Visualization of Distinct Entities of the SecYEG Translocon during Translocation and Integration of Bacterial Proteins

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The universally conserved SecYEG/Sec61 translocon constitutes the major protein-conducting channel in the cytoplasmic membrane of bacteria and the endoplasmic reticulum membrane of eukaryotes. It is engaged in both translocating secretory proteins across the membrane as well as in integrating membrane proteins into the lipid phase of the membrane. In the current study we have detected distinct SecYEG translocon complexes in native *Escherichia coli* membranes. Blue-Native-PAGE revealed the presence of a 200-kDa SecYEG complex in resting membranes. When the SecA-dependent secretory protein pOmpA was trapped inside the SecYEG channel, a smaller SecY-containing complex of ~140-kDa was observed, which probably corresponds to a monomeric SecYEG–substrate complex. Trapping the SRP-dependent polytopic membrane protein mannitol permease in the SecYEG translocon, resulted in two complexes of 250 and 600 kDa, each containing both SecY and the translocon-associated membrane protein YidC. The appearance of both complexes was correlated with the number of transmembrane domains that were exposed during targeting of mannitol permease to the membrane. These results suggest that the assembly or the stability of the bacterial SecYEG translocon is influenced by the substrate that needs to be transported.

INTRODUCTION

The targeting of extracytoplasmic proteins to their correct cellular compartment is a crucial issue for every living cell and accordingly all organisms have developed sophisticated machineries to recognize these proteins and to deliver them to their final destination. In bacteria, the majority of the extracytoplasmic proteins engage the universally conserved Sec translocon for exiting the cytoplasm (Koch *et al.*, 2003). This protein-conducting channel consists of the membrane proteins SecY, SecE, and SecG as core components. Targeting to the bacterial Sec translocon is achieved by two distinct pathways: the SecA/SecB-dependent posttranslational targeting of secretory proteins, i.e., proteins that reside in the periplasm or the outer membrane of Gram-negative cells (de Keyzer *et al.*, 2003), and the single recognition particle (SRP)-dependent cotranslational targeting of inner membrane proteins (Koch *et al.*, 2003; Halic and Beckmann, 2005). Thus, the Sec translocon has to switch in a substrate-dependent manner between two different operational modes: a transversal opening for allowing secretory proteins access to the periplasm and a lateral opening for the insertion of trans-

membrane domains. However, the molecular mechanisms that determine these two activities are largely enigmatic.

The interaction of the Sec translocon with soluble receptors appears to be one important factor for defining its “translocase” or “integrase” activity. The ATPase SecA is considered to be a peripheral subunit of the Sec translocon during the translocation of secretory proteins (van der Does *et al.*, 1996). Similarly, FtsY, the bacterial SRP receptor, has been shown to interact with the Sec translocon (Scotti *et al.*, 2000) and is implicated in the lateral release of a transmembrane domain from the protein-conducting channel into the lipid phase (Beck *et al.*, 2001; Urbanus *et al.*, 2001). YidC probably does not bind directly to the SecYEG core components but rather via the SecDFYajC complex (Nouwen and Driessen, 2002; Xie *et al.*, 2006), which at least transiently associates with the SecYEG complex (Duong and Wickner, 1997).

An important and still controversially discussed issue concerning the Sec translocon is its oligomeric state during active protein transport. Electron microscopy (EM) images of purified Sec complexes from different species have revealed ring-like structures probably consisting of three to four SecYEG protomers (Meyer *et al.*, 1999; Manting *et al.*, 2000). Multimeric, ring-like structures were also observed for the eukaryotic Sec61 translocon complex (Hanein *et al.*, 1996; Beckmann *et al.*, 2001). On the other hand, biochemical approaches using Blue-Mative-PAGE (BN-PAGE), fluorescent resonance energy transfer (FRET), and chemical cross-linking suggest that in *E. coli* the Sec translocon predomi-

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Abbreviations used: BN-PAGE, Blue-Native-PAGE; DDM, dodecyl-maltoside; INV, inner membrane vesicles; MtlA, mannitol permease; OmpA, outer membrane protein A; RNC, ribosome-associated nascent chain; TM, transmembrane domain; wt, wild type.

nantly exists as a monomer or dimer (Yahr and Wickner, 2000; Bessonneau *et al.*, 2002; Mori *et al.*, 2003). The equilibrium between the monomeric, dimeric, and tetrameric states of the SecYEG/Sec61 translocon appears to be influenced by its interaction with ligands, like SecA or the ribosome (Hanein *et al.*, 1996; Manting *et al.*, 2000; Scheuring *et al.*, 2005).

Structural information on the Sec translocon has been provided by x-ray crystallography of a detergent-purified Sec complex from *Methanococcus jannaschii* (van den Berg *et al.*, 2004). The structure revealed a single trimeric complex of one subunit each. Importantly, the channel pore appears to be located in a single copy of the SecY molecule, which displays an hourglass shape with a central constriction (the pore ring) in the middle of the membrane. The constriction has an opening of 5–8 Å and appears to be closed on the periplasmic side by a small helix that is suggested to form a plug, thereby sealing the channel (van den Berg *et al.*, 2004). Permanently locking the plug in the open state appears to be lethal to the cells (Harris and Silhavy, 1999), but deleting the plug does not interfere with cell viability (Junné *et al.*, 2006; Maillard *et al.*, 2007), presumably because a new seal is formed by recruiting additional residues to the pore ring (Li *et al.*, 2007).

Cross-linking data using the SecA-dependent substrate OmpA support the hypothesis that a single SecYEG copy is sufficient to form a protein-conducting channel during post-translational translocation (Cannon *et al.*, 2005), although a second SecYEG copy might be required for binding of the motor protein SecA (Osborne and Rapoport, 2007). A dimeric SecYEG translocon has also been deduced from cryo-EM studies of ribosome-nascent chain (RNC) complexes bound to detergent-solubilized SecYEG (Mitra *et al.*, 2005).

Protein transport in bacteria is probably a highly dynamic process and the conflicting results observed by using different approaches for determining the oligomeric state of the Sec translocon might reflect these dynamic changes. The analysis of the oligomeric state of the SecYEG translocon is further complicated by the observation that both the expression level of SecYEG as well as the solubilization conditions appear to influence its oligomerization (Manting *et al.*, 2000; Yahr and Wickner, 2000; Bessonneau *et al.*, 2002; Scheuring *et al.*, 2005). In the current study we have analyzed the SecYEG translocon in wild-type *E. coli* membranes in its resting and in its active state by combining BN-PAGE with two-dimensional (2D) SDS-PAGE at a defined protein:detergent ratio.

MATERIALS AND METHODS

Strains, Plasmids, and Protein Purification

The following *E. coli* strains were used: TY0, TY1, and TY22 (Taura *et al.*, 1997), MRE600 and MC4100 (Koch *et al.*, 1999), BL325 (Duong and Wickner, 1997), CM124 (Traxler and Murphy, 1996), and JS7131 (Samuelson *et al.*, 2000). SecYEG and YidC purification was achieved using the strains TY0/pHis-EYG and XL1-Blue/pROEX-HTB-yidC, respectively (Collinson *et al.*, 2001; Koch *et al.*, 2002) and purification of SecYEG and YidC were performed following the protocols from Nishiyama *et al.* (2006) and Koch *et al.* (2002), respectively. The in vitro transcription/translation of OmpA, MtlA, and SecY was performed using the plasmids pDMB (Behrmann *et al.*, 1998), p717-MtlA (Beck *et al.*, 2000), and pJM8CS7 (Koch *et al.*, 1999).

In Vitro Synthesis of Ribosome-associated Nascent Chains

The in vitro system of *E. coli* and the purification of its components were described before (Koch *et al.*, 1999). The synthesis of ribosome-associated nascent chains (RNCs) using the following oligodeoxynucleotides OmpA-126 (5'-TAAACGTTGGATTAGTGTG-3'; 3.0 µg/25 µl), MtlA-189 (5'-ACCGTG-GTTAATGGCGTGTGT-3'; 3.0 µg/25 µl), MtlA-304 (5'-AGCACCTTTTGTGTCATCGC-3'; 3.0 µg/25 µl), MtlA-348 (5'-CATGTCCTGCATA-CGAC-GAGT-3'; 3.0 µg/25 µl), MtlA-385 (5'-CGCACTGGAACCCATACCGGC-3'; 3.0 µg/25 µl) was performed in a 10-fold up-scaled reaction mixture. For the synthesis of OmpA-126 and MtlA-304 RNCs, RNaseH (3U/25 µl) and 10SA-

RNA antisense oligodeoxynucleotide (Beck *et al.*, 2000; 0.3 µg/25 µl) were added and for the synthesis of MtlA-189, MtlA-348 and MtlA-385 RNCs only 10SA-RNA.

Solubilization of Native and ³⁵S-SecY-containing Membranes and Their Analyses on BN-PAGE and 2D SDS-PAGE

Membranes (100 µg protein) were resuspended in lysis buffer (50 mM Imidazole/HCl, pH 7.0, 5 mM 6-aminocaproic acid, 50 mM NaCl) and solubilized with 100 µg DDM (dodecyl-maltoside; Roche Diagnostics, Mannheim, Germany) for 5 min at 25°C. After centrifugation (30 min at 45,000 rpm, 4°C; TLA-45 Beckman rotor) solubilized material was analyzed on 4–15% BN-gels. For SDS treatment, samples after centrifugation were incubated with 0.1% SDS final concentration for 10 min at 56°C and only then analyzed on BN-PAGE. For 2D SDS-PAGE gel stripes from 1D BN-PAGE were incubated in equilibration buffer (50 mM Tris/HCl, pH 6.8, 2% [wt/vol] SDS, 6 M urea, and 30% [wt/vol] glycerol) for 30 min at room temperature. Membrane protein complexes were cut out from the gel stripes and mounted onto a 12% SDS-PAGE. After separation, proteins were blotted onto a PVDF membrane, and the membrane was first analyzed by immunodetection and subsequently by phosphorimaging for detecting the radioactively labeled OmpA-tRNA and MtlA-tRNA derivatives.

Preparation of tRNA Translocation Intermediates

The isolation of RNCs was performed as detailed in Behrmann *et al.* (1998). The RNCs were resuspended in RANC buffer (Beck *et al.*, 2000; with 40 mM TeaAc, pH 7.5, instead of 40 mM HEPES-NaOH, pH 7.5) containing 8 M urea and incubated on ice for 30 min to dissociate the ribosome and to create tRNA-tethered substrates. The urea concentration was then reduced to 0.8 M, and the OmpA-tRNA was subjected to an in vitro transport assay in the presence of 250 µg inner membrane vesicles (INVs), 2.5 µg SecA, and an ATP-regenerating system (Beck *et al.*, 2000) for 15 min at 37°C. For the production of MtlA-tRNA translocation intermediates, in vitro synthesis was performed in the presence of INVs (250 µg) and the INV-bound RNCs were subjected to urea treatment as described above. Translocation intermediates were then either subjected to flotation gradient analyses (Neumann-Haefelin *et al.*, 2000) or solubilized with 250 µg DDM and subjected to BN-PAGE (Schägger, 2001). Because of the instability of the covalent linkage between the tRNA and the protein, samples for SDS-PAGE were denatured at 37°C in SDS loading buffer, unless otherwise stated. Radioactively labeled proteins were visualized by phosphorimaging using a PhosphorImager (GE Healthcare, Munich, Germany) and quantified using the ImageQuant software. For mock treatment 100 µg of INVs were treated with 1 mM puromycin for 10 min at 37°C and subsequently with 1 M potassium acetate, pH 7.5, for 15 min on ice. INV were collected by a 30-min ultracentrifugation step (45,000 rpm at 4°C; TLA 45 rotor, Beckman, Krefeld, Germany) and resuspended in lysis buffer after solubilization with 100 µg of DDM. Alternatively, INVs were urea-treated as described above but in the absence of in vitro-synthesized substrates.

Antibody Blocking Assay

For antibody blocking (Wiedemann *et al.*, 2003), INVs were incubated with lysis buffer, preimmune serum, or antibodies for 60 min at 4°C and subsequently isolated by centrifugation (30 min at 45,000 rpm, 4°C; TLA-45 rotor, Beckman). The isolated INVs were then incubated with the tRNA-tethered substrates as described above.

Immunoblotting

After BN-PAGE or SDS-PAGE, samples were electroblotted onto PVDF membranes and decorated with polyclonal antibodies directed against Ffh, FtsY, or YidC or with peptide antibodies directed against SecY, SecE, SecG, SecA, SecD, or SecF. Detection was performed with horseradish peroxidase-conjugated goat anti-rabbit antibodies (Caltag Laboratories, Burlingame, CA) as secondary antibodies with ECL (GE Healthcare) as substrate.

RESULTS

The Resting Translocon in Wild-Type *E. coli* Membranes Is Organized as a SecYEG Dimer

To characterize the SecYEG translocon in its resting state in wild-type *E. coli* membranes, we used BN-PAGE, a technique that has been widely used to analyze the oligomerization and subunit composition of membrane protein complexes (Schägger, 2001). Crude *E. coli* membranes were separated by sucrose-density gradient centrifugation to obtain purified INVs. These INVs, containing only endogenous amounts of SecYEG, were then solubilized with DDM. After

Figure 1. The SecYEG translocon forms a 200-kDa complex under resting conditions. (A) Wild-type (wt) *E. coli* and SecE-depleted CM124 INVs were solubilized with DDM (1 μ g DDM/ μ g protein), separated by linear gradient BN-PAGE (4–15%), and transferred to PVDF membranes. For each lane 100 μ g of protein was loaded. Immunostaining was performed with the indicated antibodies. (B) SecE-containing and SecE-depleted INVs from *E. coli* CM124 were separated by SDS-PAGE. Immunodetection was performed with the indicated antibodies. (C) SecY was in vitro synthesized using a coupled *E. coli* transcription/translation system in the presence of either wild-type (wt) membranes, or membranes from the *secY* mutant strain TY1 or TY22. Membranes containing the radioactively labeled in vitro-synthesized SecY were reisolated by centrifugation, solubilized by DDM, and separated by BN-PAGE. Radioactively labeled SecY complexes were visualized by phosphorimaging. (D) Coomassie staining of wild-type INVs and purified SecYEG, separated on SDS-PAGE. INVs, 25 μ g, and 0.2 μ g of purified SecYEG were loaded. (E) Western blot of BN-PAGE-separated wild-type INVs (100 μ g protein) and purified SecYEG (0.2 μ g) using antibodies against SecY. When indicated, DDM solubilized INVs were further treated with SDS (0.1% for 10 min at 56°C) in order to dissociate the SecYEG complex. (F) Western blot of SDS-PAGE-separated wild-type INVs (25 μ g protein) and purified SecYEG (0.2 μ g) using the indicated antibodies.

separation on BN-PAGE, proteins were blotted onto a PVDF membrane and decorated with antibodies against SecY, SecE, and SecG, the core subunits of the SecYEG translocon. All three antibodies recognized a single complex running at ~200 kDa, which was significantly reduced in membranes from the conditional SecE mutant strain CM124 (Figure 1A). In this strain the expression of the essential *secE* gene is under the control of the arabinose promoter, and growth in the absence of arabinose leads to reduced SecE levels and enhanced proteolysis of SecY (Akiyama *et al.*, 1996). However, because SecE is essential, it cannot be completely depleted, and therefore residual amounts of SecY, SecE, and SecG were still detectable by Western blotting of both SDS gels (Figure 1B) and BN-PAGE (Figure 1A). Nevertheless, the reduced amounts of the 200-kDa complex in SecE-de-

pleted membranes suggest that the 200-kDa complex corresponds to the SecYEG complex.

In previous studies using BN-PAGE, multiple SecYEG complexes have been observed in membranes from SecYEG-overexpressing strains and depending on the protein/detergent ratio (Bessonneau *et al.*, 2002; Duong, 2003). In the current study we focused on the SecYEG complex in wild-type membranes and at a defined 1:1 protein/detergent ratio and found only the 200-kDa complex by immunodetection methods. Whether additional, less abundant complexes were also present under these conditions was analyzed by using radioactively labeled SecY, which provides a higher sensitivity in comparison to immunodetection. We have previously shown that when SecY is in vitro synthesized and integrated into *E. coli* membranes, it assembles with endog-

enous SecE and SecG into a functional SecYEG translocon (Koch and Müller, 2000). Thus, integrating in vitro-synthesized SecY into *E. coli* membranes should subsequently result in radioactive labeling of the 200-kDa complex and possibly, also in labeling of less abundant complexes. After in vitro synthesis of SecY and its integration into INVs, membranes were solubilized and separated by BN-PAGE. Radioactively labeled SecY complexes were then detected by phosphorimaging. With this approach we were able to detect the 200-kDa SecYEG complex, but importantly no additional complexes were visible (Figure 1C). To ensure that the labeling of the 200-kDa complex was dependent on the functional integration of the in vitro-synthesized SecY, we analyzed the formation of the 200-kDa complex in membranes from two SecY mutants. The TY1 mutant (*secY205*) carries a single amino acid replacement at the C-terminal end of SecY, which impairs a functional interaction with the motor protein SecA without significantly reducing the amount of SecY in the membrane (Matsumoto *et al.*, 1997). As a consequence of this mutation, the transport of SecA-dependent proteins is impaired, whereas SecA-independent proteins like SecY can still be integrated into these membranes. In agreement with this, in vitro-synthesized SecY assembled into the 200-kDa complex in TY1 membranes. In contrast, the formation of the 200-kDa complex was drastically reduced in the presence of TY22 (*secY40*) membranes. This mutant carries a single amino acid substitution within the fifth cytoplasmic loop of SecY that does not reduce the cellular concentration of SecY but interferes with the integration of SRP-dependent membrane proteins like SecY (Newitt and Bernstein, 1998; Angelini *et al.*, 2005). Thus, even when SecY was radioactively labeled and integrated into *E. coli* membranes, the highly sensitive phosphorimaging technique revealed only the 200-kDa complex, suggesting that this is the predominant organization of the resting translocon in wild-type *E. coli* membranes.

The predicted molecular weight (MW) of a monomeric SecYEG complex is 75 kDa, suggesting that the 200-kDa band reflects either an oligomeric SecYEG complex or a complex of SecYEG with additional proteins. To discriminate between these two possibilities, we purified a His-tagged SecYEG complex from *E. coli* via metal affinity chromatography. In the purified SecYEG sample, Coomassie-blue staining revealed three bands (Figure 1D). The 37-kDa band corresponds to SecY, which has a predicted molecular weight of 48-kDa but displays irregular migration behavior on SDS-PAGE (Ito, 1984). The two closely spaced bands at ~15 kDa represent His₆-tagged SecE (predicted MW 15 kDa) and SecG (predicted MW 12 kDa). When purified SecYEG was separated on BN-PAGE and after Western transfer decorated with α SecY-antibodies, we observed like in *E. coli* membranes the 200-kDa complex (Figure 1E). Because the purified SecYEG preparation did not contain detectable amounts of proteins shown to interact with the SecYEG translocon (Figure 1F), these data suggest that the 200-kDa complex is composed of only SecY, SecE, and SecG. Furthermore, this composition implies that the 200-kDa complex probably represents an oligomeric SecYEG complex. This was analyzed by treating DDM solubilized samples with SDS in order to dissociate possible complexes before their separation on BN-PAGE. When DDM-solubilized INVs were treated with SDS, the 200-kDa complex became significantly weaker, and an additional 100-kDa complex was observed with antibodies against α SecY (Figure 1E), SecE, or SecG (Supplementary Figure S1, Supplementary Material). Thus, the 200-kDa complex most likely represents a SecYEG dimer, which dissociates into a SecYEG monomer by adding

SDS. This interpretation is also in line with previous studies, pointing to the presence of a 200-kDa dimeric SecYEG complex in *E. coli* membranes (van der Does *et al.*, 2003; Angelini *et al.*, 2006; Alami *et al.*, 2007). The purified SecYEG complex was more sensitive toward SDS treatment and completely dissociated as indicated by the occurrence of a single band running below the 69-kDa marker band on BN-PAGE, which probably reflects SecY (Figure 1E). Because of their small size, we were unable to detect SecE and SecG in the SDS-treated purified SecYEG complex by BN-PAGE.

The SecYEG translocon has been shown to interact at least transiently with accessory membrane proteins like SecDFYajC and YidC. We therefore also analyzed wild-type *E. coli* membranes for SecDFYajC and YidC complexes. Unfortunately, the quality of the available antibodies against SecD and SecF did not allow us to make definite statements about the presence of SecDFYajC complexes in wild-type *E. coli* membranes, which is probably because of the rather low concentration of this trimeric complex in *E. coli* (Pogliano and Beckwith, 1994). In contrast, YidC was detected as a single band running at ~140 kDa in wild-type INVs on BN-PAGE (Figure 2A), which was not detectable in YidC-

Figure 2. YidC forms a 140-kDa dimer in wild-type *E. coli* cells. (A) Wild-type (wt) INVs and YidC-containing or -depleted INVs from the *E. coli* strain JS7131 were solubilized with DDM, separated by BN-PAGE, and decorated with α YidC antibodies. Where indicated, the sample was treated with 0.1% SDS for 10 min at 56°C before loading to BN-PAGE. (B) Immunodetection of YidC after separation of INVs (100 μ g protein) or purified YidC (0.75 μ g) on BN-PAGE.

depleted membranes from the conditional YidC mutant strain JS7131 (Samuelson *et al.*, 2000). When DDM-solubilized INVs were further treated with SDS, the 140-kDa band dissociated into a 60-kDa band (Figure 2A). We also separated purified YidC on BN-PAGE and compared it with wild-type INVs. A weak band at ~140 kDa was also detected with purified YidC, but the majority of the purified YidC was found in the 60-kDa band (Figure 2B), which suggests that during purification of YidC, the 140-kDa complex largely dissociated into monomeric YidC. The predicted molecular weight of YidC is 60 kDa, which suggests that the 140-kDa complex probably reflects a YidC dimer.

In conclusion, our data suggest that the resting SecYEG translocon in wild-type *E. coli* membranes is organized as SecYEG dimer, which runs at ~200 kDa on BN-PAGE. The difference to the calculated MW of 150 kDa for the SecYEG dimer, most likely reflects binding of detergent and Coomassie blue to the SecYEG complex, which is a common limitation of using BN-PAGE for completely accurate molecular-weight determination (Schägger, 2001; Heuberger *et al.*, 2002; Kulajta *et al.*, 2006). Furthermore, our results indicate that YidC does not seem to be a stable component of the resting translocon but that it forms a separate dimeric complex in wild-type *E. coli* membranes, which is in agreement with previously published data (Nouwen and Driessen, 2002).

A Tool for Analyzing Actively Transporting SecYEG Complexes

For analyzing whether different assemblies were observed for the actively transporting SecYEG complex, we used tRNA-tethered translocation intermediates of the SecA-dependent outer membrane protein pOmpA and the SRP-dependent polytopic inner membrane protein mannitol permease (MtlA). Because of the bulky tRNA at the C-terminus, these intermediates are trapped in the SecYEG channel (Matlack *et al.*, 1997; Figure 3A). This approach has been used to jam the SecYEG translocon (Koch *et al.*, 1999) and to determine the contact sites between a trapped substrate and the SecYEG translocon (Beck *et al.*, 2000; Cannon *et al.*, 2005). The tRNA-tethered pOmpA derivative was generated by treatment of a 126-amino acid long pOmpA RNC (pOmpA-126) with 8 M urea. This treatment dissociates the ribosome but leaves the covalent linkage between the peptide and the tRNA intact (Matlack *et al.*, 1997; Beck *et al.*, 2000).

The validity of the pOmpA/MtlA:tRNA approach was verified by performing flotation gradient analyses. In this assay, proteins that are targeted to the membrane-bound SecYEG translocon are recovered from the membrane fractions (fractions 2 and 3) of the flotation gradient, whereas nonbound proteins stay in the pellet fraction (fraction 5; Neumann-Haefelin *et al.*, 2000). To verify that the SecYEG translocon was indeed localized in the predicted membrane fractions of the flotation gradient, INVs were separated on a flotation gradient and the distribution of SecY within the gradient was analyzed by Western blotting. This experiment confirmed that the majority of SecY was found in fraction 3 of the flotation gradient (Figure 3B).

Because the SecA-dependent targeting of pOmpA is a posttranslational process, pOmpA-RNCs do not bind to the membrane unless the ribosome is released. This is shown in Figure 3C; irrespective of whether INVs had been present or not, the vast majority of the OmpA-126 RNCs were recovered from the pellet fractions, suggesting no significant membrane binding. In contrast, the pOmpA-126:tRNA derivative was found predominantly in the membrane fraction suggesting efficient binding of the pOmpA-126:tRNA to the

Figure 3. Membrane binding of tRNA-tethered substrates. (A) Schematic showing a tRNA-tethered substrate trapped in the SecYEG translocon. (B) Western blotting of INVs (250 μ g protein) separated by flotation gradients using antibodies against SecY. (C) OmpA-126 ribosome-associated nascent chains (OmpA-126 RNCs) or pOmpA-126:tRNA, which were generated by treatment of OmpA-126 RNCs with 8 M urea, were incubated posttranslationally in the presence of SecA and an ATP-regenerating system with or without wild-type INVs. The samples were subjected to flotation gradient analyses and subsequently separated on SDS-PAGE. Fraction 5, the pellet fraction containing the non-membrane-bound material; fractions 2 and 3, the INV-bound substrates. For calculating the percentage of membrane binding, the signal present in the five fractions was summed up and set as 100%. pOmpA corresponds to the unprocessed form and OmpA to the processed form, i.e., after signal sequence cleavage. (D) OmpA-126:tRNA and OmpA-126 RNCs were incubated with or without INVs under the same conditions as in C. Samples were separated after denaturation at 96°C under alkaline conditions, which releases the tRNA from the protein (Beck *et al.*, 2000). Lane 1 contains 10 times more material than lane 2 in order to visualize the residual amounts of the pOmpA-tRNA derivative. For the posttranslational incubation of pOmpA-126 RNCs puromycin was added (1.1 mM) in order to release the ribosome. (E) For flotation gradient analyses of MtlA-385 RNCs, *in vitro* synthesis was performed in the presence or absence of INVs, and the MtlA-385:tRNA derivative was generated by urea treatment of the membrane-bound MtlA-385 RNCs. Samples were then subjected to the flotation gradient centrifugation. Radioactively labeled material was visualized by phosphorimaging.

SecYEG translocon, which is in agreement with data showing that pOmpA:tRNA constructs can be cross-linked to SecY residues located within the proposed channel (Beck *et al.*, 2000; Osborne and Rapoport, 2007). A functional interaction of the pOmpA-126:tRNA intermediate with the SecYEG translocon is further suggested by the occurrence of two bands, which presumably indicated that the signal sequence of the pOmpA-126:tRNA derivative was cleaved off (Figure 3C). This was further verified by comparing signal sequence cleavage of the pOmpA-126:tRNA derivative with signal sequence cleavage of puromycin-treated pOmpA-126 RNCs. The covalent linkage between OmpA and the tRNA is rather sensitive to denaturation at alkaline conditions and at high temperature (Beck *et al.*, 2000). When pOmpA-126:tRNAs were synthesized in the absence of membranes and denatured in alkaline SDS-loading buffer at 96°C, most of the tRNA was cleaved off. Only when a large excess of the pOmpA-126:tRNA was loaded on the SDS gel, residual amounts of the tRNA containing pOmpA were still visible (Figure 3D, lane 1). Next, OmpA-126:tRNA was posttranslationally incubated with INV's under the same conditions as for the flotation gradient analyses. After the targeting, membrane bound material was isolated by centrifugation and only then denatured at 96°C under alkaline conditions to release the tRNA. With the amount of pOmpA-126:tRNA used in this experiment we were unable to detect residual amounts of tRNA-tethered pOmpA but observed only the pOmpA-126 and a second band migrating slightly faster. The same bands were observed when pOmpA-126 RNCs were posttranslationally incubated with INV's and then puromycin treated to dissociate the ribosome (Figure 3D, lane 3). These data confirm that the signal sequence of the pOmpA-tRNA derivative is cleaved off, which indicates a functional interaction with the SecYEG translocon.

In contrast to SecA-dependent secretory proteins, SRP-dependent membrane proteins are cotranslationally targeted to the SecYEG translocon. Thus, in the presence of INV's, ~45% of MtlA-385 RNCs were recovered from the membrane fractions of the flotation gradient (Figure 3E). Because of the cotranslational targeting of MtlA, the generation of MtlA:tRNA derivatives required that MtlA-RNCs were first synthesized in the presence or absence of INV's and only then treated with urea to dissociate the ribosome. This post-targeting urea treatment did not dissociate the MtlA:tRNA translocon interaction, because binding of MtlA-385:tRNA was about the same as observed for MtlA-385 RNCs (Figure 3E).

Thus, trapping a tRNA-tethered transport intermediate of either pOmpA or MtlA inside the SecYEG channel should allow us to determine whether 1) the resting and the active SecYEG channel exist in different assemblies and 2) whether also the translocation (pOmpA) and integration (MtlA) functions of SecYEG require distinct SecYEG complexes.

A Secretory Protein Is Trapped in a 140-kDa SecYEG Complex

In a first approach we incubated radioactively labeled pOmpA-126:tRNA with wild-type INV's, in the presence of the motor protein SecA and an ATP-regenerating system. Solubilization of the SecYEG complexes was performed under the same conditions as with the resting translocon, e.g., with 1 mg DDM/mg protein, and the solubilized samples were separated on BN-PAGE. Phosphor imaging revealed the presence of a single complex of ~140 kDa (Figure 4A), which was drastically reduced in both SecE-depleted CM124 INV's and in membranes from the TY1 strain (Figure 4A), in which the SecY-SecA interaction is impaired. It is important

Figure 4. OmpA-126:tRNA intermediates assemble into a 140-kDa complex. (A) pOmpA-126:tRNA derivatives were generated by urea treatment and posttranslationally incubated with either wild-type (wt) INV's, SecE-depleted CM124 INV's, or INV's derived from the *secY* mutant strain TY1, in the presence of SecA and an ATP-regenerating system. Membranes were isolated by centrifugation and solubilized with DDM and separated on BN-PAGE. Radioactively labeled complexes were visualized by phosphorimaging. (B) Wild-type (wt) INV's were preincubated with either lysis buffer, preimmune serum (pre-IS) or α SecY-antibodies before the addition of pOmpA-126:tRNA. The membranes were reisolated by centrifugation, solubilized, and separated by BN-PAGE followed by visualization using phosphorimaging. (C) The 140-kDa complex was excised from the BN-PAGE and separated on a 2D SDS-PAGE. After transfer to a PVDF membrane, SecY was detected by antibodies and the OmpA intermediate by phosphorimaging. As control, INV's were separated directly on SDS-PAGE; asterisk (*) indicates proteolysis products of SecY. (D) As in C, but in addition to the 140-kDa complex observed in wild-type INV's, also the corresponding area of SecE-depleted membranes was separated on SDS-PAGE and analyzed by Western blotting using antibodies against SecY, SecE, and SecG.

to note that with the low amounts of in vitro-synthesized pOmpA-126:tRNA we were not able to saturate the SecYEG translocon and therefore Western blotting revealed that the 200-kDa complex was still the dominating species even in the presence of the substrate (Supplementary Figure S2A, Supplementary Material). Nevertheless, these data indicate that the formation of the 140-kDa complex was dependent on the presence of the SecYEG translocon as well as on a functional SecY-SecA interaction, as expected for the targeting of the secretory protein OmpA. Our BN-PAGE analyses do not allow to differentiate whether the 140-kDa complex contains only the processed OmpA:tRNA derivative or also the signal sequence containing pOmpA:tRNA derivative (see Figure 3B), because the difference of only 21 amino acids cannot be resolved by BN-PAGE. On the other hand, multiple studies have shown that signal sequence cleavage is not a prerequisite for complete SecYEG-dependent translocation of pOmpA in vitro (Beha *et al.*, 2003; Mori and Ito, 2003; de Keyser *et al.*, 2007).

The presence of SecY in the 140-kDa complex was further confirmed by preincubating INV with α SecY-antibodies, which should prevent or at least impair the pOmpA-126:tRNA-SecYEG interaction. When pOmpA-126:tRNA were incubated with INV that had been pre-treated with α SecY-antibodies, the formation of the 140-kDa complex was significantly reduced (Figure 4B). In contrast, pre-incubating INV with pre-immune serum or buffer had no effect on the formation of the 140-kDa complex. For directly demonstrating that SecY, SecE, and SecG were present in the 140-kDa complex, the complex was excised from the BN-PAGE and separated on a 2D SDS-PAGE. By phosphorimaging the radioactively labeled OmpA-126:tRNA was clearly visible in the 140-kDa complex (Figure 4C, top panel) and Western blotting revealed also the presence of SecY in this complex (Figure 4C, bottom panel). In both the INV control lane and the separated 140-kDa band, smaller bands recognized by the α SecY antibodies were visible (Figure 4C, bottom panel, *). It is likely that the lower bands reflect proteolytic cleavage products of SecY because SecY has been shown to be highly susceptible toward proteolytic cleavage (Ito, 1984; Chiba *et al.*, 2002), which is probably further enhanced by the lengthy procedure of combining 1D BN-PAGE with 2D SDS-PAGE. This experiment was further controlled by using the 2D approach also with SecE-depleted membranes, in which the 140-kDa complex is significantly reduced (Figure 4A). SecY, but also SecE and SecG were detectable in the 140-kDa complex formed in wild-type INV (Figure 4D). If, however, OmpA-126:tRNA was incubated with SecE-depleted membranes and subjected to the 2D analysis, we were unable to detect SecY and SecE and only a very low signal for SecG. These data further confirm that the 140-kDa complex represents a SecYEG complex carrying the radioactively labeled OmpA:tRNA intermediate.

These data imply that the size of the actively translocating SecYEG channel is actually smaller than the 200-kDa resting SecYEG channel (Figure 1). However, the experimental procedure required the use of urea for generating the pOmpA-126:tRNA derivative and the addition of SecA and an ATP-regenerating system for the subsequent targeting to the SecYEG translocon. We therefore had to exclude that it is the experimental setup that causes the dissociation or destabilization of the 200-kDa SecYEG complex. However, we still observed the 200-kDa complex but not the 140-kDa complex when wild-type *E. coli* membranes were treated with urea, SecA and the ATP-regenerating system in the absence of the pOmpA-126:tRNA substrate (Supplementary Figure S2B, Supplementary Material). These data also support our as-

sumption that the 200-kDa SecYEG complex reflects a resting translocon. If secretory substrates were trapped in the SecYEG channel during INV preparation, they should have been translocated by adding SecA and the ATP-regenerating system. The urea treatment should have also dissociated RNCs that might have been bound to SecYEG during INV preparation. In agreement with this, the 200-kDa complex was also unchanged when INV were treated with puromycin and 1M potassium acetate, a treatment that is commonly used for dissociating RNCs (Supplementary Figure S2B, Supplementary Material).

Collectively, our data indicate that the 140-kDa complex is assembled upon the SecA-dependent interaction of pOmpA-126:tRNA with the SecYEG translocon. Strikingly, this active, substrate-containing Sec translocon is significantly smaller than the 200-kDa resting translocon (Figure 1) and fits best with the association of the 36-kDa pOmpA-126:tRNA with a 100-kDa monomeric SecYEG complex (Figure 1E). This assembly is also supported by cross linking data, which show a pOmpA:tRNA derivative inside of a monomeric SecYEG complex (Cannon *et al.*, 2005).

Higher Ordered SecYEG Complexes Are Detectable during Membrane Protein Integration

For analyzing the SecYEG translocon during the integration of an SRP-dependent membrane protein, we applied the tRNA approach to the polytopic membrane protein MtlA. We first analyzed the MtlA-385:tRNA construct, which encompasses all six transmembrane domains (TMs) of mannitol permease. In comparison to the pOmpA:tRNA complexes, the separation of the MtlA:tRNA complexes on BN-PAGE revealed less focused bands, which probably suggests some instability of the SecYEG complexes that are formed during the integration of a polytopic membrane protein. Nevertheless, the integration of MtlA-385:tRNA into the SecYEG translocon resulted in two visible complexes, running on BN-PAGE at ~250 and 600 kDa, respectively (Figure 5A). The detection of two complexes with the MtlA-385:tRNA construct prompted us to analyze whether complex formation was influenced by the length or the number of TMs of a membrane protein. The MtlA-348:tRNA construct encompasses like MtlA-385 six TMs of MtlA. However, during the initial targeting of MtlA-348 RNCs to the membrane, the last TM is still inside of the ribosomal peptide tunnel and thus unable to make contact with the SecYEG translocon. Strikingly, with this construct we only observed the 250-kDa complex but not the 600-kDa complex. The 250-kDa complex was also observed with the MtlA-304:tRNA construct, which exposes only four TMs and with the MtlA-189:tRNA, which exposes only three TMs (Figure 5A).

By chemical cross-linking it was previously shown that MtlA RNCs are found in close proximity not only to the SecYEG translocon but also to YidC (Beck *et al.*, 2001). YidC itself is supposed to bind to the SecYEG translocon via the trimeric SecDFYajC complex (Nouwen and Driessen, 2002; Xie *et al.*, 2006). Such a composite assembly of the SecYEG translocon during MtlA integration could in principle explain the existence of different SecYEG subassemblies. We therefore analyzed whether complex formation was impaired in different *E. coli* mutant membranes. In *E. coli* BL325, the expression of SecDFYajC is under control of the arabinose promoter and in membranes from arabinose grown cells, MtlA-385:tRNA was again found in both the 600- and the 250-kDa complexes (Figure 5B). Although both complexes were still detectable in SecDFYajC-depleted membranes, they were significantly reduced (Figure 5B). This was not the result of reduced SecY or YidC concentra-

Figure 5. tRNA-tethered mannitol permease intermediates assemble into two distinct complexes of 250- and 600 kDa. (A) MtIA-RNCs of different length were in vitro synthesized in the presence of wild-type (wt) INVs. Membrane-bound MtIA-RNCs were then urea treated to generate the MtIA:tRNA derivatives and solubilized with DDM. After separation on BN-PAGE, the bands were visualized by phosphorimaging. Both the length of the RNCs and the number of transmembrane domains (TMs) are indicated. (+1) indicates that during the initial targeting of the MtIA-RNCs to the membrane, one additional TM is located inside the ribosomal exit tunnel, which is exposed only after the dissociation of ribosome by urea treatment. (B) MtIA-385 RNCs were in vitro synthesized in the presence of INVs derived from indicated

tions in these membranes, because Western blotting revealed that although SecD and SecF were depleted, the amounts of SecY and YidC were not significantly changed (Figure 5C). Different from SecDFYajC depleted membranes, the 600- and the 250-kDa complex were not detectable in SecE-depleted membranes or in TY22 membranes, which are impaired in SRP-dependent membrane protein integration (Figure 5B). Finally, also in YidC-depleted membranes, both complexes were undetectable (Figure 5B). Again, this was not a secondary effect due to reduced SecY concentrations because Western blotting revealed no significant differences in the SecY content between YidC-containing and YidC-depleted membranes (Figure 5C). Whether the weak radioactively labeled band at ~440 kDa reflects an additional intermediate or corresponds to unspecific binding is currently unknown.

To demonstrate directly whether SecYEG, YidC and possibly also SecDFYajC are components of the 250- and 600-kDa complexes, both complexes were excised from the BN-PAGE and separated on 2D SDS-PAGE, as described before for the 140-kDa SecYEG-pOmpA complex (Figure 4). Phosphor imaging revealed equal amounts of the radioactively labeled MtIA-385:tRNA in both complexes (Figure 6A, top panel). Subsequent immunodetection with α SecY-antibodies revealed a single SecY band in the INV control lane (Figure 6A, middle panel), but two SecY bands for the 600- and the 250-kDa complexes, one running below the 50-kDa marker band and a second band running below the 37-kDa band. Both bands did not exactly align with the SecY band in the INV sample, because the BN-PAGE gel slices were mounted on top of the SDS gel. As observed for the 2D separation of the 140-kDa OmpA:tRNA complex (Figure 4D), the lower band probably reflects a proteolytic cleavage product of SecY. Nonetheless, SecY was detectable in both the 600- and the 250-kDa complex, although the signal in the 250-kDa complex was significantly weaker than in the 600-kDa complex. In addition to SecY, Western blotting also revealed the presence of SecE and SecG in both complexes; but significantly reduced signals if SecE-depleted membranes were analyzed (Figure 6B). In both the 250- and the 600-kDa complexes we also detected YidC and again the YidC signal was slightly weaker in the 250-kDa complex (Figure 6A, bottom panel). It is important to emphasize that both complexes contained the same amount of radioactively labeled MtIA (Figure 6A, top panel), which suggests that the 250-kDa complex contains less SecY and probably also less YidC than the 600-kDa complex. However, we were unable to obtain unambiguous results as to the presence of SecDFYajC in these complexes. Whether this was due to the extremely low concentration of SecDFYajC in wild-type *E. coli* membranes (Pogliano and Beckwith, 1994) or whether this indicates that SecDFYajC only transiently interacts with the 250- and 600-kDa complexes, is currently unknown. The observation that the 250- and the 600-kDa complex were detectable even in SecDFYajC-depleted membranes, albeit at a reduced level (Figure 5B), probably indicates that SecDFYajC itself is not a component of the complexes but rather required for their assembly or stability. Collectively, our data strongly suggest that the 250- and the 600-kDa complexes represent true SecYEG-YidC complexes that are spe-

strains. After urea treatment and solubilization, the samples were separated on BN-PAGE and visualized by autoradiography. (C) Western blot of SecDFYajC-containing and -depleted membranes derived of *E. coli* BL325 and of YidC-containing and -depleted membranes from *E. coli* JS7131.

Figure 6. The 250- and the 600-kDa complexes contain SecY, SecE, SecG, and YidC. (A) The 250- and the 600-kDa complexes were excised from the BN-PAGE, separated on a 2D SDS-PAGE, and analyzed by Western blotting using SecY/YidC antibodies and by phosphorimaging. Asterisk (*) indicates proteolysis products of SecY. (B) As in A but in addition to the 250- and 600-kDa complexes observed in wild-type (wt) INVs, also the corresponding areas of SecE-depleted membranes were separated on SDS-PAGE and analyzed by Western blotting using antibodies against SecY, SecE, and SecG. The arrow indicates the weak SecY signal in the 250-kDa complex.

cifically assembled during the integration of the polytopic membrane protein MtlA.

DISCUSSION

Our analyses using *E. coli* membranes harboring wild-type SecYEG amounts have revealed distinct entities of the SecYEG translocon: 1) a 200-kDa resting complex, 2) a 140-kDa complex that was observed during the translocation of the SecA-dependent protein OmpA, and 3) two complexes of 250 and 600 kDa, which were detectable during the integration of the SRP-dependent membrane protein MtlA.

The Resting Translocon

The monomeric SecYEG complex migrates on BN-PAGE at ~100 kDa (van der Does *et al.*, 2003; Alami *et al.*, 2007, Figure 1) and thus the 200-kDa complex that is observed for both the purified SecYEG complex and in wild-type membranes (Figure 1) suggests that the inactive SecYEG translocon is organized as a SecYEG dimer containing only the core subunits SecY, SecE, and SecG. In particular, YidC which has been found in complex with SecYEG in a SecYEG overex-

pressing strain (Scotti *et al.*, 2000), appears to form at least in wild-type membranes a separate complex of 140 kDa, probably corresponding to a YidC dimer (Figure 2). Although previous studies have revealed monomeric, dimeric and tetrameric SecYEG assemblies in *E. coli* membranes, depending in particular on the expression level and the solubilization conditions (Yahr and Wickner, 2000; Bessonneau *et al.*, 2002; van der Does *et al.*, 2003; Duong, 2003; Scheuring *et al.*, 2005), there now seems to be consensus that the resting SecYEG channel predominantly exists as a dimer (Duong, 2007), which is further supported by our data.

The Translocase State of the SecYEG Translocon

During transport of the secretory protein pOmpA, a 140-kDa complex was detectable, which was only assembled upon SecA-dependent targeting of OmpA to the SecYEG complex (Figure 4). Because the monomeric SecYEG complex migrates on BN-PAGE at ~100 kDa (Figure 1E; van der Does *et al.*, 2003), the 140-kDa complex fits best with the 36-kDa OmpA:trRNA trapped in a monomeric SecYEG channel. This is in line with cross-linking data showing OmpA in close proximity to the interior of the predicted monomeric SecY channel (Cannon *et al.*, 2005; Osborne and Rapoport, 2007). By using two covalently fused SecYEG protomers, it has recently been suggested that although a single SecYEG copy appears to be sufficient for providing the pore, a second SecYEG molecule is required for SecA binding (Osborne and Rapoport, 2007). In this scenario, SecA would bind to one copy of SecYEG, but translocation would proceed through the other. However, the recent crystal structures of SecY in complex with SecA (Tsukazaki *et al.*, 2008; Zimmer *et al.*, 2008) in combination with cross-linking data (Erlandson *et al.*, 2008; Tsukazaki *et al.*, 2008) suggest that a single copy of SecY is sufficient for simultaneously binding SecA and translocating the substrate. It is important to note that our data cannot exclude that the actively translocating SecYEG is organized as a dimer. However, in contrast to the resting SecYEG, which forms a stable dimer under our experimental conditions (Figure 1), an actively translocating SecYEG dimer appears to be not stable enough to be visualized by BN-PAGE. This could indicate that the contacts between the two SecYEG protomers are destabilized during the translocation process. Two conformations of a SecYEG dimer have been proposed: a back-to-back orientation, in which both protomers interact via a SecE–SecE interface. This orientation probably reflects the resting state because it is deduced from 2D crystals of purified, reconstituted SecYEG (Breyton *et al.*, 2002; Bostina *et al.*, 2005). In the front-to-front orientation the predicted exit gates of the two SecY channels are facing each other (Mitra *et al.*, 2005, 2006). This orientation is based on cryo-EM studies of SecYEG reconstituted with a RNC complex and thus presumably reflects the active state. If the front-to-front orientation is the active conformation, signal sequence release would require opening of the proposed lateral exit gate. This opening would probably result in changes at the SecY–SecY interface, which could destabilize the SecYEG dimer and explain why the actively translocating SecYEG complex is detectable as a monomer in our BN-PAGE analyses.

The Integrase State of the SecYEG Translocon

Our data provide also information about the bacterial SecYEG translocon during the cotranslational integration of a polytopic membrane protein. The detection of SecY and YidC containing 250- and 600-kDa complexes (Figure 5) indicates that during membrane protein integration the SecYEG translocon assembles together with YidC into higher

ordered structures that are clearly different from the assemblies observed during the translocation of a secretory protein. This suggests that the composition and oligomeric state of the SecYEG translocon is not static but dynamically adjusted to its cargo. This appears to be a general feature of the Sec translocon because dynamic oligomerization has also been suggested for the Sec61 complex in eukaryotes (Hamman *et al.*, 1997; Beckmann *et al.*, 2001; Wirth *et al.*, 2003).

Currently we are unable to make definite statements as to the copy number of SecYEG and YidC in these complexes. Nevertheless, previous data have shown that a SecYEG tetramer migrates at ~440 kDa on BN-PAGE (Duong, 2003) and a (SecYEG)₄-MtIA:tRNA complex would be expected to run at ~500 kDa. By recruiting a YidC dimer the 500-kDa complex would be converted into a 640-kDa complex, which would be in line with the complex running at ~600 kDa on BN-PAGE (Figure 5). This speculative assembly scheme would be similar to the proposed assembly of the eukaryotic Sec61 complex, which has been shown to assemble upon ribosome contact as a dimer of dimers, to which a dimer of TRAP, the translocon-associated protein, is bound (Menetret *et al.*, 2005; Schaletzky and Rapoport, 2006). On the other hand, it is important to emphasize that the detection of complexes by BN-PAGE primarily depicts their stability and not necessarily the order of their formation. Thus, the 250-kDa complex might correspond to an assembly intermediate which is formed by recruiting additional proteins to a SecYEG core in a length/TM-dependent manner. Alternatively, the 250-kDa complex might reflect a disassembly intermediate of the larger 600-kDa complex, which dissociated during the BN-PAGE. The analysis of the composition of the 250-kDa complex by Western blotting revealed the presence of both SecY and YidC; however, in particular the amount of SecY appeared to be significantly lower than in the 600-kDa complex (Figure 6A), suggesting a lower copy number of SecY in this complex. However, as for the 600-kDa complex, the exact copy number of SecYEG and YidC in the 250-kDa complex remains to be determined.

Binding of YidC to the SecYEG translocon probably depends on SecDFYajC (Nouwen and Driessen, 2002; Xie *et al.*, 2006). In agreement with this, the 600- and the 250-kDa complexes were reduced in the absence of SecDFYajC (Figure 5), but we did not observe the appearance of smaller complexes. This suggests that SecDFYajC is not a stable component of both complexes but rather that it has a transient function in complex formation. Alternatively, it is also possible that complexes lacking SecDFYajC are completely unstable and that the 250- and 600-kDa complexes detectable under SecDFYajC-depleted conditions represent those that still contain the residual amounts of SecDFYajC.

Although the translocation pore may be formed by a single SecYEG/Sec61 heterotrimer and the SecYEG monomer is sufficient for ribosome binding (Menetret *et al.*, 2007), multiple studies suggest a highly dynamic and flexible translocon. In particular, the ability of the Sec translocon to bind multiple TMs and hydrophilic segments simultaneously (Nagamori *et al.*, 2003; McCormick *et al.*, 2003; Heinrich and Rapoport, 2003; Sadlish *et al.*, 2005; Kida *et al.*, 2007) and to allow the reorientation of TMs once they have been inserted into the translocon (Goder and Spiess, 2003) is difficult to reconcile with a rather static translocon. Instead, our data support the idea of a composite translocon that dynamically adjusts to a given substrate.

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