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Protein translocation across the inner membrane of Gram-negative bacteria: the Sec and Tat dependent protein transport pathways

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Abstract

Gram negative bacteria possess a large variety of protein transport systems, by which proteins that are synthesised in the cytosol are exported to destinations in the cell envelope or entirely secreted into the extracellular environment. The inner membrane (IM) contains three major transport systems for the translocation and insertion of signal sequence containing proteins: the Sec translocon, the YidC insertase, and the Tat system. The heterotrimeric SecYEG translocon forms a narrow channel in the membrane that serves a dual function; it allows the translocation of unfolded proteins across the pore and the integration of α-helical proteins into the IM. The YidC insertase is a multi-spanning membrane protein that cooperates with the SecYEG translocon during the integration of membrane proteins but also functions as an independent insertase. Depending upon the type of protein cargo that needs to be transported, the Signal Recognition Particle (SRP), the SRP receptor, SecA and chaperones are required to coordinate translation with transport and to target and energise the different transport systems. The Tat system consists of three membrane proteins (TatA, TatB and TatC) which in a still unknown manner accomplish the transmembrane passage of completely folded proteins and protein complexes.

Keywords: Sec-dependent pathway; Tat-dependent pathway; Inner membrane; Gram-negative bacteria; Protein translocation; Chaperone proteins

1. Introduction

One of the major challenges that prokaryotic and eukaryotic cells face is efficiently transporting proteins from their site of synthesis in the cytosol to their sites of function. As 20%–30% (Pugsley, 1993; Holland, 2010) of all proteins in bacterial cells are localised outside the cytosol, it is evident that protein transport is vital for the sustenance of cells. The inner membrane in Gram negative bacteria like *Escherichia coli* separates the cytosolic translation machinery from extra-cytosolic sections such as the periplasmic space or the outer membrane, forming a barrier against protein trafficking. To facilitate protein transport across this barrier, bacteria are equipped with membrane embedded protein transport systems that allow transport of proteins across the membrane into the periplasm or insertion of proteins into the membrane. There is a remarkable array of protein transport systems found in bacteria (Papanikou et al., 2007), but only three systems appear to be present in most bacterial species and are focused on in this review (Fig. 1):

a) The **Sec translocon** is the most characterised protein transport system and is thought to function as the major protein transport site in bacteria. It is present in all bacteria, archaea, and in the endoplasmic reticulum membrane of eukaryotic cells. It is also present in chloroplasts but absent in the mitochondrial membrane of most

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organisms, with the exception of the protist *Reclinomonas americana* and related protozoa (Tong et al., 2011). The Sec translocon has two functions; it transports secretory proteins across the inner membrane and inserts membrane proteins into the inner membrane. This dual role makes it functionally distinct from the YidC insertase, which has so far been shown to mainly aid the insertion of membrane proteins.

b) The **YidC insertase** is probably the simplest system for integrating membrane proteins into the cytoplasmic membrane (Dalbey et al., 2011). YidC is found in all bacterial species, many archaea and in bacteria-derived

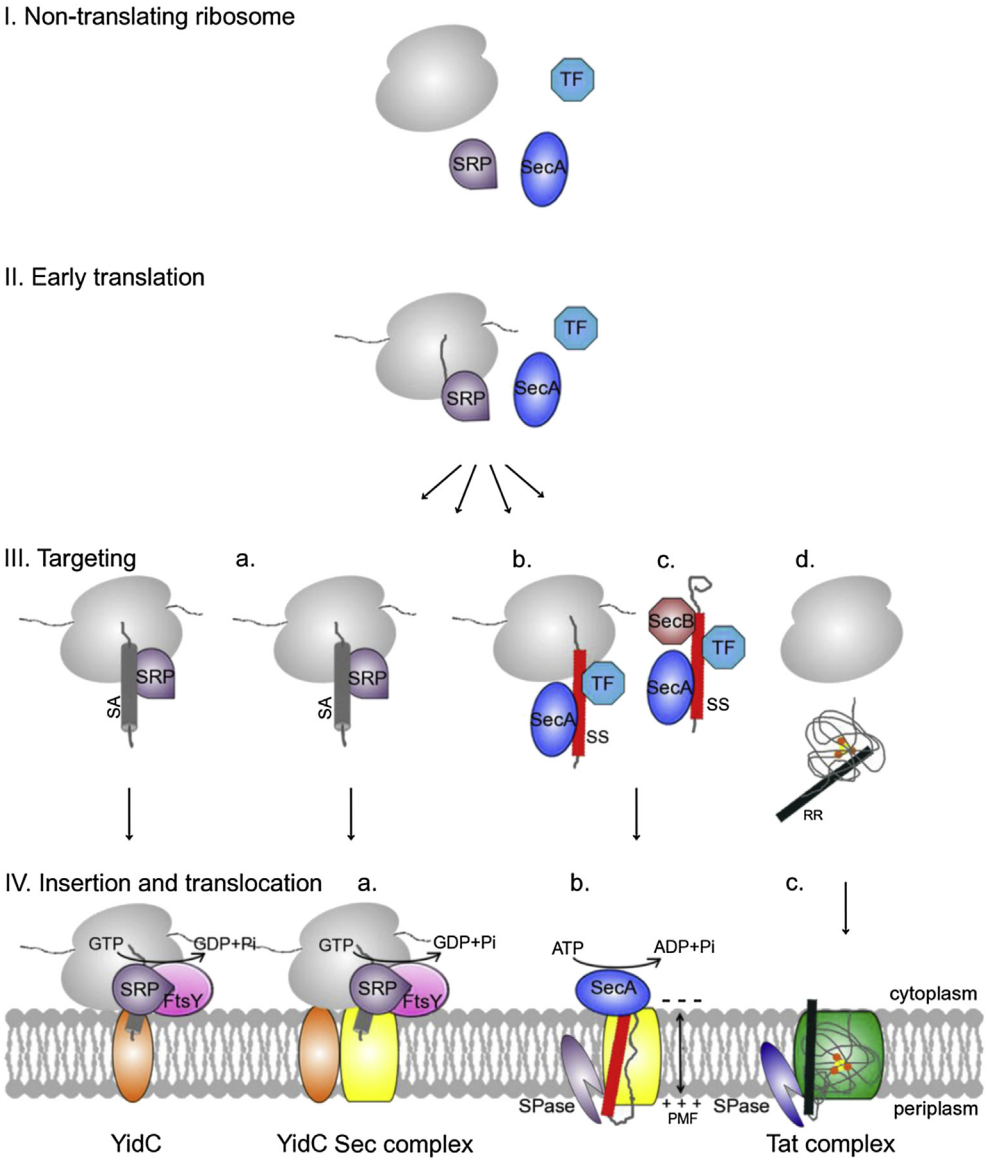

Fig. 1. Overview of the major protein transport pathways in Gram negative bacteria. I: Non-translating ribosomes have a basal affinity for the targeting factors SRP and SecA and for the chaperone Trigger Factor (TF). Whether all non-translating ribosomes have TF bound, as initially suggested (Deuerling et al., 1999) is questioned by recent data suggesting that TF binds preferentially to ribosome-associated nascent chains (RNCs) exposing at least 100 amino acids (Hoffmann et al., 2012). II: SRP has a high affinity for translating ribosomes. Although binding of SRP and TF to translating ribosomes appears to be non-exclusive, the coordination between SRP binding to RNCs and binding of other proteins like SecA or processing enzymes like peptide-deformylase (PDF) or methionine aminopeptidase (MAP) remains unclear. III: If a Signal Anchor (SA) sequence emerges from the ribosomal exit tunnel, SRP remains bound to the ribosome (3a) and targets the ribosome-nascent chain (RNC) complex to the membrane-anchored SRP receptor FtsY. If a signal sequence of a soluble protein (SS) is exposed in the growing nascent chain, SecA can replace SRP on the ribosome. Co-translational targeting starting with SecA binding to RNC (3b) is one of the two alternate targeting pathways, which have been suggested for secretory proteins. Another model (3c) favours post-translational targeting, where SecB holds the substrate in a translocation competent state with subsequent signal sequence recognition by SecA. The signal sequence of a protein transported via the Tat translocase contains a twin arginine motif (3d). The Tat complex exclusively transports folded substrates. However, signal recognition and targeting processes remain to be elucidated. IV: After targeting of an RNC by SRP, the RNC is transferred to the SecYEG translocon, YidC insertase, or to the complex of both. The SRP-FtsY complex is thought to disassemble upon GTP hydrolysis simultaneously with this event. Proteins with a SA sequence are laterally inserted to the lipid bilayer of the inner membrane (4a). Secretory proteins are translocated through the SecY pore with the aid of SecA, which acts as an ATP-powered motor (4b). Signal sequences of both Sec and Tat translocated proteins are removed in periplasm by inner membrane embedded signal peptidases (SPases).

organelles like mitochondria (Oxa1) or chloroplasts (Alb3, Alb4) (Funes et al., 2011). YidC can function as a stand-alone insertase (Samuelson et al., 2001; Chen et al., 2002), but it also cooperates with the Sec translocon during membrane protein insertion (Beck et al., 2001; Nagamori et al., 2004).

c) The **Tat system** transports folded proteins across the inner membrane, making it different from the two above mentioned transport systems, which are specific for unfolded proteins. Tat substrates are often co-factor containing proteins and the insertion of co-factors is mainly restricted to the cytosol. The Tat system is present in most bacterial and archaeal species, and chloroplasts in plants, but absent in mitochondria.

The Sec protein transport system cooperates with cytosolic partner proteins like SecA or the Signal Recognition Particle (SRP), which recognise signal sequence/signal anchor (SA) sequence containing substrates and initiate their membrane targeting (Koch et al., 2003). SRP also binds to the YidC insertase and is required for the membrane targeting of many YidC substrates (Welte et al., 2012). The Tat system appears to lack a general signal sequence recognition protein in the cytosol. Instead, for some Tat-dependent redox proteins specific signal sequence binding chaperones like TorD or DmsD are present, and often encoded in the same operon as Tat substrates themselves (Oresnik et al., 2001; Jack et al., 2004).

2. Sec-dependent protein transport

Most of the components of the Sec transport machinery were intially identified via elegant genetic screens. Mutations in *secA*, *secD*, *secE*, *secF* and *secY* conferred protein secretion defects and were referred to as *sec* alleles, while *prl* alleles (*prlD* = *secA*; *prlG* = *secE*; *prlA* = *SecY*, *prlH* = *SecG*) were identified as suppressors that allowed the secretion of signal-sequence defective pre-proteins (Schatz and Beckwith, 1990; Bieker and Silhavy, 1990). Refining these initial screens also identified components of the SRP pathway as essential players of the bacterial protein transport (Tian and Beckwith, 2002). A detailed correlation of the *prl* and *sec* mutants with the X-ray structure of the SecYEG translocon can be found in Smith et al. (2005).

2.1. The SecYEG translocon: structure and function

The SecYEG translocon is an essential, heterotrimeric and evolutionarily conserved protein complex embedded in the IM of Gram negative bacteria. The first X-ray structure was solved for an archaeal homologue (Van den Berg et al., 2004), which proved to be a major breakthrough in the protein transport field (Fig. 2). The SecY subunit (Sec61α in eukaryotes and archaea) has a molecular weight of 48 kDa, and its 10 α-helical transmembrane (TM) domains form the aqueous protein transport channel of the translocon. When visualised from the top, the 10 TMs are organised like a clam-shell with TMs 1–5

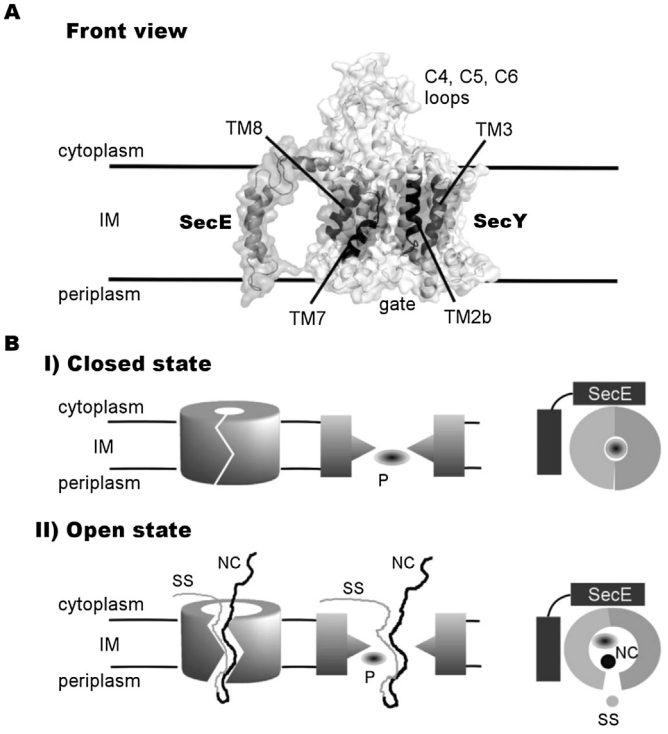

Fig. 2. Structure of the SecYEG translocon. (A) Front view of the SecYE Cryo EM reconstruction within the membrane plane (adapted from Frauenfeld et al., 2011; pdb: 3J01) showing the lateral gate of SecY ('front' of the translocon). SecY is coloured white and SecE dark grey. The TMs of the lateral gate are coloured black. The cytoplasmic loops C4, C5 and C6 of SecY are the major cytoplasmic binding sites of SecY. (B) Schematic front view of SecYE; transverse section through the middle of the pore in the membrane plane and top view from the cytoplasmic side. TMs 1–5 of SecY are indicated in light grey and TMs 6–10 in grey. SecE is coloured dark grey. The plug (P) is indicated. The transverse section shows the pore ring. I) SecY in the closed conformation i.e. the lateral gate is closed and II) SecY in the open conformation i.e. the lateral gate is opened, depicting the current model for the mode of insertion.

forming one half and TMs 6–10 the second half. A side view from the plane of membrane reveals that cytosolic loops 4–6 of SecY are well exposed to the cytosol, which is consistent with their important role as a docking site for targeting factors and the ribosome (Fig. 2B; Cheng et al., 2005; Chiba et al., 2002; Kuhn et al., 2011). The SecY channel has an hour-glass shape with a central constriction that is formed by 6 isoleucine residues. The side chains of these isoleucine residues point towards the centre of the pore and are suggested to be important for maintaining a permeability barrier during preprotein translocation by forming a seal around the translocating polypeptide (Park and Rapoport, 2011). In the 'closed' state of the SecYEG translocon, the periplasmic side of the constriction is blocked by an α-helical plug domain which is an extension of TM2a (Van den Berg et al., 2004; Tsukazaki et al., 2008). The plug domain is displaced during preprotein translocation resulting in the 'open' state of the translocon. Studies indicate displacement of the plug domain towards the SecE subunit of the SecYEG complex (Tam et al., 2005); however conflicting cross-linking data (Lycklama et al., 2010) and Molecular Dynamics simulations (Zhang and

Miller, 2010) suggest that it remains near its original position. While deleting the plug domain does not result in significant defects in protein translocation (Maillard et al., 2007), electrophysiology experiments have shown that plug deletion mutants fluctuate between the open and closed state of the translocon (Saparov et al., 2007). It has also been proposed that in the absence of the plug domain, neighbouring SecY loops can substitute for it (Li et al., 2007). A permanently displaced plug domain however, is toxic to *E. coli* as was shown by disulphide cross-linking (Harris and Silhavy, 1999).

To facilitate insertion of membrane proteins into the IM, the SecYEG translocon opens laterally into the lipid bilayer. An initial indication of this lateral opening came from a study that demonstrated that the signal sequence contacts lipids during insertion (Martoglio et al., 1995; Higy et al., 2005). The crystal structure from *Methanocaldococcus jannaschii* indicates that the insertion of TM domains of membrane proteins into the lipid bilayer occurs via the 'lateral gate', an opening between SecY TMs 2b & 3 and TMs 7 & 8 (Fig. 2B). The lateral gate region is often referred to as the 'front' of the translocon. Lipids have not been observed to enter a SecY channel with an open lateral gate and it appears likely that exiting TMs prevent lipid influx into the channel (Gumbart and Schulten, 2007). Protein translocation is also thought to induce conformational changes at the lateral gate (du Plessis et al., 2009) as the signal sequence of a preprotein intercalates between TM2b and TM7 of the lateral gate (Plath et al., 1998).

The two halves of SecY are embraced at the 'back' by the SecE subunit (Sec61γ in eukaryotes and archaea) of the SecYEG translocon, which is presumed to clamp the two halves together (Fig. 2B). In *E. coli*, SecE is a 14 kDa protein consisting of 3 TM domains. SecE is essential for protein transport because in its absence SecY is instable and rapidly degraded by the membrane protease FtsH (Kihara et al., 1995). As SecE is located at the back of the SecYEG translocon, it was presumed that it is involved in lateral gate opening. However Molecular Dynamics simulations suggest that neither the presence nor the absence of SecE influences gate closure (Gumbart and Schulten, 2007). Much of the N-terminal part of *E. coli* SecE can be deleted without compromising its function (Schatz et al., 1991; Nishiyama et al., 1992), which suggests that at least one half of the proposed clamp is not required for translocon function. This is also observed in other bacteria where their SecE homologues consist of only one TM that is homologous to TM3 of *E. coli* SecE (Murphy and Beckwith, 1994; de Gier and Luirink, 2001).

The third subunit of the Sec translocon differs between the three domains of life. In eukaryotes and archaea this subunit is Sec61β, while a distinct protein SecG constitutes the third subunit of the bacterial Sec translocon (Pohlschröder et al., 2005). The SecG subunit in *E. coli* is a 12 kDa protein with 2 TM helices that occupies a position close to the N-terminal half of SecY (van der Sluis et al., 2002; Satoh et al., 2003). Although SecG is not essential for cell viability or protein translocation in *E. coli*, it was shown to stimulate protein translocation *in vitro* at low temperatures or when the proton-motive force was compromised (Nishiyama et al., 1994; Hanada et al., 1996). The function of SecG was proposed to be directly linked to the SecA-dependent translocation of secretory proteins (Nishiyama et al., 1996). This is consistent with the fact that both SecA and SecG are exclusively present in bacteria (Pohlschröder et al., 2005) and that SecG is not required for the insertion of SecA-independent membrane proteins (Koch and Müller, 2000). The SecA–SecG interaction has been shown to change the proteolysis pattern of SecG and its accessibility to chemical modifications (Nishiyama et al., 1994). Whether this reflects a SecA induced transient topology inversion of SecG or other major conformational changes is still a matter of debate (van der Sluis et al., 2006; Sugai et al., 2007; Morita et al., 2012). However, a topology inversion is not visible in the crystal structures of SecYEG neither in the presence nor in the absence of SecA (Zimmer et al., 2008).

2.2. The oligomeric state of the SecYEG translocon and its modular organisation

A single SecYEG complex is sufficient *in vitro* and *in vivo* for protein translocation and for membrane protein insertion (Cannon et al., 2005; Becker et al., 2009; Kedrov et al., 2011; Frauenfeld et al., 2011; Park and Rapoport, 2012). Nevertheless, monomeric, dimeric and higher oligomeric states of the SecYEG translocon have been observed by native gel electrophoresis (Bessonneau et al., 2002; Boy and Koch, 2009), cross-linking (Veenendaal et al., 2001; Deville et al., 2011), electron microscopy (Hanein et al., 1996; Breyton et al., 2002; Scheuring et al., 2005; Mitra et al., 2005) and other methods (Mori et al., 2003; Tziatzios et al., 2004). The physiological significance of these higher ordered states is still unresolved but it is evident that both lipids (Gold et al., 2010) and the SecYEG concentration within the membrane influence oligomerisation (Manting et al., 2000). Substrate dependent oligomeric states of the SecYEG translocon have been observed even at native concentrations of SecYEG (Boy and Koch, 2009).

The orientation of two SecYEG copies in the proposed SecYEG dimer is controversially discussed. A 'back to back' model has been proposed based on crosslinks between two facing SecE subunits (Kaufmann et al., 1999) and on cryo-EM analyses of two dimensional SecYEG crystals (Breyton et al., 2002). In this orientation, both SecYEG monomers contact each other via a tilted TM of SecE. The alternate 'front to front' view has been suggested by flexible chain fitting of SecYEG into a low resolution electron density map (Mitra et al., 2005). In this orientation, two SecYEG monomers contact each other via the TMs at the lateral gate of SecY, which could result in one large pore formed by two SecYEG molecules (Mitra et al., 2006). Biochemical evidence for a front-to-front orientation comes from an *in vivo* cross-linking study (Das and Oliver, 2011), while another *in vivo* site directed cross-linking study has not found any evidence for SecY–SecY interaction at the lateral gate (Sachelaru et al., unpublished). Finally, a recent *in vivo* cross-linking study

indicates that both orientations can exist transiently in the resting state of the SecYEG translocon (Park and Rapoport, 2012).

The different oligomeric states of the SecYEG translocon that have been reported probably reflect substrate dependent dynamic and modular organisation of the SecYEG translocon. The modular organisation of the translocon is also supported by the number of proteins that have been found to interact at least transiently with the SecYEG translocon (Table 1, Fig. 3). In addition to the well-studied SecY–SecA and SecY-ribosome interactions (Fig. 3A), several additional proteins were found to contact the SecYEG translocon during protein transport:

a) **SecA**, the motor protein of the post-translational pathway, functions as a SecY-bound receptor for secretory proteins and has been found to crosslink to several residues within the cytosolic loops C2–C6 (Mori and Ito, 2006) (Fig. 3A and B). However, it remains unclear as to whether SecA binds as dimer or as monomer to the SecYEG translocon (Sardis and Economou, 2010).
b) The **ribosome** contacts SecY at multiple sites during co-translational targeting. These include both protein–protein contacts as well as protein-RNA contacts (Frauenfeld et al., 2011). The SecY regions contacted by the ribosome include the C4 and C5 loops (Fig. 3A and B; Raden et al., 2000; Cheng et al., 2005; Kuhn et al., 2011) and contacts of SecE to ribosomes have also been observed (Frauenfeld et al., 2011). These interactions appear to be largely conserved between bacteria and eukaryotes.
c) **FtsY**, the SRP-receptor occupies the C4 and C5-loops of SecY (Fig. 3B; Kuhn et al., 2011) and uses the same binding site as SecA. In addition, FtsY makes contact to TM2b of the lateral gate of SecY (Sachelaru et al., unpublished). FtsY occupies the ribosome binding site of SecY and recent data demonstrates that translating ribosomes displace FtsY from SecY (Kuhn et al., unpublished).
d) **SecDFYajC** is a trimeric membrane protein complex that associates with SecY (Sagara et al., 1994; Duong and Wickner, 1997). SecD shows many features of the RND (*resistance-nodulation-cell division*) family of transporters and based on a recent crystal structure of SecDF (Tsukazaki et al., 2011), it has been suggested that SecDF conduct protons from the periplasm to the cytosol. Conformational changes associated with this transfer potentially allow SecDF to pull out substrates from the periplasmic side of the SecYEG channel (Tsukazaki et al., 2011). Details on how SecDFYajC interacts with the SecYEG translocon are missing but due to its low abundance (Pogliano and Beckwith, 1994) only a small fraction of SecYEG translocons are probably in contact with SecDFYajC.
e) **YidC** was initially found to co-purify with over-expressed and purified SecYEG-SecDFYajC (Scotti et al., 2000) and so it was subsequently suggested that YidC binds to SecYEG via SecDFYajC (Nouwen and Driessen, 2002). YidC is believed to facilitate the exit of transmembrane domains from the SecY channel (Urbanus et al., 2001; Beck et al., 2001) and consistent with this, YidC was found to contact the lateral gate at multiple sites independently of SecDF (Fig. 3C; Sachelaru et al., unpublished). Interestingly, the same residues of SecY that contact YidC were also found in contact with the membrane-bound chaperone PpiD (Fig. 3C). PpiD has been implicated in protein translocation (Antonoaea et al., 2008), but details on its function are lacking.
f) **Syd** was found to interact with SecY when over-expressed (Shimoike et al., 1995). Syd is a small protein that binds via a negative surface to the positively charged C4 loop of SecY (Dalal et al., 2009). The exact function of this interaction is unknown but it has been proposed that Syd dissociates the SecYEG complex when SecY–SecE interaction is compromised. Thus, it appears possible that Syd together with FtsH is involved in quality control of the SecYEG translocon.

Table 1
Protein-protein interactions of key components of the Sec transport machinery. Affinities when available are indicated in nM; note that different experimental setups were used for determining these values. (+) indicates that interaction has been observed but affinities not yet determined; (–) interaction has not been observed so far. (n.a.) not applicable. *binding has been observed but functional relevance is unclear. Adapted from: [1]Bornemann et al. (2008), [2]Zhang et al. (2010), [3]Holtkamp et al. (2012), [4]Bahari et al. (2007), [5]Jagath et al. (2000), [6]Peluso et al. (2000), [7]Patzelt et al. (2002), [8]Rutkowska et al. (2008), [9]Huber et al. (2011), [10]Woodbury et al. (2002), [11]Randall and Hardy (2002), [12]Topping et al. (2001), [13]Prinz et al. (2000), [14]Beck et al. (2001), [15]Frauenfeld et al. (2011), [16]Kuhn et al. (2011), [17]Angelini et al. (2005), [18]Douville et al. (1995), [19]Bessonneau et al. (2002), [20]Scheuring et al. (2005), [21]Sagara et al. (1994), [22]Duong and Wickner (1997), [23]Kohler et al. (2009), [24]Welte et al. (2012), [25]Sachelaru et al. (unpublished), [26]Jiang et al. (2003), [27]Lotz et al. (2008).

	Ribo some	RNCs	SRP	FtsY	TF	SecA	SecB	SecYEG	SecDF	YidC
Ribo some	n.a.	n.a.	50–100[1,2]	+*[4]	1000–2000[7,8]	900[9]	–	6[13]	–	+[23, 24]
RNCs	n.a.	n.a	0.5–1[1,2,3]	+*[4]	100–500[8]	200–300[9]	–	+[14, 15]	–	+[23]
SRP	50–100[1,2]	0.5–1[1,2,3]	n.a.	220[6], 24–36[5]	n.a.	n.a.	n.a.	–	–	+[24]
FtsY	+*[4]	+*[4]	220[5], 24–36[6]	n.a.	n.a.	n.a.	n.a.	+[16,17]	–	+[24]
TF	1000–2000[7,8]	100–500[8]	n.a.	n.a.	18,000[7]	n.a.	–	–	–	–
SecA	900[9]	200–300[9]	n.a.	n.a.	n.a.	100[10]	1000–1600[11]	0.02–0.04[18]	–	–
SecB	–	–	–	–	–	1000–1600[11]	+[12]	–	–	–
SecYEG	6[13]	+[14,15]	–	+[16,17]	–	0.02–0.04[18]	–	+[19,20]	+[21,22]	+[25]
SecDF	–	–	–	–	–	–	–	+[21,22]	–	+[26]
YidC	+[23,24]	+[23]	+[24]	+[24]	–	–	–	+[25]	+[26]	+[23,27]

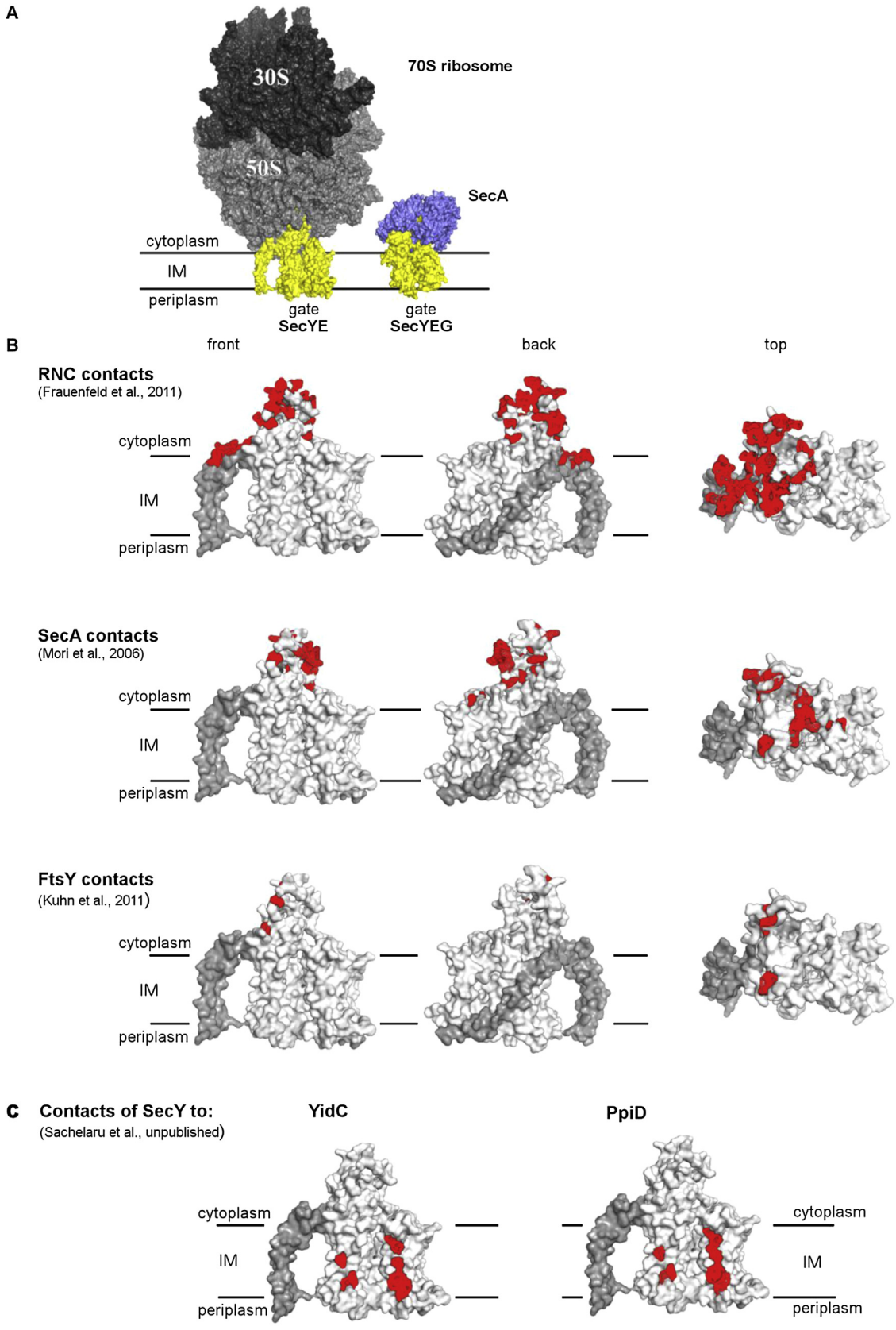

Fig. 3. Protein−Protein interactions at the SecYEG translocon. (A) Cryo EM reconstruction of an *E. coli* ribosome and SecYE (adapted from Frauenfeld et al., 2011; pdb: 3j00, 3J01) on the left and the SecYEG-SecA crystal structure (adapted from Zimmer et al., 2008, pdb: 3DIN) from *Thermotoga maritima* on the right. Note the size differences between the ribosome-SecY complex (co-translational transport) and the SecA−SecY complex (post-translational transport). (B) RNC, SecA and FtsY contacts to SecYE mapped on SecYE (adapted from Frauenfeld et al., 2011, pdb: 3J01) inferred from structural (Frauenfeld et al., 2011) and crosslinking data (Mori and Ito, 2006 and Kuhn et al., 2011). SecY is coloured white, SecE grey and their contact sites black. Front view and back view of the SecYE and top view from the cytoplasmic side. **(C)** Two of the contact partners at the lateral gate of SecY are YidC and PpiD. SecY has been adapted from a cryo EM reconstruction of SecYE (Frauenfeld et al., 2011, pdb: 3J01) and is coloured white, SecE grey and the contact sites black.

A recent report on the integration of the membrane-bound Rieske iron-sulfur protein of *Streptomyces coelicolor* indicates that the Sec translocon also cooperates with the Tat translocase during integration (Keller et al., 2012). Although it is unknown whether this involves a physical contact between the two translocases, it further substantiates that protein transport in bacteria is organised very flexibly and employs different modules depending on the exact properties of the substrate protein.

2.3. Protein targeting to the SecYEG translocon and membrane transport

The SecYEG translocon is engaged by two different targeting pathways; inner membrane proteins are predominantly targeted co-translationally by the signal recognition particle (SRP), while secretory proteins, i.e. proteins that are destined to reside in the periplasm or the outer membrane are targeted post-translationally by the SecA/SecB pathway (Fig. 1). The decision as to whether a protein is routed into either the co-translational or the post-translational pathway is probably made at the ribosomal tunnel exit, which is why translating ribosomes associate with targeting factors in the cytosol (Fig. 4). Routing of proteins into either of the two pathways is aided by the presence of a unique identification tag, the signal sequence, which is recognised by targeting factors and initiates the process of protein transport. Secretory proteins that are transported across the inner membrane via the SecYEG translocon have an N-terminal signal sequence that consists of a positively charged N-region, a central hydrophobic H-region, and a polar C-terminal region that contains the signal peptidase cleavage site (von Heijne, 1990). Signal sequences are first inserted into the membrane with the positively charged N-terminal side oriented towards the cytoplasm. The soluble part of the protein is then transported across the membrane via an interaction between SecA and SecYEG and ATP hydrolysis by SecA. The signal sequence is subsequently cleaved by membrane embedded signal peptidases (Paetzel et al., 2002).

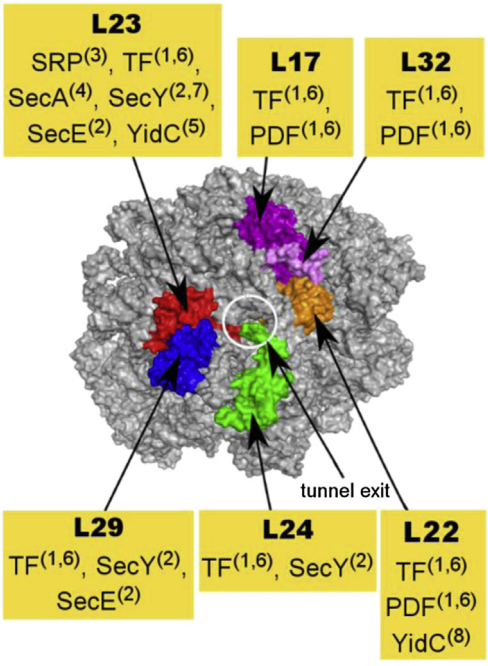

Fig. 4. The ribosome as a docking site of targeting factors, chaperones and processing enzymes. Cryo EM reconstruction of an *E. coli* ribosome (adapted from Frauenfeld et al., 2011; pdb: 3j00, 3j01) showing the ribosomal tunnel exit and its surrounding proteins L17, L22, L23, L24, L29 and L32. Protein–protein interactions have been deduced from structural and cross-linking data and are indicated by arrows (PDF, peptidyl deformylase; SRP, signal recognition particle; TF Trigger Factor). Contacts to RNA have been omitted for better understanding. Adapted from [(1)]Bingel-Erlenmeyer et al., 2008, [(2)]Frauenfeld et al., 2011, [(3)]Gu et al., 2003, [(4)]Huber et al., 2011, [(5)]Kohler et al., 2009, [(6)]Kramer et al., 2002, [(7)]Kuhn et al., 2011, [(8)]Welte et al., 2012.

Signal sequences of membrane proteins are generally more hydrophobic α-helical transmembrane domains and in most cases do not contain a signal peptidase cleavage site. Instead, the uncleaved signal sequence serves to anchor an inserting membrane protein into the lipid bilayer and is hence referred to as a signal anchor (SA) sequence. SA sequences of membrane proteins are recognised by the bacterial SRP, which binds hydrophobic stretches of amino acids (Luirink et al., 2005; Valent et al., 1997) and α-helical transmembrane (TM) domains of membrane proteins close to the N-terminal region of the protein (Beck et al., 2000; Houben et al., 2002; Welte et al., 2012). The affinity of SRP for ribosome-associated nascent chains (RNCs) varies between 0.5 and 1 nM for the SA sequence of the membrane protein leader peptidase (Lep) (Table 1; Bornemann et al., 2008) and 80–100 nM for the cleavable signal sequence of the periplasmic protein alkaline phosphatase (Zhang et al., 2010). High-affinity binding of SRP to RNCs is therefore determined at least in part by hydrophobicity, which complements early cross-linking studies (Valent et al., 1998; Neumann-Haefelin et al., 2000). Binding of SRP to hydrophobic SA sequences also influences complex formation with the SRP receptor FtsY. It appears likely that SRP scans all translating ribosomes until the emergence of a signal anchor sequence facilitates the targeting reaction by accelerated binding of SRP to FtsY (Holtkamp et al., 2012).

2.3.1. The ribosome as docking site for targeting factors and chaperones

Proteins are synthesised at a rate of 10–20 amino acids per second (Talkad et al., 1976), the ribosomal tunnel has a length of approx. 100 Å (Nissen et al., 2000) and a single amino acid occupies approx. 3.5 Å. Thus, within a few seconds after translation is initiated, the polypeptide, given that it is in extended conformation, starts to emerge from the ribosomal exit tunnel. After an additional 1–2 s the N-terminal signal/SA sequences should be fully exposed to the outside. To avoid undesired interactions of these hydrophobic sequences, ribosomes have evolved a mechanism for early substrate recognition by acting as a platform for kinetic and spatial coordination of modifying enzymes, targeting factors and chaperones (Fig. 4). This guarantees the correct subcellular localisation of a substrate.

Substrate discrimination could perhaps even occur before the polypeptide emerges from the tunnel. It has been suggested that the initial signal for downstream targeting from a translating ribosome could be the organisation of a nascent polypeptide into secondary structures within the ribosomal tunnel (Woolhead et al., 2004; Peterson et al., 2010). Hydrophobic signal sequences of proteins form helix-like structures within the ribosomal exit tunnel (Woolhead et al., 2004; Halic et al., 2006) and this could be the initial step towards substrate discrimination within the ribosome. Consistent with this hypothesis is data from recent studies, which indicates that intra-tunnel structural differences of the nascent chain differentially affect the recruitment of SRP or the chaperone Trigger Factor (TF) to the ribosome (Peterson et al., 2010; Lin et al., 2012). Signalling from the interior of tunnel to the surface may therefore be an important determinant for the subsequent targeting reaction. The deletion of an intra-tunnel loop of the protein L23 which probably interacts with a translating protein, delays translation sensing by SRP (Bornemann et al., 2008), and subsequently interferes with protein translocation (Huber et al., 2011). Downstream of the initial recognition event, studies in eukaryotes suggest that the ribosome-bound translocon already opens when the first TM of a nascent membrane protein is still buried inside the ribosomal tunnel (Liao et al., 1997).

SRP in *E. coli* has a high affinity for translating ribosomes, regardless of whether the synthesized protein bears a SA sequence (Table 1; Bornemann et al., 2008). However, when a translating protein further emerges from the tunnel and bears no SA sequence, the affinity of SRP for translating ribosomes decreases rapidly to >200 nM, which is lower than its affinity for empty ribosomes (K_d = 50–70 nM) (Bornemann et al., 2008).

The molecular motor protein SecA has a lower affinity for ribosomes than SRP, binding with a 900 nM affinity to non-translating ribosomes, which increases 3–5 fold for translating ribosomes (Table 1; Huber et al., 2011). The latter finding is unexpected since SecA has so far been considered to function strictly post-translationally, i.e. after the substrate is released from the ribosome (Driessen and Nouwen, 2008; Rapoport, 2007). On the other hand, this observation explains previous reports that have shown that SecA binds to ribosome-associated nascent chains of a secretory protein in an ATP-independent manner (Chun and Randall, 1994; Eisner et al., 2003; Karamyshev and Johnson, 2005). It is not yet entirely clear how SecA targets substrates to the translocon after an initial co-translational recognition event. Huber et al. (2011) have observed retardation in protein translocation across the membrane when the ribosome binding residues of SecA are mutated. SecA has been found to compete with RNCs for the common ribosome binding site on SecYEG (Wu et al., 2012), consistent with a study that shows that SecA and the ribosome have common binding sites on the SecYEG translocon (Kuhn et al., 2011). It now remains to be determined whether co-translational recognition of substrates by SecA also induces their co-translational targeting to the SecYEG translocon. These recent findings corroborate that protein transport in bacteria does not follow a 'black or white' scheme but flexibly adjusts to the cell's requirements.

The cytosolic chaperone Trigger Factor (TF) has been previously proposed to be the first protein to contact an emerging polypeptide (Deuerling et al., 1999; Eisner et al., 2003). TF functions as a holdase and defoldase, which prevents premature folding of the RNC (Hoffmann et al., 2012) and also has downstream activities of chaperoning proteins released from ribosomes that are either cytosolic or need to be translocated across the inner membrane post-translationally. The activities of TF are promoted by ribosome binding, which maintains the chaperone in the vicinity of emerging nascent proteins (Hoffmann et al., 2012). TF binds to empty ribosomes with an approximate K_d of 1 μM (Paetzel et al., 2002). The affinity of TF for ribosomes increases around 30 fold during translation (Rutkowska et al., 2008), indicating its role in co-translational folding of proteins. Trigger Factor has been proposed to cooperate with downstream chaperones such as DnaK and GroEL (Lakshmipathy et al., 2007) during the folding of soluble proteins. It is suggested to hold substrates in a translocation competent state, which allows their channelling into the post-translational transport pathway. This would complement a recent quantitative proteomics approach, which identified mainly outer membrane proteins as TF substrates (Calloni et al., 2012). On the other hand, *in vitro* studies have argued that TF binds to all substrates and gets substituted by SRP in the presence of a SA sequence (Beck et al., 2000; Houben et al., 2005; Ullers et al., 2003; Eisner et al., 2003). Finally, recent TF-specific ribosome profiling data indicate that TF binds to all newly synthesised proteins except for those that are recognised by SRP (Oh et al., 2011). The same study also indicates that the recruitment of TF to nascent chains *in vivo* occurs only after 100 amino acids are synthesised (Oh et al., 2011). This is in contrast to previous *in vitro* crosslinking data that had indicated that the signal sequence contacts TF at a length of 70–80 amino acids (Valent et al., 1995; Hesterkamp and Bukau, 1996; Eisner et al., 2003).

SRP, SecA and TF all contact the conserved ribosomal protein L23 located close to the ribosomal tunnel exit (Fig. 4; Gu et al., 2003; Kramer et al., 2002; Ullers et al., 2003; Ferbitz et al., 2004; Baram et al., 2005; Schlünzen et al., 2005; Huber et al., 2011). L23 is also involved in binding peptide deformylase (PDF), the first enzyme to process nascent chains (Kramer et al., 2009), SecY (Kuhn et al., 2011) and YidC (Kohler et al., 2009). L23 therefore appears to constitute a major docking site for nascent chain interacting proteins.

How exactly the ribosome co-ordinates the binding of targeting factors, chaperones, processing factors and the translocon in time and space is currently unknown. Translation speed regulated via codon usage or stalling (Zalucki et al., 2011) might be an important determinant (Zhang and Ignatova, 2011), as the binding of SRP to SA sequences appears to be influenced by translation speed (Zhang and Shan, 2012). Translation speed also probably determines the orientation of TMs inside the translocon (Goder and Spiess, 2003; Zhang and Miller, 2012).

2.3.2. Co-translational targeting by SRP and its receptor FtsY

SRP is a universally conserved and essential ribonucleoprotein complex that in *E. coli* consists of the protein Ffh and the 4.5S RNA (Koch et al., 2003; Kuhn et al., 2013). Ffh and the SRP receptor FtsY belong to the SIMIBI-family (after signal recognition particle (SRP), MinD and BioD) of nucleotide-hydrolysing enzymes and are similarly organised. Both Ffh and FtsY are composed of three domains each and their respective N and G-domains are highly conserved (Fig. 5A). The N-domain forms a four-helix bundle, while the G-domain harbours a Ras-like GTPase-domain. The G-domain also contains a unique insertion box (I-box), which facilitates nucleotide exchange and stabilises the nucleotide-free protein (Moser et al., 1997; Rapiejko and Gilmore, 1997). As a result neither Ffh nor FtsY depends on external Guanine nucleotide Exchange Factors (GEFs). Complex formation between SRP and FtsY proceeds via their NG-domains (Fig. 5B) and leads to the formation of an active site that promotes reciprocal GTP hydrolysis (Focia et al., 2004; Egea et al., 2004). Thus, FtsY and Ffh belong to a growing number of GTPases that are activated by nucleotide-dependent dimerisation (GADs, Gasper et al., 2009). The third domains of both proteins i.e. the M-domain of Ffh and the A-domain of FtsY show no similarity to each other, but serve as function-related moieties that are fused to the conserved NG-core.

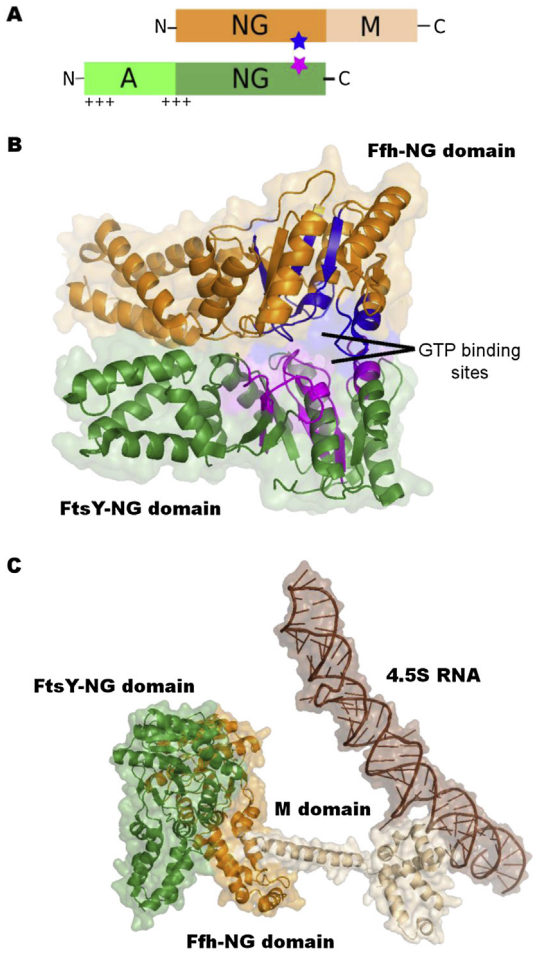

Fig. 5. Structure of the signal recognition particle (SRP) and its receptor FtsY. (A) Schematic representation of the domain organisation and interaction of Ffh, the protein component of the bacterial SRP and of FtsY. The proteins interact via their NG domains. The GTP binding sites of both proteins are indicated by stars. The charged lipid binding domains of FtsY are represented by (+++). (B) The crystal structure shows the Ffh NG domain (orange) complexed with FtsY NG domain (green) (adapted from Focia et al., 2004; pdb: 1OKK). (C) SRP-FtsY crystal structure (adapted from Ataide et al., 2011; pdb: 2XXA), where Ffh is coloured orange, 4.5S RNA brown and FtsY green.

The C-terminal M-domain of Ffh is responsible for binding substrates via a substrate binding groove (Fig. 5C; Zheng and Gierasch, 1997; Batey et al., 2000) and it also interacts with the 4.5S RNA via a helix-turn-helix motif, located opposite the hydrophobic substrate binding groove. The substrate binding region is characterised by an unusually high number of methionine residues in mesophilic bacteria (Bernstein et al., 1989), and these are suggested to provide a flexible hydrophobic surface for interaction with signal sequences.

Cryo-EM studies on SRP-RNCs have identified an electron dense region in the signal sequence binding groove, which most likely can be mapped to a signal sequence (Halic et al., 2004; Schaffitzel et al., 2006). Two recent crystal structures of a signal sequence in complex with SRP have in fact confirmed that an α-helical signal sequence binds to the hydrophobic groove and that there are significant conformational changes of SRP upon signal sequence recognition (Janda et al., 2010; Hainzl et al., 2011). SA sequence bound SRP has an extended conformation bringing the GTPase domain closer to the tetraloop region of the 4.5S RNA (Janda et al., 2010; Hainzl et al., 2011). In this conformation, the RNC-bound SRP is probably primed for subsequent interaction with FtsY (Zhang et al., 2009; Ataide et al., 2011).

The high affinity binding of SRP-RNCs to the SRP receptor FtsY facilitates membrane delivery of the RNCs. Although FtsY lacks a TM domain, approx. 90% of all FtsY molecules are found at the membrane (Mircheva et al., 2009). The A-domain of FtsY which is fused to the N-terminus of the conserved NG-domains is intrinsically unfolded, highly charged and is responsible for strong membrane association (de Leeuw et al., 2000; Weiche et al., 2008; Braig et al., 2009; Parlitz et al., 2007). FtsY in *E. coli* is equipped with two lipid binding helices; one of which is present at the N-terminus and the other at the interface of the A and N domains. These lipid binding helices have been found to bind to negatively charged lipids like phosphatidylglycerol and cardiolipin (Braig et al., 2009). One of the several FtsY-SecY binding sites is sandwiched between the two lipid binding helices (Kuhn et al., 2011).

FtsY occupies part of the ribosome binding site on SecY, which ensures efficient delivery of the RNCs directly to the integration site. Recent data demonstrate that incoming SRP-RNCs displace FtsY from SecY (Kuhn et al., unpublished), facilitating co-translational protein insertion via ribosome binding to the SecY channel. Co-translational protein insertion is further promoted by the interaction between SRP-RNCs and FtsY, resulting in the formation of a SRP-FtsY heterodimer, which in turn induces reciprocal GTPase activation. GTP hydrolysis leads to the dissociation of the two proteins and

binding of the ribosome-nascent chain complex to the SecYEG translocon (Miller et al., 1994; Grudnik et al., 2009). Due to the limited number of SecYEG translocons in *E. coli* (Table 2; Drew et al., 2003), only approx. 5% of all FtsY molecules can be in contact with SecY, while the majority are bound to the negatively charged phospholipid surface of the membrane. The large fraction of soluble FtsY that has been observed after cell fractionation (Luirink et al., 1994) is most likely an artefact, because FtsY *in vivo* appears to be exclusively membrane-bound (Mircheva et al., 2009). It is also important to emphasise that the A-domain is found mainly in proteobacteria, while other prokaryotes have shorter FtsY-derivatives, consisting of only the NG-domain and one lipid-binding helix (Weiche et al., 2008; Braig et al., 2009; Mircheva et al., 2009).

A certain degree of flexibility exists in the classical SRP pathway of protein targeting. Braig et al. (2011) have shown that pre-existing complexes of SRP and FtsY are present at the membrane and are formed in the absence of SA sequences. These pre-formed complexes are able to accept RNCs and transfer them to the SecYEG translocon. Thus, substrate recognition can occur either in the cytosol via ribosome-bound SRP or at the membrane via a preformed SRP-FtsY complex. Studies also indicate that mRNA of membrane proteins can be targeted and localised to the inner membrane where ribosomes are present in the environment of the translocon (Nevo-Dinur et al., 2011). This data is consistent with earlier data from eukaryotes where mRNA was observed to be localised at the ER membrane even upon removal of ribosomes (Lande et al., 1975). mRNA targeting also had been shown for proteins in neuronal cells, where an anterograde transport takes place along the axon (Manseau, 2001; Yoon et al., 2009). However, mRNA targeting raises the question as to how ribosomes are targeted to the membrane in order to translate the membrane localised mRNA. One possibility is put forth by Bibi (2012), who proposes that ribosomes are targeted to the inner membrane while translating FtsY. These ribosomes then sit at the SecYEG translocon, translate the targeted mRNA of membrane proteins and protein insertion occurs subsequently. This targeting pathway would bypass the need for SRP mediated targeting and needs further investigation.

Table 2
Estimated number of proteins associated with protein transport in the *E. coli* cell. As the amount of ribosomes, translocases, targeting factors etc. is most likely need based, numbers will vary depending on the cell and growth phase. The numbers refer to the monomer. Adapted from [(1)]Drew et al., 2003, [(2)]Jensen and Pedersen, 1994, [(3)]Teter et al., 1999, [(4)]Woodbury et al., 2000, [(5)]Sachelaru et al., unpublished, [(6)]Matsuyama et al., 1992, [(7)]the concentration was calculated assuming a volume of $1 \cdot 10^{-15}$ L for the bacterial cytoplasm (cytosol + inner membrane). (Moran et al. (2010) *dependent on the number of ribosomes.

		Molecules/cell	Concentration
Ribosomes		20,000–30,000[(1)]	33–50 μM[(7)]
SRP	FFh	200–300*[(2)]	0.3–0.5 μM[(7)]
	4.5 sRNA	800–1200*[(2)]	1.3–2 μM[(7)]
FtsY		10,000[(1)]	17 μM[(7)]
TF		20,000[(3)]	33 μM[(7)]
SecA		2000–5000[(1,6)]	4 μM[(4)]
SecB		12,000[(7)]	20 μM[(4)]
SecYEG		200–600[(1,6,7)]	1 μM[(5)]
SecDF		20–40[(1,6)]	0.03–0.06 μM[(7)]
YidC		2500–3000[(1,7)]	5 μM[(5)]

2.3.3. Mechanisms of membrane protein insertion via SecYEG

Once RNCs are targeted to the inner membrane via the SRP-FtsY pathway, the translating ribosome aligns with the SecY channel (Figs. 1 and 3; Beckmann et al., 1997; Becker et al., 2009; Frauenfeld et al., 2011) via the C4–C6 loops of SecY (Cheng et al., 2005; Ménétret et al., 2007; Becker et al., 2009) and the ribosomal proteins L23, L24 and L29. Additionally, the C4 and C5 loops of SecY contact the 23S rRNA in the ribosomal exit tunnel. Ribosomes also contacts lipids, which may prepare the membrane environment in front of the lateral gate of the SecY translocon for protein insertion (Frauenfeld et al., 2011). These contacts are believed to result in the switching of the translocon to the pre-open state, where the lateral gate partially opens, but the central channel remains closed by the plug domain (Becker et al., 2009). Simultaneously the central pore in SecY also starts to widen (Egea and Stroud, 2010). The SA sequence of the inner membrane protein (IMP) then inserts into the SecY channel and moves towards the lateral gate at helices 2b and 7 in SecY. This is probably followed by displacement of the plug domain and switching of the translocon to the open state. The protein then exits via the lateral gate and inserts into the lipid bilayer co-translationally.

Lipid insertion of membrane proteins has been proposed to be mediated by two factors: (1) By pulling forces exerted by the translocon and its surrounding lipid bilayer on the SA sequence and the subsequent TM domains (Ismail et al., 2012) and (2) By YidC, which has long been thought to interact with SecYEG to mediate the lipid insertion of multi-spanning membrane proteins (Beck et al., 2001; Houben et al., 2004). Consistent with this idea, recent data demonstrate that YidC is indeed located in front of the lateral gate and undergoes displacement upon emergence of a SA sequence (Sachelaru et al., unpublished).

YidC also facilitates the assembly and folding of membrane proteins (Nagamori et al., 2004; Wagner et al., 2008). YidC is an essential protein, yet most multi-spanning membrane proteins insert correctly into SecYEG proteoliposomes in the absence of YidC (Braig et al., 2011; Welte et al., 2012). It appears likely that YidC exerts a kinetic effect on lipid insertion of TMs that exit the SecYEG translocon without being essential *in vitro*. However, other membrane proteins strictly depend on both SecYEG and YidC (Yi et al., 2003; du Plessis et al., 2006; van Bloois et al., 2006). It has been suggested that the hydrophobicity of a TM and the charge of the periplasmic loop determines whether a membrane protein requires the assistance of YidC during insertion (Price and Driessen, 2010; Gray et al., 2011; Neugebauer et al., 2012).

TM domains are usually inserted into the lipid bilayer sequentially as they are synthesised (Sadlish et al., 2005; Houben et al., 2005), but weakly hydrophobic TMs can also

insert together with a more hydrophobic preceding TM (Heinrich and Rapoport, 2003). In this case, more than one transmembrane domain needs to be assembled at the translocon before being released *en bloc* into lipids. A recent FRET approach shows that TMs can be retained at the translocon by protein–protein interactions until their release is triggered by translation termination or by arrival of a new nascent chain (Hou et al., 2012).

The insertion of membrane proteins with long periplasmic loops has been shown to be dependent on both SRP and SecA (Sääf et al., 1995; Neumann-Haefelin et al., 2000; Deitermann et al., 2005; Welte et al., 2012). Periplasmic loops longer than 30 amino acids require the coordinated activity of the ribosome and SecA for their translocation. This coordinated activity of SecA and the ribosome in aiding translocation is puzzling, especially as SecA and the ribosome use overlapping binding sites on SecY (Kuhn et al., 2011) and their binding has been shown to be mutually exclusive (Wu et al., 2012). Thus, for a single-spanning membrane protein, SecA probably translocates the periplasmic domain after translation termination. How SecA mediates translocation of periplasmic loops of multi-spanning membrane proteins is currently unknown, but it appears possible that SecA binds to periplasmic loops while the ribosome is still attached to the nascent membrane protein (Deitermann et al., 2005; Antonoaea et al., 2008). This suggests that the ribosome could dissociate and subsequently re-bind to the SecY translocon during co-translational membrane insertion. A similar situation has been envisioned for some periplasmic proteins that are targeted co-translationally by SRP, but depend on SecA for complete transport (Huber et al., 2005).

2.3.4. Post-translational targeting by SecA/SecB

Secretory proteins are transported across the inner membrane post-translationally, i.e. they are fully synthesised prior to transport. The classical post-translational pathway proposes that these proteins are in contact with chaperones such as SecB or TF, which maintain them in a translocation competent state in the cytosol, as they are targeted to the inner membrane (Fig. 1; Watanabe and Blobel, 1989). SecB is a 17 kDa, tetrameric chaperone (Xu et al., 2000) that is exclusively present in proteobacteria (de Cock and Tommassen, 1991; van der Sluis and Driessen, 2006). It has been suggested to hold nascent proteins in a translocation competent state (Randall et al., 1998) until they are delivered to SecYEG. The binding of SecB to nascent proteins was thought to assist in the targeting process, since SecB has a high affinity for SecA (Table 1; Hartl et al., 1990) and was shown to bind to the C-terminus of SecA. The interaction between SecB and SecA potentially dissociates the preprotein from SecB and binding of the signal sequence to SecA is then required to ensure efficient transfer of the preprotein to SecYEG (Fekkes et al., 1998). SecB has also been thought to be released from the translocase at an early stage of translocation when SecA binds to ATP (Fekkes et al., 1997).

SecA is a 100 kDa molecular motor protein that is essential for the translocation of proteins across the inner membrane. It exists in a soluble state in the cytosol, but is also membrane-bound (Cabelli et al., 1991). Membrane binding of SecA is similar to the membrane binding of FtsY, i.e. it involves negatively charged phospholipids (Lill et al., 1990) and the cytosolic loops of SecY (Hartl et al., 1990; Mori and Ito, 2006; Das and Oliver, 2011). The affinity of SecA for SecY is much higher than its affinity for a pre-protein in the cytoplasm (Gouridis et al., 2009) and SecA is therefore considered to function as a soluble receptor subunit of the SecYEG translocon. Once at the inner membrane, proteins are secreted via the SecYEG translocon following repeated cycles of ATP hydrolysis by SecA. The proton-motive force (PMF) serves as an additional driving force, and is thought to determine the correct orientation of the signal sequence inside the channel and to influence channel opening (Tani et al., 1989).

Each SecA molecule is organised into six distinct domains (Fig. 6) (Kusters and Driessen, 2011; Zimmer and Rapoport, 2009; Papanikolau et al., 2007). The motor function of SecA is executed by the N-terminal nucleotide-binding domain I (NBD1) together with a second nucleotide-binding domain 2 (NDB2). The NBD1/NBD2 interface forms the binding site for a single ATP molecule. ATP hydrolysis triggers conformational changes in the motor domain as well as in the peptide-binding domain (PBD; also called *preprotein-cross-linking domain*, PPXD). The PBD consists of an antiparallel β-strand and a globular region and is involved in substrate and translocon binding (Zimmer et al., 2008). The PBD is located between the two NBDs and forms the substrate binding clamp together with NBD2 and helical scaffold domain (HSD), (Cooper et al., 2008; Zimmer and Rapoport, 2009). The HSD is followed by the helical wing domain (HWD) and finally by the C-terminal domain (CTD). The latter is not essential for catalysis, but has been suggested to inhibit futile cycles of ATP-hydrolysis in the absence of the SecYEG translocon (Keramisanou et al., 2006). CTD is also important for SecB and phospholipid binding (Lill et al., 1990; Breukink et al., 1995). All SecA domains with the exception of HWD are involved in the interaction with SecY (Fig. 3B) (Mori and Ito, 2006; Zimmer et al., 2008; Das and Oliver, 2011). In most structures, SecA forms an antiparallel dimer, but whether the SecA-dimer is the active species in protein translocation is still controversially discussed (Jilaveanu et al., 2005; Or and Rapoport, 2007; Kusters and Driessen, 2011).

2.3.5. Mechanism of SecA-dependent translocation of secretory proteins through SecYEG

After initial ATP binding to SecA and pre-protein release from SecB, the signal sequence of the pre-protein acquires a α-helical conformation (Chou and Gierasch, 2005) that probably binds to the substrate binding clamp of SecA (Zimmer et al., 2008). Simultaneous conformational changes cause SecA to penetrate deeper into the channel, where the signal sequence is intercalated at the lateral gate of SecY (Papanikou et al., 2007), while the downstream segment resides inside the pore. The interaction of the signal sequence with the lateral gate probably also induces conformational changes of the plug

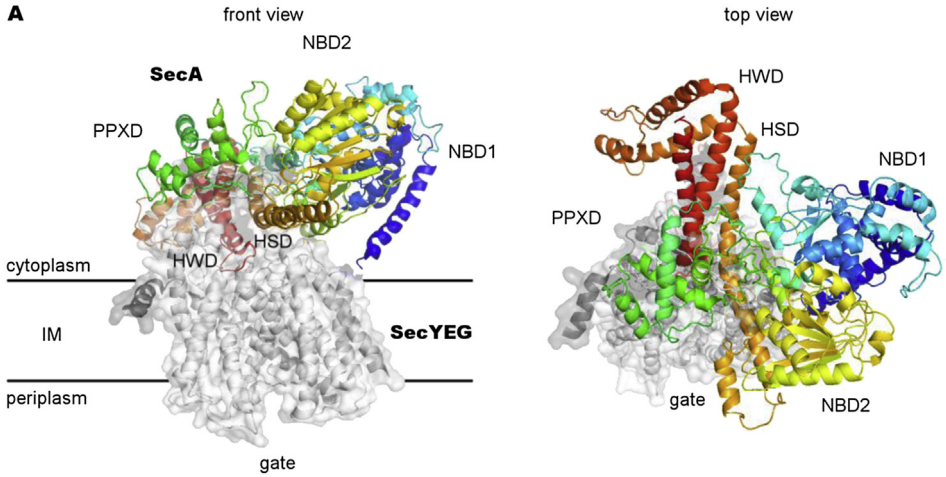

Fig. 6. Structure of SecA in complex with the SecYEG translocon. Crystal structure (adapted from Zimmer et al., 2008; pdb: 3DIN) of a SecY–SecA complex from *Thermotoga maritima*. SecY is coloured white, SecE dark grey and SecG black. The domains of SecA are indicated (NBD: Nucleotide binding domain, HWD: helical wing domain, HSD: helical scaffold domain, PPXD: prepeptide-cross-linking domain). The figure on the left shows a view from the membrane plane and the figure on the right a top view from the cytoplasmic side.

and the pore-ring, which results in channel opening. ATP is then hydrolysed, the substrate is released and the SecA–SecY interaction weakened. Whether SecA completely dissociates from SecY at this stage is unclear. This translocation initiation phase is then followed by the next ATP binding and hydrolysis cycle of SecA, which pushes approx. 5 kDa (~30–40 amino acids) of the preprotein across the channel (Schiebel et al., 1991). Repeated cycles of ATP hydrolysis are thought to cause a step-wise translocation of the pre-protein across the channel, which would be consistent with the observation that the time required for translocation is proportional to the length of a substrate (Tomkiewicz et al., 2006).

Different models have been proposed for explaining how conformational changes within SecA drive protein translocation:

a) The *power-stroke model*: In this model, the two-helix finger motif of the SecA HSD is proposed to reach into the SecY channel and provide the mechanical force that pushes the substrate across the channel (Erlandson et al., 2008). However, recent data indicate that movement of the two-helix finger is not required for protein translocation (Whitehouse et al., 2012).
b) The *Brownian ratchet* model: In this model, SecA is thought to primarily trap the retrograde movement of an unfolded translocating peptide, which moves via Brownian motion through the channel. ATP-dependent trapping by SecA would determine the directionality of transport (Tomkiewicz et al., 2007).
c) The *piston or molecular peristalsis* model: This model is based on the SecA-dimer structure from *Mycobacterium tuberculosis* (Sharma et al., 2003) and proposes that conformational changes at the SecA dimer interface drive polypeptide movement and channel opening (Mitra et al., 2006). A major difference to the power stroke model is that the substrate would probably be trapped in a central pore, which is formed by a SecA-dimer. Translocation of the substrate would then depend on both Brownian motion and conformational changes in SecA dimer.

None of these models is currently accepted, because (1) ATP-dependent movement of the two-helix finger has not been shown so far, (2) The Brownian ratchet model does not explain step-wise translocation and (3) The presence of a front-to-front orientation and a SecA dimer during translocation is controversially discussed. Thus further experimental evidence is required for validating these models.

Protein transport channels in eukaryotic cells usually employ additional motor proteins that function *in trans* of the translocases, *e.g.* the HSP70 homologue BiP in the ER lumen (Zimmermann et al., 2011) or the Pam machinery in the mitochondrial matrix (van der Laan et al., 2006). ATP-dependent chaperones do not exist in the bacterial periplasm, but the periplasm of *E. coli* contains a number of chaperones that show a high degree of functional redundancy. The chaperones include Peptidyl Prolyl Isomerases (PPIases) such as SurA, FkpA, PpiA and PpiD and small chaperones like Skp. Skp has been shown to contact a secreted outer membrane protein as it is transported through the SecY channel (Schäfer et al., 1999). It is thought to function as a pair of prongs that prevent aggregation of proteins in the periplasm, thereby functioning as a periplasmic 'holdase'. Other chaperones such as PpiA, FkpA and PpiD have so far not been demonstrated to influence OMP transport or assembly. However, data indicate that PpiD is present at the lateral gate of SecY (Sachelaru et al., unpublished). Its location suggests that it might be involved in protein transport. However, it has no role in the insertion of membrane proteins *in vitro* (Renuka Kudva, unpublished).

3. The YidC insertase

While it is generally believed that the majority of inner membrane proteins are inserted via SecYEG, some inner

membrane proteins seem to use YidC as an alternate insertion site (Fig. 1; Wang and Dalbey, 2011; Dalbey et al., 2011; Welte et al., 2012). This was first described for small phage proteins (Samuelson et al., 2000) and later for native *E. coli* inner membrane proteins (Dalbey et al., 2011). Recent *in vitro* data demonstrates that SRP-dependent multi-spanning membrane proteins that were so far considered to be exclusively inserted via SecYEG can also be inserted via YidC (Welte et al., 2012). Only SecA-dependent membrane proteins appear to be strictly dependent on SecYEG for insertion. This suggests that the percentage of membrane proteins that is inserted via YidC is probably much higher than previously anticipated. The use of YidC as alternate integration site would also prevent a situation in which the majority of SecYEG complexes would be occupied by translating ribosomes and thus not accessible for secretory proteins. The observation that multi-spanning membrane proteins can be inserted via YidC is not completely unexpected, given that the depletion of YidC in cells preferentially affects the insertion of multi-spanning membrane proteins without long periplasmic loops (Wickström et al., 2011). The members of the YidC family of proteins have been described and characterised in chloroplast membranes (Alb3), mitochondria (Oxa1) and bacteria (Funes et al., 2011). In archaea, hypothetical proteins with sequence homology to YidC have been identified (Yen et al., 2001), but not yet characterised. YidC is essential in bacteria and its depletion in *E. coli* results in global changes in cell physiology (Wang et al., 2010; Price et al., 2010; Wickström et al., 2011). *E. coli* YidC consists of 6 TM domains and TM2, TM3 and TM5 are involved in substrate binding (Klenner and Kuhn, 2012). Structural information about YidC is so far limited to the X-ray structure of the large periplasmic loop (Ravaud et al., 2008; Oliver and Paetzel, 2008), which connects TM1 and TM2. The periplasmic loop was seen to crystallise as a dimer, where each monomer was characterised by a β-supersandwich folding motif and a C-terminal α-helical region. A potential substrate binding site was occupied by polyethylene glycol in the X-ray structure, which could indicate that peptides or acyl side chains interact with the periplasmic loop (Ravaud et al., 2008). Tryptophan fluorescence measurements show conformational changes of the periplasmic loop upon binding of Pf3, a phage protein that is inserted via YidC (Imhof et al., 2011). Nevertheless, all available data indicate that the periplasmic loop is not essential for YidC function in *E. coli* (Jiang et al., 2003) and its exact function still needs to be defined.

Targeting of many membrane proteins to YidC is mediated by the SRP/FtsY pathway (Facey et al., 2007; Neugebauer et al., 2012; Welte et al., 2012), consistent with the theory that most membrane proteins are recognised by SRP and then targeted to the next available insertion site which is either SecYEG or YidC (Welte et al., 2012). A co-translational, SRP-dependent targeting of substrates to YidC is also supported by a cryo-EM study on RNC-YidC complexes (Kohler et al., 2009) and by cross-linking studies, which show that ribosomal subunits, SRP and FtsY interact with the C-terminus of YidC (Table 1; Welte et al., 2012). For those membrane proteins that are exclusively inserted via YidC, the targeting pathway is less clear. The phage proteins M13 and Pf3 are too short to allow co-translational SRP interaction and their targeting does not require SRP/FtsY. Nevertheless, the transmembrane helix of Pf3 can be cross-linked to SRP if it is fused to leader peptidase (Chen et al., 2002). The F_oc subunit of ATPase is also inserted exclusively via YidC, but SRP dependence of this substrate has been controversially discussed (van Bloois et al., 2004; Yi et al., 2004; van der Laan et al., 2004). Thus it is possible that substrates, which can be inserted via either YidC or SecYEG are generally recognised by SRP, while substrates which are exclusively inserted via YidC escape SRP recognition.

The mechanism of membrane insertion via YidC is largely unknown. A YidC monomer or dimer could contain an insertion pore as suggested based on a low-resolution projection structure of YidC (Lotz et al., 2008). It is also possible that YidC just provides a hydrophobic docking surface that allows TMs to insert at the YidC-lipid interface. A high-resolution structure of YidC is most likely the only way to differentiate between the two possibilities.

4. The Tat system

4.1. Unique characteristics of the Tat pathway

4.1.1. Tat-specific signal sequences

Tat-specific signal sequences (Fig. 7A) share the canonical tripartite structure with Sec-targeting signal peptides (see above) and are also cleaved by signal peptidase I (Lüke et al., 2009; Yahr and Wickner, 2001). They are however distinguished by the consensus motif S-R-R-x-F-L-K with the name-giving arginine pair (twin arginine precursors or RR-precursors) (Berks, 1996), which is located at the distal end of their n-region. The RR-pair is almost invariant and a conservative substitution by a KK-pair usually abolishes translocation. A few naturally occurring exceptions have been described, in which one Arg is replaced either by a Lys, Asn, or Gln (Hinsley et al., 2001; Ignatova et al., 2002; Molik et al., 2001; Widdick et al., 2008). Accordingly, when those mutations are introduced into various Tat precursors, they are are partially tolerated (DeLisa et al., 2002; Halbig et al., 1999; Ize et al., 2002b; Kreutzenbeck et al., 2007; Stanley et al., 2000).

In addition to the consensus motif, further specificity determinants of Tat signal sequences are an overall lower hydrophobicity compared to Sec signal sequences (Cristobal et al., 1999; Ize et al., 2002a) as well as positive charges proximal and distal of the signal peptide cleavage site (Blaudeck et al., 2003; Bogsch et al., 1997; Ize et al., 2002a; Tullman-Ercek et al., 2007). This might be reflected by recent findings that high external salinity of a bacterial cell can lead to a re-direction of a Tat substrate to the Sec translocon (van der Ploeg et al., 2011; van der Ploeg et al., 2012).

4.1.2. Tat client proteins

The number of secreted proteins that use the Tat route varies considerably between different organisms and species

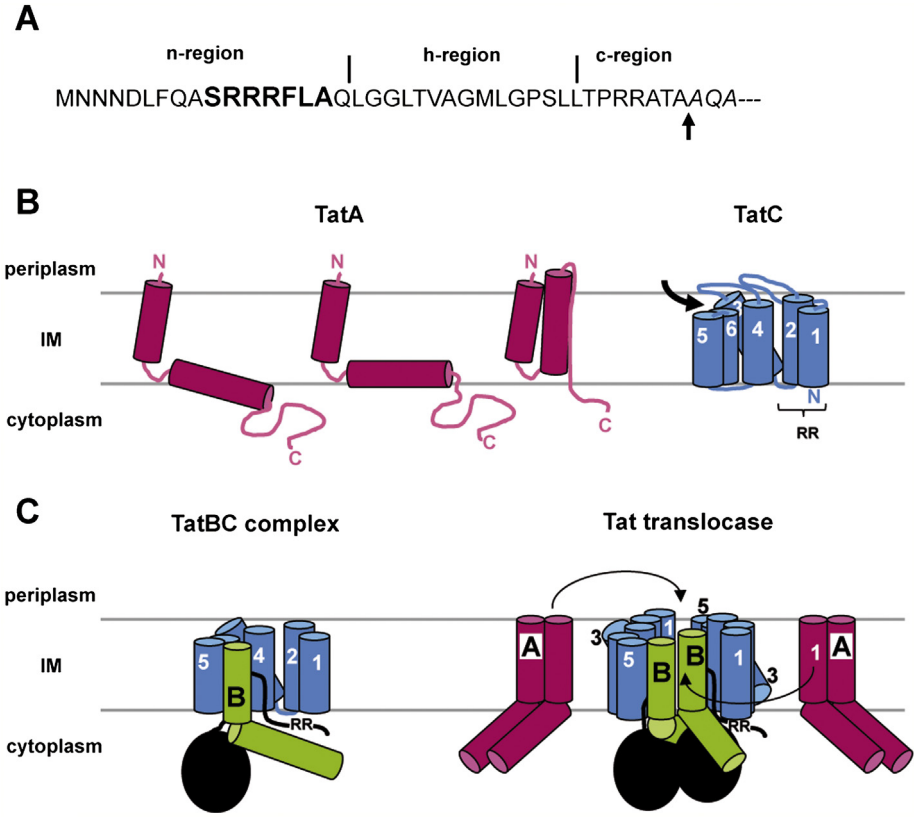

Fig. 7. The Tat protein transport system. (A) Typical Tat-specific signal sequence with the RR-containing consensus motif shown in bold letters. The canonical tripartite structure that is shared with Sec signal sequences is indicated by the boundaries between the three regions and the signal sequence cleavage site by an arrow. Depicted is the amino acid sequence of the TorA signal peptide derived from *E. coli* TMAO reductase, which has a third arginyl residue in its consensus motif. (B) Three different topologies of TatA. Left, NMR structure of an N-terminal fragment of *B. subtilis* $TatA_d$ reconstituted into planar bicells (Walther et al., 2010). The N-terminal transmembrane domain is followed by a partially membrane-embedded amphipathic helix. This basic composition is shared by TatB and TatE. Middle, full immersion of the amphipathic helix of TatA into the membrane that could cause destabilising of the bilayer ("membrane-weakening hypothesis"). Right: Flipping of the amphipathic helix into the membrane to form a hydrophilic channel from a ring of TatA protomers ("trapdoor hypothesis", "charge zipper mechanism"). Structure of TatC drawn after Rollauer et al. (2012). The arrow indicates access from the periplasm to the concave face of the molecule. The bracket encompasses the RR-binding site (N-terminus and first cytosolic loop of TatC). **(C)** Left: binding of a Tat substrate to the TatBC receptor complex. Due to its insertase activity, TatC (blue cylinders, most interhelical loops were omitted) embeds the signal sequence (black line) between helices of TatC and TatB (green cylinders). Interaction between the TM of TatB and TM five of TatC was experimentally verified (Kneuper et al., 2012). The black ellipse represents the folded domain of the Tat substrate. Right: several precursor-TatBC-complexes assemble to an oligomeric complex that recruits TatA protomers (red cylinders) to form a large functional Tat translocase. The TatBC complex was turned counterclock-wise by about 90° compared to the left-hand cartoon.

(Cline and Theg, 2007; Palmer et al., 2010; Rose et al., 2002; van Dijl et al., 2002). Most Tat substrates undergo cytosolic folding, maturation (*e.g.* insertion of cofactors) or oligomerisation events prior to export (DeLisa et al., 2003; Halbig et al., 1999; Panahandeh et al., 2008; Sanders et al., 2001; Santini et al., 1998). Others are oligomeric proteins, in which only one subunit possesses a Tat signal sequence ("hitchhiker transport") (Rodrigue et al., 1999; Sambasivarao et al., 2000). On the other hand, monomeric and cofactor-less proteins might be Tat clients, because they exhibit fast folding kinetics. Such a situation seems to prevail in halophilic Archaea which in fact export the majority of their secreted proteins via the Tat route (Hutcheon and Bolhuis, 2003; Rose et al., 2002).

Most Tat substrates are secretory proteins released after membrane passage, whereas some others remain anchored by either N or C-terminal TMs (Bachmann et al., 2006; De Buck et al., 2007; Hatzixanthis et al., 2003; Keller et al., 2012). Functionally, many bacterial Tat substrates are redox proteins involved in anaerobic respiration. In addition, Tat substrates are involved in the biogenesis and re-modelling of the cell envelope. Their lack causes the *tat* phenotype characterised by the formation of long, non-separated cell chains and outer membrane permeability defects. Several pathogenic bacteria use the Tat system to secrete virulence factors.

4.2. The components of Tat machineries

4.2.1. The TatA/B and TatC protein families

Tat translocases assemble from two sorts of membrane proteins: a polytopic TatC-type protein with six transmembrane domains (TMs) and one or more single spanning TatA-type proteins denoted TatA, TatB or TatE.

Gram-positive bacteria (for a detailed description see Chapter VI of this issue by R. Freudl) possess so called minimal Tat translocases composed of one TatA and one TatC orthologue each. On the contrary, the Tat machineries of

Gram-negative bacteria typically require in addition to a TatC orthologue, two functionally distinct TatA-type proteins usually termed TatA and TatB. The same holds true for the thylakoid membranes of plant chloroplasts, where TatA, TatB, TatC are denominated Tha4, Hcf106, cpTatC, respectively. Most proteobacteria express the three Tat proteins from *tatABC* operons, which often contain a downstream *tatD* gene encoding a cytosolic protein (Wexler et al., 2000). TatD orthologues are found in all kingdoms of life, even when no other Tat proteins are expressed. Most of them harbour deoxyribonuclease activity (Centore et al., 2008; Qiu et al., 2005; Wexler et al., 2000).

TatB very likely arose by a relatively early gene duplication of *tatA* and therefore evolved into a functionally independent protein (Yen et al., 2002). *Enterobacteria* express an additional paralogue of TatA, denoted TatE, which probably resulted from a rather late gene duplication event (Yen et al., 2002), since it can functionally replace TatA (Baglieri et al., 2012; Sargent et al., 1998, 1999).

a) **TatA** is a small protein consisting of an N-terminal TM, a short hinge region, an amphipathic helix (APH), and a C-terminal tail (Fig. 7B). This sequence-predicted structure was recently verified by NMR analysis (Chan et al., 2011; Hu et al., 2010; Walther et al., 2010). These studies also revealed that the TM of the *B. subtilis* paralogue $TatA_d$ is tilted and with its 14 amino acids so short that it causes both the hinge region and the proximal part of the APH to be immersed in the lipid bilayer (Fig. 7B). Whereas earlier studies suggested an N_{in}-C_{out} orientation of the TM of TatA (Chan et al., 2007; Gouffi et al., 2004), recent data strongly suggest that the N-terminus of TatA is located in the periplasm (Aldridge et al., 2012; Koch et al., 2012).

Except for one phenylalanine in the APH of *E. coli* TatA (F39), mutagenesis studies have not revealed any other residue being essential for function, but mutations within the APH and its flanking region usually impair the activity of TatA (recently summarised in (Fröbel et al., 2012b)).

Predominantly for the TatA orthologues of Gram-positive organisms and thylakoids of plant chloroplasts, soluble and cytosolic forms have been described (Barnett et al., 2009; Berthelmann et al., 2008; De Keersmaeker et al., 2005; Frielingsdorf et al., 2008; Mehner et al., 2012; Pop et al., 2003; Schreiber et al., 2006; Westermann et al., 2006). Whether these findings merely reflect an unusually loose membrane association of TatA isoforms or rather a particular functional state of TatA is still a matter of debate.

b) **TatB** shares the same modular structure with TatA but usually has a longer C-tail. *E. coli* TatA and TatB display 20% sequence identity (Hicks et al., 2003). No single mutation has thus far been identified that would completely inactivate *E. coli* TatB (Fröbel et al., 2012b). A lack of TatB abolishes the transport of endogenous Tat substrates in *E. coli*, while allowing low levels of export of some fusions between an RR-signal sequence and reporter proteins (Blaudeck et al., 2005; Chanal et al., 1998; Ize et al., 2002b). These findings could reflect some residual TatB-like activity of *E. coli* TatA, consistent with the idea that ancestral orthologues of TatA were as bifunctional as those of nowadays Gram-positive bacteria. Interestingly, bifunctionality can be restored to *E. coli* TatA by discrete mutations in the first N-terminal residues of TatA preceding its TM (Barrett et al., 2007; Blaudeck et al., 2005).

c) **TatC** orthologues are polytopic membranes proteins exhibiting six TMs (Behrendt et al., 2004; Ki et al., 2004; Punginelli et al., 2007). Very recently the first crystal structure of a TatC paralogue from *Aquifex aeolicus* was published (Rollauer et al., 2012) demonstrating that the six TMs are arranged as "curved wall overhung by a periplasmic cap" that is formed by the first two periplasmic loop regions. The cap delineates a groove leading from the concave face to the periplasm (Fig. 7B, arrow).

Mutational analyses of *E. coli* TatC consistently identified its cytosolic N-terminus, first cytosolic loop as well as the first two periplasmic loops as being critical for activity (Allen et al., 2002; Barrett and Robinson, 2005; Buchanan et al., 2002; Holzapfel et al., 2007; Kneuper et al., 2012). Recently, inactivating mutations were also found in the TM5 of *E. coli* TatC (Kneuper et al., 2012).

4.2.2. Oligomerization tendencies of Tat proteins

Both the TatC and the TatA/B family members have a conspicuous tendency to form homo-oligomeric and hetero-oligomeric complexes. From Gram-negative bacteria and plant chloroplasts, Tat proteins are usually isolated as separate oligomeric TatBC and TatA complexes (Cline and Mori, 2001; de Leeuw et al., 2002; Oates et al., 2005). A TatBC complex free of any TatA however, seems to be unique to plant chloroplasts, whilst the bacterial TatBC complexes usually also contain some TatA (Tat(A)BC complexes). There are no data available as to the possible association of TatE with either Tat(A)BC or TatA complexes but homo-oligomeric TatE assemblies have been isolated (Baglieri et al., 2012). Although the propensity of Tat proteins to self-associate seems to be related to the formation of functional Tat translocases from protomeric Tat subunits (see below), it remains questionable whether the Tat(A)BC and TatA complexes that can be isolated from cells represent functional units as such (Barrett et al., 2007).

4.3. Recognition and membrane targeting of Tat substrates

4.3.1. Contacts between Tat signal sequences and cytosolic proteins

As detailed below, the first dedicated step along the Tat pathway seems to be the recognition of a Tat signal peptide by TatC. There is less clarity about molecular events that precede recognition by TatC. No specific Tat signal sequence-recognising targeting factor like SRP has been identified. Whether or not membrane-targeting of Tat signal sequences

depends on general cytosolic chaperones is an unsettled question. Consistent with the signal sequences of RR-precursors prevailing in an unfolded and unstructured conformation before contacting the membrane (Kipping et al., 2003; San Miguel et al., 2003), the chaperones DnaK, Trigger factor, SlyD and other FK506-binding proteins were found to interact with signal sequences of Tat substrates (Graubner et al., 2007; Holzapfel et al., 2009; Jong et al., 2004). On the other hand, deletion of these chaperones does not negatively affect Tat-dependent export, except in the case of the *E. coli* Tat substrate CueO, which remains cytosolic in the absence of DnaK (Graubner et al., 2007). However, this effect was not due to a specific interaction of DnaK with the signal sequence of CueO (Graubner et al., 2007) but might rather be explained by a general stabilising effect that DnaK seems to exert on Tat substrates (Perez-Rodriguez et al., 2007). Consistent with general chaperones not being requisite to the Tat pathway, Tat-dependent translocation was observed in the *bona fide* absence of any cytosolic chaperone of *E. coli* (Holzapfel et al., 2009).

A different situation exists for several Tat-dependent redox proteins of bacteria that undergo co-factor insertion, folding and even hetero-oligomerisation in the cytosol before they become export-competent by the Tat machinery. These modifications require dedicated chaperones that are called REMPs (for redox enzyme maturation protein) (Sargent, 2007; Turner et al., 2004). Some of these REMPs were shown to directly interact with the signal sequences of their cognate substrates (Dubini and Sargent, 2003; Genest et al., 2006; Grahl et al., 2012; Jack et al., 2004; Maillard et al., 2007; Oresnik et al., 2001) and thereby are likely to prevent membrane targeting (Jack et al., 2004). Only after completion of the maturation process, REMPs would be released from the RR-signal sequences to allow for a subsequent targeting to the Tat translocase. In this way, REMPs would serve as proof-reading chaperones for co-factor containing Tat substrates. For one particular REMP (DmsD) a function in mediating contact of its client protein DmsA to TatBC was suggested (Papish et al., 2003).

4.3.2. Global and Tat-specific membrane targeting of Tat substrates

a) **Lipid targeting**. Although the specific recognition of the RR-signal occurs via the TatBC proteins (see below), RR-precursors were found to also into protein-less lipid bilayers (Hou et al., 2006; Schlesier and Klösgen, 2010; Shanmugham et al., 2006). Furthermore, the conversion of membrane-targeted RR-precursors from a lipid-bound state to a Tat protein-associated form was described (Bageshwar et al., 2009). On the contrary, co-elution assays using Sepharose columns did not reveal binding of an RR-precursor to *E. coli* membrane vesicles lacking the TatABC proteins (Panahandeh et al., 2008). Moreover, in the presence of Tat-deficient membrane vesicles, RR-signal sequences were found to cross-link to the same soluble protein as they did in the complete absence of membranes (Holzapfel et al., 2007). These results argue against lipid-binding being a critical step in membrane-targeting of all RR-precursors.

b) **RR-signal sequence recognition by TatC**. TatC has now been identified as the primary specific recognition site for RR-signal sequences. Initially, several experimental strategies had suggested that TatB and TatC form a receptor complex for Tat signal sequences. Thus, precursor binding to the Tat translocase is blocked by antibodies directed against TatB and TatC (Cline and Mori, 2001), and a Tat precursor co-migrates with a TatBC complex on BN-PAGE (Richter and Brüser, 2005) and co-purifies with TatB and TatC upon membrane solubilisation (McDevitt et al., 2006; Tarry et al., 2009). In accordance with TatC harbouring a recognition site for the RR-consensus motif, the site-specific introduction of photo-crosslinkers into RR-signal peptides revealed a close proximity between the RR-pair and TatC. On the contrary, downstream parts of Tat signal peptides were found to be located more in the vicinity of TatB (Alami et al., 2003; Gerard and Cline, 2006; Panahandeh et al., 2008). Whilst the signal peptide-TatC cross-links were obtained even in the absence of TatB, cross-linking to TatB strictly depended on the presence of TatC and was also quenched by high amounts of TatC (Alami et al., 2003) suggesting a dominant role of TatC in precursor binding.

In further support of a signal sequence receptor function of TatC, inactivating single alanine substitutions in the N-terminal half of TatC encompassing periplasmic, cytosolic and transmembrane sites of the molecule all impair membrane-targeting of RR-precursors (Holzapfel et al., 2007). A more precise hint as to the precursor recognition site on TatC came from the isolation of *tatC* mutants that suppress the defective transport of Tat substrates with an inactive consensus motif. The suppressor mutations mapped to the cytosolic N-terminus and the first cytosolic domain of TatC (Kreutzenbeck et al., 2007; Lausberg et al., 2012; Strauch and Georgiou, 2007). Using cross-linker scanning, the same cytosolic domains of TatC were confirmed as primary binding site for Tat signal sequences, which is therefore superficially exposed on the cytosolic leaflet of the membrane (Zoufaly et al., 2012). The crystal structure of *A. aeolicus* TatC fully confirmed this surface-exposed binding site (Fig. 7B) with two negatively charged residues being juxtaposed to coordinate the RR-motif of Tat signal peptides (Rollauer et al., 2012). In full agreement with a superficial attachment site, binding of RR-precursors to the thylakoid Tat translocase had been found to still render signal sequences (Gerard and Cline, 2007), including their N-termini (Fincher et al., 1998), accessible to proteases.

c) **TatBC-binding pocket**. Whilst the RR-consensus motif thus interacts with a cytosolic docking site of TatC, the entire RR-signal peptide is recognised by TatC and TatB in a concerted fashion. The participation of TatB in signal sequence binding can be concluded from several findings. Thus, although inactive KK-signal sequences cannot be cross-linked to TatC (Alami et al., 2003; Gerard and Cline,

2006), they were found to interact with the Tat(A)BC receptor complex (Alder and Theg, 2003b; McDevitt et al., 2006; Panahandeh et al., 2008). Moreover, several single amino acid exchanges in *tatC* do not only affect cross-linking of RR-precursors to TatC but also to TatB (Holzapfel et al., 2007). Consistent with a juxtaposition of TatB and TatC in a joint binding pocket, the N-terminus of TatC cross-links to TatB (Zoufaly et al., 2012). Furthermore, translocation defects of RR-precursors resulting from mutations in their consensus motif are suppressed by mutations in the TM of TatB (Kreutzenbeck et al., 2007; Lausberg et al., 2012)

4.3.3. Post-targeting and pre-translocation events

a) **Loop-like insertion of an RR-signal sequence**. The above mentioned *tatB* mutations that suppress inactive variants of Tat signal sequences map close to the *trans*-sided N-terminus of TatB. This would suggest that a TatB-bound RR-signal peptide must extend far into the membrane (Fig. 7C). In fact, the same N-proximal residue of the TM of TatB that upon mutation suppresses defective signal sequences, was found to cross-link to RR-precursor proteins (Maurer et al., 2010). *Vice versa*, the hydrophobic region of Tat signal peptides located downstream of the RR-consensus motif also preferentially cross-links to TatB (Alami et al., 2003; Gerard and Cline, 2006; Panahandeh et al., 2008).

The question then arises how at this stage of the Tat pathway a Tat signal sequence would move deeply into the plane of the membrane. This was addressed in a recent study (Fröbel et al., 2012a), in which TatB-independent functions of TatC were analysed and which revealed that TatC has the properties of a signal peptide insertase. By virtue of this function, RR-signal sequences are integrated into the membrane in a loop-like fashion that can even result in the premature exposure of the cleavage site to signal peptidase I, if TatB is not present and if the sequence context around the cleavage site of the RR-precursor allows for the formation of a fully extended signal peptide. In support of an RR-signal peptide accommodated in the plane of the bilayer between TatC and TatB, the TM of TatB was recently found to specifically interact with TM5 of TatC (Fig. 7C) (Kneuper et al., 2012; Rollauer et al., 2012).

b) **PMF-dependent contacts to the Tat machinery**. Further pre-translocational interactions of RR-precursors that follow the initial Tat-targeting step could be identified on the basis of their dependence on the proton-motive force (PMF). The PMF is the only source of energy that powers Tat-dependent protein translocation (Mould et al., 1991). There is still some controversy as to the essential involvement of the PMF in all Tat systems (Di Cola et al., 2005; Finazzi et al., 2003) and whether or not both components of the PMF, the pH gradient (ΔpH) and the electrical potential ($\Delta\Psi$) are equally involved or even mutually exchangeable (Bageshwar and Musser, 2007; Braun et al., 2007; Theg et al., 2005). Physical binding of RR-precursors to thylakoids (Ma and Cline, 2000) and *E. coli* inner membrane vesicles (Alami et al., 2002; Panahandeh et al., 2008), cross-linking of Tat signal sequences to TatB and TatC (Alami et al., 2003), and FRET between the folded domain of a Tat substrate and TatB/C (Whitaker et al., 2012) were shown to proceed in the absence of the PMF. Moreover, the TatC-mediated insertion of Tat signal sequences was observed to occur in the absence of the PMF (Fröbel et al., 2012a). These results suggest that initial targeting events do not require energy from the PMF.

On the contrary, more advanced binding stages were identified that are definitely dependent on the PMF. Thus, implementation of the PMF moves a thylakoid-bound RR-precursor from a protease-accessible to a membrane-protected environment (Gerard and Cline, 2007). Furthermore, site-specific photo cross-linking between an RR-signal peptide and TatA, and *vice versa* between the transmembrane helix of TatA and RR-precursors, both disappear or become weaker when the PMF is dissipated by CCCP (carbonyl cyanide m-chlorophenyl-hydrazone)(Alami et al., 2003; Fröbel et al., 2011). Incidentally, cross-linking of TatBC-bound precursors to TatA invoke a close proximity between TatA and TatBC, for which experimental evidence in fact has been provided (Fröbel et al., 2011; Kostecki et al., 2010; Mangels et al., 2005; Zoufaly et al., 2012).

Collectively, advanced yet still pre-translocational interactions of RR-precursors with the Tat machinery seem to involve a positional change of the signal peptide and first contacts to TatA. The PMF-dependent nature of some of these events would be consistent with the finding by Bageshwar and Musser that transport of an *E. coli* RR-precursor into membrane vesicles requires two ($\Delta\Psi$)-dependent steps, one that is necessary early and one later during transport (Bageshwar and Musser, 2007).

c) **Oligomerisation events**. Consistent with a concerted recognition of RR-signal peptides by TatB and TatC (see above), both Tat proteins associate into 1:1 complexes (Bolhuis et al., 2001). In addition, TatB and TatC each have the individual property to homo-oligomerise (Behrendt et al., 2007; de Leeuw et al., 2001; Kostecki et al., 2010; Lee et al., 2006; Maldonado et al., 2011a, 2011b; McDevitt et al., 2005; Orriss et al., 2007; Punginelli et al., 2007) suggesting that a functional TatBC complex actually consists of several TatBC dimers. This was confirmed by single-particle electron microscopy of TatA-free TatBC complexes from *E. coli* revealing structures, into which up to seven protomeric TatBC complexes could be fitted (Tarry et al., 2009). When these TatBC complexes were produced in the presence of a natural *E. coli* RR-precursor, one or two extra densities were obtained that asymmetrically associated with the surface of

the particles and that by size could represent one or two precursor molecules superficially bound to a heptameric TatBC complex. Using chemical cross-linking two or four precursor molecules were found associated with the chloroplast Tat translocase (Ma and Cline, 2010). Surprisingly, the bound precursor molecules were translocated even if they had been covalently tethered together, suggesting that oligomeric TatBC assemblies might allow for a simultaneous membrane passage of several cargo molecules (Ma and Cline, 2010). More recent binding studies showed that in plant chloroplasts, a functional TatBC complex seems to be octameric with eight precursor binding sites that in the presence of sufficient TatA are concurrently active (Celedon and Cline, 2012). Hence functional Tat translocases seem to encompass oligomeric TatBC complexes but it is not clear if these assemblies simply reflect the intrinsic oligomerisation tendencies of TatB and TatC (see above) or whether their formation is strictly substrate-induced. The latter is suggested by a recent *in vivo* analysis involving fluorescently labelled Tat components (Rose et al., in preparation).

An obvious yet controversially discussed aspect of having up to eight precursor molecules simultaneously bound to TatBC is that of a potential cooperative effect on downstream events (Alder and Theg, 2003b; Celedon and Cline, 2012; Hauer et al., 2013). If this is not the case, what would be the functional advantage of an octameric TatBC-precursor assembly over eight individually functioning TatBC complexes? A clue might come from the finding that the folded domains of *E. coli* RR-precursors, after membrane targeting but prior to translocation are in close proximity to more than a single TatB monomer (Maurer et al., 2010). In other words, neighbouring TatB molecules, most likely via their APHs, seem to associate to form a multivalent binding structure for a single folded RR-precursor. Recruitment of several TatB monomers to a folded, TatBC-bound RR-precursor could be the trigger for a substrate-induced formation of the octameric complex. In support of such a model, a disulphide-mapping analysis of TatB suggested that TatB monomers might associate into a central core structure of an oligomeric TatBC complex with the TatC protomers being peripherally associated (Lee et al., 2006).

4.3.4. Transmembrane translocation of Tat substrates: pore forming versus membrane-destabilizing effects of TatA

a) **TatA pores**. Thus far the actual translocation event has remained the least understood step of the Tat pathway. A major role of TatA in the translocation process was originally derived from the finding that binding of precursor proteins to the TatBC-receptor complex is followed by the recruitment of TatA oligomers (Dabney-Smith et al., 2006; Leake et al., 2008; Mori and Cline, 2002). Two basically different ideas were developed how TatA might achieve the ensuing membrane passage of Tat substrates. In the one model, TatA is assumed to form size-fitting pores for folded Tat substrates and in the other TatA would help to destabilize the lipid bilayer and thereby its permeability barrier. Underlying both models is the homo-oligomerisation tendency of TatA orthologues. This became first apparent by the isolation of higher order TatA structures of various sizes (Barrett et al., 2005; Gohlke et al., 2005; McDevitt et al., 2006; Oates et al., 2005), but could later on be shown to also exist *in vivo* (Greene et al., 2007; Kostecki et al., 2010; Leake et al., 2008), even at wild-type expression levels of TatA (Leake et al., 2008). Oligomerisation of TatA occurs via all sections of the molecule (Dabney-Smith and Cline, 2009; Dabney-Smith et al., 2006; de Leeuw et al., 2001; Greene et al., 2007; Porcelli et al., 2002; Warren et al., 2009). On Blue Native-PAGE, purified TatA resolves into a ladder of bands with a step size of about 40 kDa suggesting a TatA tetramer (Gohlke et al., 2005; Oates et al., 2005) as the protomeric subunit.

The strongest experimental support for TatA oligomers forming a pore comes from single particle electron microscopy of isolated TatA (Gohlke et al., 2005). The obtained particles had a pore-like appearance and could be grouped into size classes with different diameters that would well fit with the size range (20–70 Å) of known folded Tat substrates from bacteria (Berks et al., 2000). The walls of the pore-like particles were suggested to consist of the TMs of TatA, which in fact can form a ring-like array as observed by EPR spectroscopy of spin-labelled *E. coli* TatA variants (White et al., 2010). The TatA particles possess a lid-like density on their presumed cytosolic face, which was suggested to represent the APHs of the circularly arranged TatA monomers and to function as a potential gating device (Gohlke et al., 2005). Not fully consistent with the view of customised TatA pores with variable diameters is, however, the finding that at least in plant chloroplasts oligomerisation of TatA can be initiated merely by an RR-signal peptide (Dabney-Smith and Cline, 2009; Mori and Cline, 2002).

b) **Functions of the amphipathic helix of TatA**. The ability of the APHs of TatA to adopt different topologies within the plane of the membrane has been the subject of several investigations (Aldridge et al., 2012; Chan et al., 2007; Dabney-Smith et al., 2006; Gouffi et al., 2004). Such a topology switch would be the basis of alternative pore models (trapdoor model, charge zipper mechanism) (Chan et al., 2007; Cline and Theg, 2007; Dabney-Smith et al., 2006; Gouffi et al., 2004; Greene et al., 2007; Walther et al., 2013), in which rather than the hydrophobic TMs of TatA, the polar surfaces of the APHs and C-tails of TatA after adopting a perpendicular orientation in the membrane would line a hydrophilic pore. Based on complementary charge patterns on the APH of TatA and its proximal C-tail, the formation of hairpins by "charge zippers" was postulated (Walther et al., 2013). These TatA hairpins (Fig. 7B) could self-assemble into a transmembrane pore by similar electrostatic interactions between neighbouring hairpins (Walther et al., 2013).

Alternatively, the APH of TatA might undergo only minor changes in its orientation, such as a transition from the slanted, partially membrane-embedded alignment revealed by NMR (Walther et al., 2010) to a flat, fully lipid-immersed position (Fig. 7B). This would be sufficient to account for the substrate-induced decrease in the accessibility of the APH observed for the thylakoidal TatA (Aldridge et al., 2012). Locally accumulating TatA APHs could then exert a destabilising effect on the membrane bilayer allowing a direct passage of Tat substrates through the membrane lipids as proposed by the membrane weakening hypothesis (Brüser and Sanders, 2003). This model further postulates that TatC pulls the whole Tat substrate across the destabilised membrane in an energy-dependent manner. An insertase activity of TatC was in fact recently described, which however only mediates insertion of RR-signal sequences but no translocation of the folded substrate and was also independent of the PMF (Fröbel et al., 2012a). Consistent with a membrane-destabilising effect of TatA, the bacterial phage shock protein PspA was recently shown to associate with *E. coli* TatA (Mehner et al., 2012). PspA is involved in maintaining the PMF under cellular stress conditions (Kleerebezem et al., 1996) and, like its homolog in plant chloroplasts VIPP1, was found to improve the efficiency of Tat translocation (DeLisa et al., 2004; Lo and Theg, 2012; Vrancken et al., 2007). Therefore, PspA might function in counteracting membrane stress induced by TatA (Mehner et al., 2012), be it via a destabilising effect by TatA or also the formation of large TatA pores.

In favour of the membrane weakening hypothesis, it has thus far not been possible to generate membrane-spanning translocation intermediates trapped by TatABC subunits (Cline and McCaffery, 2007). In contrast, a partially membrane-protected translocation intermediate of a folded RR-precursor was found to accumulate in the vicinity of TatA (Panahandeh et al., 2008). Furthermore, quite distantly located sites on the surface of a membrane-targeted RR-precursor were shown to contact TatA, much like one would expect if a circular Tat structure encapsulates the precursor (Maurer et al., 2010).

c) **An extended pore model**. If the Tat-specific protein conduits across membranes would in fact consist of proteinaceous channels, it is not clear if they would be made exclusively from TatA subunits. It could be speculated that the observed oligomerization of TatB at a membrane-targeted RR-precursor (Maurer et al., 2010), actually serves as a nucleation center for the recruitment of TatA protomers (Fig. 7C). If so, the final pore might not constitute a separate entity but rather assemble around TatBC-bound precursors. Such a scenario would provide a plausible explanation for how an RR-precursor could cross the bilayer through a proteinaceous structure and yet remain anchored via its signal sequence at TatC as it was experimentally demonstrated to occur (Gerard and Cline, 2006).

d) **Involvement of the PMF in translocation-related events**. Irrespective of the nature of the transmembrane passageway, experimental data has accumulated suggesting that the translocation step is somehow dependent on the PMF. For the Tat system of chloroplasts, it has been established that TatA subunits are recruited to the TatBC precursor complexes in a PMF-dependent manner (Dabney-Smith et al., 2006; Mori and Cline, 2002). Similarly, surface contacts of a folded RR-precursor with TatA are not obtained after dissipation of the PMF (Maurer et al., 2010; Pal et al., 2013). Except for those events, it is not really understood, how a late step of the Tat pathway such as translocation would be powered by the PMF. The N-proximal transmembrane domain of the chloroplast TatA homologue Tha4 harbours a conserved and essential Glu residue (Dabney-Smith et al., 2003), which could become protonated following the trans-sided accumulation of protons. However, there is no acidic residue at equivalent positions of bacterial TatA orthologues. In agreement with experimental data (Alder and Theg, 2003a; Musser and Theg, 2000b), a coupled Tat substrate/proton flow has also been postulated (Theg et al., 2005).

e) **Signal peptide cleavage**. It seems reasonable to assume that the time-consuming steps of the Tat pathway are the various assembly processes of a functional Tat machinery, whereas crossing the lipid bilayer might be very rapid. It is an unsolved question when during these events the signal peptide is cleaved off. Mutation of the signal sequence cleavage site was reported to lead to the accumulation of non-translocated precursor in the thylakoidal membrane of plant chloroplasts (Di Cola and Robinson, 2005), whereas in several other cases signal peptide processing was not found to be a prerequisite for a complete translocation (Frielingsdorf and Klosgen, 2007; Maurer et al., 2009; Panahandeh et al., 2008). In fact, the finding that translocation might occur while an RR-signal peptide remains covalently linked to TatC (Gerard and Cline, 2006) suggests that cleavage of the signal peptide step is executed rather late and after translocation is completed. From this result, one could even speculate that the association of a Tat signal peptide with TatBC is maintained until after translocation, because otherwise the protein conduit might fall apart. If so, the cleavage site should not become accessible to the signal peptidase prior to translocation. Interestingly, TatB was recently suggested to play an important role in controlling the access of the cleavage site to signal peptidase 1 (Fröbel et al., 2012a).

4.4. Quality control

As detailed above, many bacterial Tat substrates can be exported only in a completely folded state. This refers particularly to those proteins, which receive cofactors by a strictly cytosolic process and to those heteromeric complexes, in which only one subunit carries an RR-signal peptide. Although the thylakoidal Tat system of plant chloroplasts had long been known to also transport unfolded proteins (Hynds

et al., 1998), a key question has been how the folding state of Tat substrates is sensed leading to a ceased transport of improperly folded substrates.

A sensor of folding was strongly suggested by experimental data obtained with artificial Tat substrates that allow a direct comparison of folded versus unfolded conformations. Thus disulphide-containing proteins whose folding varies with the redox state of the environment were shown both *in vivo* and *in vitro* to be exported by the Tat machinery only under oxidising, i.e. folding-promoting conditions (DeLisa et al., 2003; Panahandeh et al., 2008; Richter and Brüser, 2005). Similarly, Tat-dependent export of a cytochrome *c* variant harbouring an RR-signal sequence required the pre-translocational incorporation of heme (Sanders et al., 2001). In addition, mutations and truncations with seemingly minor impacts on conformation were reported to impair Tat-dependent transport in bacteria and chloroplasts (Maurer et al., 2009; Roffey and Theg, 1996).

The molecular nature of such a sensor rejecting improperly folded Tat substrates has largely remained elusive. One possibility is that it is the Tat translocase itself that performs quality control of folding. This idea was nourished by the recent isolation of Tat mutants that suppress rejection of misfolded proteins (Rocco et al., 2012). In contrast, the fact that translocation-incompetent Tat substrates still associate with Tat translocases (Musser and Theg, 2000a; Panahandeh et al., 2008; Richter and Brüser, 2005), even though with different binding-characteristics (Panahandeh et al., 2008), would suggest that malfolded Tat substrates rather than being rejected by the Tat machinery would be eliminated by an efficient degradation system (Brüser and Sanders, 2003; Richter and Brüser, 2005) as demonstrated to occur for incompletely assembled Fe/S proteins in *E. coli* (Matos et al., 2008).

Notably, Tat translocases in general seem to be capable of also transporting unstructured peptides provided that those do not exceed a length of about 100–120 amino acids and are deficient in hydrophobic residues (Cline and McCaffery, 2007; Richter et al., 2007). Likewise, Tat substrates that fail to be transported by the *E. coli* Tat machinery, because they are incompletely folded, become partially tolerated when they are C-terminally shortened (Maurer et al., 2009). In all these cases, the actual determinants of acceptance by the Tat machineries are not well understood. It has been proposed that the threshold parameter could be the total surface area of an RR-precursor (Cline and McCaffery, 2007).

5. Conclusion

Studies on protein transport in bacteria have gained momentum in the past decade especially since X-ray structures of many of the components involved are available. Biochemical and structural studies have allowed us to potentially trace the route taken by a protein from its inception within the ribosome to its destination via the SecYEG translocon. However, the sequence of events and details on the mechanisms which finally lead to protein transport remain ambiguous.

Some of the processes that are yet not understood include the co-translational recognition of secretory proteins by SecA and how the coordinated activity of SecA and SecY lead to ATP-dependent translocation of substrates. Likewise elucidating the co-ordination between SRP-RNCs and SecA during the insertion of multi-spanning membrane proteins with long periplasmic loops is an unresolved issue and needs investigating. Decoding these processes would help to understand the mechanisms involved in targeting and transporting substrates across the SecY channel.

While the basic principles of SRP-dependent co-translational targeting appear to be resolved, it is still puzzling how the vast array of translating ribosomes are scanned, recognised and targeted by the small number of SRP molecules. Alternative targeting routes have been proposed, including ribosome targeting by FtsY (Bibi, 2012), recognition of RNCs by pre-formed SRP-FtsY complexes at the membrane (Braig et al., 2011), and membrane targeting of mRNAs encoding membrane proteins (Nevo-Dinur et al., 2011). It appears likely that *in vivo* the repertoire of targeting mechanisms is much higher and more flexible than the available *in vitro* data suggest.

An interesting and yet puzzling issue has developed recently from data showing that many multi-spanning membrane proteins can be delivered by SRP to either SecYEG or YidC for insertion (Welte et al., 2012). As the concentration of YidC exceeds that of SecYEG (Table 2), membrane protein insertion via YidC may be more prevalent than previously anticipated. This would also explain why the limited number of SecYEG molecules is sufficient for handling both secretory proteins and a sub-set of inner membrane proteins. However, the mechanism of dual targeting of substrates to either SecYEG or YidC has to be confirmed in live cells.

Another highly debated issue is the oligomeric state of the translocon which has been studied for decades. While the current view is that a monomeric SecY functions as the translocon, it seems plausible that SecYEG can form higher orders of oligomers to accommodate different substrates. There is also recent evidence that the assembly of translocases and their co-ordination with targeting factors is “need-based” and potentially highly flexible. This stems from data showing that some proteins might use a combination of the Sec and Tat translocases during their transport.

As for the Tat system, the major challenge remains to elucidate the structure and composition of a functional protein conduit across the membrane. Future approaches will have to address whether or not the conduit consists of TatA alone, and if so which parts of TatA would line a hydrophilic path, how membrane leakage would be prevented should TatA destabilise the lipid bilayer, and if soluble TatA is of functional relevance. Central to a molecular understanding of the Tat mechanism are issues such as the involvement of the proton-motive force and the discrimination between apt and faulty Tat substrates.

Finally, what are lacking are high-resolution X-ray structures of an active SecYEG channel translocating or inserting a substrate, and of a ‘holo-translocon’ with the accessory

subunits SecDFYajC and YidC. Also needed is structural information about the YidC insertase and the Tat translocase. Elucidating these structures would guide further biochemical analyses and potentially lead to a thorough understanding on how cells manage to deliver proteins across and into the membrane.

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