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Review

The Sec translocase ☆

David J.F. du Plessis [1], Nico Nouwen [2], Arnold J.M. Driessen *

Department of Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute and the Zernike Institute for Advanced Materials, University of Groningen, 9751 NN Haren, The Netherlands

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ABSTRACT

The vast majority of proteins trafficking across or into the bacterial cytoplasmic membrane occur via the translocon. The translocon consists of the SecYEG complex that forms an evolutionarily conserved heterotrimeric protein-conducting membrane channel that functions in conjunction with a variety of ancillary proteins. For posttranslational protein translocation, the translocon interacts with the cytosolic motor protein SecA that drives the ATP-dependent stepwise translocation of unfolded polypeptides across the membrane. For the cotranslational integration of membrane proteins, the translocon interacts with ribosome-nascent chain complexes and membrane insertion is coupled to polypeptide chain elongation at the ribosome. These processes are assisted by the YidC and SecDF(yajC) complex that transiently interacts with the translocon. This review summarizes our current understanding of the structure–function relationship of the translocon and its interactions with ancillary components during protein translocation and membrane protein insertion. This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

Contents

1. Introduction

Many proteins that catalyze essential cell functions are embedded in the cytoplasmic membrane or function on the outside of the cell. These proteins are synthesized at ribosomes in the cytosol and are directed to the Sec translocase as the major facilitator in the

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* Corresponding author. Tel.: +31 50 3632164; fax: +31 50 3632154.
E-mail address: a.j.m.driessen@rug.nl (A.J.M. Driessen).
[1] Current address: Adolf-Butenandt-Institute für Physiologische Chemie, Ludwig-Maximilians Universität München, Butenandtstrasse 5, 81377, München, Germany.
[2] Current address: Laboratoire de Symbiosis Tropicales et Méditerranéennes, Campus International de Baillarguet TA 10/J 34398 Montpellier cedex 5, France.

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translocation and insertion of these proteins across or into the inner membrane of prokaryotes, the endoplasmic reticulum membrane in eukaryotes, and the thylakoid membrane of photosynthetic Eukarya [172] (Fig. 1). The substrates for the Sec translocase range from very hydrophilic to very hydrophobic proteins, yet all contain a hydrophobic N-terminal region, i.e., a signal sequence for secretory proteins (preproteins) and a membrane anchor signal for inserted inner membrane proteins (IMPs). Translocated proteins are processed by signal peptidase that removes the signal sequence and allows the release and subsequent folding of the mature protein on the outer side of the inner membrane. Lipoproteins are processed by a specific signal peptidase once the cysteine at the +1 position of the mature domain has been lipid modified. This ensures lipid membrane anchoring before maturation. The signaling domain of IMPs most often remains associated with the inserted protein.

The Sec translocon is conserved across all three domains of life. Its core consists of a heterotrimeric protein complex designated as SecYEG in Bacteria and Sec61αβγ in Eukaryotes. Ancillary components associated with the translocon provide the energy for translocation and insertion. The translocon can facilitate the movement across or integration of proteins into the membrane in a cotranslational or posttranslational manner. In Bacteria, the cotranslational pathway is mainly employed by inner membrane proteins, while the posttranslational pathway is utilized by proteins that are secreted across the membrane [222]. The selection step for either pathway lies at an early stage of translation once the nascent chain emerges from the ribosomal exit tunnel [147,224]. Ligand crowding at the exit tunnel allows signal recognition particle (SRP) to be the first to interact with the ribosome nascent chain (RNC) [61]. If the emerging signal sequence displays a high level of hydrophobicity [242] and helicity [27], SRP binds the RNC tightly and it has been suggested that this binding reaction results in a pausing or slow down of translation (see also below). The entire SRP-RNC complex is then targeted to the membrane-associated signal–particle receptor (FtsY) that itself is bound to a competent translocon [147]. SRP and FtsY form a heterodimeric complex, and this stimulates GTP hydrolysis on both proteins, whereupon the RNC is transferred to the translocon with the ribosomal exit tunnel in close proximity to the translocon pore [17]. Polypeptide chain elongation at the ribosome provides the energy for the cotranslational insertion of membrane proteins.

When a signal sequence emerging from the ribosome does not display a high level of hydrophobicity, it is bound by a trigger factor which shields it for further binding by SRP [61]. Next, the polypeptide is translated through its full length by the ribosome in the cytosol. In a subset of Bacteria (mostly Proteobacteria), the newly synthesized preprotein is maintained in an unfolded state by the cytosolic molecular chaperone SecB as firmly demonstrated by recent single molecule measurements [14] (For a review on SecB, see Reference 66). Next, the SecB–preprotein binary complex is targeted to the translocon where SecB binds to the ATPase motor protein SecA. After the release of SecB, and the transfer of the preprotein to SecA, translocation is initiated at the expense of ATP [197]. In contrast to bacterial protein translocation that is strictly dependent on SecA, translocation in the Eukaryotic ER can also occur cotranslationally [73]. During posttranslational translocation in Eukaryotes, the ER luminal protein BiP provides the energy for translocation at the anterior or exit site of the translocon as opposed to SecA which is found at the interior face of the translocon [165]. For Archaea, posttranslational translocation has been suggested to occur; however, Archaea lack a SecA homologue. Although they contain BiP

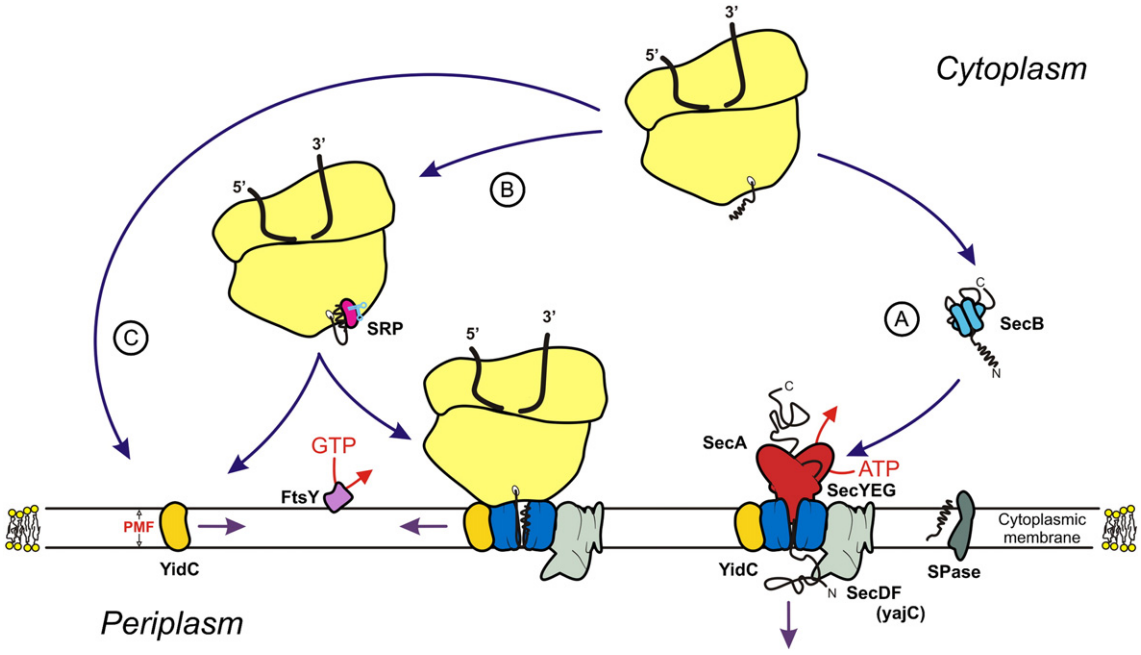

Fig. 1. Schematic representation of protein targeting to the Sec translocase. The bacterial Sec translocon (*blue*) spans the cytoplasmic membrane (CM) and consist of SecY, SecE and SecG. SecA (*red*) acts as the peripherally associated motor protein on the cytoplasmic side. Other ancillary proteins interacting with the translocase include YidC (*yellow*) and the SecDF(yajC) complex (*light grey*). Signal sequences of preproteins are cleaved at the periplasmic face of the membrane by Signal peptidase (SPase). (A) Proteins synthesized at the ribosome (*light yellow*) destined for secretion are mostly post-translationally targeted to the Sec translocase by a targeting sequence which is recognized by the motor protein SecA. Alternatively, the molecular chaperone SecB (*light blue*) binds the preprotein, keeps it in a translocation competent state and targets it to the Sec translocon for translocation. (B) Cotranslational targeting of the ribosome with the nascent chain to the translocase complex is attained by the binding of the signal sequence of some preproteins or the signal anchor sequence of membrane proteins by SRP (*pink*) and the SRP receptor FtsY (*purple*). Membrane proteins with large hydrophilic periplasmic domains require the presence of SecA in the insertion process to translocate these domains across the membrane. YidC interacts with TMs as they emerge from the proposed lateral gate of SecYEG; however, the importance here is low and not clearly understood. Some proteins such as CyoA and Lep require the translocon, SecA and YidC for its proper insertion; however, limited data exist on how this is achieved precisely. (C) A subset of membrane proteins can insert into the cytoplasmic membrane via YidC after targeting of the ribosome nascent chain to YidC. Conflicting evidence exist for the involvement of the SRP pathway. Some studies indicated the targeting of the ribosome nascent chain to YidC via the SRP pathway, whereas SRP depletion studies showed no effect on membrane insertion suggesting a direct targeting of the ribosome nascent chain to YidC.

homologues, i.e., Hsp70 proteins, these chaperones reside in the cytosol and thus cannot bind at the exit site of the translocon like in Eukaryotes. Therefore, a major unresolved question is how posttranslational translocation in Archaea is energized. IMPs with large periplasmic domains represent a special class. These proteins insert into the membrane in a cotranslational manner but require SecA for the translocation of their large polar domains. This involves a mechanism in which the ribosome while actively engaged in the synthesis of a polar domain must be released from the translocon to allow SecA binding.

To date, various requirements and conditions have been described for the translocation of proteins and the insertion of membrane proteins. Particularly, a great advancement in the understanding of these processes has come with the development of biochemical *in vitro* assays to determine the minimal components required. There is no other protein translocation system to date that has been studied at such advanced level, including crude membrane systems up to liposomes reconstituted with the purified components performing the key activities of the translocon [28]. Here we will give an overview of the factors involved in translocation of polar polypeptide domains across the membrane, and the insertion of apolar polypeptide domains into the membrane. The focus is on the role of the Sec translocase acting as a multipurpose device that facilitates these two seemingly opposing activities. The term translocon is used to indicate the protein conducting channel while the term translocase includes the complex of the translocon with one of its energy supplying ligands, i.e., in Bacteria, SecA, or the ribosome.

2. The translocase—components and structure

2.1. Organization of the translocon

The Sec translocon exhibits the distinct ability to both translocate substrates across a membrane as well as to insert them into the membrane laterally. Its functional properties have been studied in great detail. The translocon in Bacteria consists of three proteins, namely SecY, SecE, and SecG [28]. The Sec61p of the endoplasmatic reticulum (ER) is homologous to the SecYEG complex with an identical trimeric arrangement of Sec61α (homologous to SecY), Sec61β and Sec61γ (homologous to SecE) [74]. Sec61β is not homologous to SecG in either structure or function. Rather, Sec61β has been implicated in acting as a guanine nucleotide exchange factor for the SRP receptor [84]. SecG stimulates the activity of SecYE by attenuating the SecA activity [149,150,152,233]. In the yeast ER (*Saccharomyces cerevisiae*), two homologous Sec systems exist. One Sec61p complex with Sec61p (Sec61α), Sbh1p (Sec61β), and Sss1p (Sec61γ) as subunits and a second complex consisting of Ssh1p (Sec61α) and Sbh2p (Sec61β) [67]. While the components of the two translocons can interact, the Ssh1p complex is not essential for viability and has been shown to be involved in cotranslational protein translocation only [67,186,250]. The Sec61p complex can interact with the Sec62p complex that consists of three membrane subunits Sec62, Sec63, and Sec70/72 which are involved in the BiP-mediated posttranslational translocation of preproteins across the endoplasmic reticulum membrane [97,165,170]. In the thylakoid, membranes of chloroplasts homologues to SecY and SecE have been characterized, that together with SecA, mediate the translocation of preproteins across the thylakoid membrane [121,140,194,195,199]. SecY homologues have also been found in cyanelles [46,68,241], cyanobacteria [141], and certain mitochondria of primitive protists [29]. In Archaea, homologues for the translocon exist as SecY, Sec61β, and SecE with notably the absence of a SecA or possible BiP homologue that could be involved in posttranslational translocation [7,9,104,110]. The archaeal subunits are more closely related to those of the Eukaryotes than the bacterial ones [60].

SecY has a molecular mass of 48 kDa, spans the membrane 10 times in an α-helical configuration, and is highly hydrophobic. It is the largest component of the translocon and essential for viability and translocation. In *Escherichia coli*, SecE is a small integral membrane protein of 14 kDa with three predicted transmembrane segments (TMs). Other Bacteria have SecE proteins with a single TM that is homologous to the third TM of the *E. coli* protein [137]. Despite its small size, SecE is essential for viability and translocation. Only the third TM of the *E. coli* SecE is required for a fully functional translocon corresponding with the observation that most SecE homologs consist only of a single TM [38,182]. SecY and SecE form a stoichiometric complex, and in the absence of SecE, SecY is unstable and readily degraded by FtsH [108]. SecY seems to be toxic to cells when not associated with SecE as the overproduction of SecY in a conditional lethal FtsH mutant background leads to the inhibition of cell growth as well as protein export [108]. SecG is a 12-kDa protein containing two TMSs. While not essential for viability or translocation, SecG displays some remarkable biochemical properties. SecG has been found *in vitro* to stimulate preprotein translocation, particularly at low temperatures [149] or when the proton–motive–force (PMF) is reduced [77]. SecG has been hypothesized to facilitate the binding and insertion of SecA into the translocon by undergoing transient topological inversions during protein translocation. This hypothesis is based on biochemical studies by Tokuda and coworkers that showed an altered proteolysis pattern of SecG [152] as well as a change in accessibility of loop residues for chemical modifications [138] during protein translocation. However, another study showed that a topologically fixed SecG is fully functional in protein translocation [233]. No evidence has been found for a physical interaction between SecE and SecG, and SecG has been found to bind SecY in the absence of SecE [89] thereby weakly improving the stability of SecY [151] and of the SecYE complex [89].

2.2. Structure of the translocon

The first high-resolution structure reported was of the archaeal SecYEβ complex of *Methanocaldococcus jannaschii* [228]. The structure has provided many new insights in the structure–function relationship of the translocon and the possible mechanism of channel opening (Fig. 2). Superimposition of the crystal structure onto the three-dimensional reconstruction of the *E. coli* SecYEG based on electron cryoelectron microscopic imaging of two-dimensional crystals [26] revealed that the two complexes differ only slightly in conformation [24]. The 10 TMs of the main subunit SecY are arranged like a clamshell in which the two halves TMs 1–5 and TMs 6–10 are hinged at the cytoplasmic loop between TM5 and TM6 (Fig. 2B). The SecE protein embraces the two SecY halves at the so-called “back” end of the translocon with its TM crossing through the membrane diagonally. The surface-exposed amphipathic helix of SecE lies flat onto the cytoplasmic side of the cytoplasmic membrane [240]. The SecY channel has the shape of an hourglass, with at its centre a constriction that narrows the channel to a few angstroms suggesting that the *M. jannaschii* structure represents the closed conformation of the translocon. The centre of this constriction contains six hydrophobic amino acid residues which have their hydrophobic side chains directed towards the centre of the channel. Proteins are thought to pass the translocon through the central pore and the hydrophobic residues within the constriction have been proposed to form a seal around the translocating protein thereby maintaining the permeability barrier of the membrane during translocation. At the cytoplasmic side, the water-filled channel has an opening of 20 to 25 Å where SecA, the ribosome and the polypeptide likely interact with the channel. At the external or periplasmic face of the membrane, TM2a forms a re-entry loop that folds back in the outer funnel to block the channel. TM2a is also referred to as the “plug” domain. A previous cross-linking study [80] has led to the suggestion that upon binding of a signal sequence to the translocon, the plug is displaced allowing preprotein translocation to occur [228]. Signal sequences of

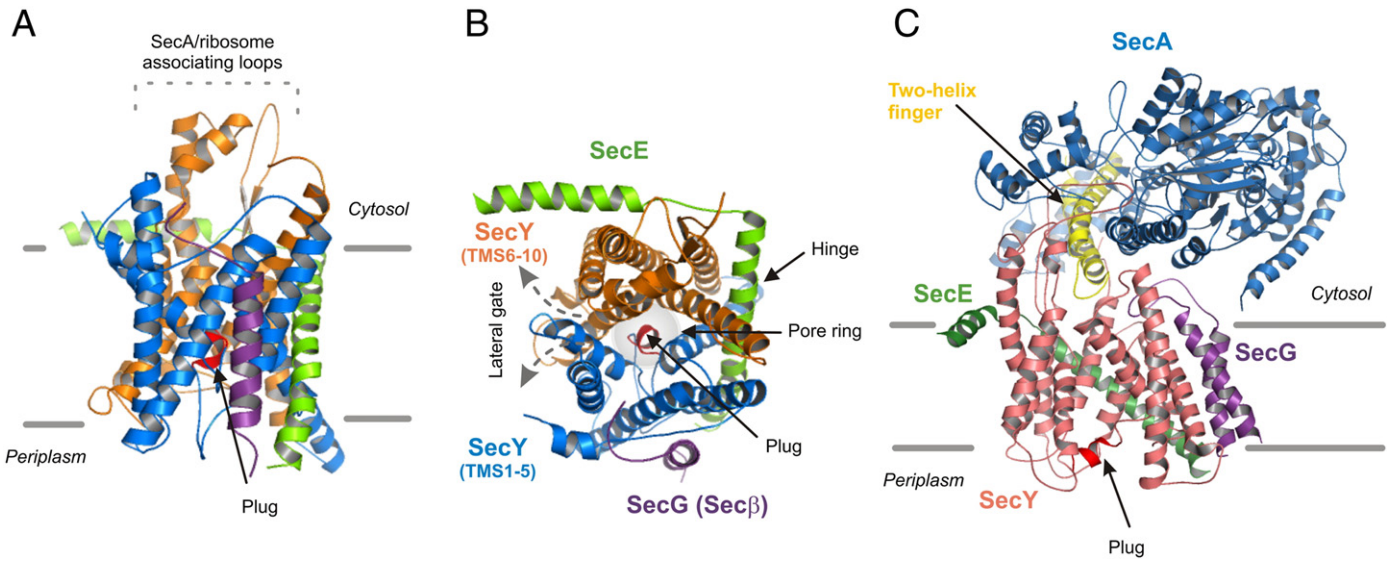

Fig. 2. Structure of the Sec translocase. Side view (A) and top view (cytosolic face) (B) of the crystal structure obtained from SecYEβ of *M. jannaschii* based on coordinates deposited at the Protein Data Bank as 1RHZ [228]. The hourglass shaped translocon consists of SecY (Sec61α) as the core of the translocon, SecE (Sec61γ) that embraces SecY in a supportive manner and SecG (Sec61β) which is peripherally bound to SecY. At the centre of the SecY channel a pore ring, consisting of hydrophobic amino acids, forms a constriction. A small alpha-helix (TM2a) that folds back as a re-entry helix acts as a plug (*red*) from the periplasmic side and together with the pore ring serves to close off the channel and prevent ion leakage. The central pore formed by SecY is arranged in a clamshell like arrangement with TMS 1–5 (*blue*) and TMS 6–10 (*orange*) forming the two halves. The clamshell is hinged at the back towards SecE. It has been proposed that the front of the clamshell at the intersection between TM7 and TM2b can open up and act as a gate to laterally release TMs into the lipid bilayer. Proteins that are translocated bind with their signal sequence at the TM2b and TM7 interface leading to the widening of the central pore and destabilization of the plug. As a consequence the central pore opens to accept proteins for translocation across the lipid bilayer in a concerted effort together with SecA insertion [228]. (C) Side view of the crystal structure for the SecA/SecYEG complex from the gram-negative Bacteria *T. maritima* based on coordinates deposited at the Protein Data Bank as 3DIN [260]. The binding of SecA (*blue*) to SecYEG (*pink*, *green*, and *purple*) induces a conformational change in SecYEG whereby the lateral gate of SecYEG opens up to the lipid interface, the plug (*red*) moves away from the centre of the pore and SecA inserts a two-finger helix (*yellow*) which has been proposed to move up and down the channel with cycles of ATP hydrolysis.

preproteins can be cross-linked to the TM2 and TM7 [169,170,248]. These two helices are at the 'front' of the translocon, and it has been proposed that insertion of the signal sequence into the TM2/TM7 interface induces a separation of the two halves of SecY which would further facilitate the displacement of the plug from its central position. A recent molecular dynamics study has shown that the opening the channel by this mechanism creates an opening that is large enough for the passage of unfolded and even larger α-helical domains in proteins [213]. The opening between TM2 and TM7 would also allow the lateral partitioning of TMs and signal sequences into the lipid bilayer and thereby expected to play a very important role in the insertion of membrane proteins via the Sec translocon. For this reason, this region was termed the 'lateral gate'. Based on a cross-linking study [80], it has been suggested that in the open state, the plug may completely vacate the central channel and move to a position close to the C-terminus of SecE [228]. Besides liberating the exit site, the hydrophobic surface of the plug domain may guide the unfolded protein towards the periplasm [22] and in combination with the hydrophobic constriction ring involved in sealing of the preprotein–translocon junction [228].

In recent years, two independent studies presented the structure of the Sec translocon in a 'pre-open' state. The structure of the *Thermus thermophilus* SecYE complex is with a Fab fragment bound to the cytoplasmic loops C4 and C5 of SecY [217]. This Fab fragment was proposed to induce a conformation that would correspond to the SecA bound state of SecYEG. Herein, TM6, TM8 and TM9 were displaced by 10 Å relative to the *M. jannaschii* SecYEβ structure. The structure of the *Thermotoga maritima* SecYEG translocon with bound SecA (Fig. 2C) was solved at a medium resolution of 4.5 Å [260]. The SecA protein had bound ADP-berrylium fluoride, which is a transition state analog of ATP hydrolysis. In this structure, the tightly bound SecA causes the lateral gate of SecY between TM2 and TM7 to open, with the TM2a plug domain moving away in the direction of the periplasmic side of the translocon. Furthermore, a two-helix finger of SecA was found to insert near the "front" entrance of the channel (see next section). Again, this structure appears to adopt a 'pre-open' state. Importantly, the structure indicates that lateral gate opening and SecA function might be allosterically linked. Indeed, biochemical studies showed that the opening of the lateral gate of SecYEG is essential for SecA-mediated protein translocation and linked to the activation of the SecA ATPase activity [52]. In the 'pre-open' state of SecYEG, the plug domain moves towards the C-terminus of TM7 but still closes the central pore. This is different from the proposed 'open' state where the plug domain is suggested to be completely moved towards the C-terminal tip of SecE [80,228]. A recent cross-linking study demonstrated that only a small displacement of the plug occurs during channel opening [127]. Interestingly, a molecular dynamics simulation [258] suggests that the plug domain may function as a kind of 'ruler' that senses the polarity of the incoming polypeptide. For polar polypeptide domains that need to cross the channel, plug displacement would result in the formation of a vectorial aqueous pore. In contrast, an apolar polypeptide domain that needs to insert into the membrane would not induce such plug displacement. However, a true function of the plug as ruler remains to be demonstrated biochemically.

2.3. SecA, a preprotein-stimulated translocation ATPase

SecA functions as a motor protein both in protein translocation as well as in the translocation of hydrophilic domains of membrane proteins across the membrane. SecA associates with SecYEG, and this interaction involves the major cytosolic loops of SecY [100,130,187]. The interaction of SecA with the translocon occurs at a much higher affinity than the interaction with preproteins in the cytoplasm [75,81]. In addition, SecA interacts with anionic phospholipid headgroups at a low affinity [125]. The structures of SecA proteins have been characterized by X-ray crystallography in great detail employing proteins derived from various organisms [93,154,163,166,205,238,259,261]. Most of these structures display SecA packed as a dimer (Fig. 3) with an antiparallel orientation except for the *T. thermophilus* SecA that was

crystallized as a parallel dimer [238]. SecA belongs to the superfamily 2 DExH/D proteins [115] and contains a motif that is also found in DNA/RNA helicases. The conserved DEAD helicase motor, made up by the two nucleotide binding subdomains (NBF1 and NBF2), is the site for ATP binding and hydrolysis (Fig. 3). The ATP hydrolysis cycle drives conformational changes in the motor domain [71,93,208] that are transferred to the helical wing domain (HWD) and the preprotein cross-linking domain (PPXD) [71,208]. SecA interacts with preproteins via its PPXD domain [109,167]. The helical scaffold domain (HSD) lies at the centre of the protomer, where one of the α-helices interacts with all other subdomains of SecA. The C-terminal linker domain of SecA (CTL) has been shown to constitute a zinc finger and shown to be involved in both SecB binding and the interaction with phospholipids [25,64].

Several functionally important regions in the SecA protomer have been defined. Within the HSD domain, a helix–loop–helix structure that contacts both the PPXD and NBF2 subdomains has been identified to act as a global regulator of ATP turnover. This region is indicated as the intramolecular regulator of ATP hydrolysis 1 (IRA1) (Fig. 3). This domain most likely prevents promiscuous ATP hydrolysis in the cytosol, as a deletion or mutations in this domain lead to an ATPase activity that is uncoupled from preprotein interaction [102]. A conserved salt bridge known as Gate 1 controls the opening and closing of the nucleotide binding groove in concert with the binding signal observed at the PPXD domain [101]. However, this mechanism seems to be only active once SecA is bound to SecYEG thereby leading to a synchronized preprotein binding and release cycle coupled to ATP hydrolysis and resulting in the stepwise translocation of the preprotein across the membrane [197,234].

Experimental evidence to date favors a model where SecA inserts itself partially into the translocon during protein translocation [52,59,62]. This inserting domain corresponds to a two-helix finger of SecA that binds and interacts with the preprotein substrate during translocation [62]. It was proposed that the two-helix finger may drive translocation by insertion into the cytosolic funnel-like opening.

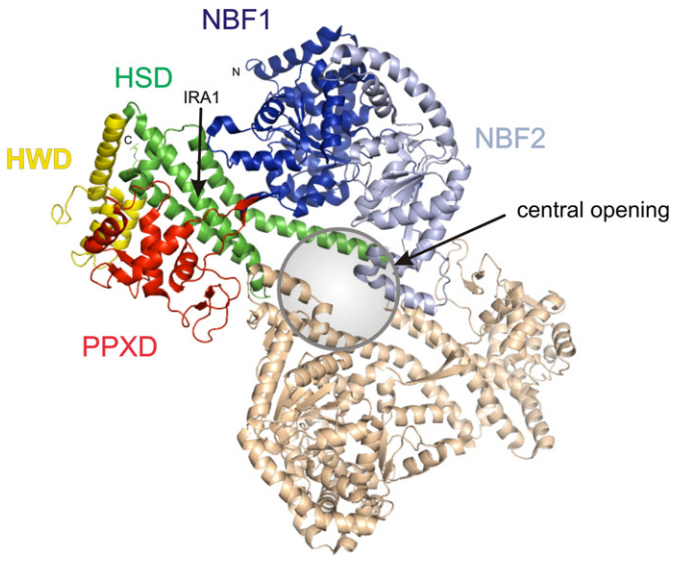

Fig. 3. Structure of SecA, the motor protein of the Sec translocase. Graphic representation of the *Mycobacterium tuberculosis* SecA based on coordinates as deposited at the Protein Data Bank as 1NKT [205]. The antiparallel crystal structure as obtained is displayed here with the second SecA protomer in its antiparallel arrangement in beige. Also indicated is the formation of a central pore at the centre of the dimer. Nucleotide binding folds 1 and 2 (NBF1, NBF2) are shown in dark blue and light blue, respectively. The preprotein cross-linking domain (PPXD) is shown in red, while α-helical scaffold domain (HSD) is shown in green. Finally, the α-helical wing domain (HWD) is shown in yellow. The C-terminal linker domain (CTL) was not resolved in this structure. The intramolecular region of ATP hydrolysis 1 (IRA1) which is responsible for the hydrolysis of ATP at NBF1 is indicated.

Cross-linking data indicate that SecA captures the preprotein in a clamp-like manner, whereupon the preprotein may move through the clamp as an extended protein and enter the SecYEG translocon [13]. This clamp-like structure has recently been resolved for the *Bacillus subtilis* SecA in complex with a peptide [261]. Similarly, the signal peptide binding domain within *E. coli* SecA has been visualized utilizing FRET measurements [8]. The signal peptide binding domain within SecA seems to comprise part of the PPXD as well as regions from NBF1 and the HSD. From these two studies, it has become clear that preprotein binding and translocation in SecA occur at a multidomain interface further indicating the high flexibility of SecA during protein translocation.

With regards to the role of SecA in insertion of membrane proteins, it has been shown that inner membrane proteins with periplasmic domains larger than 60 amino acids require SecA for their correct and complete insertion [3]. Membrane proteins with smaller periplasmic loops do not require SecA or SecG for their insertion [111]. One can envisage a membrane protein with periplasmic loops of varying size that would intermittently require SecA. How this is achieved together with cotranslational membrane protein insertion involving the ribosome is not understood. It has been suggested that SecA and the ribosome bind the translocon simultaneously during cotranslational translocation [262], but since SecA and the ribosome bind to overlapping binding sites of the SecY protein, it is not clear how simultaneous binding might occur. Moreover, in the cytosol, SecA also binds directly to ribosomes [103]. The exact role of SecA in membrane protein insertion and, in particular, the dynamics of the interaction of SecA with the ribosome and translocon requires further investigation.

2.4. YidC and SecDF(yajC)

SecDFYajC is a membrane protein complex that associates in a transient fashion with the Sec translocon [55] and stimulates preprotein translocation. Although SecD and SecF are not essential, their inactivation in *E. coli* results in a severe pleiotropic protein secretion defect as well as a severe growth inhibition [171]. Interestingly, some Bacteria such as *Lactococcus lactis* lack a SecDF complex and the introduction of a heterologous SecDF protein results in improved preprotein translocation. Mutations in SecDF may result in a cold-sensitive growth phenotype. SecDF has initially been implicated in the cycling of SecA during preprotein translocation [56], whereas its role in membrane protein insertion is less clear [32] and possibly even indirect as depletion of SecDF affects the SecG levels *in vivo* [105]. SecDF have also been implicated in PMF-dependent translocation [56], but even in the absence of SecDF, translocation remains PMF-dependent [160]. Recently, a preliminary X-ray diffraction study has been reposed on the SecDF protein from *T. thermophilus* [218]. Future structural studies will likely shed more light on the role of this mysterious subunit of the translocase. Especially, the role of SecDF in membrane protein biogenesis warrants further investigation.

In the last decade, another essential protein has been identified that interacts with the translocon but that appears to fulfill a specific role in membrane protein insertion (Fig. 1). This protein, YidC, is a member of the Oxa family of membrane proteins consisting of YidC in Bacteria [190], Oxa1p in mitochondria, and Alb3 in the thylakoid membranes of chloroplasts [118]. YidC plays an essential role in the insertion of a subset of membrane proteins via the translocon [53,113,226], while it also has been shown to crosslink to membrane proteins exiting the translocon that are not dependent on YidC for their insertion [15,90,201,223]. Interestingly, YidC has also been implicated in the insertion of SecE [256]. Cross-linking studies suggest that YidC transiently interacts with the translocon during the insertion of IMPs and this interaction may involve SecD and SecF through a heterotetrameric YidC–SecDFYajC complex [158]. Importantly, YidC is also able to act as an insertase on its own [176,177,190,230]. To date, the YidC substrates identified are rather limited, although most appear

to be membrane subunits of large respiratory complexes, such as CyoA of the bo_3 cytochrome oxidase, subunits a and c of the F_1F_0-ATPase, and NuoK of the NADH dehydrogenase I. Phage M13 and Pf3 coat proteins also require YidC for their insertion [31,190,191]. Several membrane proteins depend on YidC for folding rather than membrane insertion, while the observation that the entire F_1F_0-ATPase complex can be copurified with YidC homologs in *B. subtilis* [189] suggests that YidC fulfils additional roles in folding and complex assembly. For a recent review describing the role of YidC in inserting and assembling subunits of large respiratory complexes, see Reference 114, and for a review on the role of YidC homologues in gram-positive Bacteria as well as Archaea, see Reference 257. Also, a recent review discusses the biogenesis of respiratory chain complexes with a focus on the role of YidC in these processes [178].

The structure of YidC is unknown. Remarkably, the large periplasmic domain does not contribute significantly to the function of YidC [98]. This domain has a β-fold sandwich structure with a possible substrate binding cleft [161,183]. Interestingly, while the five terminal TMs are essential for YidC activity, systematic mutagenesis of residues in TM2, TM3, and TM6 as well as swapping TM4 and TM5 with unrelated TMs proved to have little effect on YidC activity [98]. Thus, it seems that YidC is rather invariant for mutagenesis, indicating a role as an insertion platform rather than an active insertase. On the other hand, YidC seems to undergo conformational changes upon substrate binding [251]. A subset of Bacterial YidC homologs contains a C-terminal extension that shows some homology to the C-terminal region of Oxa1p that has been implicated in ribosome binding [69]. Various Bacteria contain more than one YidC homolog that may differ in the presence of this C-terminal extension. Possibly, there is a functional differentiation of YidC-like proteins that act in cotranslational membrane protein insertion requiring an interaction with the ribosome, and the insertases that may act posttranslationally. A recent cryo-EM study proposed that YidC, in association with a translating ribosome, forms a dimeric pore [112]. The interface of interaction may involve TM2 and TM3. On blue-native gels, YidC migrates as a monomer or dimer, while Oxa1 is found as a tetramer [145,231]. It would be interesting to investigate the oligomeric state of YidC as it interacts with the SecYEG translocon, especially in context of its larger structure that it seems to form with SecDF(yajC). In this respect, a structural analysis of YidC will be essential for our understanding of this seemingly promiscuous yet essential protein.

3. Oligomeric state of the translocon

The oligomeric state of the translocon and its ancillary proteins during protein translocation and membrane protein insertion is a controversial topic. As many of these components are interacting, we will give a brief overview of the discussion on the oligomeric states of SecA and the translocon.

3.1. Monomeric versus dimeric SecA

SecA can be found in the cell either in a soluble or membrane-bound form. Cytosolic SecA exists in a dynamic equilibrium between a monomeric and dimeric state [252] with a dissociation constant of around 1 nM (Ilja Kusters, unpublished results). Since the cellular concentration of SecA is close to 8 μM [2], the dimer will be the predominant species in the cell. High salt and detergent promote dimer dissociation, whereas the equilibrium is also affected by translocation ligands such as synthetic signal peptides and nucleotides. Although these studies indicate a fragile monomer–dimer equilibrium for SecA in solution, the oligomeric state of SecA bound to the SecYEG complex has been a major topic of controversy. In detergent solution using methods such as native gel-electrophoresis and gel-filtration studies, both the monomeric and dimeric SecA have been shown to bind SecYEG [18,54,220]. By chemical cross-linking and surface plasmon resonance (SPR) analysis [39,99], it appears that SecA, while bound to SecYEG, remains dimeric throughout the translocation reaction. At a very low concentration, SecA can also be cross-linked as a monomer to SecYEG [162], but under those conditions, the system is essentially inactive. By means of mutagenesis and truncation, the monomer–dimer equilibrium can be shifted to yield mostly the monomeric species. However, such mutants are severely compromised in preprotein translocation [99,162,181]. Another study investigated the functional oligomeric state of heterodimeric SecA consisting of combinations of inactive and active SecA monomers [49]. Here, heterodimers were found to be completely inactive, lending strong support that SecA functions as a dimer.

Crystallization of different SecA proteins resulted in crystals in which in most cases SecA was present as a dimer in an antiparallel orientation as shown in Fig. 3. Only one SecA dimer, SecA from *T. thermophilus*, has been crystallized in a parallel dimer orientation [238]. However, the recent crystal structure of monomeric SecA bound to SecYEG [260] demonstrates that a stable complex can be formed between monomeric SecA and SecYEG. Since the crystallization was performed in detergent solution at high salt concentration, both conditions that cause a dissociation of the SecA dimer, the obtained crystal structure does not exclude the possibility that the SecA dimer is the active state during protein translocation as suggested by functional studies. While many of the crystallized SecA dimers have also been observed as dimers using other biochemical approaches [45,162,259], it is still unclear if the SecA dimer is a physiologically relevant state. Suggested roles for the dimeric form of SecA are the following: 1) a cytosolic chaperone that guides preproteins to the translocon, 2) the inactive state of the motor protein, and 3) the physiological relevant conformation for interacting with the translocon. For a recent overview on the state of the art in the SecA monomer versus dimer hypotheses, see Reference 192.

3.2. Oligomeric state of SecYEG

Also with respect to the oligomeric state of SecYEG, a considerable controversy exists in the field. The oligomeric state of detergent-solubilized SecYEG in the absence of any ligand revealed, similar to SecA, that the translocon can be found in a dynamic equilibrium between monomers, dimers, and even higher-order oligomers. These different oligomeric states have been observed using a variety of techniques such as density centrifugation [24], analytical ultracentrifugation [34], native gel-electrophoresis [18], gel-filtration [220], as well as negative stain electron microscopy [78,129,132]. Also, higher-order oligomers were found with SecYEG reconstituted into proteoliposomes [196]. Functional studies with a tandem SecY–SecY fusion construct that yielded covalently linked SecYEG dimers suggests a functional asymmetry in the translocase with one of the SecYEG channels acting as a binding frame for SecA and the other channel acting as a translocation pore [164]. Remarkably, this dimeric SecYEG orientation is not supported by a structural analysis of the monomeric SecA–SecYEG complex that was suggested to suffice for protein translocation [260]. Another study suggests that the phospholipid cardiolipin fulfils a crucial role in driving the dimerization of SecYEG. It was suggested that monomeric SecA is bound to both protomers of the SecYEG dimer wherein one of the protomers only functions as a supporting platform [72]. It is difficult to assess the functional significance of these observations as cardiolipin is not required for protein translocation and viability.

Interestingly, in other studies, SecA has been found to bind only to dimeric SecYEG [54,220]. It has been reported that the binding of SecA to SecYEG shifts the equilibrium of monomeric SecYEG towards the oligomeric state [129,196]. Also, covalently linked SecYEG dimers [54,164] as well as disulfide cross-linked translocons [232] were all found to be active in protein translocation. Electron microscopy (EM) has also been utilized to determine the oligomeric state of SecYEG and

the Sec61p complex. Earlier low-resolution EM images showed ring-like structures of translocons associated with ribosomes or SecA, and these were suggested to be oligomeric states of the translocon, such as dimers, trimers, or tetramers [17,78,129,132,136]. Interestingly, a more recent higher-resolution analysis indicates the presence of a monomeric SecYEG to be bound to ribosomes charged with a nascent membrane protein [16].

Although current evidence suggests that a monomeric SecYEG suffices for protein translocation, an interesting question with the alternatively proposed SecYEG dimer is how it is oriented. Currently, two models for the orientation of the dimeric translocon exists (Fig. 4), the 'front-to-front' and 'back-to-back' orientation. In the 'front-to-front' orientation, the lateral gates of the two SecYEG complexes are facing each other. In this orientation, there is the possibility that the two channels fuse to form a consolidated channel. On the other hand, in the 'back-to-back' orientation, the two SecYEG complexes are in contact via the transmembrane segment of SecE that embraces the SecY subunit as a clamp. A cryo-EM study on a ribosome-nascent chain–SecYEG proposed that the two SecYEG complexes in the dimer are in a 'front-to-front' manner [136] (Fig. 4A). It was suggested that the ribosome senses at an early stage the presence of a nascent preprotein or IMP in the tunnel and that this results in conformational changes within the ribosome that are transduced to the translocon [134]. This may result in an assembly of a dimer and even induce the opening of the channel. It was suggested that one channel of the dimer functions as an exit site for the inserting transmembrane domain, whereas the other channel acts as the translocation pore [135]. Interestingly, in a biochemical study, various single-cysteine residues introduced in the "front" of the translocon resulted in a very efficient cross-linking of SecYEG into a dimeric complex [232]. However, no further biochemical or other cryo-EM experiments have been put forward to test the front-to-front model. Again, higher-resolution structures from cryo-EM are required so that TMs can be assigned in the structure unambiguously, but a recent cryo-EM study of the ribosome-bound Sec61p complex suggests the presence of a monomeric translocon excluding the dimer hypothesis [16].

The 'back-to-back' model (Fig. 4B) for the translocon has been suggested on the basis of a very effective cross-linking of unique cysteine positions between neighboring SecE proteins [106,239]. With this SecE–SecE cross-link [106] translocation is strongly inhibited, suggesting either that this orientation of the dimer is not compatible with protein translocation or that some form of flexibility is required that is impaired by the introduced crosslink. Interestingly, an 8-Å resolution structure of the *E. coli* SecYEG, as determined by cryo-EM from two-dimensional crystals [26,34], showed two SecYEG translocons in a twofold symmetry axis at the third TM of the SecE protein in close contact. An atomic homology model of the 'back-to-back' crystal structure [26,34] was built by incorporating the atomic structure of the *M. jannaschii* SecYEG [24,228]. The recent X-ray crystal structure of SecYEG from the gram-negative *T. maritima* bound to SecA (Fig. 2C) shows a tight interaction between SecA and a single SecYEG complex. An extensive cross-linking analysis of the sites of interaction between SecA and SecY included many positions on SecA that remained unaccounted for in the monomeric SecA–SecYEG structure [99,128]. Although docking attempts with a second SecYEG complex could not provide evidence for such sites of interaction [260], it was argued that the most plausible orientation in the proposed SecYEG dimer is the 'back-to-back' orientation. However, in a recent docking study, it was argued that major domain movements in the SecA protein will allow for 'back-to-back' orientation of the SecYEG dimer that takes the observed cross-links into account [72].

4. Ribosome targeting by SRP

The targeting of ribosome-nascent chain complexes (RNCs) to the translocon is universally conserved over all domains of life [173]. The signal recognition protein (SRP) binds the RNC once a hydrophobic signal sequence or transmembrane segment has emerged from an actively translating ribosome. At the membrane, the complex is recognized by the SRP receptor FtsY (SR) whereupon a heterodimeric SRP–SR complex is formed. Upon binding and hydrolysis of GTP, SRP is released and the RNC is transferred to the translocon. Under those circumstances, continued translation is coupled to the insertion of membrane proteins and in Eukaryotes also in the translocation of proteins (Fig. 1).

In *E. coli*, SRP is a ribonucleoprotein with a conserved structure. The protein component is known as Ffh (fifty-four homologue), which is associated with the 4.5S RNA [175,184]. Ffh is homologous to SRP54 in eukaryotes while 4.5S RNA is partly homologous to eukaryotic 7SL RNA. The eukaryotic SRP has a more complicated domain organization as compared to the bacterial SRP (see, for review, Reference 76). In Bacteria, the SRP system is mainly involved in the targeting of IMPs to the translocon, while in the endoplasmic reticulum (ER) of eukaryotes, the SRP targets both IMP and preproteins [38]. SRP is a multidomain protein with an intrinsically unfolded acidic (A–) domain, a conserved GTPase (G–) domain and a signal sequence binding M-domain [76]. The bacterial SRP lacks the key subunits SRP9 and SRP14 that are involved in translational arrest or pausing in eukaryotes [247] and thereby prevent premature synthesis of the IMP until the RNC–SRP complex has reached the ER. In *E. coli*, both SRP and FtsY are essential for growth suggesting their important role in inserting membrane

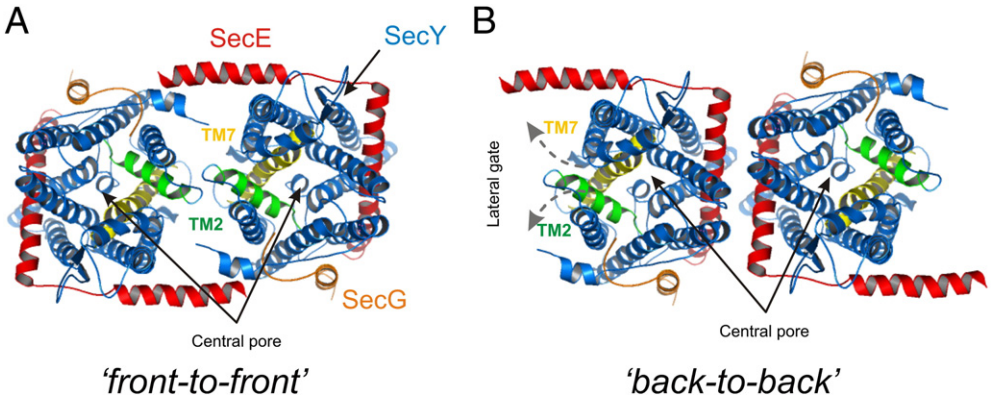

Fig. 4. Schematic representations of the proposed oligomeric states of the Sec translocon. (A) The front-to-front model for the Sec translocase with the lateral gates formed by TM2 (*green*) and TM7 (*yellow*) opposing each other. SecE (*red*) braces the two translocons on either side. It has been proposed that in this state the two SecY channels (*blue*) can from a single consoled pore [135]. (B) Some biochemical data have suggested a back-to-back arrangement for two SecYEG translocons. In this instance, the two translocons are aligned with the large TM of SecE. Both translocons can act independently of each other with membrane protein insertion and protein translocation. Indicated are the locations of the central pores as well as the TM2/TM7 lateral gate.

proteins [126]. FtsY associates with the SRP–RNC whereupon the GTPase activity is activated, and the RNC is transferred to the translocon [41,225]. FtsY binds to lipids but also directly to the translocon [4,5]. SRP–FtsY complex formation at the translocon prevents premature release of the RNC and may ensure the efficient transfer of the RNC to the translocon as an elaborate rearrangement of the SRP–FtsY complex is required before the RNC can unload the substrate to initiate translocation [12,204]. A recent study suggests that the SRP targeting pathway is nondiscriminatory and also targets nascent cytosolic proteins to the translocon [23]. According to this model, the nascent chains are selected at the translocon, and the signal-free nascent chains are rejected by the translocon for translocation initiation followed by release of the RNC into the cytoplasm.

The ribosome may not only be involved in polypeptide chain elongation and membrane protein insertion, it may also modulate and/or activate the translocon. The ribosomal exit tunnel is 100 Å long and about 10–20 Å wide [11,17,131,153]. Nascent proteins seem to fold already in the ribosomal tunnel [44,79,116,117,219]. Furthermore, the formation of hydrophobic folded TM domains in the ribosome exit tunnel may promote ribosome-induced changes in the translocon itself [124]. Recently, it has been shown that the presence of a TM in the exit tunnel leads to the recruitment of the small tail-anchored membrane protein RAMP4 to the Sec61 translocon supporting the notion that there is communication between the ribosome and the translocon, preparing it for the arrival of a nascent TM segment [174].

Other proteins have been shown to interact with the ribosomal tunnel and thereby causing translational pausing [212]. SecM is a preprotein that is encoded by a gene that localizes upstream of the *secA* gene. A specific polypeptide sequence in SecM causes the translational arrest in the absence of an available translocon [142,144]. This specific motif binds to the ribosomal tunnel close to the polypeptide entry site, and the translational arrest results in unfolding of an mRNA hairpin that result in enhanced expression of the downstream encoded SecA protein. Thus SecM functions as a sensor to detect translocation defects in the cell (for review, see Reference 143). Recently, a similar kind of regulatory cascade was proposed for YidC-dependent membrane protein insertion in *B. subtilis* that contains two YidC homologs, SpoIIIJ and YqjG. A gene located upstream of *yqjG*, termed *mifM*, seems to act as sensor of the SpoIIJ activity and that regulates *yqjG* expression. Decreased levels of SpoIIIJ results in an arrest in mifM translation causing the unfolding of an mRNA hairpin that blocks initiation of yqjG expression [33].

It has been suggested that in *E. coli* the ribosome interacts with YidC [92]. Thus, the SRP pathway would direct IMPs not only to the translocon but also to the YidC-only pathway. How in such dual mechanism specificity is maintained by the SRP pathway is currently unclear.

5. Mechanism of protein translocation

5.1. ATP and PMF-driven translocation

In *E. coli*, the synthesis and translocation of the preproteins are uncoupled events [180], and preprotein translocation is mediated by the motor protein SecA. After a considerable portion of the preprotein has been synthesized at the ribosome, it can be bound by SecB that prevents it from stable folding and aggregation. SecB then targets the preprotein to the translocon where upon hydrolysis of ATP the preprotein is handed over to SecA [65]. Some preproteins are targeted to the translocon by SRP, but they still require SecA for translocation [198,209]. Both SecA, utilizing ATP, and the PMF are the driving forces of translocation across the membrane [48]. Not only is ATP essential for the initiation of translocation, it is also utilized throughout the translocation reaction as the energy source. After binding of ATP to SecA as the initial step, a hairpin loop of the signal sequence is inserted into the lateral gate of the translocon. While this step is solely dependent on ATP, it can be stimulated by the PMF. The PMF most likely plays a role in determining the correct orientation of the signal sequence within the channel [159,227], but alternatively, it may affect the conformation of the SecY protein and facilitate opening of the lateral gate region. The latter suggestion is inspired by the observation that so-called PrlA mutations in SecY that cause a destabilization of the translocation pore, also cause translocation to be less dependent on the PMF [157]. Following translocation initiation, the hydrolysis of ATP leads to a dissociation of SecA from the preprotein and a weakening of the SecA–SecYEG binding affinity. SecA is then likely released from the translocon [197] but may rebind to the partially translocated preprotein thereby causing a translocation of approximately 5 kDa of the preprotein through the channel [197,234]. Next binding of ATP to SecA causes a further translocation, likely of another 5 kDa, whereupon the ATP is hydrolyzed and a new catalytic cycle of SecA can be initiated. It is believed that multiple rounds of ATP binding and hydrolysis lead to the stepwise translocation of the preprotein. While the exact step size has not been defined, it has been demonstrated that duration of translocation is directly proportional to the length of the preprotein [215]. Various other factors such as the hydrophobicity of segments in the translocated protein may influence the kinetics of translocation [193]. Interestingly, once SecA has dissociated from the translocon, the PMF can continue to drive the translocation of the preprotein across the translocon [51,197,211,234]. While the PMF has been shown to participate and aid in translocation, it has also been shown to be involved in stimulating the release of ADP from SecA [207] as well as inducing conformational changes in SecA during protein translocation [148]. The PMF has also been implicated in channel opening [157,211]. Intermediate stages of translocation are reversible in the absence of ATP, SecA, or the PMF, and the preprotein shows hysteresis movements in the channel likely driven by folding at the *cis* and/or *trans* side of the membrane [51,197]. While tightly folded proteins can block translocation [6], it has been shown that SecA can display a sort of chaperone function by unfolding tightly folded proteins such as human I27 when presented at the C-terminal end of a preprotein [156].

5.2. Translocation models

Various models have been proposed for the SecA motor function, in particular the power–stroke and Brownian ratchet model [214]. With the power–stroke model, the binding and hydrolysis of ATP result in the conformational change of the motor protein in such a manner that it imposes a mechanical insertion force on the translocating preprotein thereby pushing it through the protein conducting channel. It would seem that the two helix-finger shown to contact preproteins during translocation would fit with a power–stroke model [8,62], but currently, there is no evidence that this region indeed moves in response to the nucleotide-bound state of SecA [260]. Moreover, it is difficult to envision how a movement of the two-helix finger can affect the translocation of about 25 amino acids per stroke. On the other hand, stepwise translocation might not depend on a large conformational change of the two-helix finger domain. A mechanical force on the translocating protein may also be affected by binding and release of SecA, which might involve a positioned interaction between the two-helix finger and the translocating preprotein. To effectively interact with an unfolded nascent chain, the two-helix finger needs to contact the amino acid side chains. Remarkably, the translocase can translocate long stretches of polyglycine of even up to 25 amino acids [155], and currently, it is not clear how the two-helix finger would be able to interact with such polypeptide sequences.

The Brownian ratchet model suggests that SecA utilizes and directs the random Brownian motion of an unfolded translocating peptide

[210]. Here, the retrograde movement of the preprotein in the channel is trapped by SecA in an energy-dependent manner. This then leads to a directed translocation of the preprotein in a unidirectional fashion. Stepwise translocation involving distinct translocation intermediates [197,216,234] seems most consistent with a power–stroke mechanism, but an exact definition requires more accurate measurements of the translocation progression during the catalytic cycle.

Another model involves the dimeric structure of SecA [49]. In the antiparallel dimeric structure of SecA (Fig. 3), there is a central opening between the two SecA protomers. The piston model proposes that this central pore aligns with the translocon and with ATP-binding traps the preprotein in a SecA-bound state. Utilizing a power–stroke mechanism, the preprotein would be pushed into the translocation channel [205]. This proposed mechanism was further adjusted and refined into the molecular peristalsis model [135] as discussed recently [50]. Briefly, this model requires the docking of a dimeric SecA onto a dimeric front-to-front oriented translocon (Fig. 4). Herein, translocation might occur through a consolidated channel formed by the two front-to-front translocons that would align with the central opening in the SecA antiparallel dimer. Conformational changes in SecA would not only affect preprotein trapping and translocation but also cause the opening and closure of the translocon for translocation. The model proposes that there is an alternating opening and closure of the central SecA dimer channel that is synchronized with closure and opening of the protein conducting channel, thus leading to alternate trapping and release. Actual translocation in this model would be driven by Brownian motion, while the nucleotide binding and hydrolysis at SecA would be linked to channel opening and closure. A recent cross-linking study, however, suggests that the formation of a consolidated channel does not occur (FP, unpublished results).

5.3. Proofreading by the translocon

An interesting set of mutants of the translocon are the so-called protein localization (*prl*) mutants. These mutations in the *sec* genes suppress the translocation defect of preproteins that have a defective targeting signal [19,20,43]. In SecY, most of these mutations are localized at the inside of the pore of the translocon or are located on the plug domain (TM2a). Various explanations have been provided for the *prl* mutations in the SecY and SecE proteins. It has been suggested that these cause the loosening of the association of subunits of the SecYEG translocon [58]. While the *prl* mutants compensate for the defective signal sequence, it has also been suggested that these mutants either stabilize the open state or destabilize the closed state of the translocon. In *prl* mutants, the interaction between SecYEG and SecA is stabilized [235]. In particular, there is a tighter binding of SecA in its ADP-bound state, resulting in suppression of the release of SecA from the translocation site. Consequently, translocation initiation is much more effective in the *prl* mutants as compared to the wild-type, and therefore translocation is more efficient in these mutants. In this respect, *prl* mutants are also seen as mutants with a defective proofreading allowing for more efficient translocation at the expense of specificity (defective signal sequences). This proofreading function seems related to the establishment of an actively primed state of SecA [146] that normally is dependent on the presence of a translocation competent preprotein with a functional signal sequence. In the *prl* mutants, this primed state is no longer dependent on the presence of a preprotein and possibly corresponds to the 'pre-open' state of the translocon. Along these lines, these mutants are also less dependent on PMF for translocation [157] as discussed in a previous section. Overall, it appears that *prl* mutants mimic a SecA–SecYEG interaction where the SecA is in a constitutively active state [75,235]. In this respect, the signal sequence may indirectly activate SecA for ATP hydrolysis by inserting into the translocon, thereby promoting the open state of the channel which in turn may lead to an activation of the SecA ATPase.

6. Mechanism of membrane protein integration

6.1. Signals for membrane protein insertion

For membrane proteins, integral signals in their TMs are read and decoded by the translocon or alternatively by YidC. Typically, TMs of membrane proteins in the inner membrane consist of hydrophobic α-helices that contain around 20 to 27 residues. These helices are inserted perpendicular to the membrane (Fig. 2A) and often are found tilted in the membrane. While most proteins destined for secretion contain a cleavable signal sequence, very few integral membrane proteins contain a cleavable signal peptide. Those that do contain a cleavable signal peptide contain a second hydrophobic sequence also known as a stop–transfer sequence [244,246]. Deletion of this stop–transfer sequence can lead to a conversion of the membrane protein into a secreted protein [1,36]. The reverse is also possible where a secreted protein is converted into a membrane protein by the addition of a stop–transfer sequence [37]. For a review of typical topologies of membrane proteins and a summary of their requirements for insertion, see References 253 and 63. For multispanning membrane proteins, the TM domains need to fold and pack to form a functional protein. Various factors have been proposed to affect the folding of large multispanning proteins; in particular, YidC has been suggested as folding chaperone. For instance, YidC has been shown to be essential for the correct folding of the 12 TM spanning LacY [139]. As YidC has been shown to contact TMs that exit the translocon [91], it has been proposed that it can act as a chaperone for membrane protein folding. However, with LacY, SecY mutations have been identified that affect the correct folding of the protein [206], suggesting that SecY together with YidC synergistically affect the folding of membrane proteins. Once inserted, membrane proteins may assemble into multimeric protein complexes. This process requires that subunits are present in the correct stoichiometry and assembly likely occurs in a specific order. Moreover, for complexes like the F_1F_0 ATPase, various subunits may utilize different insertion routes. For instance, subunit c of the F_0-sector inserts into the membrane via YidC [230], while subunit a requires both YidC and SecYEG [113,255]. Furthermore, subunit c needs to assemble first into the c-ring before it associates with the ab_2 subcomplex. It could well be that the assembly process also depends on YidC, a concept which is further re-enforced by observations that the entire F_1F_0 ATPase copurifies with the *B. subtilis* YidC homologs [189]. The control and regulation of the assembly of these large energy-transducing complexes remain a subject of further study.

The insertion and final topology of membrane proteins is also influenced by the PMF, in particular the transmembrane potential, $\Delta\psi$. In the early 1980s, it was shown that the membrane insertion of the procoat protein into the *E. coli* cytoplasmic membrane strictly depends on the $\Delta\psi$ [35]. While binding to the membrane occurs in the absence of a $\Delta\psi$ [70], translocation of the periplasmic loop that connects the two TM-like domains of procoat is dependent on this force. Mutagenesis of the negatively charged amino acid residues in this loop into neutral or positively amino acids results in $\Delta\psi$-independent membrane insertion [30,119,200]. Since YidC is responsible for the insertion of M13 procoat [31,118,190], it remains a major question as to whether $\Delta\psi$ acts by electrophoresis or whether it functions via YidC. In this respect, Pf3 variants that exhibit a reduced $\Delta\psi$ dependence still require YidC for membrane insertion. Moreover, other membrane proteins also depend on the $\Delta\psi$ for insertion by as yet unknown mechanisms.

A major topology-determining factor for membrane protein insertion is the "positive inside rule" of von Heijne et al. This rule states that the positively charged amino acid residues flanking the

transmembrane domains are topology determining and remain in the cytosol during biogenesis [243,245]. However, introduction of negatively charged amino acids at the cytosolic face of the membrane of a membrane protein can negatively influence insertion and topology [122]. Here, the negatively charged amino acid residues seem to respond to the presence of $\Delta\psi$, whereas $\Delta\psi$ supports the translocation of negatively charged amino acid residues and inhibits the translocation of positively charged amino acid residues [107]. Recently, Seppälä et al. [203] investigated the insertion of multispanning membrane proteins with regard to the role that positive charges play in the topology of this class of proteins. Surprisingly, they found that the topology of EmrE, a topological sensitive protein, comprising of four or five transmembrane helices could be controlled by the placement of a single positively charged amino acid residue at various locations within the protein. While EmrE is sensitive to its orientation, this study highlights the effect that a single positive charge can have on the overall topology of a protein, even when this charge is found on the very C-terminus of the protein. To understand the mechanism how this is accomplished, a detailed investigation of the insertion and release of TMs from multispanning membrane proteins via the translocon is required.

6.2. Thermodynamic mechanism of translocon-dependent partitioning of transmembrane domains

How does the translocon identify and select TMs for insertion? Membrane proteins are highly prone to aggregation when released into the cytosol by the ribosome. Therefore, the ribosome and the translocon work together with the SRP targeting pathway to insert membrane proteins into the lipid bilayer. When a TM that is exposed from the ribosome associates with the translocon, it needs to be recognized such that it can be released laterally in the membrane. Furthermore, the TM segments of multispanning membrane proteins need to assemble into a functionally folded protein. Newly synthesized membrane proteins rapidly equilibrate with the lipid bilayer after their insertion [123,249]. The thermodynamics of this process was recently studied utilizing model TMs [86]. Insertion is determined by the average hydrophobicity of TM segments quantitatively described by the Gibbs free energy of insertion (ΔG_{app}) [86]. This suggests that insertion involves partitioning between a polar and apolar environment, possibly involving the lateral diffusion of the inserting TM segment into the lipid bilayer [83]. Alternatively, the hydrophobicity of the TM segment controls the gating of the translocation channel [258]. To discriminate between above possibilities, it has to be determined whether TM domains first insert into the aqueous translocation pore before their lateral release into the lipid bilayer. Importantly, insertion is also kinetically controlled [221], and moderately hydrophobic polypeptide domains may insert into the membrane when translocation (or translation) is slow [57]. In another study, the effect of the position of a specific amino acid in the TM was investigated [86]. When the polar amino acid arginine is positioned closer to the centre of the TM segment, the greater the energy cost to insert the TM. This suggests that protein–lipid interactions are crucial for the translocon to recognize the inserting TM segment. Interestingly, a significant number (25%) of TMs in multispanning membrane proteins exhibit a predicted ΔG_{app} greater than 0 [87], suggesting that elements other than the translocon or mere water–lipid partitioning contribute to their insertion. Insertion of TM helices also depends on the presence and composition of the neighboring TM helices for proper insertion. Membrane insertion via YidC seems to follow similar principles [254].

A study on the insertion of aquaporin which consists of 4 TM segments, via the Sec61 translocon [188], has revealed that the TM segments leave the translocon in the same order as they are released from the ribosome. However, it was also observed that TM segments once released by the translocon can return at a later stage when other TM segments enter the lipid bilayer. This suggests a mechanism by which TM segments help each other in the lateral release into the lipid bilayer, possibly by promoting proper protein folding. Von Heijne et al. [82] studied the phenomenon of marginally hydrophobic TMs. They screened 16 TM domains with marginal hydrophobicity for their insertion as individual TMs or in the context of flanking TMs and loops. Most of the marginally hydrophobic TM domains appeared insufficient to stably insert on their own and required flanking hydrophobic TMs for insertion. A study on the insertion of short TMs [96] revealed that the efficiency of insertion of TMs via the translocon is determined by the length of the TM, its amino acid composition and the positional arrangement of amino acids within the TM domain. However, the variety of structural elements found in membrane proteins that might influence the insertion of TMs is far from understood as insertion seems to depend not only on the physicochemical properties of the TM domains but also on the activity of the translocon. In this respect, mutations have been described in SecY (Sec61α) that affect the final topology of the inserting membrane protein. This suggests an active role of the translocon in membrane protein insertion.

Finally, membrane insertion being tightly linked to polypeptide chain elongation at the ribosome must synchronize with the SecA motor function. When large polar domains emerge from the ribosome tunnel that need to be translocated, SecA needs to bind to the nascent chain that is exposed to the cytosol and dissociate the ribosome from its SecYEG bound state. Interestingly, under in vitro conditions where posttranslational membrane insertion was enforced, SecA was released from the polypeptide chain once the SecA encountered a hydrophobic transmembrane segment [88]. Future studies should address the exact mechanism of the interplay between the ribosome and SecA, and how their binding to SecYEG is coordinated.

7. The role of lipids in translocation and membrane protein insertion

Typically, the inner membrane of *E. coli* consists of roughly 75% PE (phosphatidylethanolamine), 20% PG (phosphatidylglycerol), and around 5% of cardiolipin [179]. Interestingly, PE has the propensity to form inverted nonbilayer structures when in isolation. Therefore, being the most abundant lipid in the membrane, it creates a certain level of curvature stress, a condition which has been proposed to play a significant role in protein function [40]. *In vivo*, a strain lacking PE is viable only in the presence of a high concentration of divalent cations such as Mg^{2+} or Ca^{2+}. In this strain, the lack of PE is compensated by increased levels of PG and cardiolipin and it has been argued that the presence of divalent cations enforces the type II hexagonal phase structure of cardiolipin consistent with a strong requirement for nonbilayer lipids. Indeed, membrane vesicles from the same strain show a severe defect in protein translocation in the absence of divalent cations, and this could be rescued by the reintroduction of PE into the vesicles [185]. A strain in which the phosphatidylglycerol synthetase gene was depleted is devoid of PG and cardiolipin shows a lethal phenotype but accumulates increased levels of the negatively charged phospholipids phosphatidylserine and phosphatidic acid. This phenomenon can be attributed to a loss of the anionic phospholipid PG as the inactivation of the cardiolipin synthetase genes has no effect on growth. Remarkably, a recent study suggested that cardiolipin stimulates the formation of the SecYEG dimer [72] despite the fact that cardiolipin is nonessential for protein translocation. Both *in vivo* and *in vitro* studies demonstrate that in the absence of PG, protein translocation is severely impaired [42]. By reintroducing either PG or any other anionic phospholipid, this deleterious effect could be rescued, indicating that the negative charge on the polar head group of the phospholipid is essential for protein transport [120]. Furthermore, anionic lipids have also been found to be essential for the activity of SecA by increasing the affinity

of binding to SecY and stimulating the hydrolysis of ATP [81,85,125]. Reconstitution studies with the purified SecYEG complex confirmed the requirement for nonbilayer lipids as well as the need for anionic phospholipids for protein translocation [229]. Interestingly, these studies suggest a bulk requirement for such lipids as the optimal protein translocation activity with the *E. coli* and *B. subtilis* SecYEG complexes is observed with a synthetic mixture of phospholipids that corresponds to the polar head group composition of the respective species. The role of lipids in the SRP targeting pathway has also been investigated. Lipids are important for the release of SRP molecule from the nascent chain [202,225]. Moreover, FtsY interacts specifically and peripherally with the head groups of PE (or PC) via its AN domain [133]. Recently, it has been shown that lipids stimulate the GTPase activity of FtsY [10]. Analysis of FtsY mutants indicated that the N-domain of FtsY contains an amphipathic lipid-binding domain that is essential for its function *in vivo* [168]. Leader peptidase requires anionic membrane lipids for its insertion [237] and for obtaining the correct topology [236]. This phenomenon has been attributed to an anionic lipid requirement for SecA activity needed to translocate the polar catalytic domain of leader peptidase across the membrane. Obviously, many catalytic and structural aspects of membrane proteins are affected by lipids in the bilayer. For a recent review on the role of lipids in determining membrane protein topogenesis, see References 21 and 47. For a review specifically on the interaction of lipids with membrane proteins, with references to the structure, see References 94 and 95. The proposed model of a lateral gate opening of the translocon and the possible mechanism of insertion of TM domains suggests that lipids might play a more direct role in the insertion process. This possible function, however, needs to be studied in further detail.

8. Concluding remarks

Here, we have given an overview of the recent insights in the process of protein translocation and membrane protein biogenesis in Bacteria. Although in recent years, significant insights have been obtained in the structural and functional roles of the various components of the translocase, major questions still remain unresolved as for instance the mechanism by which TM domains exit ribosomes and the translocon. Obviously, any proposed mechanism will need to prevent the uncontrolled leakage of ions through an aqueous pore, while a laterally opened channel should not compromise the water-filled integrity of the translocon. Despite the multitude of approaches employed, the debate concerning the oligomeric state of the translocon has still not been resolved. This is mostly due to fact that in many of the experimental conditions used for the analysis of the oligomeric state of the translocon, the functionality of the translocon is not guaranteed. Another concept emanating from this review is the amazing complexity observed for the insertion of membrane proteins into the lipid bilayer. In particular, the question whether the ribosome fulfils a role in early recognition and in controlling the opening and closure of the translocon remains to be investigated. YidC has so far resisted structural elucidation, and this has hampered our insights in the molecular basis of the YidC function, which ranges from membrane protein insertion, assembly and folding. While there is a high level of conservation between the essential components of the translocase in Eukaryotes, Bacteria and Archaea, there are significant differences between these systems that warrant independent investigations of catalytic mechanism of protein translocation and membrane protein insertion. For instance, Archaea lack a clear homolog of the SecA translocation ATPase suggesting that translocation in these organisms is either cotranslationally or involves some novel motor protein(s). Mechanistic studies on the translocase will benefit from investigations at the single molecule level to reveal intimate features of the translocation reaction and the coupling between ATP and translocation progress.

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