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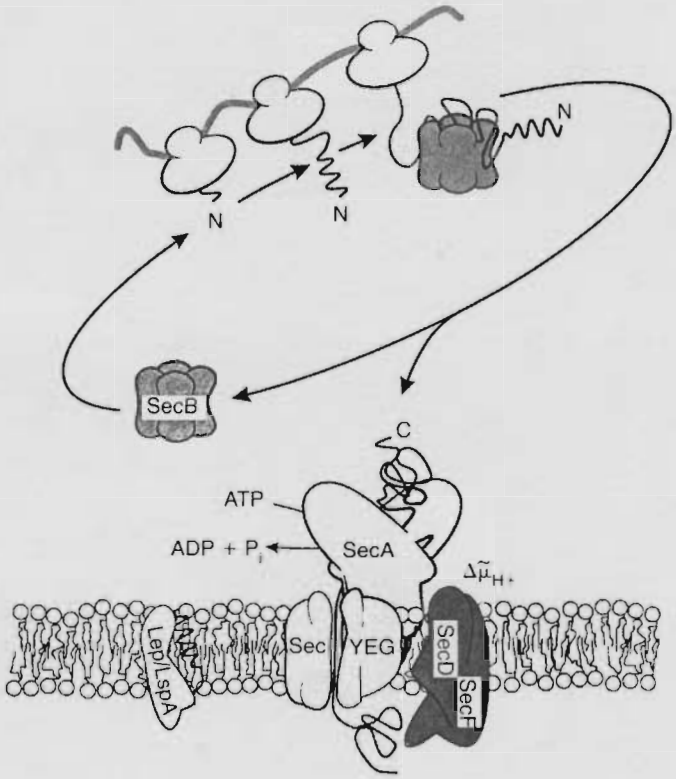
Introduction

In prokaryotes, proteins are exported across the cytoplasmic membrane and subsequently targeted to the periplasm, the outer membrane and/or the external medium. These proteins are synthesized at the ribosomes, mostly as cleavable signal-sequence-bearing precursors (see Chapter 3). The signal sequence acts as a targeting and recognition signal for the translocase, a complex of integral and peripheral membrane proteins that catalyses the energy-dependent translocation of preproteins across the cytoplasmic membrane (see also Chapter 7).

During the past few years, our understanding of the mechanism of protein translocation has advanced rapidly with *Escherichia coli* as a model organism. By ingenious genetic screening methods, and in some cases by reversed genetics possible after biochemical analysis, so-called *sec* (i.e. secretion) genes have been identified. The *secA*, *secB*, *secD*, *secE*, *secF*, *secG* and *secY* genes each code for a component of the translocation machinery (Figure 1) [1,2]. Many of these genes are essential for viability, and conditional lethal mutants exhibit pleiotropic protein export defects. Sec proteins have been overproduced and purified to homogeneity, and an authentic energy-dependent protein translocation system has been reconstituted into liposomes using only a minimal number of purified components [3]. The Sec pathway is widely distributed, and is present in all Eubacteria and in the thylakoid of higher plants ([4]; Chapter 14). In addition, the translocation of proteins across the cytoplasmic membrane of Archaea and the endoplasmic reticulum (ER) of mammals, yeast and algae requires the Sec61p complex, of which the α, β and γ subunits are ('remotely') homologous to SecY, SecG and SecE respectively ([5,6]; Chapter 10). Translocation may therefore occur by similar mechanisms in Eubacteria, Archaea, and Eukarya.

Figure 1 **Schematic overview of the Sec components involved in the general protein export pathway in *E. coli***



Signal peptidase cleaves the signal sequence from the preprotein, but is not required for translocation.

The protein translocation cascade

Preprotein translocation is an ordered process (Figure 1). Short nascent secretory proteins may interact with a signal recognition particle (SRP), and at a later stage with SecB. SecB targets preproteins to SecA, a preprotein-stimulated ATPase that is bound to a holoenzyme heterotrimeric membrane protein complex having SecY, SecE and SecG as stable subunits, and SecD and SecF as dissociable subunits. The preprotein translocase mediates the ATP- and protonmotive force (Δp)-dependent translocation of preproteins across the membrane.

The SRP: a remnant of an alternative pathway or an early targeting route?

In mammals, most membrane and secretory proteins are synthesized on free cytosolic ribosomes that are subsequently targeted co-translationally to the membrane of the ER by the SRP ([7,8]; Chapter 10). After binding the signal peptide and the ribosome, the SRP provokes translational arrest and binds to the SRP receptor at the ER membrane surface. The SRP receptor is composed of a peripheral subunit (SRα) and a transmembrane GTPase (SRβ) [9]. Upon GTP binding, the SRP receptor releases SRP from the ribosome–nascent-chain complex [10], and the signal sequence of the nascent chain and ribosome bind to Sec61p, the ER translocase [11]. GTP hydrolysis subsequently releases SRP from the SRP receptor so that it recycles into the cytosol. SRP consists of one RNA (7 SL RNA) and six protein subunits. The 54 kDa subunit of SRP (SRP54) contains an N-terminal GTPase domain and a C-terminal domain that binds the signal sequence and SRP RNA.

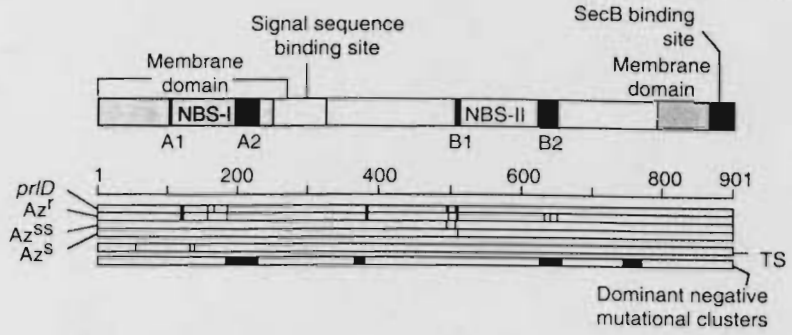
In *E. coli*, the *ffh* (Fth), *ffs* (4.5 S RNA) and *ftsY* genes code for a homologue of SRP54, the small motif in SRP RNA and SRα respectively [12–15]. The 4.5 S RNA is involved in general protein synthesis [16], but may also function in protein translocation, as it associates with Fth to form a ribonucleoprotein complex that interacts with the signal sequence of nascent chains [17] and with FtsY in a GTP-dependent manner [18]. Fth can substitute for SRP54 in a mixed reconstitution system with the eukaryotic SRP with respect to the particle formation and translation arrest activity [13], but it does not interact with the eukaryotic SRP receptor or facilitate translocation. Depletion or overexpression of Ffs results in retardation of the export of pre-β-lactamase, but not of other proteins [19,20]. Pre-β-lactamase is noteworthy, as it shows the slowest post-translational translocation kinetics in *E. coli*. Depletion of Fth [21] or FtsY [22], or overexpression of FtsY [22], causes growth arrest and retards the export of several preproteins. *In vitro*, however, FtsY depletion has only a minor effect on translocation [22]. The precise role of the SRP in bacteria is not understood [12,23]. This pathway may fulfil an early targeting role, as the SRP can be cross-linked to the signal sequence of preproteins and hydrophobic domains of membrane proteins provided that about 80 amino acids of the nascent chain have emerged from the ribosome [24]. The SRP may transfer nascent chains to SecB or, alternatively, function in co-translational translocation. This may, in particular, be relevant for membrane proteins that are difficult to stabilize in the cytosol in a soluble form. These proteins require SecY for translocation [25–27], but it is not clear if SecA is also needed [27]. SRP- and chaperone (represented by SecB)-dependent pathways may therefore co-exist for general protein export and converge at the SecYEG protein complex (i.e. the complex of SecY, SecE and SecG) and/or differ in the timing of their association with nascent chains.

SecB: a folding catalyst with a targeting role

Bacterial protein translocation can occur post-translationally, i.e. upon completion of polypeptide biosynthesis [28]. SecB, a tetramer of identical subunits [29], stabilizes some preproteins in the cytosol [30–33]. SecB is a true folding catalyst [34] and uses the energy of polypeptide binding to stabilize the preprotein in an unfolded, non-aggregated state [33,35–39]. SecB-bound preproteins have a native-like secondary structure and tertiary folding [39], but seem not to have the tight packing of the side chains typical of native proteins [34]. The specificity of the SecB–preprotein interaction has remained elusive [40,41]. The signal sequence is not required for SecB interaction, but slows down the folding of the mature domain [37] and stabilizes the complex [42]. SecB binds large nascent chains [43], presumably in the middle of the preprotein [40]. SecB binds a variety of unfolded proteins *in vitro*, and it has been suggested that the binding specificity is determined by kinetic partitioning that excludes fast-folding proteins from interacting with SecB [44]. However, SecB associates with polypeptides at a collision-limited rate [45] which can be greater than the rate at which nascent chains (or domains) fold [46]. SecB seems to be optimized to interact with unfolded polypeptide segments at a high rate but with low specificity [45], yet *in vivo* SecB is highly selective and interactions are thus specific [43]. Some mutations in SecB interfere with complex-formation, but have only mild effects on translocation [47,48]. The pattern of these mutations suggests that the preprotein binding site is hydrophobic, with β-sheet structure [48]. Various other chaperones (including DnaK and GroEL) are involved in stabilizing newly synthesized preproteins, but none of them is able to replace SecB [49–52]. SecB is essential only when preproteins tend to queue for translocation, as in fast-growing cells [38]. SecB is also needed for the translocation of signal-sequence-less (pre-)proteins in *prlA* (where *prl* stands for protein localization) suppressor strains (see below) [53,54].

SecB targets preproteins to the membrane surface by binding specifically to SecA [55–57]. The SecA–SecB interaction is weak in solution, but occurs with high affinity (K_D 150–250 nM) at the membrane surface [55,57], and is promoted by preproteins [55,58]. Deletion of the C-terminus of SecA (Figure 2) destroys the SecB–SecA interaction [58,59]. This region of SecA is important for viability [58], and fusion proteins bearing only the last 22 amino acids of SecA have been shown to genuinely bind SecB [60]. This highly conserved domain corresponds to a flexible, strongly electropositive surface (pI 9.5–10.8). It may interact with a putative β-structured acidic bristle on SecB that has been identified by the analysis of mutants that are defective in translocation but still bind the preprotein [48]. SecB therefore functions as a coupling factor that binds to nascent polypeptide chains when they emerge from the ribosome, stabilizes the precursors in a translocation-competent conformation and guides them to the SecA subunit of the translocase.

Figure 2 Domain structure of the SecA protein



Indicated are the interacting regions that bind the membrane, signal sequence and SecB. NBS-I and NBS-II are the high- and low-affinity NBSs respectively. A1 and B1, and A2 and B2, correspond to regions with similarity to the Walker A- and B-motifs found in NTP-binding proteins. Marked are clusters of dominant-negative mutations and deletions, signal sequence suppressor defects (prlD), azide-resistance (Az^r), -sensitivity (Az^s), and -super-sensitivity (Az^{ss}) mutations, and temperature-sensitivity mutations (TS).

SecA: a preprotein-stimulated ATPase

SecA is a central component in protein translocation [61] and functions as a dimer [62,63]. SecA exhibits three distinct ATPase activities [64,65], i.e. low endogenous, lipid-stimulated and translocation ATPase activities. The activity is coupled to preprotein translocation, as it requires the SecA protein to interact with translocation-competent preproteins, the SecYEG protein complex and anionic phospholipids [3,64,66]. Photoreactive nucleotide analogues inactivate SecA at two distinct nucleotide-binding sites (NBSs) [64]. These sites have been localized by cross-linking and site-directed mutagenesis studies [67–71]. The high-affinity NBS-I (K_D 0.13 μM) is confined to the N-terminal region, while the low-affinity NBS-II (K_D 340 μM) is located in the C-terminal half of the protein (Figure 2). 8-Azido-ATP-treated inner membranes are inactive for translocation, and activity can be restored by adding purified SecA [64,72]. Both NBS-I and NBS-II are required for the translocation activity of SecA. In contrast with ATP binding, NBS-I and -II function in a co-operative manner [68,70], and many of the dominant-negative mutants in SecA cluster around these sites [73] (Figure 2).

SecA recognizes preproteins via the positive charge at the N-terminus of the signal peptide [74–76], and also interacts with the mature domain [66]. Synthetic signal peptides inhibit translocation [77], compete with preproteins for binding to SecA [76] and activate SecA ATPase activity when added in conjunction with, and thus physically separated from, the mature domain of a preprotein [66]. Soluble SecA liganded with ADP binds preproteins with high affinity [74,78], whereupon it releases the bound ADP at NBS-I [78]. At the membrane, ADP is exchanged for ATP and translocation is initiated.

SecA membrane interaction and topology

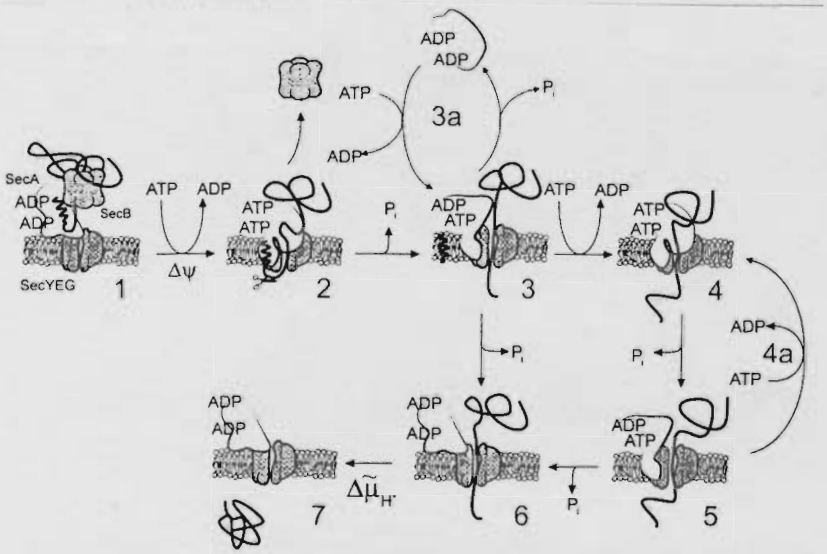
SecA cycles between free cytosolic (including ribosome-bound forms [79]) and membrane-bound states [80]. At the membrane, it binds to sites of low [81] and high [55] affinity. Acidic phospholipids are required for translocation [82,83] and they promote the SecA membrane interaction [66,84], as well as stimulating the SecA translocation ATPase activity [66]. The unabated, low-affinity interaction of SecA with anionic phospholipids, however, renders the protein thermolabile [66], causing it to unfold [85] and aggregate (A.J.M. Driessen, unpublished work). ATP, preproteins and the SecYEG complex stabilize the lipid-bound SecA, which then exhibits SecA lipid ATPase activity [66]. SecA is able to penetrate far into the hydrophobic acyl-chain region of the membrane [85–87]. ATP hydrolysis at NBS-I prevents this insertion [69,86]; thus, in the ADP-bound state, SecA associates with the membrane surface, while in the presence of non-hydrolysable ATP analogues the inserted state dominates [86]. SecA binds with high affinity to the SecYEG complex (K_D 40 nM) [55]. Some SecA proteins, in particular those bearing mutations in NBS-I, associate tightly with the membrane and cannot be removed by urea or carbonate [80,88–90]. Along with an increased number of high-affinity SecA binding sites [91], overproduction of the SecYEG complex also increases the amount of urea-resistant membrane-bound SecA [90]. SecA penetrates the membrane [92] and, in native vesicles, exposes a C-terminus to the periplasmic face of the membrane [90]. This region of SecA has been implicated in lipid binding [58] and shown to bind SecB [60]. In the presence of ATP and preprotein [93], or with a non-hydrolysable ATP analogue [94], SecA inserts into the membrane with a 30 kDa domain. In the presence of preprotein, membrane insertion of SecA results in the release of bound SecB. Since SecB binds SecA at the C-terminus, it appears that membrane insertion of SecA results in altered accessibility of this domain. Monoclonal antibodies against the C-terminal half, but not those against the N-terminal half, of SecA prevent membrane insertion [95]. In the presence of ADP, a stable tryptic 23 kDa fragment of SecA can be formed [96]. NBS-I seems to regulate SecA membrane insertion [89,94], and therefore circumstantial evidence suggests that it is the early N-terminal domain that integrates into the membrane. For instance, N-terminal fragments of SecA that functionally complement the *secA51(ts)* mutant [80,97–99] associate readily with the membrane [80]. Taken together, these data suggest that various domains of SecA may integrate into the membrane during preprotein translocation.

SecA is an ATP-driven force generator

SecA undergoes nucleotide-modulated cycles of insertion into and excision from the membrane [69,86,93,94]. It is thought that small polypeptide domains of the bound preprotein are translocated due to co-insertion with SecA upon ATP binding (Figure 3). ATP hydrolysis dissociates the SecA–preprotein complex bound at the SecYEG complex [100], excises the SecA from the membrane [92] and allows rapid exchange with cytosolic SecA [69,93]. This phenomenon requires ATP hydrolysis

at NBS-I [69]. NBS-I mutants [68] that still bind ATP and drive the limited translocation of a polypeptide domain [69] through membrane co-insertion upon ATP binding [93,94] are unable to cycle, as they are defective in excision from the membrane [89,94]. *In vitro* translocation is arrested immediately by depletion of ATP or the addition of non-hydrolysable ATP analogues [100,101], presumably due to inhibition of the excision of SecA [93]. NBS-II is not needed for SecA cycling [94], but appears to be involved in coupling of SecA movement to preprotein translocation. Differential-scanning calorimetric studies suggest that

Figure 3 **Model for the initial and intermediate stages of protein translocation**



SecA with bound SecB and preprotein at the SecYEG complex (step 1) is activated for ADP:ATP exchange. Binding of ATP at NBS-I and -II (unknown order) facilitates the insertion of SecA into the membrane at the SecYEG complex (step 2), releases SecB into the cytosol and allows membrane insertion of an N-terminal loop of the signal sequence and the mature domain for processing by leader peptidase. Subsequent hydrolysis of ATP at NBS-II by SecA drives the release of the bound preprotein and promotes the excision of a SecA domain from the membrane-bound state (step 3). At this stage, hydrolysis of ATP at NBS-I cycles the membrane-bound SecA and exchanges it for cytosolic SecA (step 3a), or ADP:ATP exchange at NBS-II drives another preprotein domain across the membrane by co-insertion with a SecA domain (step 4), followed by preprotein release and de-insertion of the SecA domain from the membrane (step 5). When SecA has de-inserted from the membrane due to hydrolysis of ATP at NBS-I (step 6), further translocation may be driven by the Δp (step 7) and/or by SecA-dependent cycles of ATP binding and hydrolysis (step 4a), until the preprotein has been translocated completely.

SecA unfolds thermally as a two-domain protein [96]. Saturation of NBS-I by ADP increases the stability of the protein and promotes the interaction between the two domains. Saturation of NBS-II with ADP further enhances these effects and increases dramatically the compactness of the protein. SecA seems to adopt a more extended conformation in the presence of non-hydrolysable ATP analogues. These nucleotide-induced compact and extended conformations may correspond to the membrane-excised and -inserted states of SecA respectively. The C-terminal region of SecA seems to facilitate the coupling of ATPase activity to cycles of protein translocation [59]. Binding of ADP and ATP at NBS-I seems to provoke opposite conformational changes in the C-terminus of SecA [96]. ATP binding and hydrolysis may therefore cause alterations in the relative positions of the two domains of SecA, alternating the protein between the extended and compact states. These events may provide a mechanical explanation for preprotein translocation driven by SecA membrane insertion (Figure 3).

Membrane-integrated domains of the translocase

The membrane-integrated domain of the translocase consists of three polypeptides, i.e. SecY, SecE and SecG [1,3,102,103]. Although opposing views have been expressed [104], this complex is required for translocation [3,91,105–110]. Unlike SecY and SecE, SecG is not essential, but disruption of its gene confers cold-sensitivity to protein export ([111]; Chapter 7). SecY [102], SecE [103,112] and SecG ([113]; Chapter 7) are polytopic membrane proteins with ten, three and two transmembrane segments (TMSs) respectively. The C-terminal TMS plus attached sequences of SecE suffices for activity [108,114,115], and is the size of SecE in most bacteria [6,112]. SecY and SecE were identified genetically as translocase subunits, while SecG (previously termed 'band 1' [3] or 'pl2' [116]) was identified biochemically. SecG co-purifies and co-immunoprecipitates together with SecY and SecE as a stable heterotrimeric membrane protein complex [3] that is labile at ambient temperature [117]. This complex has been purified to homogeneity from wild-type *E. coli* cells using the SecA translocation ATPase assay [3,118,119]. In reconstituted form, SecYEG mediates multiple turnovers of SecA-dependent preprotein translocation [120]. This complex has also been reconstituted functionally from the individually isolated subunits [121], and from a SecYEG-overproducing strain [90,122]. SecG enhances dramatically the translocation activity of proteoliposomes containing only SecE and SecY [107,116]. The *secG null* mutant can be complemented by phosphatidylglycerol synthetase, which may alter the membrane lipid composition [123]. SecG seems to reverse the orientation of its TMS during translocation [113], and it has been proposed that it acts as a lubricant to facilitate the membrane insertion of SecA. This activity would be particularly important at lower temperatures when the membrane viscosity is high [111].

SecE stabilizes SecY [124,125] and protects it against proteolysis by FtsH, a membrane-bound ATP-dependent protease [126]. Newly synthesized SecY associates immediately with SecE to form a complex that does not dissociate during translocation, while SecG is a less stable subunit [125,127]. Genetic experiments argue that SecE and SecY are dissociable subunits [128]. Based on the synthetic defect of pairs of *prlA* (SecY) and *prlG* (SecE), it appears that the SecY–SecE interaction takes place between the first N-terminal periplasmic loop of SecY and the C-terminus of SecE, and between TMS3 of SecE and TMS7 and TMS10 of SecY [129]. Other mutations in SecY that affect the association between SecY and SecE are clustered in the second cytosolic loop [130,131]. The SecYEG complex functions as a high-affinity membrane binding site for SecA [90,91] that binds at or near SecY [55,105,132,133].

Most conditional lethal mutations in *secY* and *secE* are cold-sensitive (*cs*). Temperature-sensitive mutations are known only for *secA* and *secY*. Protein translocation may include some intrinsic cold-sensitive steps, and it has been suggested that a lowering of the activity in a step that follows the cold-sensitive step invariably leads to the *cs* export phenotypes [134]. Membrane insertion of SecA is cold-sensitive [93,94], but the insertion of the signal sequence domain could be cold-sensitive as well. SecD and SecF are membrane proteins that were identified by *cs* mutations that cause the pleiotropic accumulation of precursors in the cell [135,136]. Both proteins have large periplasmic domains positioned between the first two of six TMSs [137]. SecD and SecF are not essential for viability [136] or translocation [3,138,139], or for high-affinity membrane binding of SecA [94]. SecD and SecF seem to function as dissociable subunits of the translocase at a late stage of translocation [140,141], and stabilize the membrane-inserted state of SecA [92,94]. The latter may explain the *cs* phenotype of *secD* and *secF* mutants, as SecA membrane insertion is cold-sensitive [93,94]. Protein export in spheroplasts is inhibited by an anti-SecD antibody [142], and it has been suggested that SecD is required to release the proteins into the periplasm. However, the proteins are not required for clearing of the translocation site *per se*, as the reconstituted translocase mediates multiple rounds of preprotein translocation even in their absence [120].

Signal sequence proofreading

Suppressors of signal sequence mutations have been found in the *secA* (*prlD*) [143–145], *secY* (*prlA*) [54,114,146] and *secE* (*prlG*) [54,114] genes. *prl* suppressors may function not by restoring the recognition of altered signal sequences but rather by preventing the rejection of defective preproteins [54,146]. According to this hypothesis, SecA, SecY and SecE are involved in proofreading. The majority of strong signal sequence defect suppressing *prl* mutations map to *prlA* (SecY), whereas the weaker suppressors map to *prlG* (SecE) and to *prlD* (SecA) [54,143].

Most *prl* mutations cluster in TMS7 of SecY and TMS3 of SecE, suggesting that these helices are involved in a signal sequence proofreading process.

Many of the azide-resistant (Az^r) mutants coincide with *prlD* mutations [143]. Sodium azide is an inhibitor of Sec-dependent protein translocation, and blocks the SecA translocation ATPase activity [147,148]. Az^r mutants show an elevated translocation ATPase activity, and mainly cluster around NBS-I (Figure 2) [143,144,148]. This site has been implicated in SecA membrane insertion [94], suggesting that signal sequence proofreading may occur while SecA inserts into the membrane [143]. Another class of *prlD* (SecA) mutants are azide-super-sensitive (Az^{ss}), and these are clustered around NBS-II (Figure 2) [143]. The *prl* and Az^{ss} phenotypes caused by these mutants are dominant in diploid analysis [143]. Az^{ss} mutants may be unable to hydrolyse ATP once they have inserted in the membrane, thereby blocking access to the translocation sites for wild-type SecA. An Az^r phenotype is also conferred by suppressor mutations in the vicinity of NBS-II. The suppressor phenotype of these mutants is either weak or unknown, indicating that NBS-II might not be essential for signal sequence proofreading.

Translocation pathway

Preproteins may translocate through the lipid bilayer, through a proteinaceous channel or at the interface between the lipid and the protein complex. In the initial stages of translocation, the signal-peptide–lipid interactions seem to be important (Chapter 3). In short, the positively charged signal sequence may interact electrostatically with anionic phospholipids in the membrane, thereby favouring insertion of the α-helical hydrophobic core into the bilayer in a loop-like conformation [149]. Subsequent stretching of the signal sequence into a transmembrane orientation would pull the N-terminus of the mature domain into the membrane. These steps are most probably mediated by the translocase. Binding of the signal sequence at sites on SecY and SecE may trigger a series of events, and allow the N-terminal mature domain of the preprotein to enter the translocation channel laterally from the lipid phase, as suggested for the Sec61p complex ([150]; Chapter 10). The SecYEG complex most likely acts as a protein-conducting pore, permitting preproteins to translocate either through its centre or along a proteinaceous surface provided by membrane-inserted SecA and the SecYEG complex. Translocation intermediates can be photocross-linked specifically to SecA and SecY in an environment devoid of phospholipids [151]. Systematic probing of the environment of a polypeptide chain translocating through the ER membrane indicates that its surrounded by Sec61α [152], and fluorescent probes incorporated into nascent secretory proteins have shown directly that nascent chains move through the translocase via an aqueous pore [153]. Conductivity experiments are also consistent with the presence of an aqueous pore for protein translocation in the ER ([154]; Chapter 10). Curiously, conductivity measurements in *E. coli* membranes and spheroplasts have shown the presence of a

signal-peptide-induced pore only in the presence of a non-physiological polarity of the transmembrane electrical potential ($\Delta\Psi$), i.e. negative on the *trans*-side instead of positive [155]. The ion-permeability of the membrane increases dramatically when preprotein translocation intermediates are trapped in the translocase ([156,157]; A.J.M. Driessen, unpublished work). The reconstituted SecYEG complex allows water movement across the membrane; this activity is suppressed by SecA, but increases when preprotein translocation is initiated (A.J.M. Driessen, unpublished work). Taken together, these data suggest that SecYEG is a SecA-gated channel, and that preproteins are translocated across the membrane along a proteinaceous surface with water as a lubricant.

The translocase allows the translocation of preproteins that bear internal non-polypeptide stretches, indicating that peptide-backbone recognition is not required throughout the translocation reaction [158]. It is not known how the translocase recognizes stop-transfer sequences (or TMSs) in translocating membrane proteins. Such recognition is required to allow release of these proteins into the lipid bilayer. Recognition of such sequences could be confined to SecA as part of the proofreading event. An attractive model is that SecA inserts into the membrane and translocates the polypeptide segments in a co-translational fashion along a proteinaceous surface at the SecA/SecYEG interface. When a TMS is encountered that is flanked by a positively charged region, SecA may dissociate temporarily from the translocating polypeptide chain, undergo excision and thereby release the TMS into the lipid bilayer. SecA may re-engage the protein translocation reaction by binding to the exposed nascent chain and then continue with translocation. In this model, membrane proteins are inserted into the membrane according to the 'positive-inside rule' [159]. Moreover, there is no need for opening of a translocation channel, as this would be effected by the SecA moving away from the proteinaceous surface along which the preprotein translocates. Recent evidence gained with the ER Sec61p indicates that the TMSs of polytopic membrane proteins assemble at first within the translocase and are not allowed to diffuse into the bilayer until translation at the ribosome is terminated [160,161].

Initiation of preprotein translocation

The energetics and catalysis of bacterial preprotein translocation have been studied in great detail [162–170]. Preprotein translocation proceeds in a stepwise manner, with discreet translocation intermediates [100,101,171,172] and energy needs (ATP and Δp) (Figure 3). Translocation may commence with a lipid, surface-bound [81], freely diffusible ternary complex of SecA, SecB and a preprotein [55]. This complex will diffuse laterally to the SecYEG complex, where SecA is activated for ADP:ATP exchange [69,89,94]. SecA changes its conformation upon ATP binding [78,96], inserts into the membrane [93,94] and drives the membrane insertion of the signal

sequence and early mature domain of the bound preprotein by co-insertion [100]. SecB is redundant at this stage [55], and is released from the complex when SecA inserts into the membrane [60]. The signal sequence may adopt a loop-like structure ([173]; Chapter 3) that is in contact with anionic phospholipids. Under the influence of a $\Delta\Psi$ (inside negative), it may stretch into a transmembrane configuration [173]. The ATP-dependent membrane insertion of SecA may serve to properly expose the signal-sequence domain to binding sites on SecY and SecE to allow proofreading (see above). If the signal sequence is rejected, no functional translocase is formed, and ATP hydrolysis will force SecA to release the defective preprotein into the cytosol [143]. When the signal sequence is properly recognized, a functional translocase is formed, and the signal-sequence domain will be processed by signal peptidase [143], while the mature region presumably associates with SecYEG [151] in order to prevent it from diffusing away. SecA releases the bound preprotein upon the hydrolysis of ATP, and undergoes excision from the membrane [93,94].

The order of events ensures that the preprotein is released by SecA at the translocation site, and precludes unproductive reactions with the lipid bilayer [100]. Further ATP-dependent translocation proceeds stepwise and, in the case of proOmpA, a series of defined intermediates has been detected [100,174]. SecA can re-bind the translocation intermediate, and through membrane insertion drive the forward translocation of a 2.0–2.5 kDa polypeptide stretch, which has been defined as one 'quantum', or about 20–30 amino acid residues [100,174]. Another quantum of translocation occurs upon ATP binding to SecA. Subsequently, the translocase seems to stall, as stable intermediates appear to occur at two-quanta spacings [170]. This may be the result of two subsequent translocation steps, one driven by preprotein binding by SecA and one driven by ATP binding to SecA. The preprotein segments that are initially associated with SecA move into contact with SecY as they traverse the membrane, and remain bound to SecA until they have emerged on the periplasmic face of the membrane [151]. The bound preprotein segment may unfold when it binds to SecA, and/or when SecA inserts into the membrane. A fusion protein consisting of cytosolic dihydrofolate reductase linked to the C-terminus of proOmpA translocates up to the folded dihydrofolate reductase moiety when stabilized by NADPH and methotrexate [175]. Removal of these ligands allows the spontaneous translocation of about a quantum, concomitant with the unfolding of dihydrofolate reductase. The folded moiety of dihydrofolate reductase may prevent SecA from inserting into the membrane and, when the ligands are removed, SecA membrane insertion may force the dihydrofolate reductase to unfold. A similar phenomenon may explain the 'backward and forward' translocation of unstable proOmpA intermediates that translocate to full length when freed from a synthetically imposed translocation arrest [100]. Since translocation (and possibly unfolding) of the preprotein is driven by ATP-dependent SecA membrane insertion, translocation seems to occur through force generation rather that via 'ratcheting'; the latter assumes translocation through diffusion and Brownian motion in polypeptides [176].

Catalysis of later stages of translocation

Multiple cycles of SecA-dependent translocation ultimately lead to the complete translocation of the preprotein [100]. This process is slow and requires the hydrolysis of numerous ATP molecules [162,177]. Δp permits a rapid and efficient translocation of polypeptide segments once SecA has released the preprotein to the SecYEG complex [100,162]. A collapse of Δp results in the accumulation of early translocation intermediates [170], while Δp-driven translocation can be blocked completely by non-hydrolysable ATP analogues [100] or when SecA is unable to hydrolyse ATP due to a mutation in NBS-I [69]. This SecA mutant cannot undergo excision from the membrane and is unable to release the preprotein. Excess SecA suppresses Δp-dependent preprotein translocation [88,100,169], as it favours the rapid re-binding of released preproteins by SecA. At limiting SecA concentrations, translocation is more dependent on Δp [178]. Δp modulates the activity of SecA by reducing the apparent K_m of the translocation reaction for ATP, allowing Δp-driven translocation at low ATP concentrations [179]. Under these conditions, Δp promotes membrane insertion of SecA [94] and thus may indirectly affect the K_m by allowing faster recycling of the translocation site.

A detailed analysis of the intermediate stages of translocation has only been carried out for proOmpA. For prePhoE, and for some proOmpA constructs that bear mutations in the mature N-terminal region [180,181], it appears that the initiation of translocation is critically dependent on Δp [173,182]. SecA not only may be needed for the initial stages, but may also be required throughout the translocation reaction [100,183]. It is likely that ATP- and Δp-driven preprotein translocation are alternating events. Intermediate stages of translocation are readily reversible, and SecA-mediated ATP hydrolysis is not strictly coupled to net preprotein movement [100]. Futile cycles of ATP hydrolysis occur when translocation is prevented by a stable tertiary structure in the preprotein [100,183], or when a Δp of reversed polarity is imposed [162,184]. In the absence of SecA association, hysteresis movement of translocation intermediates takes place [100]; this is prevented by Δp [163]. Futile cycling presumably causes poor coupling between translocation and ATP hydrolysis *in vitro*. Δp indirectly improves the coupling [162], as it allows the translocation of long polypeptide stretches that would otherwise have required multiple cycles of SecA-mediated ATP hydrolysis [100]. Δp is also needed to release the translocated preprotein into the periplasm [185].

Mechanism of Δp-driven translocation

Δp-driven translocation involves both the $\Delta\Psi$ and ΔpH [162,184,186], while in the marine bacterium *Vibrio alginolyticus* protein translocation appears to be coupled to the sodium-motive force [187]. This suggests that protons (or sodium ions) are involved in protein translocation directly, but a demonstration of vectoral proton

fluxes has been difficult due to proton leaks that accompany the *in vitro* translocation reaction [156,157]. The detection of a kinetic solvent isotope effect in the $\Delta\Psi$-driven chase of a translocation intermediate of proOmpA suggests that critical proton-transfer reactions are involved in a rate-limiting step during translocation [167]. It is uncertain if these concern vectoral proton movements. Scalar protons are involved in translocation, as the activity of the translocase is adversely affected by a lowering of the pH at the cytosolic face of the membrane [167].

Preproteins need to be in a so-called 'loosely' folded state in order to be translocated [188], but the translocase can mediate the translocation of short segments of proOmpA with a stable tertiary fold, e.g. segments as large as 2 kDa that are stabilized by a disulphide bridge [172,183,185]. This translocation requires both ATP and Δp. In the absence of Δp, translocation stops at the stable fold, and it has been suggested that Δp drives the translocation of large folded domains by widening the size of the translocation channel. A reversed Δp would restrict its size, thereby preventing 'forward' translocation [162]. In *prlA* suppressor strains, the Δp-dependency of translocation seems to be suppressed [182], and it has been argued that the translocation channel in these strains is already in an open conformation. Preproteins also vary in their requirement for Δp for translocation [169]. This may be related to the presence or absence of stable folded tertiary-structure elements, the number and affinity of SecA-binding sites in the polypeptide chain (assuming that there is some specificity in these interactions), and the charge distribution along the polypeptide chain. Preproteins bearing a mature domain devoid of ionizable residues still require Δp for translocation [189], again implying that Δp performs a mechanistic function rather than acting upon the preprotein itself, as for instance through protonation/deprotonation or electrophoresis. The same seems to be the case with the Δp-driven insertion of the signal peptide domain into the membrane [173,180,181,190], as the Δp requirement of this process is also suppressed in *prlA* strains [182].

SecD and SecF have been implicated in Δp-driven translocation, as the depletion of SecD and SecF severely affects the ability of cells to maintain a Δp [138]. *In vitro* studies demonstrated that SecD and SecF are not needed for ATP-dependent translocation, but that they affect kinetically the Δp-dependent portion of the translocation reaction in a manner that resembles the action of protonophores [138]. SecD and SecF are not, however, essential for coupling of the Δp to translocation [3]. Since translocation is slow in *secD* and *secF* null strains, it is possible that leakiness arises from translocase units jammed by translocation intermediates. These units would act as energy sinks, causing the dissipation of Δp (C. van der Does, unpublished work). The translocation of various preproteins is affected in different ways in *secD* and *secF* mutant strains, but this may be related to the Δp-dependency of their translocation.

Concluding remarks

During the last decade, many salient features of the mechanism of bacterial protein translocation have been resolved [2,191]. The complicated process of protein translocation as it occurs in the cell can now be reconstituted with purified Sec proteins. As Sec proteins can be purified in large quantities, the bacterial system will be amenable to biophysical studies and crystallization attempts that will reveal details of the molecular mechanism of protein translocation.

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