STRUCTURAL BIOLOGY

Insertion and folding pathways of single membrane proteins guided by translocases and insertases

Tetiana Serdiuk[1], Anja Steudle[2], Stefania A. Mari[1], Selen Manioglu[1], H. Ronald Kaback[3,4,5], Andreas Kuhn[2], Daniel J. Müller[1]*

Biogenesis in prokaryotes and eukaryotes requires the insertion of α-helical proteins into cellular membranes for which they use universally conserved cellular machineries. In bacterial inner membranes, insertion is facilitated by YidC insertase and SecYEG translocon working individually or cooperatively. How insertase and translocon fold a polypeptide into the native protein in the membrane is largely unknown. We apply single-molecule force spectroscopy assays to investigate the insertion and folding process of single lactose permease (LacY) precursors assisted by YidC and SecYEG. Both YidC and SecYEG initiate folding of the completely unfolded polypeptide by inserting a single structural segment. YidC then inserts the remaining segments in random order, whereas SecYEG inserts them sequentially. Each type of insertion process proceeds until LacY folding is complete. When YidC and SecYEG cooperate, the folding pathway of the membrane protein is dominated by the translocase. We propose that both of the fundamentally different pathways along which YidC and SecYEG insert and fold a polypeptide are essential components of membrane protein biogenesis.

INTRODUCTION

The biogenesis of most membrane proteins is governed by specific interactions between the newly synthetized nascent polypeptide chain and the evolutionary conserved and essential insertases and translocases (*1*–*3*). Insertases and translocases recognize their substrate and lower the free-energy barrier for inserting and folding the polypeptide into cellular membranes (*3, 4*). This insertion and folding can occur cotranslationally as the polypeptide exits the ribosome or posttranslationally after the polypeptide has been released by the ribosome. The bacterial translocase SecYEG has a eukaryotic homolog, Sec61, in the endoplasmatic reticulum (*1*), whereas the bacterial insertase YidC has Oxa1 and Oxa2 homologs in mitochondria, Get1 in endoplasmatic reticulum, and Alb3 in chloroplasts (*5*–*7*). In Gram-negative bacteria, SecYEG folds α-helical membrane proteins into the inner membrane and translocates precursors of soluble periplasmic and β-barrel outer membrane proteins to the periplasm (*1, 8*). Independently of SecYEG, YidC inserts certain membrane proteins into the bacterial inner membrane (*3, 9*). Alternatively, SecYEG and YidC can cooperate to insert and fold α-helical proteins into inner membranes (*3*). In principle, these combinations provide at least three pathways to insert and fold proteins into cellular membranes. However, it remains poorly understood whether insertases and translocons guide the folding of membrane proteins along different pathways and what the distinct folding steps of these pathways look like. One reason for this lack of knowledge is the complexity of the insertion and folding process, which is sensitive to the composition of the phospholipid membrane, temperature, pH, and osmolarity, and thus should be studied under physiological conditions (*4, 10, 11*). Another barrier is the lack of experimental techniques capable of following the structural segments a single polypeptide inserts into a membrane.

More than two decades ago, atomic force microscopy (AFM)–based single-molecule force spectroscopy (SMFS) was introduced to mechanically unfold membrane proteins from native membranes and to quantify mechanical, kinetic, and energetic properties of their fold (*12*–*14*). The SMFS unfolding pattern is characteristic of the conformation, ligand or substrate binding, assembly, and misfolding of a membrane protein and so serves as a fingerprint to identify the native fold and state of membrane proteins (*15*–*18*). Unfolded membrane proteins are prone to misfolding, although chaperones affect this and promote folding (*18*). Recently, we introduced an SMFS-based assay to pick up the polytopic α-helical lactose permease (LacY) from *Escherichia coli* by the C-terminal end using an AFM cantilever and to mechanically unfold and extract the protein from the membrane (*17, 19*). The unfolded polypeptide could then be brought into close proximity (~5 to 10 nm) to another phospholipid membrane (*20*) and allowed to insert and fold starting from the free N-terminal end (*4*). We observed that the LacY polypeptide could not insert without assistance and misfolded. The insertase YidC suppressed misfolding and enabled the polypeptide to insert stepwise into the membrane (*20, 21*). However, it remains unclear how YidC and SecYEG individually facilitate insertion of the LacY polypeptide, whether and how the folding pathways guided by YidC and SecYEG differ, and how YidC and SecYEG work together to fold native membrane proteins.

To address this basic question of membrane protein biogenesis, here, we use SMFS-based assays to characterize the insertion and folding pathways guided by YidC and SecYEG alone and by both together. As a model membrane protein, we use LacY, which folds 12 transmembrane α-helices organized in two pseudosymmetrical C- and N-terminal six-helix bundles (*22, 23*). LacY is of particular interest because it serves as a paradigm of the large and conserved major facilitator superfamily (*24*). Our assays unravel the working principles of how insertases and translocases, both individually and collectively, initiate the insertion of polypeptides into the membrane and stepwise fold the polypeptide toward the native membrane protein.

[1]Department of Biosystems Science and Engineering, Eidgenössische Technische Hochschule (ETH)–Zürich, 4058 Basel, Switzerland. [2]Institute of Microbiology and Molecular Biology, University of Hohenheim, 70599 Stuttgart, Germany. [3]Department of Physiology, University of California, Los Angeles, Los Angeles, CA, USA. [4]Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, Los Angeles, CA, USA. [5]Molecular Biology Institute, University of California, Los Angeles, Los Angeles, CA, USA.
*Corresponding author. Email: daniel.mueller@bsse.ethz.ch

RESULTS

Preparing insertase, translocase, and LacY

To set up a single-molecule folding assay of LacY, we wanted to pick up LacY by the stylus of the AFM cantilever, to mechanically unfold and extract a single LacY from a membrane, and to transport the extended polypeptide to another membrane containing YidC, SecYEG, or YidC and SecYEG. At each of the target membranes, the polypeptide had to be kept in close proximity (~5 to 10 nm) to allow the insertion and folding for sufficiently long time periods (~1 to 10 s). Because α-helical membrane proteins exit the ribosome and start insertion and folding from the N-terminal end (*4*), we searched for ways to attach the C terminus of LacY to the stylus. As shown earlier, engineering a 36–amino acid–long unstructured "polyGly" polypeptide to the C-terminal end of LacY considerably enhances the probability of mechanically attaching the AFM stylus to the C terminus (*17*). We hence elongated the C-terminal end of wild-type LacY with the polyGly polypeptide, followed by an eight–amino acid–long His-tag (His_8-tag; fig. S1A), which both did not affect LacY structurally or functionally (*17*). Then, we reconstituted this LacY construct into phospholipid membranes consisting of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (PE)/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-rac-(1-glycerol) (PG) at 3:1 (PE/PG) ratio (fig. S1B), which roughly mimics the lipid composition of the *E. coli* inner membrane and maintains the full functionality of LacY (*22, 25*).

Next, we reconstituted YidC, SecYEG, or YidC and SecYEG into phospholipid (PE/PG, 3:1) liposomes (Materials and Methods and figs. S1 and S2). To ensure that coreconstituted YidC and SecYEG were in close proximity, we engineered a fusion construct having a 14–amino acid–long (QLLEVLFQGPELHL) linker (*26*), fusing the C-terminal end of SecY and the N-terminal end of YidC. The linker, which encoded a specific cleavage site LEVLFQ/GP for the PreScission protease, ensured that insertase and translocon showed the same stoichiometry, were in close proximity, and both oriented the same way toward the membrane surface. After overexpression and purification of each protein, SDS–polyacrylamide gel electrophoresis (SDS-PAGE) confirmed their abundance and showed that SecY and SecY-YidC fusion construct copurified with SecE and SecG (fig. S2A). After reconstitution, we imaged the LacY, YidC, SecYEG, and SecYEG-YidC proteoliposomes by transmission electron microscopy (TEM) and AFM (figs. S1, B to E, and S2, B to E). Together, TEM and AFM showed the reconstituted LacY, YidC, SecYEG, or SecYEG-YidC construct homogeneously distributing in phospholipid membranes.

Functionality of insertase and translocase construct

The functionality of YidC, SecYEG, and SecYEG-YidC construct used in our study was examined for complementation in *E. coli* strains lacking either functional SecY or YidC (fig. S3). To test whether SecYEG and SecYEG-YidC fusion construct were functional, we used a cold-sensitive *E. coli* AF659 SecYcs strain that expresses no functional SecY at 20°C (*27*). In this strain, SecY is depleted at 20°C, thus inhibiting bacterial growth unless introducing a functional SecY plasmid (*27, 28*). The strain was transformed with a vector encoding SecYEG, SecYEG-YidC fusion construct, or no insert as a control. Only in the presence of SecYEG or SecYEG-YidC plasmid, the bacteria grew at 20°C. Further, comparison to bacterial growth at 37°C showed that the plasmids encoding SecYEG or SecYEG-YidC fusion construct functionally substituted SecYEG. To evaluate functionality of YidC, we used the *E. coli* MK6 strain, in which chromosomally encoded YidC is under control of the arabinose promoter (*29*). In the absence of arabinose, YidC depletes, thus inhibiting bacterial growth unless presenting a plasmid encoding the functional YidC gene. The strain was transformed with a vector carrying YidC, SecYEG-YidC construct, or no insert as a control. Only in the presence of YidC or SecYEG-YidC construct, the bacteria grew in the absence of arabinose. Comparison to bacterial growth in the presence of arabinose showed that YidC and SecYEG-YidC construct functionally substituted the YidC depleted in the absence of arabinose. In summary, these complementation experiments show that insertase and translocase alone and within the SecYEG-YidC fusion construct are functional.

YidC catalyzes stochastic insertion and folding

To characterize how YidC assists the insertion and folding of the LacY polypeptide, we adsorbed YidC proteoliposomes to mica in buffer solution and imaged the sample by AFM to localize YidC membranes (fig. S4A). We then coadsorbed LacY proteoliposomes and imaged the sample again to colocalize membranes embedding LacY (fig. S4B). To tether a single LacY, we gently pushed the AFM stylus onto the LacY membrane (700 pN for 500 ms). Then, we retracted the stylus, recording a force-distance curve (Fig. 1, A and B). In most cases (99.9%, n = 8,063,324), the curve detected no force peaks, indicating that no LacY attached to the stylus. However, in ~0.1%, a LacY attached to the stylus, and the force-distance curve recorded a sawtooth-like pattern of force peaks, with each force peak representing an unfolding step of the transporter (*17*). Repeating the experiment several hundred times revealed a highly reproducible force peak pattern of 10 force peaks (Fig. 1C). Previous measurements showed this force peak pattern to be specific for the C-terminal attachment of LacY to the AFM stylus (*17, 19*) and assigned each of the 10 force peaks to the unfolding of one distinct structural segment of LacY (Fig. 1D). It was also shown that this force peak pattern, which describes the stepwise unfolding of structural segments, is sensitive to the native fold of LacY and changes upon misfolding (*17, 19*). The pattern can thus serve as a fingerprint of LacY residing in the natively folded state (*21*).

Next, we followed a previously published protocol to characterize how YidC assists LacY polypeptides to insert and fold into the membrane (*20*). In each experiment, we attached the C-terminal end of a LacY to the AFM stylus, unfolded and extracted the LacY from the membrane, and brought the LacY polypeptide in close proximity (~5 to 10 nm) to the surface of a YidC membrane (Fig. 2A). After a given folding time, we retracted the AFM stylus and recorded a force-distance curve (Fig. 2B). We recorded more than 160 single LacY polypeptides inserting and folding into YidC membranes at folding times ranging from 1 to 10 s. To further increase the statistics necessary to gain deeper insight into the folding process, we included our previously published dataset (*20*). Together, we analyzed 478 experiments recording a single LacY inserting and folding in the presence of YidC, applying advanced protocols (Materials and Methods). From 155 experiments detecting the insertion and folding of a LacY after 1 s, 72% recorded only one force peak (Fig. 2B), which could occur at varying positions (fig. S5A). The position of each force peak correlated with 1 of the 10 force peaks recorded in the fingerprint pattern of native LacY (Fig. 1C and fig. S6). The experiments thus suggested that YidC enabled the LacY polypeptide to start the insertion process with any of the 10 structural segments S1 to S10 (Fig. 2C). Thereby, segments of the C-terminal bundle of LacY showed slightly enhanced preference to insert with segment S4 showing the highest values.

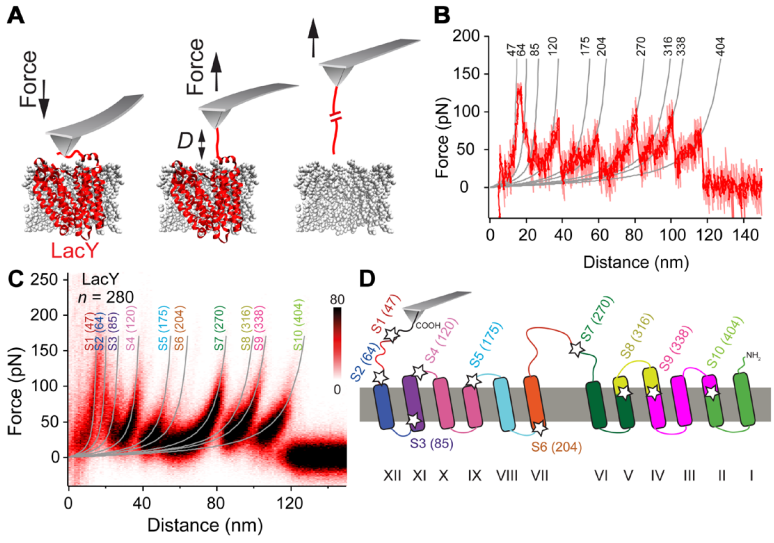

Fig. 1. Unfolding fingerprint pattern of native LacY. (**A**) Schematics of the mechanical unfolding of native LacY from the phospholipid (PE/PG) membrane. The AFM stylus is pushed onto LacY (PDB 1PV7) to nonspecifically attach the elongated C-terminal end (polyGly LacY). Then, the cantilever is retracted to apply mechanical pulling force to the terminus. During retraction, LacY stepwise unfolds structural segments until being completely unfolded and extracted from the membrane (*17*). (**B**) Force-distance curve recorded upon unfolding a single LacY. The force-distance curve is shown as raw data (pale red) and smoothed (Savitzky-Golay filter, dark red). To obtain the contour lengths of mechanically unfolded polypeptide stretches (in amino acid), we fit every force peak using the worm-like chain (WLC) model (gray curves; Materials and Methods). (**C**) Density plot of 280 superimposed force-distance curves each showing the mechanical unfolding of one LacY. Mean contour lengths given at the top of each WLC curve define the ending of the previously unfolded structural segment and the beginning of the next segment to be unfolded. (**D**) Structural segments S1 to S10 mapped to the secondary structure of LacY as unfolded beginning from the C terminus. The C terminus is shown at the left, and transmembrane α-helices are numbered I to XII.

At folding times of 2 s, the force-distance curves recorded more force peaks, and the comparison with the native fingerprint pattern of LacY showed that the polypeptide inserted more structural segments into the YidC membrane (Fig. 2D and fig. S5B). The force peaks appeared in random order without any preference; hence, the probability to insert distributed equally among all structural segments (Fig. 2E). At folding times of 5 s, the number of force peaks and thus of inserted structural segments increased further (Fig. 2F and fig. S5B). In average, the LacY polypeptide inserted six structural segments, although some experiments recorded all force peaks such as described for native LacY (10%, n = 60). The probability to insert into the membrane distributed uniformly among all structural segments (Fig. 2G). In summary, the experiments suggest that the LacY polypeptide brought in close proximity to YidC inserted structural segments into the membrane, the number of which increased with the folding time until, in some cases, all structural segments have inserted and folded such as observed for the native LacY. The order in which the segments inserted distributed randomly, thus suggesting their stochastic insertion.

LacY requires assistance to insert and fold into membranes

To test whether the insertion and folding steps of the LacY polypeptide were specific to YidC, we attached the C terminus of LacY to the AFM stylus, retracted the stylus to mechanically unfold and extract LacY from the membrane, and approached the polypeptide to another position (~20 to 100 nm in distance) in close proximity (~10 nm) to the same membrane, an empty phospholipid membrane, or a phospholipid membrane embedding the betaine transporter BetP, which has no function in assisting the insertion and folding of membrane proteins (fig. S7). After folding times of 1 to 5 s, we retracted the stylus to test whether the polypeptide inserted and folded into either one of the membranes. Even at 5-s folding time, most of single-molecule folding experiments (~99.995%, n = 374,141) detected no force peaks (fig. S7), thus indicating that LacY alone could not insert into the membranes. Rarely, force-distance curves detected force peaks (~0.005%), which positions did not compare with the native fingerprint pattern of LacY, and thus suggested a misfolded or aggregated polypeptide (*20*). These controls, which are supported by previous experiments showing that bovine serum albumin or lysozyme has no effect on the insertion and folding of LacY (*20, 21*), show that the fully unfolded and extracted LacY polypeptide cannot insert and fold into the membrane without assistance.

SecYEG inserts structural segments sequentially

Next, we used our assay to investigate how SecYEG inserts and folds LacY polypeptides into the membrane. Hence, we colocalized SecYEG and LacY proteoliposomes coadsorbed to mica using AFM, extracted a single LacY by mechanically pulling its C terminus, and brought the LacY polypeptide in close proximity (~5 to 10 nm) to a SecYEG membrane (Fig. 3A). After a folding time of 1 s, we retracted the stylus and recorded a force-distance curve (Fig. 3B). Most of experiments, which detected at least one insertion and folding event of the LacY polypeptide (n = 172), recorded only one force peak (64%). These single force peaks could occur at varying positions. Because each force peak matched the position of a force peak of the fingerprint pattern of native LacY (fig. S8A), the experiments documented that the polypeptide inserted one structural segment of native LacY. However, the varying positions of the force peaks indicated that the polypeptide could start insertion from any among the 10 structural segments of LacY (Fig. 3C). Among all structural segments, S2 to S4, which located in the C-terminal bundle of LacY, showed preference to insert.

At 2-s folding time, the LacY precursor inserted about one to three structural segments (Fig. 3D and fig. S8B). However, the force peaks were grouped in individual force-distance curves, suggesting

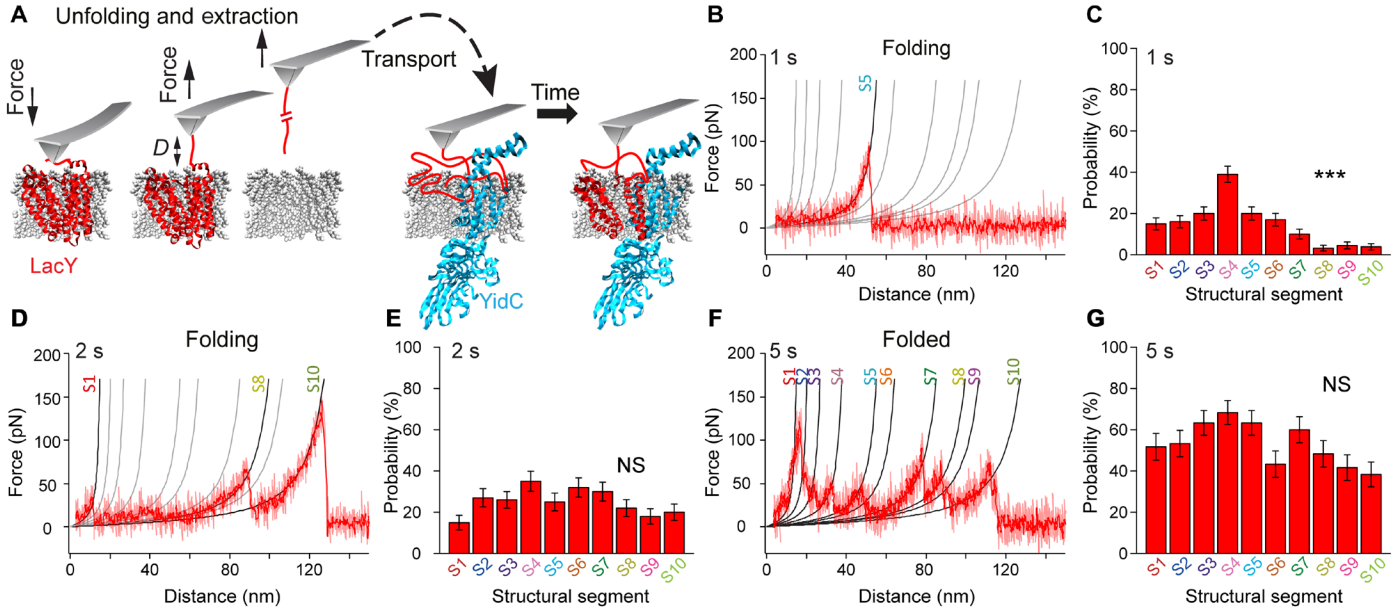

Fig. 2. YidC promotes stochastic insertion of LacY until having folded the native structure. (**A**) Schematic folding experiment of LacY in the presence of YidC. First, the AFM stylus attached to the C terminus is used to mechanically unfold and extract LacY from the membrane. Then, the unfolded LacY polypeptide is transported by the stylus in close proximity (~5 to 10 nm) to a phospholipid membrane embedding YidC. (**B**) After a folding time of 1 s, the stylus is retracted, recording a force-distance curve. To reveal whether the polypeptide folded structural segments, force peaks of the curve are fitted with the WLC model. WLC curves matching the fingerprint pattern of native LacY in terms of means ± SD are represented in black, and fits not matching are gray (fig. S6). (**C**) Probability of structural segments S1 to S10 inserting after 1-s folding time [number of structural segments (n_{ss}) = 231]. (**D**) Force-distance curve recorded in the presence of YidC after 2 s. (**E**) Probability of segments inserted after 2 s (n_{ss} = 266). (**F**) Force-distance curve recorded in the presence of YidC after 5 s. (**G**) Probability of segments inserted after 5 s (n_{ss} = 319). χ^2 tests indicate (non)uniform distributions (***P < 0.001). NS, nonsignificant. Error bars indicate SE. More force-distance curves are shown in fig. S5.

that the polypeptide inserted neighbored structural segments. Among all segments, S2 to S5 showed highest probability to insert (Fig. 3E). Further, increasing the folding time to 5 s increased the number of force peaks (fig. S8B), which appeared in groups, thus suggesting that the number of inserted neighbored structural segments increased. As the polypeptide did not complete folding of LacY, we increased the folding time to 10 s (Fig. 3F). The average number of structural segments inserted per LacY polypeptide approached ~5.2. In ~21% of all folding events (n = 38), the force-distance curve detected the full fingerprint pattern described for native LacY (Fig. 3F and fig. S8B), thus documenting the completion of the folding process. The probability histogram showed that the structural segments S1 to S5 of the C-terminal bundle inserted with priority (Fig. 3G). Together, our data indicate that, after having inserted the first structural segment, SecYEG supports the LacY polypeptide to sequentially insert structural segments until folding of LacY has been completed.

Insertion assisted by SecYEG and YidC progresses sequentially

We then tested how SecYEG and YidC together facilitate insertion and folding of the LacY polypeptide. Hence, we extracted and unfolded single LacY polypeptides from the C terminus and brought it in close proximity (~5 to 10 nm) to a membrane embedding the SecYEG-YidC construct (Fig. 4A). After a folding time of 1 s, we retracted the AFM stylus, recording a force-distance curve. Most of force curves recording a folding event (55%, n = 110) showed one force peak (Fig. 4B and fig. S9A). The force peaks varied in position, each of which indicating the insertion of 1 of the 10 structural segments described for native LacY. Among all structural segments, S2 to S4 showed higher priority to insert (Fig. 4C). By increasing the folding time to 2 s, we detected more force peaks appearing in groups (Fig. 4D and fig. S9B). The grouped force peaks correlating in position to the unfolding force peaks of native LacY indicated that the polypeptide inserted neighbored segments into the membrane. Among all structural segments, S2 to S5 showed highest insertion probability (Fig. 4E). Increasing the folding time to 5 s was insufficient to complete the folding of LacY (fig. S9B). After further increasing the folding time to 10 s, we detected folding events (~19%, n = 52) in which the polypeptide inserted all 10 structural segments observed for native LacY (Fig. 4F and fig. S9B). In average, the polypeptide inserted ~5.8 segments of which the structural segments S1 to S5 of the C-terminal bundle showed highest priority to insert (Fig. 4G).

Translocase dominates insertase

Next, we analyzed the number of structural segments inserted over time (Fig. 5A). In the presence of YidC, we could determine two insertion rates of LacY by linear regression, which were 0.96 ± 0.03 segments s^{-1} (means ± SD, n > 400) for folding times of ≤5 s and 0.13 ± 0.08 segments s^{-1} (n > 100) for folding times from 5 to 10 s. In contrast, the unfolded LacY polypeptide inserted 0.42 ± 0.06 segments s^{-1} in the presence of SecYEG and 0.42 ± 0.06 segments s^{-1} in the presence of the SecYEG-YidC construct for all folding times. This analysis suggests that, at folding times of >5 s, the YidC-assisted insertion rate slowed down, whereas SecYEG alone and the SecYEG-YidC construct kept their insertion rates constant. This indicates that after having inserted an increasing number of structural segments, YidC slows down in completing the folding process, whereas SecYEG and the SecYEG-YidC construct do not face this problem.

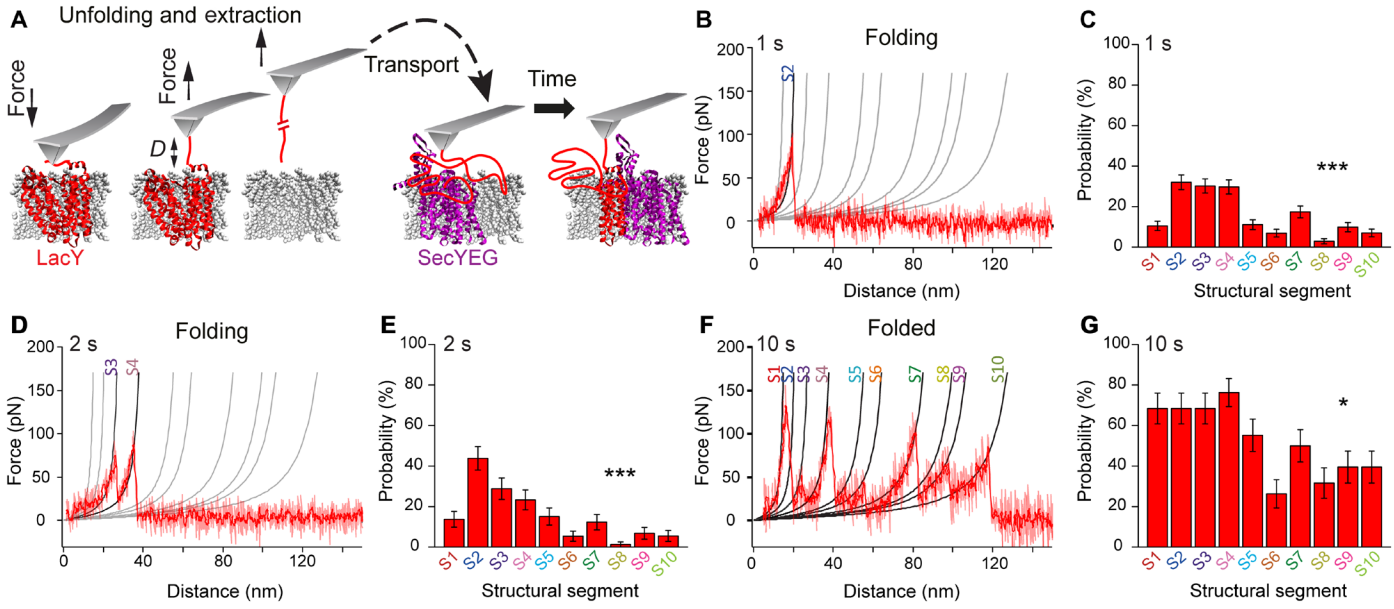

Fig. 3. SecYEG promotes sequential insertion of LacY until having folded the native structure. (**A**) Schematic LacY folding experiment in the presence of SecYEG. An AFM stylus nonspecifically attached to the C terminus mechanically unfolds and extracts LacY from the membrane. The unfolded LacY polypeptide is then transported by the AFM stylus in close proximity (~5 to 10 nm) to a phospholipid membrane embedding SecYEG where it is kept for 1 s. (**B**) After this folding time, the AFM stylus is retracted, recording a force-distance curve. To reveal whether the polypeptide folded structural segments, force peaks of the curve are fitted with the WLC model. WLC curves matching the fingerprint pattern of native LacY in terms of means ± SD are represented in black, and fits not matching are gray (fig. S6). The example shows the insertion of structural segment S2. (**C**) Probability distribution of structural segments inserted after 1 s (n_{ss} = 271). (**D**) Force-distance curve recorded in the presence of SecYEG after 2 s. (**E**) Probability distribution of segments inserted after 2 s (n_{ss} = 114). (**F**) Force-distance curve recorded in the presence of SecYEG after 10 s. (**G**) Probability distribution of segments inserted after 10 s (n_{ss} = 198). χ^2 tests indicate (non)uniform distributions (*P < 0.05, ***P < 0.001). Error bars indicate SE. More force-distance curves are shown in fig. S8.

Moreover, the insertion rate of LacY assisted by the SecYEG-YidC construct was similar to that observed in the presence of SecYEG only. This observation indicates that the insertion and folding kinetics of structural segments is dominated by SecYEG over YidC and that, in this case, YidC is not a limiting factor.

The insertion and folding behavior observed for SecYEG and YidC fused into a construct could have been affected by the linker fusing translocase and insertase. To test whether this was the case, we enzymatically separated the reconstituted construct, which fusing linker carried a cleavage site for the PreScission protease (fig. S10). After this, we characterized the insertion and folding of LacY polypeptides as described for YidC, SecYEG, and uncleaved SecYEG-YidC construct (Fig. 5A). The folding experiments showed that fused and nonfused SecYEG and YidC inserted and folded structural segments at very similar rates of 0.42 ± 0.06 and 0.38 ± 0.06 segments s^{-1}, respectively. The controls thus highlight that the fusion construct did not change the way how SecYEG and YidC together insert and fold the unfolded LacY polypeptide, which is supported by our in vivo experiments, showing that both SecYEG and YidC of the fusion construct are functional in *E. coli* (fig. S3).

The occurrence of neighbored force peaks observed in our snapshots of the folding process indicates the sequential insertion of structural segments (Fig. 5B). Intrigued by this occurrence, we quantified the probability of observing the insertion of 2 to 4 neighbored structural segments in the presence of YidC, SecYEG, SecYEG-YidC construct, and of SecYEG and YidC (Fig. 5C). Whereas neighboring force peaks were dominant for SecYEG, SecYEG-YidC construct, and for SecYEG and YidC, they were much less frequently observed for YidC. This statistical analysis underlines that SecYEG assists the sequential insertion of the LacY polypeptide, whereas YidC supports the insertion of structural segments in random order.

DISCUSSION

Folding initiates by inserting a first segment

Here, we introduced a single-molecule assay to study how the YidC insertase and the SecYEG translocon insert and fold the membrane protein LacY either alone or cooperatively under physiologically relevant conditions. In the presence of YidC and/or SecYEG, the first insertion step of the LacY polypeptide into the membrane could be initiated with any of the 10 structural segments found in native LacY. This finding is rather unexpected since it is believed that in vivo the N-terminal segments insert first (*2, 4*). The difference might be explained by our experimental approach as our bottom-up assay does not include the ribosome to cotranslationally insert membrane proteins (*2, 3*). Our experiments thus rather describe the posttranslational insertion and folding process and highlight the flexibility of insertase and translocon in initiating the folding of a polytopic membrane protein. We observed that YidC, SecYEG, or SecYEG and YidC together preferentially insert structural segments of the C-terminal bundle of LacY first. This finding is supported by in vitro LacY translation and insertion assays, showing that during cotranslational insertion, the N-terminal six-helix bundle of LacY remains in the hydrophilic milieu until the helices of the C-terminal bundle have been translated and inserted into the membrane (*30*). Complementary, it has been also shown that the N-terminal six-helix bundle of LacY alone, if translated and released from the ribosome, folds defectively and that its correct folding requires the folding of C-terminal six-helix bundle (*30*).

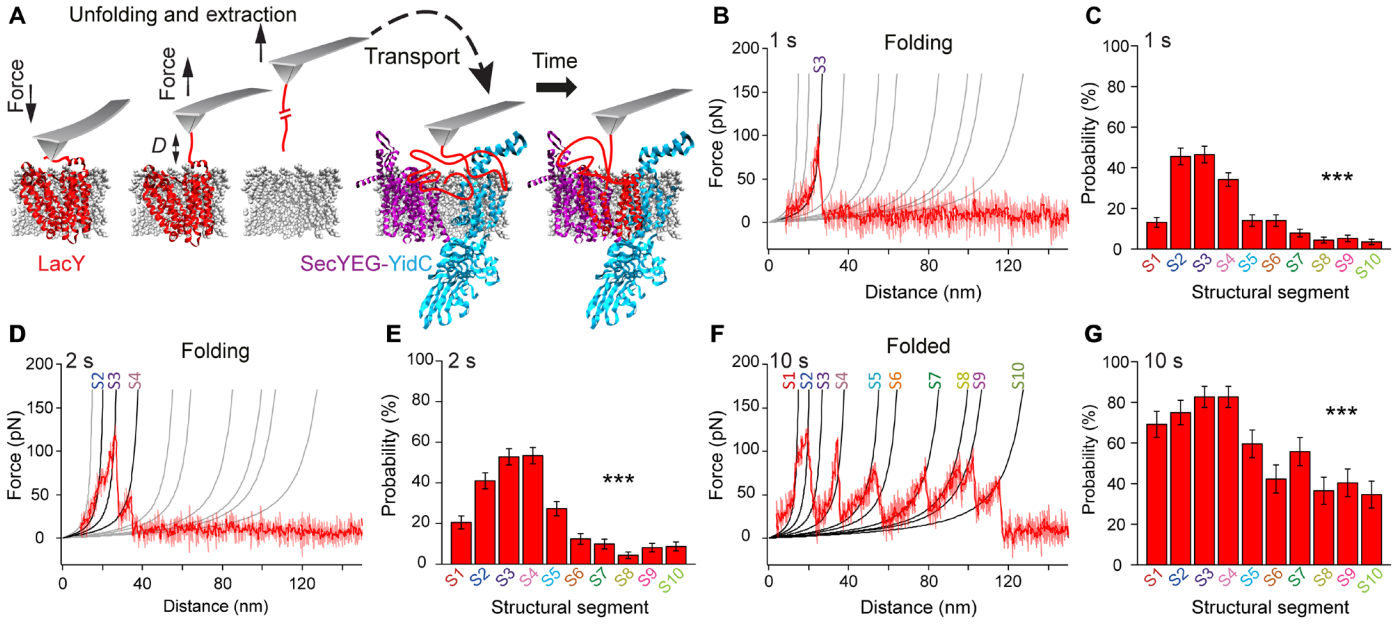

Fig. 4. SecYEG-YidC fusion construct promotes sequential insertion of LacY until having folded the native structure. (**A**) Schematic LacY folding experiment in the presence of the SecYEG-YidC fusion construct. An AFM stylus nonspecifically attached to the C terminus mechanically unfolds and extracts LacY from the membrane. The unfolded LacY polypeptide is then transported by the AFM stylus in close proximity (~5 to 10 nm) to a phospholipid membrane embedding the SecYEG-YidC construct where it is kept for 1 s. (**B**) After this folding time, the AFM stylus is retracted, recording a force-distance curve. To reveal whether the polypeptide folded structural segments, force peaks of the curve are fitted with the WLC model. WLC curves matching the fingerprint pattern of native LacY in terms of means ± SD are represented in black, and fits not matching are gray (fig. S6). The example shows the insertion of structural segment S3. (**C**) Probability distribution of structural segments inserted after 1 s (n_{ss} = 206). (**D**) Force-distance curve recorded in the presence of SecYEG-YidC after 2 s. (**E**) Probability distribution of segments inserted after 2 s (n_{ss} = 384). (**F**) Force-distance curve recorded in the presence of SecYEG-YidC after 10 s. (**G**) Probability distribution of segments inserted after 10 s (n_{ss} = 301). χ^2 tests indicate (non)uniform distributions (***P < 0.001). Error bars indicate SE. More force-distance curves are shown in fig. S9.

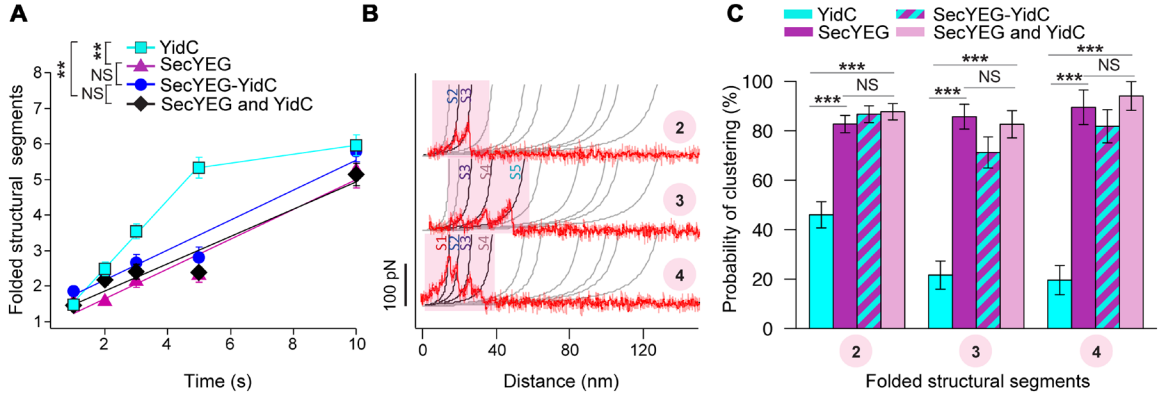

Fig. 5. In the presence of YidC and SecYEG, the translocase defines the folding pathway of the LacY polypeptide. (**A**) Folding kinetics of LacY in the presence of YidC, SecYEG, SecYEG-YidC fusion construct, or SecYEG and YidC from the SecYEG-YidC construct cleaved by the PreScission protease (fig. S10B). Colored linear fits approach the insertion and folding rate of structural segments. (**B**) Force-distance curves exemplify single LacY polypeptides inserting and folding 2, 3, or 4 neighbored structural segments in the presence of SecYEG. Force peaks matching the fingerprint pattern of native LacY are represented in black, and fits not matching are gray (fig. S6). (**C**) Probabilities of detecting the insertion of 2, 3, or 4 neighbored segments in the presence of YidC, SecYEG, SecYEG-YidC, or SecYEG and YidC. Four hundred seventy-eight experiments (force-distance curves) detecting insertion and folding events of LacY have been recorded in the presence of YidC, 395 in the presence of SecYEG, 397 in the presence of SecYEG-YidC fusion construct, and 313 in the presence of SecYEG and YidC. Statistical differences examined by analysis of covariance (A) and Z (C) tests were considered nonsignificant for P > 0.05 and significant for **P < 0.01 and ***P < 0.001. Error bars indicate SE.

Random and sequential progression of polypeptide insertion

After the insertion of the first structural segment, we detected the subsequent insertion steps of the remaining structural segments until the polypeptide completed folding of LacY. Assisted by SecYEG, the structural segments of the C-terminal bundle of LacY preferentially inserted for all folding times, but this was not the case for YidC-assisted folding. Independently of the insertion and folding mechanism, the translocon and insertase could complete the folding process either independently or cooperatively. This result agrees with the hypothesis that many membrane proteins can use either YidC or SecYEG for insertion and folding (*31*). However, whereas

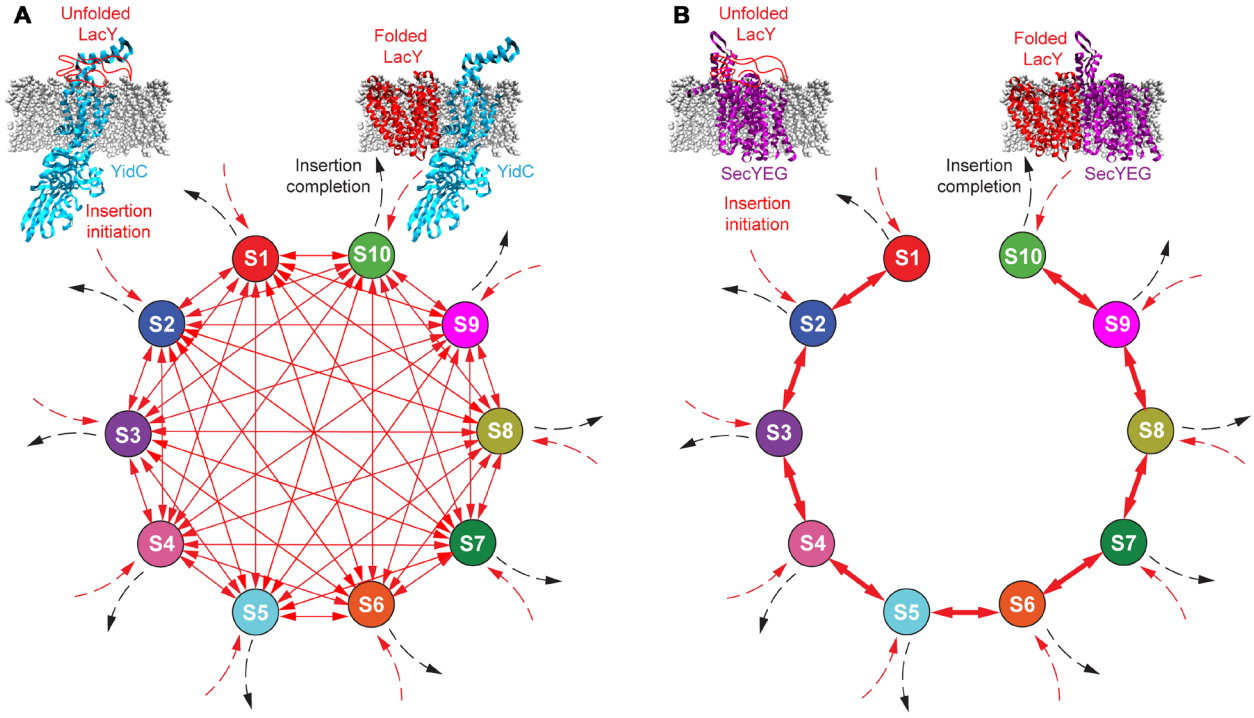

Fig. 6. SecYEG and YidC insert and fold the membrane protein LacY along different pathways. (**A**) YidC starts insertion of the LacY polypeptide from any structural segment after which it stepwise inserts the remaining structural segments S1 to S10 of LacY. Structural segments insert in random order until folding of LacY has been completed. YidC offers 10! (3,628,800) pathways to fold the 10 structural segments S1 to S10 of LacY toward the native structure. (**B**) SecYEG alone or SecYEG and YidC together start insertion of the LacY polypeptide from any structural segment after which the remaining segments are inserted sequentially until folding of LacY has been completed. SecYEG or SecYEG and YidC offer 10 principal pathways to fold the 10 structural segments toward native LacY. Red dashed arrows indicate possibilities of initiating insertion. Red double arrows highlight the insertion and folding steps of the folding pathways. Black dashed arrows indicate the completion of the insertion and folding process.

the insertase inserted structural segments in random order, the translocon inserted them sequentially. The random insertion of polypeptide segments leads to 10! (3,628,800) possible pathways, while the sequential insertion offers only 10 pathways toward folding native LacY (Fig. 6). This marked difference of the number of folding pathways describes two folding landscapes of fundamentally dissimilar complexity. It is expected that LacY can fold along different pathways as the coexpression of two nonoverlapping LacY fragments complementing into functional LacY has been demonstrated (*32*–*34*). However, in vivo YidC alone inserts rather short membrane proteins, while large polytopic membrane proteins are preferentially inserted and folded by SecYEG (*35, 36*). One may thus speculate that the random insertion of segments into cellular membranes, which results in a complex folding landscape, is preferred for smaller membrane proteins, whereas the sequential insertion of segments along a simplified folding landscape is preferred for larger polytopic membrane proteins.

SecYEG takes the lead

Our SecYEG-YidC fusion construct acts as both a functional translocon and insertase as it complements *E. coli* strains lacking either SecY or YidC. From our single-molecule folding assay, we observe that the construct preferentially starts insertion with structural segments S2 to S4 and proceeds to sequential insertion of the remaining segments of LacY. Folding follows the same pathways and kinetics as observed for SecYEG alone. SDS-PAGE confirmed that SecY or YidC alone was not present in proteoliposomes, which might have occurred as the result of digestion of the linker between SecY and YidC. However, in control experiments, we have cleaved the polypeptide linking the SecYEG-YidC fusion construct and characterized how nonfused SecYEG and YidC together insert the LacY polypeptide. The folding kinetics and pathways of LacY were the same as observed for the fusion construct. Our data thus show that SecYEG dominates YidC in shaping the folding pathway of the membrane protein. This result corresponds to in vivo observations where insertion and folding of LacY primarily depends on SecYEG and benefits from YidC in a supporting role (*37*).

SecYEG and YidC have been proposed to cooperate in a sequential or simultaneous manner (*3*). In the sequential scenario, YidC and SecYEG act merely independently so that one region of a membrane protein polypeptide is inserted by YidC (e.g., N terminus), whereas the other region is inserted by SecYEG (e.g., C terminus) or vice versa (*3, 38, 39*). In the simultaneous scenario, SecYEG and YidC act together, and transmembrane segments after entering the SecYEG channel are passed to YidC before being released to the membrane. Our results support the simultaneous model as we observe SecYEG and YidC together to insert LacY in a SecYEG manner. Therefore, when working together with SecYEG, YidC might rather serve as a folding assistant or as an assembly site to support folding intermediates along the SecYEG folding pathway.

In a bottom-up approach, we have systematically characterized the insertion and folding pathways along which YidC and SecYEG guide the LacY polypeptide. The folding pathways provide detailed mechanistic insight into the different and complementary working

principles of insertases, translocons, and the two acting in concert. Because insertase and translocon are conserved among species and LacY serves as a model among the large major facilitator superfamily of transporters, we anticipate that the working principles are of general relevance for membrane protein biogenesis. Moreover, in principle, our bottom-up assay is ready to be extended to characterize the detailed folding pathways of other membrane proteins and how they are assisted by the cellular machinery, including ribosomes, chaperones, insertases, and translocases.

MATERIALS AND METHODS

Cloning, expression, and purification of LacY, YidC, SecYEG, and SecYEG-YidC

Engineering, expression, and purification of LacY were performed as described (*40*). Briefly, the C-terminal end of wild-type LacY was extended with a 36–amino acid–long unstructured polyGly polypeptide, followed by a His_8-tag [GSM(G_{11})EAVEEAVEEA(G_{11})S(His_8)] using QuikChange II polymerase chain reaction and plasmid pT7-5/LacY as a template. PolyGly LacY was purified from *E. coli* XL1-Blue (StrataGene) transformed with pT7-5 plasmids harboring given mutant genes by using Co(II) affinity chromatography as described. LacY eluted from the Co(II)-Talon column was concentrated and washed with 50 mM sodium phosphate (NaP_i) (pH 7.5) and 0.01% (w/w) dodecyl-β-D-maltopyranoside (DDM; Maumee) on an Amicon Ultra-15 concentrator (EMD Millipore) with a 30-kDa cutoff.

Wild-type YidC with a His_{10}-tag on the C terminus was cloned in pT7-7 plasmid and expressed and purified similar to LacY with a few exceptions: The expression strain used was *E. coli* BL21 (DE3), and the concentration of DDM was 0.03%. YidC eluted from the Co(II)-Talon column was concentrated and washed with 150 mM NaCl, 50 mM sodium phosphate (NaP_i) (pH 7.5) and 0.03% (w/w) DDM on an Amicon Ultra-15 concentrator with a 30-kDa cutoff.

For cloning, expression and purification of SecYEG *E. coli* SF100 cells (*41*) bearing pTrc99a-SecYEG were grown in 2 liters of Luria-Bertani (LB) medium containing ampicillin (200 μg ml^{-1}) and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the cultures reached an optical density at 600 nm of 0.8. After 2 hours of induction, the cells where harvested and shock-frozen in buffer 1 [20 mM tris-HCl (pH 8), 300 mM NaCl, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol (DTT)]. After cell disruption by a one-shot cell disruptor (Constant Systems LTD) and separating the cellular fragments by centrifugation, the membranes were harvested by ultracentrifugation and resuspended in buffer 1 (without DTT). For solubilization, 2% DDM was added and incubated for 2 hours at 4°C. After ultracentrifugation, the supernatant was incubated rotating with 0.5 ml of equilibrated Ni–nitrilotriacetic acid (NTA) and 30 mM imidazole in a total volume of 50 ml for 1 hour at 4°C. The Ni-NTA was separated from the buffer on a minicolumn and washed with 10-column bed volume (CV) washing buffer [20 mM tris-HCl (pH 8), 300 mM NaCl, 10% glycerol, 50 mM imidazole, and 0.05% DDM]. SecYEG was eluted with 10 CV elution buffer [20 mM tris-HCl (pH 8), 300 mM NaCl, 10% glycerol, 300 mM imidazole (pH 8), and 0.05% DDM]. The elution fractions were flash-frozen and stored at −80°C.

The purification of the SecY-YidC fusion construct (fig. S2) was identical to the SecYEG purification with some modifications: The expression strain for SecYEG-YidC fusion construct was Lemo21 (DE3). The cells were induced with 0.4 mM IPTG for 1 hour at 30°C. The pH of the buffers was 7.5, and for solubilization, 1% DDM was added. For immobilized metal affinity chromatography, 1 ml of equilibrated Ni-NTA and 20 mM imidazole in a total volume of 50 ml were used.

Reconstitution of LacY, YidC, SecYEG, and SecYEG-YidC into proteoliposomes

LacY, YidC, SecYEG, or SecYEG-YidC fusion construct were reconstituted into liposomes formed from PE (Avanti Polar Lipids) and PG (Avanti Polar Lipids) (ratio, 3:1). LacY and YidC were reconstituted using the dilution method (*25*). Briefly, purified LacY or YidC was mixed with PE/PG phospholipids dissolved in 1.2% octyl glucoside (Maumee) at a lipid-to-protein ratio of 5 (w/w). The mixtures were incubated for 20 min on ice. After the incubation time, mixtures were quickly diluted 50-fold in 50 mM NaP_i at pH 7.5. Centrifugation (100,000*g*) was applied for 1 hour to collect proteoliposomes, which were then suspended in 50 mM NaP_i at pH 7.5 and flash-frozen in liquid nitrogen.

Before SecYEG or SecYEG-YidC was reconstituted, the samples were dialyzed three times for 1 hour in 20 mM tris-HCl (pH 8), 300 mM NaCl, and 0.03% DDM. Then, purified SecYEG or SecYEG-YidC was mixed with PE/PG (3:1, mol/mol) liposomes in a lipid-to-protein ratio of 10 (SecYEG) and 30 (SecYEG-YidC) (w/w) and extruded 15 times. To remove the detergent, activated Biobeads were added (twice for 1 hour at 4°C). The proteoliposomes were collected by centrifugation at 4°C (Airfuge, 10 min, 20 psi), resuspended in 50 mM NaP_i (pH 8.0), and stored on ice.

SDS–polyacrylamide gel electrophoresis

To charge the success of expression, purification, and reconstitution, LacY, YidC, SecYEG, and SecYEG-YidC samples were Coomassie Blue–stained (fig. S1) or silver-stained (fig. S2) after SDS-PAGE (4 to 12% Bis-Tris Plus gel; Invitrogen, Thermo Fisher Scientific, MA, USA).

Cleavage and Western blot analysis of the SecY-YidC fusion construct

Proteoliposomes containing the SecY-YidC fusion construct with the 14–amino acid–long linker between SecY and YidC encoding cleavage site for the PreScission protease (LEVLFQ/GP) were sonicated for 30 min and incubated with the PreScission protease (0.5 μM) for 2 hours at 14°C. The cleaved SecYEG-YidC construct was then separated by SDS-PAGE, and the gel was transferred onto a nitrocellulose membrane (0.45-μm nitrocellulose; Amersham Protran; GE Healthcare Life Sciences, Germany). After 2-hour blocking with 3% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) in tris-buffered saline (TBS) buffer [50 mM tris-HCl (pH 7.5) and 150 mM NaCl], the His_{10}-tags of the SecYEG and YidC termini were probed with Ni-NTA–horseradish peroxidase conjugate [1:1000 dilution in TBS-T buffer (TBS buffer plus 0.1% Tween 20 from Qiagen, Hilden, Germany)] overnight at 4°C. The proteins were then visualized with the Amersham ECL Western blotting detection reagents (GE Healthcare Life Sciences, MA, USA) according to the supplier's protocol (fig. S10B). After being adsorbed to mica, proteoliposomes embedding SecYEG and YidC from the cleaved SecYEG-YidC fusion constructs were incubated a second time with the PreScission protease (2 hours at 14°C). The samples were then rinsed several times with SMFS buffer [50 mM potassium phosphate (KP_i) (pH 7.2)] and used for folding experiments.

Single-molecule force spectroscopy

Proteoliposomes were adsorbed onto freshly cleaved mica for 30 min and rinsed with SMFS buffer several times to remove nonadsorbed and weakly adsorbed proteoliposomes. Membrane patches containing reconstituted proteins were localized by AFM imaging. SMFS experiments were carried out with the same AFM (Nanowizard II Ultra; JPK Instruments AG, Berlin, Germany) having an 850-nm laser detection system. SMFS was conducted using Si_3N_4 cantilevers (OMCL RC800PSA; Olympus, Tokyo, Japan) having a nominal spring constant of 0.05 $N{\cdot}m^{-1}$ and resonance frequency of 18 kHz. Cantilevers were calibrated applying the equipartition theorem (*42*) before and after of each experiment. A fresh sample and a new cantilever were used in each experimental day, and >350 days were needed to conduct all SMFS experiments described in this work. The volume of buffer, electrolyte concentration, and temperature were monitored and kept constant during the experiments.

To pick up and mechanically unfold a single LacY molecule, the AFM stylus was approached to the surface of LacY proteoliposomes until reaching a contact force of ~700 pN, which was applied for 500 ms. To enhance the probability of attaching LacY from C-terminal end, polyGly extension was engineered on the C-terminal end of LacY. This 36–amino acid–long polyGly tail followed by a His_8-tag increased the probability to pick up C-terminal end of LacY 10-fold (~0.1%, n = 2974) with an AFM stylus (*17*). Upon complete mechanical unfolding and stretching, the 461–amino acid–long LacY polypeptide (417–amino acid wild-type LacY elongated by 36–amino acid–long polyGly extension and a His_8-tag) shows the force peak pattern extending to ~120 nm (*17*). To completely unfold and extract individual LacY transporters from the membrane, the AFM cantilever was retracted by ≥190 nm at a constant speed of 0.7 $\mu m{\cdot}s^{-1}$.

SMFS unfolding data analysis

The worm-like chain (WLC) model was fitted to every unfolding force peak in each force-distance curve recorded upon unfolding individual membrane protein (*13*). A persistence length of 0.4 nm and contour length of 0.36 nm per amino acid were used for data analysis to reveal the contour length (number of amino acid) of the polypeptide unfolded and stretched in each detected force peak. Contour length histograms of all force peaks detected in all unfolding force-distance curves were fitted with a Gaussian mixture model, which revealed 10 force peak classes for LacY (*17*). The 10 force peak classes were mapped to the secondary structure of LacY (Fig. 1D) taking into account the polyGly and His_8-tag elongation of the C-terminal end. If the force peak class located the beginning/end of a stabilizing structural segment on the mica-facing side of the membrane or within the lipid membrane, then the thickness of the membrane was considered for peak mapping (*13*).

Single-molecule folding experiments and data analysis

LacY folding experiments were performed as follows (*20*): First, PE/PG (3:1) proteoliposomes containing reconstituted YidC, SecYEG, SecYEG-YidC fusion construct, or SecYEG and YidC from the SecYEG-YidC construct cleaved by the PreScission protease were absorbed onto freshly cleaved mica for 30 min. Then, the samples were gently rinsed several times with SMFS buffer to remove nonadsorbed proteoliposomes. AFM imaging revealed that upon attachment to mica, the proteoliposomes opened and adsorbed as single-layered membrane patches containing reconstituted YidC, SecYEG, SecYEG-YidC construct, or SecYEG and YidC (figs. S1, S2, and S4). Second, LacY proteoliposomes were coadsorbed for 30 min to mica supports having YidC, SecYEG, SecYEG-YidC, or SecYEG and YidC membranes already adsorbed (fig. S4). After the absorption time passed, the sample was gently rinsed several times to remove weakly adsorbed and nonadsorbed LacY proteoliposomes. AFM topographs recorded after the first and second adsorption identified LacY membranes and YidC, SecYEG, SecYEG-YidC, or SecYEG and YidC membranes (fig. S4).

A single LacY protein was first picked up, completely unfolded, and extracted from the membrane with an AFM stylus. Then, the unfolded polypeptide was transported by AFM cantilever into close proximity (~5 to 10 nm) of the surface of a membrane containing YidC, SecYEG, SecYEG-YidC construct, or SecYEG and YidC from the SecYEG-YidC construct cleaved by the PreScission protease, where it was kept for a given time (1 to 10 s) to insert and fold. After this time passed, the AFM cantilever was retracted, recording a force-distance curve. This curve was inspected to evaluate whether or not the polypeptide inserted structural segments into the membrane. In case of having detected a force peak in the force-distance curve, the peak was fitted using the WLC model to reveal its contour length. This contour length was compared with the contour lengths of the force peaks of the fingerprint of native LacY. If the force peak matched any of the force peaks of the fingerprint within their means ± SD positions, then the force peak was classified as representing the insertion of a structural segment. If the force peak did not match any force peak of the fingerprint pattern, then the force peak was classified as representing a misfolding event (fig. S6). In case of detecting no force peaks, we assumed the LacY polypeptide to having not inserted and folded. If force-distance curve indicated that the AFM stylus touched the membrane during the folding time (i.e., thermal drift), then it was discarded from the analysis.

Compared to our previously established data analysis procedures (*20*), we advanced these analysis procedures to reveal insight into (i) the structural segments initiating the LacY insertion and folding process in the presence of insertase and/or translocase. Most of folding events recorded after a folding time of 1 s showed one force peak, which indicated the insertion and folding of a first structural segment. We thus determined probability histograms to reveal the structural segments preferentially inserting first and starting folding. (ii) The folding rate at which the unfolded LacY polypeptide assisted by insertase and/or translocase inserted structural segments into the membrane. Accordingly, we determined the average number of folded segments per folding time, which was fitted with a linear regression to approach their insertion and folding rate. (iii) The structural segments inserted were analyzed to reveal whether they inserted clustered or stochastically. The same analysis procedures and classification criteria were applied to all insertion and folding experiments.

Statistics and statistical data analysis

To record an insertion and folding event of a single LacY polypeptide, we had to approach and withdraw the AFM tip to and from proteoliposomes frequently. For example, in >137,000 attempts approaching the AFM tip to proteoliposomes containing SecYEG, we detected only 172 events, showing that the LacY polypeptide inserted and folded one or more structural segments into the lipid bilayer (at a folding time of 1 s). Similarly, we needed >193,000 attempts to detect 155 insertion and folding events of the LacY polypeptide in the presence of YidC at a folding time of 1 s. These numbers do not

represent efficiencies of insertion because, in each attempt, an unfolded LacY polypeptide is approached to the surface of a proteoliposome showing relatively low densities of translocases and/or insertases (figs. S1E and S2, D and E). Under these conditions, the probability that the unfolded LacY polypeptide interacts with a translocase and/or insertase is rather low. In addition, because the polypeptide had been attached to the AFM tip unspecifically and transiently, it slipped quite often from the tip (*12*–*14*).

Datasets of 478 (in the presence of YidC), 395 (in the presence of SecYEG), 397 (in the presence of SecYEG-YidC construct), and 313 (in the presence of SecYEG and YidC from the cleaved SecYEG-YidC fusion construct) force-distance curves, each recording a folding event, were analyzed. Probability distributions were compared with the uniform distribution using χ^2 tests. Two-tailed Z tests were performed to evaluate the significance of difference between insertion probabilities for YidC, SecYEG, and SecYEG-YidC construct. Analysis of covariance was used to test the difference between the slopes for kinetic measurements. The difference between groups and distributions was considered to be statistically not significant when $P > 0.05$ and statistically significant when $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Error bars give SE. Statistical analysis was accomplished in R and Prism 7.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/1/eaau6824/DC1

Fig. S1. LacY and YidC reconstituted into PE/PG phospholipid membranes.
Fig. S2. SecYEG and SecYEG-YidC fusion construct reconstituted into PE/PG phospholipid membranes.
Fig. S3. YidC, SecYEG, and SecYEG-YidC fusion constructs are functional in vivo.
Fig. S4. Coadsorption of YidC and LacY proteoliposomes.
Fig. S5. YidC facilitates random insertion and folding of LacY.
Fig. S6. Classification criteria of LacY misfolding and folding events.
Fig. S7. Completely unfolded and extracted LacY polypeptides cannot insert and fold into phospholipid membranes without assistance.
Fig. S8. SecYEG facilitates sequential insertion and folding of LacY.
Fig. S9. SecYEG and YidC together facilitate sequential insertion and folding of LacY.
Fig. S10. SecY-YidC fusion construct cleaved by the PreScission protease.

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