the membrane in each cycle [93]. SecA insertion can also be reverted by the pmf [28], which then drives translocation of the precursor in a SecA-independent manner [94].

SecG is a component which is not absolutely essential, but renders translocation highly efficient [95]. SecG contains two transmembrane segments connected by a mildly hydrophobic domain. SecG resides in the membrane in a C-out/N-out topology. However, antibodies raised against the C-terminus of SecG inhibit translocation. This led to the discovery that SecG completely reverses its membrane topology upon insertion of SecA [24]. SecG is thought to facilitate the insertion of SecA, thereby making translocation more efficient.

Through cysteine-scanning mutagenesis it was found that SecE and SecY interact with helices 3 and 2 and with their second and first periplasmic loops respectively [25,96]. Interestingly, it was also found that a SecE dimer is formed upon insertion of SecA into the membrane [25]. These experiments demonstrate that the bacterial translocation channel also undergoes dramatic conformational changes during protein translocation. These conformational changes within the plane of the membrane would obviously be hindered if the lateral pressure profile is changed by depletion of non-bilayer-preferring lipids [14].

SecA–LIPID INTERACTIONS

Depletion of PG leads to a severe defect in translocation [13,97]. Re-addition of negatively charged lipids, independent of headgroup species, restores translocation to wild-type levels [54]. One of the components with a high potential to interact with the acidic phospholipids is the signal sequence, which is discussed above. The other component that interacts strongly with acidic phospholipids is SecA.

SecA inserts into membranes containing negatively charged lipids, which results in partial unfolding [98]. Insertion is inhibited by the presence of ATP [99,100]. Addition of liposomes containing anionic phospholipids leads to a stimulation of ATPase activity; this is termed lipid ATPase activity [97]. Under these conditions SecA is thermolabile; however, SecA activity is stabilized by the addition of precursor protein. SecA lipid ATPase activity is further stimulated by the presence of non-bilayer-preferring lipids, indicating that these may facilitate bilayer insertion [101]. Possibly the wider spacing of phospholipid headgroups facilitates insertion. SecA completely traverses the lipid bilayer during this insertion [102].

SecA binds with high affinity to the SecYEG complex in the membrane, but a substantial part is also bound with low affinity, presumably to phospholipids in the membrane [18]. SecA ATPase activity is stimulated in the presence of SecYEG, precursor protein and acidic phospholipids [103]. This ATPase activity coincides with translocation, and hence is called translocation ATPase activity. Translocation ATPase activity can be uncoupled from translocation by blocking a translocation intermediate in the translocon. Lipid ATPase activity was not found in the presence of inner membrane vesicles, presumably due to the presence of membrane proteins. Translocation of precursor proteins is highly dependent on the presence of negatively charged phospholipids. The high-affinity binding to SecYEG is lost upon depletion of acidic phospholipids, directly indicating why translocation activity is lost [104].

These observations led to the idea that SecA binding and insertion at the membrane occurs at a lipid–protein interface consisting of SecY and acidic phospholipids, similar to the binding of the signal sequence to Sec61. However, experiments with hydrophobic photolabels and photoactivatable phospholipids led to unexpected results. Upon insertion in the SecYEG translocation channel, SecA forms two protease-resistant domains of 30 and 65 kDa [22,87]. Introduction of the hydrophobic photolabel [^{125}I]TID-BE [3-trifluoromethyl-3-(*m*-[^{125}I]-iodophenyl)diazirine benzoic acid ester], which can be used to selectively label membrane-embedded segments of integral proteins, did not compromise any translocation function. Upon generation of the 30 kDa fragment and subsequent photoactivation of the label, little labelling of the 30 kDa fragment was observed as compared with SecY, which has approx. 30 kDa exposed to the bilayer [105]. Also, labelling of the 65 kDa fragment was not observed. However, labelling of several fragments derived from lipid-bound SecA molecules was observed. Although obviously SecA molecules that were fully degraded by the protease escaped detection, this study showed that the 30 and 65 kDa protease-resistant fragments were not inserted in the lipid phase of the bilayer.

Reconstitution of purified SecYEG into proteoliposomes containing the photoactivatable phospholipid [^{125}I]TID-PC/16 (1-*O*-hexadecanoyl-2-*O*-{9-[({2-[^{125}I]iodo-4-(trifluoromethyl-3H-diazirin-3-yl)benzyl}oxy)carbonyl]nonanyl}-*sn*-glycero-3-phosphocholine) resulted in a translocation system in which the interaction of SecA with phospholipids could be assayed directly [106]. Labelling of SecA by liposomes containing this lipid was dependent on the presence of acidic lipids, as reported before. Also, in proteoliposomes containing SecYEG, SecA was readily labelled by this phospholipid. However, when using substoichiometric amounts of SecA relative to SecYEG, a very low amount of labelling was observed as compared with labelling in the presence of liposomes composed of pure phospholipid. This demonstrated that the population of SecA molecules bound to the SecYEG translocation channel is largely shielded from the phospholipid bilayer. The shielding of SecA from the lipid bilayer occurs irrespective of the presence of translocation ligands.

According to both studies, SecA inserts in the membrane mainly through a proteinaceous environment and not at the interface of SecYEG and phospholipid through the lipid bilayer. This is consistent with electron microscopy studies, which show that SecYE forms ring-like structures [107]. SecA presumably inserts into the pore formed by SecYEG and pushes the precursor

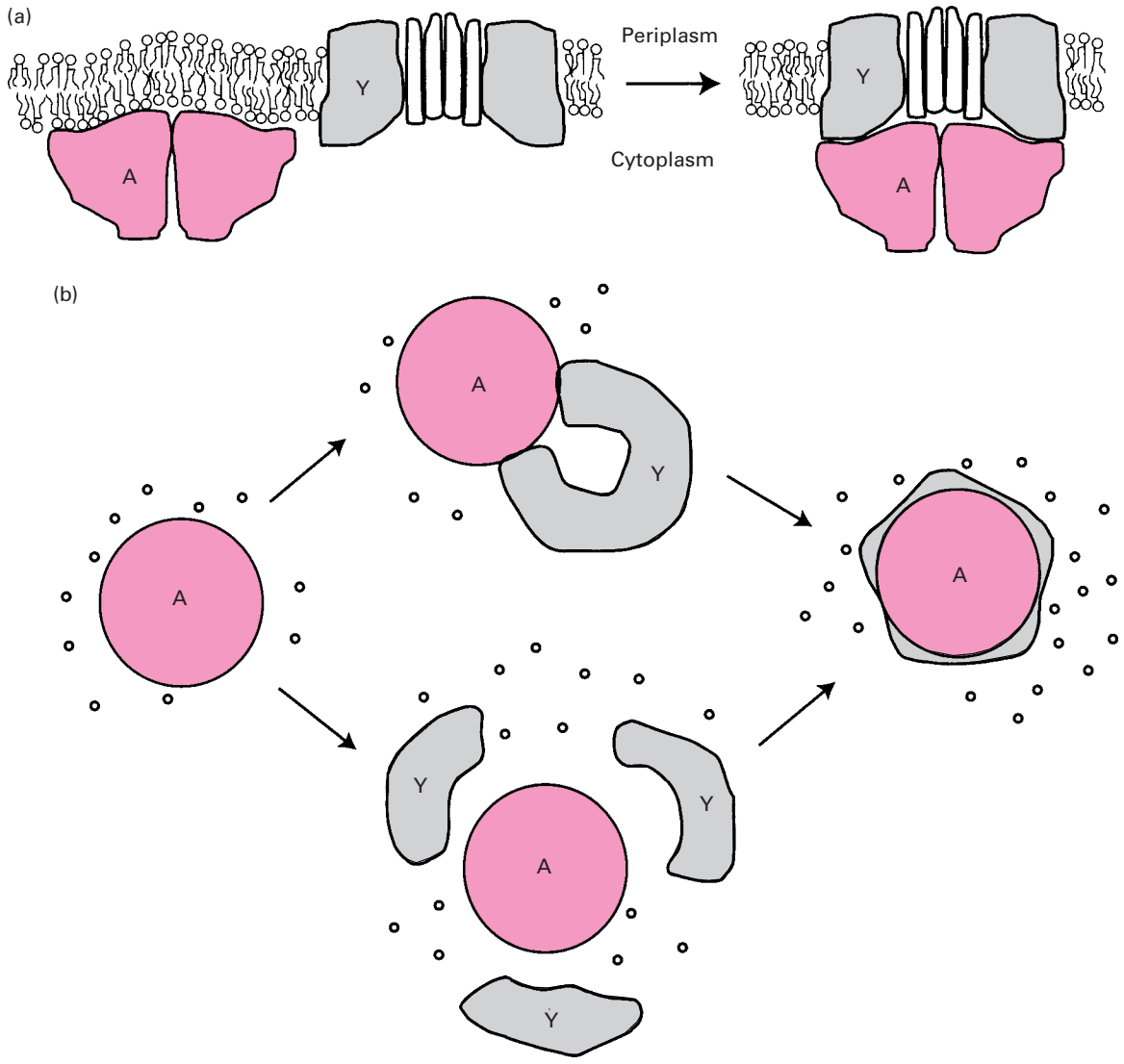

Figure 5 SecA insertion model

SecA first inserts into the lipid bilayer and subsequently integrates into the translocase (**a**). SecA can enter the translocation channel via a lateral opening (**b**, upper route) or via assembly of individual subunits (**b**, lower route).

protein through. How could one then explain the dependency on acidic lipids of SecA function?

First of all, it was shown that SecA is shielded from the phospholipid acyl-chain phase, and interaction with the negatively charged headgroups is not excluded. SecA is an elongated molecule [108] and may reach over the edge of the ring when bound to SecYEG. Interaction with acidic headgroups would then stabilize its interaction with SecYEG. Alternatively, since the binding to SecYEG is diminished in the absence of anionic phospholipids, the conformation of SecYEG itself may be disturbed and thereby prevent productive binding of SecA, which then leads to the translocation defect.

These two explanations do not include a functional role for SecA lipid insertion, as observed in model membrane systems. However, in the cell SecA is present in the cytosol and is bound to the inner membrane, to the SecYEG complex and to the phospholipids. During translocation SecA at the translocase inserts into the membrane, resulting in the protection of two domains of 65 and 30 kDa from protease digestion [22,87]. This inserted SecA exchanges with soluble SecA during translocation. This study was challenged by experiments with membranes containing saturating amounts of ^{35}S-labelled SecA. In these experiments, several other protease-resistant fragments were found next to the 65 and 30 kDa domains [109]. It was suggested that this SecA did not exchange with soluble SecA, but was permanently present in the membrane. Possibly, SecA molecules that are displaced from the translocase stay on the membrane by binding to the phospholipids. We would like to speculate that there is functional exchange between lipid-inserted SecA molecules and the SecYEG-bound species. In this model, lipid-inserted SecA binds to the hydrophobic transmembrane helices of SecY at the lipid–protein interface (Figure 5a). This binding event leads to the formation of a closed-ring structure, as seen in electron micrographs. Two possibilities exist (Figure 5b). First, the SecYEG pore may be assembled from individual SecYEG subunits in the membrane: the assembly model. In this case SecA

would nucleate assembly of the pore by binding to one subunit and subsequently collecting the others. After translocation, the SecA subunit would dissociate again, and disassembly of the pore ensures that the barrier function of the membrane stays intact. This model is similar to the Sec61 system, in which the ribosome induces the formation of oligomeric rings [83]. In the second possible model, the SecYEG pore does not disassemble in the membrane, but is open towards the lipid bilayer, allowing SecA to enter from the lipid phase, and subsequently forms a closed-ring structure. This opening might also be involved in the release of transmembrane segments in the bilayer. Dissociation of the bound SecA molecule would then lead to the opening of the translocation channel again.

CONCLUDING REMARKS

Many components of the protein-translocating machineries in the ER membrane and bacterial membrane interact with phospholipids during the translocation process. Changes in phospholipid composition will perturb these interactions and compromise the whole machinery. Furthermore, the dynamic nature of these translocases suggests the occurrence of substantial conformational changes in the membrane during the process. Alteration of the lateral pressure profile will be likely to interfere with these structural rearrangements. Much is known about the interactions of purified components with phospholipids. However, to understand the role of these interactions in the complete translocation machinery, detailed knowledge of the structure and of the conformational changes taking place is essential. Further insight into the structure of the translocase will also lead to a better understanding of the mechanism of insertion of transmembrane domains and the role of phospholipids therein.

Important questions for future research include the following: at what stages of translocation do phospholipids function, and how do phospholipids influence the structure of the relevant membrane components?

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