The Sec protein-translocation pathway

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The Sec machinery (or translocase) provides a major pathway of protein translocation from the cytosol across the cytoplasmic membrane in bacteria. The SecA ATPase interacts dynamically with the SecYEG integral membrane components to drive the transmembrane movement of newly synthesized preproteins. This pathway is also used for integration of some membrane proteins and the Sec translocase interacts with other cellular components to achieve its cellular roles. The detailed protein interactions involved in these processes are being actively studied and a structural understanding of the protein-conducting channel has started to emerge.

In all organisms, genetic messages are translated primarily by cytosolic ribosomes, yet the translation products end up in various cellular locations. In fact, no less than 10% of gene products cross a membrane before arriving at their destinations. Thus, protein translocation across a biological membrane can be viewed not only as an intriguing biochemical reaction but also as an integral part of the genome-directed biogenesis of the cell. At least three categories of translocation process occur in bacterial cells: (1) Sec-mediated translocation across the cytoplasmic membrane[1]; (2) Tat-mediated translocation across the cytoplasmic membrane[2]; and (3) more specialized mechanisms for delivery of specific proteins to the outermost surface of the cell[3]. Of these reactions, Sec-mediated translocation is most relevant to the central question of cell biology. In contrast to the Tat system, which transports a special set of already folded and cofactor-bound enzymes, the Sec pathway is generally responsible for the transport of newly synthesized proteins out of the cytosol before they acquire their final structures. One key feature of the translocation process is that it occurs without compromising the general permeability barrier of the membrane. The translocation event that occurs at the cytoplasmic membrane in prokaryotes is equivalent to a similar process at the endoplasmic reticulum (ER) membrane in eukaryotes. The prokaryotic and eukaryotic Sec systems share some similar elements, such as the components of the integral membrane 'channel'. This review is intended to discuss outstanding questions and recent advancements in the study of the Sec translocation pathway in bacteria.

The Sec pathway and the major questions that remain

The major players

In this section, Sec and related factors will be introduced briefly for the sake of clarity. SecB is a chaperone dedicated to protein export. SecA is an ATPase that drives protein movement into and across the membrane. SecY, SecE, SecG, SecD, SecF and YajC are integral membrane proteins. SecY, SecE and SecG form a hetero-trimeric complex, SecYEG, which constitutes a pathway ('channel') for polypeptide movement. These proteins span the membrane ten, three and two times, respectively. Reconstituted proteoliposomes containing SecYEG are active in translocating a preprotein in the presence of SecA and ATP. Thus, SecYEG and SecA, known as the translocase, are the primary components of the translocation machinery. SecD and SecF are required for efficient protein export *in vivo*. An intact SecYEGDFYajC complex has been isolated under appropriate solubilization conditions[4]. The leader (signal) peptidase is the enzyme responsible for signal peptide cleavage. YidC (a bacterial homolog of mitochondrial Oxa1) is a recently identified membrane protein that has been shown to participate in protein integration into the membrane[5]. In *Escherichia coli*, the signal recognition particle (SRP) is composed of a protein component, Ffh, and 4.5S RNA[6]. SRP targets certain proteins to the membrane, where FtsY is thought to be the SRP receptor.

Translocation and integration signals

Signal sequences in Sec precursor proteins are characterized by a hydrophobic core of ten or more residues flanked by a positively charged amino-terminal region and a hydrophilic carboxy-terminal region containing a consensus sequence for leader peptidase cleavage[6]. These sequences are required for the initiation of translocation but their cleavage is not essential for this process. A classical signal sequence translocates the carboxy-terminal segment adjacent to it into the periplasmic space, and this process usually depends on the Sec system.

A signal-sequence-like element can also become a transmembrane segment (TMS) of a membrane protein. A signal-anchor sequence is topologically similar to a non-cleavable signal sequence and assumes an N-in, C-out TM orientation. Membrane proteins of this orientation use the major SecA–SecYEG pathway for their membrane integration[7]. In addition, YidC has been shown to be involved in this process[8,9]. A hydrophobic region (stop-transfer sequence) that follows an export signal or signal anchor halts translocation, generating a TMS of the N-out, C-in orientation. Thus, the Sec translocation machinery not only mediates complete translocation across the membrane but can also facilitate protein integration into the membrane lipid phase[10,11]. It remains an interesting question whether YidC has any role in the stop-transfer mode of TMS establishment.

It should be noted that the orientation of a TMS is somehow governed by the 'positive-inside rule', such that the positively charged side of a hydrophobic

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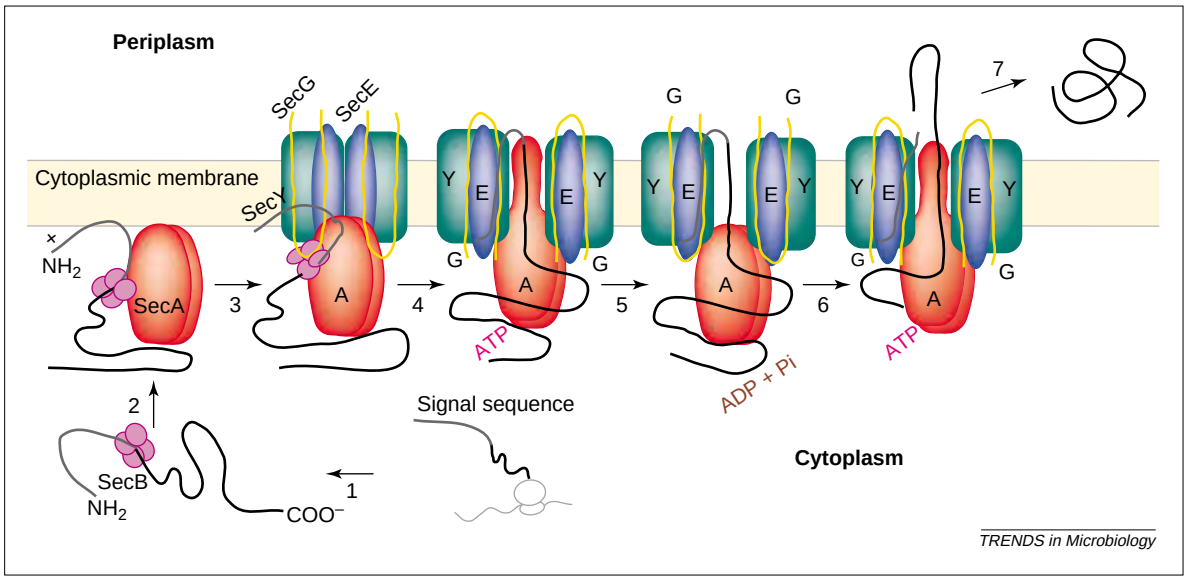

Fig. 1. Sec factors and translocation processes. The preprotein is represented by a black line, with the gray region showing the signal sequence. Steps 1–3, targeting. A signal sequence and its immediate carboxy-terminal region comprise an initiation domain that is recognized by the Sec machinery. SecB, the Sec-system-specific chaperone, channels the preprotein to the Sec translocation pathway and, additionally, actively targets the bound precursor to the translocase by its ability to bind SecA. The preprotein-bearing SecA then binds to the membrane, at a high-affinity SecA-binding site. SecY, SecE and SecG form a hetero-trimeric complex, SecYEG, which constitutes a pathway ('channel') for polypeptide movement. Steps 4 and 5, initiation. The initiation step requires ATP but not its hydrolysis. Step 6, continuation. Continued translocation requires cycles of ATP hydrolysis and/or proton-motive force across the membrane. Translocation is thought to occur in a step-wise fashion with a step of 20–30 amino acid residues. Step 7, completion. As yet, little is known about the completion process, which occurs on the periplasmic side, leading to the release and/or folding of the substrate protein into the periplasmic space.

segment is orientated towards the cytoplasm. Thus, a hydrophobic region with a positive charge on the carboxy-terminal side can assume the N-out, C-in orientation, translocating the amino-terminally adjacent region to the periplasmic side. This mode of translocation and/or integration does not depend on the Sec system; recent evidence indicates that it depends instead on YidC (Ref. 5).

Finally, Sec-independent membrane insertion can occur for a pair of hydrophobic segments with a short connecting loop that protrudes into the periplasm[6]. A classical example is M13 coat protein, membrane integration of which has been shown to depend on YidC (Ref. 5). The loop region of M13 coat protein is cleaved by leader peptidase upon integration.

Cytosolic events

The Sec translocation machinery, unlike the Tat machinery, is unable to handle tightly folded proteins. In the post-translational mode of translocation, newly synthesized presecretory proteins should be prevented from folding tightly in the cytosol. SecB is a Sec-system-specific chaperone, with a known 3-D structure. It primarily recognizes some sequence motifs present in the mature part of a precursor protein, and retards its folding[12]. In this way, SecB channels the precursor, which is in kinetic competition between folding and translocation, to the Sec translocation pathway. In addition, it actively targets the bound precursor to the translocase by its SecA-binding ability (Fig. 1, steps 1–3). It should be noted that some Sec substrates do not require SecB for efficient targeting and/or translocation[7].

Some signal sequences are recognized by the SRP. Recent evidence indicates that the SRP is involved in the translocation and/or insertion of membrane proteins in *E. coli*[13,14]. The SRP has a preference for signal sequences with higher hydrophobicity[15].

Is there a cotranslational pathway?

In an elegant earlier study, it was shown that translocation can be initiated before completion of translation, but is not coupled mechanistically to the elongation of the polypeptide chain[16]. As the SRP in mammalian cells mediates cotranslational targeting of the nascent chain–ribosome complex to the ER membrane, the bacterial SRP is also often thought to work cotranslationally. In several recent studies, nascent chain equivalents have been created by *in vitro* translation of truncated mRNAs and subjected to crosslinking with Ffh (the signal-sequence-binding protein subunit of SRP) and other components[17]. The resulting crosslinkage has been taken as experimental evidence to support the cotranslational mechanism. However, the actual timing of translocation and/or insertion has not been determined for the SRP-dependent pathways in bacteria. SecM, an unusual SRP-dependent periplasmic protein, undergoes self-translation arrest at a site close to its carboxyl terminus, and this arrest is released by translocation of the nascent SecM (Ref. 18) (Fig. 3). This system could be useful to know when the commitment to translocation is established during translation.

Cotranslational membrane insertion is an attractive mechanism for hydrophobic membrane proteins, which otherwise might aggregate before integration. Toxic aggregates are indeed formed in

SRP-depleted cells[19]. The existence of a cotranslational translocation pathway is also supported by recent biochemical evidence of high-affinity binding between ribosomes and the SecYEG complex[20]. However, the existence of a functional ribosome–translocase junction has not been demonstrated.

Initiation, continuation and completion of translocation and gating of the channel

The preprotein-bearing SecA binds to the membrane, at the high-affinity SecA-binding site made up of SecY and SecE. This is followed by initiation, continuation and completion of translocation, either all the way to the periplasmic side or into the lipid phase of the membrane. In the initiation event, the signal-peptide region inserts into the membrane in a loop-like configuration leaving the amino terminus on the cytosolic side. This leads to a state in which the signal-peptide-cleavage site has reached the periplasmic surface and approximately 20–30 mature residues are already within the membrane (Fig. 1, step 4). Positively charged residues that are within ~30 residues following the signal sequence act as an obstacle to initiation[21]. This should be contrasted with the continuation stage (Fig. 1, step 5), in which even a positively charged region (located away from the signal sequence) can be translocated without any problems. Thus, a signal sequence and its immediate carboxy-terminal region seem to comprise a special initiation domain that is recognized by the Sec machinery. The molecular nature of this recognition is a central question that must be addressed.

In vitro reactions show that the initiation step requires ATP but not its hydrolysis. Continued translocation then requires cycles of ATP hydrolysis and/or proton-motive force across the membrane. Under appropriate *in vitro* conditions, translocation takes place in a step-wise fashion with a step of 20–30 amino acid residues. These features of translocation are closely related to the SecA insertion–deinsertion cycle (discussed later)[4]. Translocating molecules can be crosslinked with SecA and SecY but not extensively with phospholipids, suggesting that they go through a SecY-containing proteinaceous pathway.

Not much is known about the completion process, which should occur on the periplasmic side, leading to the release and/or folding of the substrate protein to the periplasmic space (Fig. 1, step 7). It has been suggested that SecDF (Ref. 22) and SecY (Ref. 23) itself are involved in the late-step reactions. Crosslinking experiments have revealed that Skp, a peptidyl-prolyl *cis-trans* isomerase, interacts with a translocating molecule of an outer membrane protein precursor from the periplasmic side[24]. Still less is known about the mechanism by which a hydrophobic segment stops translocation and gets released into the lipid phase to become a TMS. Again, the SecYEG channel itself appears to have roles in the stop-transfer event[10,11]. It is a tempting proposal that YidC is a factor involved in the lateral movement from the translocase to the lipid phase[8].

When viewed from the channel side, the initiation and termination steps can be regarded as gate-opening and -closing events, respectively. The gating function of the channel, which should operate for both the vertical and lateral movements of polypeptides, is central to the problem of how the events of protein translocation can be compatible with the maintenance of the membrane permeability barrier.

How is translocation driven?

Regulation of SecA ATPase

SecA, a dimeric ATPase, interacts with ATP, SecB, the preprotein and the membrane (low-affinity binding to phospholipids and high-affinity binding to a SecYEG-containing membrane). Its protomer is composed of an amino-terminal ATPase domain and a carboxy-terminal domain with a lipid-binding site as well as a Zn^{2+}-coordinating SecB-binding site. The ATPase domain contains two proposed ATP-binding regions, NBD1 and NBD2, for high- and low-affinity binding, respectively, as well as a preprotein-binding site. The high-affinity interaction with SecYEG could also be ascribed to the ATPase domain[25,26]. Whereas SecA itself has very low ATPase activity, it is strikingly activated under the translocation conditions in the presence of a preprotein and the SecYEG membrane. NBD1 is solely responsible for the catalysis, and NBD2 has regulatory roles[27,28]. The intrinsic ATPase activity is downregulated by dual mechanisms, that is, the carboxy-terminal domain[29] and the NBD2 region[27] inhibit the activity independently. Interaction with a preprotein and the membrane will liberate SecA from this downregulation, a process in which some endothermic denaturation-like conformational change could be involved[30]. The rate-limiting step in overall ATP hydrolysis is the release of ADP for its exchange with ATP[28,30]. The successful crystallization of *Bacillus subtilis* SecA promises to start our structural understanding of this ATPase[31]. However, several different conformational states must be elucidated before a full understanding can be achieved.

Transmembrane mobility of SecA drives translocation

Although the ATP-induced conformational change of an isolated SecA molecule is rather subtle[32], SecA on the SecYEG membrane undergoes such remarkable conformational changes that it moves across the membrane[4]. The original SecA insertion model was based on the observation that a 30 kDa carboxy-terminal domain of SecA became protected from added protease by the membrane under the conditions of active preprotein translocation. This inserted form of SecA represents an equilibrium between ATP- and preprotein-dependent insertion and ATP hydrolysis-dependent deinsertion. ATP hydrolysis is also thought to induce the release of the preprotein. Repeated cycles of these movements were proposed to drive the step-wise movement of preprotein into and across the membrane (discussed earlier). This model became

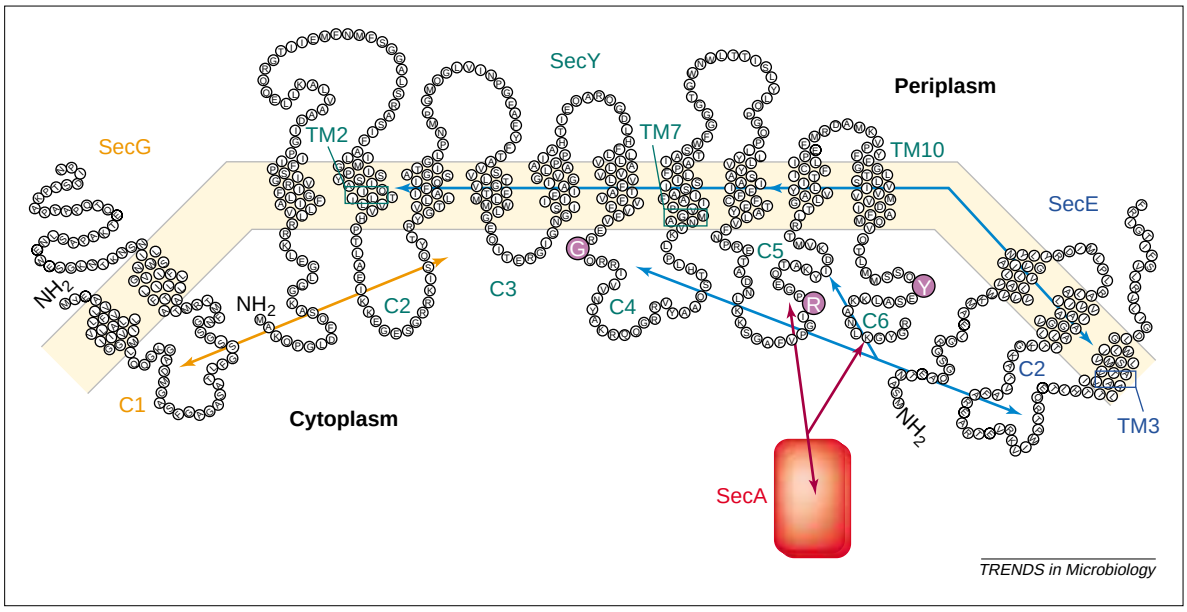

Fig. 2. An interaction map of the protein translocase. SecY, SecE and SecG are represented by the possible topological arrangement of their amino acid residues in the cytoplasmic membrane. C and TM indicate cytoplasmic and transmembrane domains, respectively, which are numbered in the N to C direction for each protein. Interactions between SecA–SecY, SecE–SecY and SecG–SecY are shown by red, blue and orange arrows, respectively, with the arrowheads pointing to approximate regions of interactions. Three residues in SecY are highlighted in purple. They are Gly240, Arg357 and Tyr429, thought to be important for SecE-binding, SecA activation and SecA insertion, respectively.

more plausible when it was later shown that the amino-terminal ATPase domain, which includes the preprotein-binding region, is also 'membrane inserted'. Several regions of SecA are indeed accessible for chemical modification from the periplasmic side[33].

Although a similar protease-resistant form of SecA is seen even in detergent solutions in the presence of a non-hydrolysable ATP analog[34], the ATP- and preprotein-dependent 'insertion' event does require membrane with functional SecY (Ref. 35). *In vivo* and *in vitro* studies using a *secY* mutation (Tyr429, Fig. 2) that impairs the SecA insertion reaction as well as *secA* mutations that suppress this *secY* defect supported the notion that SecA insertion occurs at the SecYEG channel and that this event is indeed important for driving protein translocation[35]. Consistent with the notion that SecA inserts into the SecYEG channel, the inserted SecA segments are sequestered away from phospholipids[36]. The translocation channel could have to accommodate a preprotein together with the bound SecA segments, raising a serious question about the dimensions of the channel and the structural nature of SecA insertion. In this sense, it is somewhat controversial whether 'insertion' is the right word to indicate the dynamism of SecA.

The SecDF protein might stabilize the inserted state of SecA, whereas the proton motive force could accelerate the deinsertion[37]. It should be noted, however, that the proton motive force has multiple roles in translocation, including a more direct role in the forward movement of a precursor.

Protein interactions in translocase

Recognition of a preprotein

As already discussed, the SecA–SecYEG system recognizes the signal and the following mature segment for initiation of translocation. Mutations *prlD* (in *secA*), *prlA* (in *secY*), *prlG* (in *secE*) and *prlH* (in *secG*) allow translocation of a preprotein having a mutationally defective signal sequence. Thus, these components are somehow involved in the decision to accept or reject a preprotein. Some *prlA* mutations even suppress total deletion of the signal sequence. Thus, some discriminating function is lost by these mutations. However, they do not alleviate the positive charge rejection in the initiation process[38]. Many of the *prl* alterations in SecYEG are in TMSs, consistent with an idea that they could make the channel more open (also discussed later). Studies on a yeast system have shown that the signal sequence interacts with the second and the seventh TMSs (TM2 and TM7) of Sec61p (the SecY homolog in yeast)[39]. These TM segments are the preferred sites of occurrence of *prlA* mutations in *E. coli* SecY.

SecYE–SecA cooperation

SecY and SecE constitute the high-affinity SecA-binding site on the membrane. Consistent with this notion, high-affinity SecA binding is abolished by Syd (a SecY-interacting protein), in a *secY* mutant in which SecY and SecE interact only weakly[40]. As already discussed, another *secY* mutation has defined a SecY function that is required for the productive occurrence of the SecA membrane-insertion reaction[35]. The domain containing this mutational alteration (C6) and the neighboring cytoplasmic domain (C5) are particularly important for the SecA-activating function of SecY. Several export-defective mutations here are dominant-negative *in vivo* and the mutant SecYs are unable to activate SecA. Among others, Arg357 (highlighted in Fig. 2) in C5 is a functionally crucial residue in SecY (Ref. 41) and it is conserved among the SecY/Sec61 proteins of different organisms. By contrast, a *prlA*

mutation, which can relax the translocation channel, enhances SecY–SecA interaction[42].

Inversion of SecG and its SecA-assisting role

SecG shows an unusual property of inverting its orientation in the membrane. This occurs under conditions that would stabilize the inserted state of SecA (Fig. 1, steps 4 and 6). SecG is not absolutely required for viability or protein export. However, it becomes important in a mutant with partially impaired SecA function[43] as well as in a mutant with altered phospholipid metabolism[44]. By contrast, a requirement for SecG is alleviated by a gain-of-function *secA* mutation[45]. SecG seems to assist in the SecA reaction cycles. This poses an intriguing thermodynamical question: how can the energy-costing flip-flop movements act like a lubricant for SecA?

Protein interactions in SecYEG

Primary protein interactions in the SecYEG complex are those between SecY and SecE and between SecY and SecG (Fig. 2). It should be noted that SecY can only exist stably as a complex with SecE *in vivo*, and any unassembled SecY subunits are rapidly eliminated by the FtsH protease[46]. The SecY C4 domain (in particular Gly240, highlighted in Fig. 2) is important for the SecY–SecE interaction[47]. Site-specific crosslinking experiments show that the C4 and C5 domains of SecY are indeed close to the C2 region of SecE, whereas the C2 and C3 domains are close to the cytoplasmic loop of SecG in the resting state (Y. Satoh *et al*., unpublished).

SecY–SecE interactions also occur at TM and periplasmic regions[48]. TM3 of SecE is in close proximity to TM2 and TM7 of SecY, as demonstrated by intermolecular disulfide-bond formation[49,50]. Some PrlI mutations in TMSs weaken the interaction between SecY and SecE. This is consistent with the notion that they 'relax' the SecYEG channel[51].

Molecular nature of the SecYEG channel complex

Structural information on the SecYEG complex, based on electron microscopy, has only recently begun to accumulate. A 2-D crystallographic approach has provided the first, low-resolution projection images of the SecYEG protomer, the dimensions of which seems too small to comprise a translocation channel[52]. By contrast, negatively stained images of *B. subtilis* SecYE and of *E. coli* SecYEG revealed ring-like structures with a central hole of 20–30 Å, and this entire complex was estimated to be composed of three or four SecYEG complexes[53,54]. SecYEG seems to be in equilibrium of monomer, dimer and tetramer[52,54]. It was reported that this equilibrium is affected by a *prlA* alteration in SecY (Ref. 52) as well as by interaction with SecA (Ref. 54). SecA could induce tetramerization of SecYEG.

These observations suggest that the active Sec channel is formed by a tetrameric superassembly of SecYEG. However, biochemical characterization of a translocation intermediate suggests that the functioning SecYEG is monomeric[55]. Thus, whether SecYEG undergoes higher-order assembly is a controversial issue. The superassembly model is attractive, as it provides useful guidance for our understanding of the gate-opening process. Whether this process is indeed triggered by SecA is a crucial question to be addressed further. Some evidence is available in support of the superassembly as the functional unit of SecYEG. First, a disulfide-bonded dimer of SecG is functional *in vivo* and *in vitro*[56]. Second, dominant-negative variants of SecE apparently sequester wild-type SecE (E. Matsuo *et al*., unpublished). Finally, disulfide crosslinking experiments suggest that two SecE molecules are adjacent to each other using one side of the TM3 helix as the interface[50]. It is crucial to determine the atomic structure of SecYEG while it is engaging in the translocation reaction.

Interplay of the Sec system with other factors

YidC

A fraction of YidC is found in association with the SecY complex[57]. YidC depletion results in a partial defect in integration of Sec-dependent membrane proteins as well as in stronger defects in the integration of some Sec-independent proteins[5]. The former class of proteins interact first with the Sec machinery and then with YidC. Thus, Sec and YidC could function sequentially[8,9].

SRP and ribosomes

SRP-dependent protein targeting presumably occurs to the membrane-associated FtsY, an SRP receptor homolog[17], however, the exact post-targeting pathways have not been established. The involvement of SecA is particularly controversial[17,58,59]. In this context, it should be noted that SecM exhibits clear dependence on both SRP and SecA, as well as on SecYEG (Ref. 18) (Fig. 3). Its signal sequence is unusually long for both the hydrophobic core and the amino-terminal hydrophilic region[60]. Although SecM is exported to the periplasm, its signal sequence could direct it to a pathway that is shared by SRP-dependent membrane proteins. Thus, SecM monitors both the protein export and protein integration activities of the cell to modulate the translation level of SecA (Refs 18,60) (Fig. 3).

What is driving the movement of proteins that depend on both SRP and SecA? The *B. subtilis* SRP interacts with SecA (Ref. 61). Thus, a ribosome-associated nascent chain might still be translocated by the SecA ATPase in bacteria. This is consistent with the fact that tight translation–translocation coupling has not been demonstrated in bacteria[16]. However, it still remains possible, given the presence of a high-affinity ribosome–SecYEG interaction[20], that some special membrane proteins could be directly targeted to SecYEG and inserted without aid from SecA. A *secY* mutation (in the C5 domain) was reported to be specifically defective in membrane protein integration without significant defect in protein export[62]. This class of mutations could affect the interaction of SecY

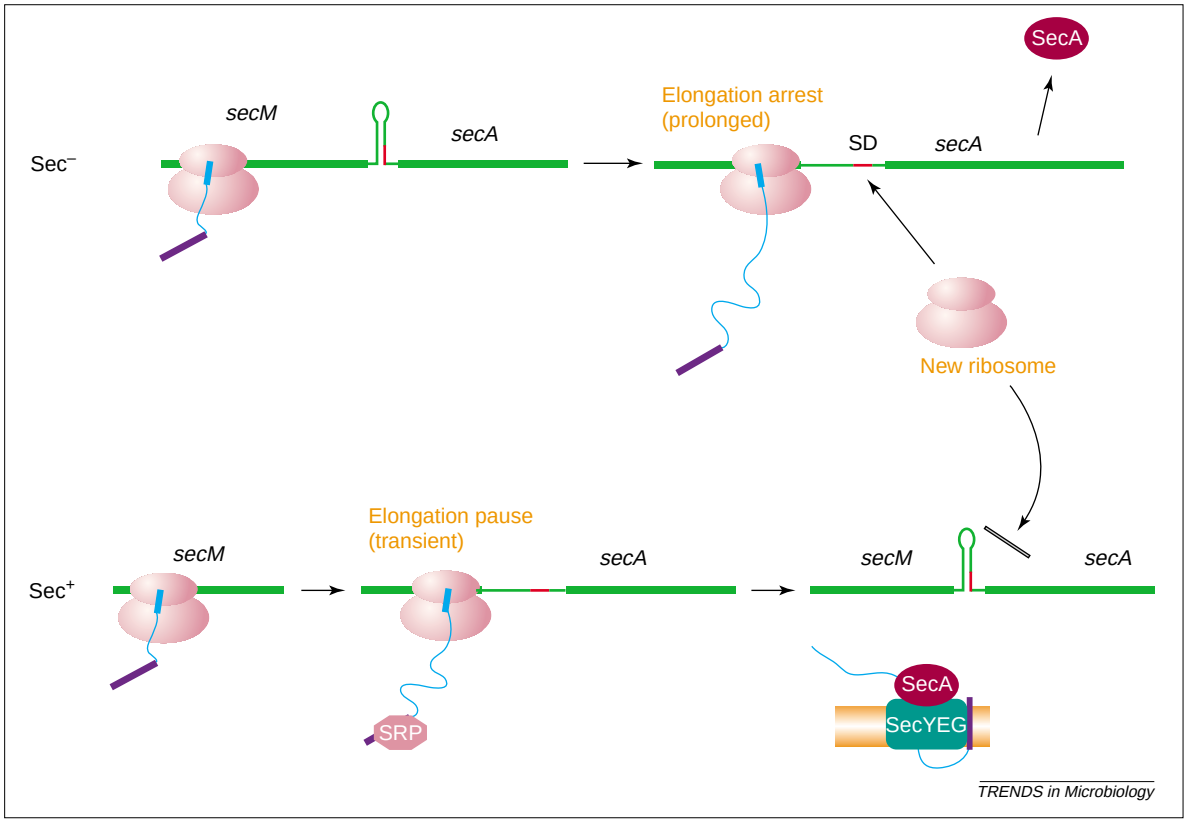

Fig. 3. SecM is subject to translation arrest, is translocated by a signal recognition particle (SRP)–SecA–SecYEG pathway, and regulates SecA translation. The translation of SecM is subject to transient or prolonged elongation arrest, under Sec⁺ or Sec⁻ conditions, respectively. Under the latter conditions, the stalled ribosome can disrupt the secondary structure of the *secM–secA* mRNA (shown by horizontal green line, with a stem-loop and the *secA* Shine–Dalgarno (SD) sequence shown in red), leading to enhanced SecA translation.

with the ribosome, FtsY, SRP, YidC or the integration signal on the substrate protein.

Degradation pathways and quality control of Sec factors

Abnormal ER proteins are exported back via the Sec61 translocon to the cytosol, where they are captured by the proteasomes for degradation[63]. In a somewhat similar pathway of protein degradation in *E. coli*, some abnormal cytoplasmic membrane proteins have been proposed to be translocated back to the cytoplasm to be captured by FtsH (Ref. 46). This system is interesting in two contexts. First, it raises the question of whether the SecYEG channel is involved in this retrograde protein translocation. Second, this system is relevant to the quality-control mechanism of the SecYEG channel (as already stated, SecY is a substrate of FtsH).

Regulation of sec *genes*

Not much is known about the regulation of expression of the Sec factors. SecA expression is subject to SecM-dependent translational control in response to the cellular activity of protein translocation[18,60] (Fig. 3). It is also subject to autogenous translational repression. Both SecY and SecE are within operons encoding translation-related factors and their expression levels might be coordinated with those of the translational apparatus.

Conclusions and perspectives

With the wealth of genomic information now available, it will become possible to obtain a more integrated picture of the interrelationships of different, but related, cellular activities, such as translation, folding, translocation and degradation. Thus, we will be able to view the Sec translocation machinery as an integral part of the cell. Another important task will of course be to elucidate the molecular mechanisms of the structural changes. As discussed in several places in this review, the functioning of Sec factors can be accompanied by dynamic conformational changes, including apparent movements across the membrane. Therefore, structural determination might not be as straightforward as we anticipate. To circumvent this difficulty effectively, different areas of expertise should be combined. We believe that this is a subject that deserves focused collaborations between structural biology, genetics, cell biology and chemistry.

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