Glycolipozyme MPIase is essential for topology inversion of SecG during preprotein translocation

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Presecretory proteins are translocated across biological membranes through protein-conducting channels such as Sec61 (eukaryotes) and SecYEG (bacteria). SecA, a translocation ATPase, pushes preproteins out with dynamic structural changes through SecYEG. SecG, a subunit of the SecYEG channel possessing two transmembrane stretches (TMs), undergoes topology inversion coupled with SecA-dependent translocation. Recently, we characterized membrane protein integrase (MPIase), a glycolipozyme involved in not only protein integration into membranes but also preprotein translocation. We report here that SecG inversion occurs only when MPIase associates with SecYEG. We also found that MPIase modulates the dimer orientation of SecYEG. Cysteine-scanning mutagenesis mapped SecG TM 2 to a relatively hydrophilic environment. The dimer formation of SecG, crosslinked at TM 2, was not observed on SecG inversion, indicating that SecYEG undergoes a dynamic structural change during preprotein translocation.

Presecretory proteins with an N-terminally attached signal sequence are translocated across the endoplasmic reticulum (ER) membranes of eukaryotes and the cytoplasmic membranes of *Escherichia coli* with the aid of the conservative protein-conducting channels called the Sec61 and SecYEG complexes, respectively (1–3). In *E. coli*, SecYEG, a translocon, and SecA, a translocation ATPase, play central roles in preprotein translocation. According to the X-ray crystal structures and the results of cryo-electron microscopic analyses of SecYEG and SecA, detailed molecular mechanisms for preprotein translocation have been proposed and discussed (4–8). However, the structures that have so far been determined are those of the resting or preactive states; the structure of the translocating channels is largely unknown. Biochemical studies also have revealed that *E. coli* SecYEG forms oligomers, such as a dimer and a tetramer (9–12). In some crystals, SecYEG forms a dimer, of which the interface is either SecE (back-to-back) (4, 6) or SecY (front-to-front) (8), with the back-to-back interface being predominantly formed (13). In contrast, it has been proposed that only a single copy of SecYEG functions as a channel (14, 15). Moreover, it has been reported that the translocon is present as a monomer (5, 16). Thus, it is still a matter of debate as to whether SecYEG functions as a monomer or an oligomer. Furthermore, as the translocon undergoes dynamic structural changes during the catalytic cycle of the reaction, it can be proposed that SecG undergoes structural changes, including the monomer–dimer transition or even the transition between different dimers.

One of the most dynamic structural changes of the translocon so far proposed is the topology inversion cycle of SecG (17–20). In the absence of preprotein translocation, both the N- and C-terminal regions are exposed to the periplasmic space (Fig. 1*A*, *Left*). In contrast, during SecA-dependent translocation, SecG undergoes a repeat of the topology inversion cycle to accelerate the SecA-dependent translocation (Fig. 1*A*). Soluble SecA is also important for SecG inversion (18, 19). SecG inversion is supported by three independent lines of evidence; namely, (*i*) preprotein translocation is inhibited by an anti-SecG antibody that recognizes the periplasmic region of SecG when added from the cytoplasmic side (17, 22), (*ii*) proteinase K digests SecG on the opposite side of membranes when preprotein translocation occurs (9, 17–19), and (*iii*) membrane-impermeable reagents react with SecG regions on the opposite side of membranes on preprotein translocation (20). However, a report that disfavors this model of SecG inversion stated that SecG, crosslinked to SecY through Cys residues introduced into both proteins at appropriate positions, is fully functional (23). The major difference between this report and our study is that inverted membrane vesicles (IMV) either with an overproduced amount (23) or the WT level (our study) of SecYEG were used. Later, we found that SecG inversion cannot be reproduced under SecYEG overproduction conditions (18, 23). Therefore, it is suggested that a factor or factors other than known Sec factors is required to invert SecG, as the factor should be limiting on SecYEG overproduction.

Recently, we identified a glycolipid named membrane protein integrase (MPIase) that is essential for membrane protein integration in *E. coli* (24, 25). We have determined the complete structure of MPIase and studied its structure–function relationship, and propose that MPIase is a glycolipozyme (25). As MPIase is not only involved in Sec-dependent integration but also stimulates preprotein translocation (26), it is highly suggested that MPIase interacts with SecYEG. In this article, we report that MPIase is essential for SecG inversion, modulating the dimer orientation of SecYEG.

Results

MPIase Is Essential for SecG Inversion. Under nontranslocation conditions, a C-terminal fragment of 9 kDa derived from SecG was detected on proteinase K digestion of IMV (Fig. 1*B*, −ATP). SecG inversion was reproducibly observed when IMV prepared from WT cells (WT IMV) were used and pOmpA (precursor form of outer membrane protein A) translocation was inhibited by the addition of adenylylimidodiphosphate (AMP-PNP), a nonhydrolyzable analog of ATP, as revealed by the digestion of the SecG band material without generation of the 9-kDa fragment (WT IMV, ATP/AMP-PNP). In contrast, SecG inversion was not observed in the case of IMV with an overproduced amount of SecYEG (SecYEG^{++} IMV), as reported (23). When proteoliposomes (PL) reconstituted with SecYEG were used, no inversion was observed (SecYEG PL). However, when MPIase was co-reconstituted with SecYEG, SecG inversion was clearly observed (SecYEG + MPIase PL), indicating that MPIase is essential for SecG inversion. Therefore, it is highly likely that a lack of MPIase explains why SecG was not inverted in SecYEG^{++} IMV or SecYEG PL.

Next, a soluble MPIase derivative lacking its lipid part (PP-MPIase), which is active in integration of Sec-independent substrates (25), was subjected to the SecG inversion assay. Essentially, PP-MPIase was inactive in SecG inversion (SecYEG PL +

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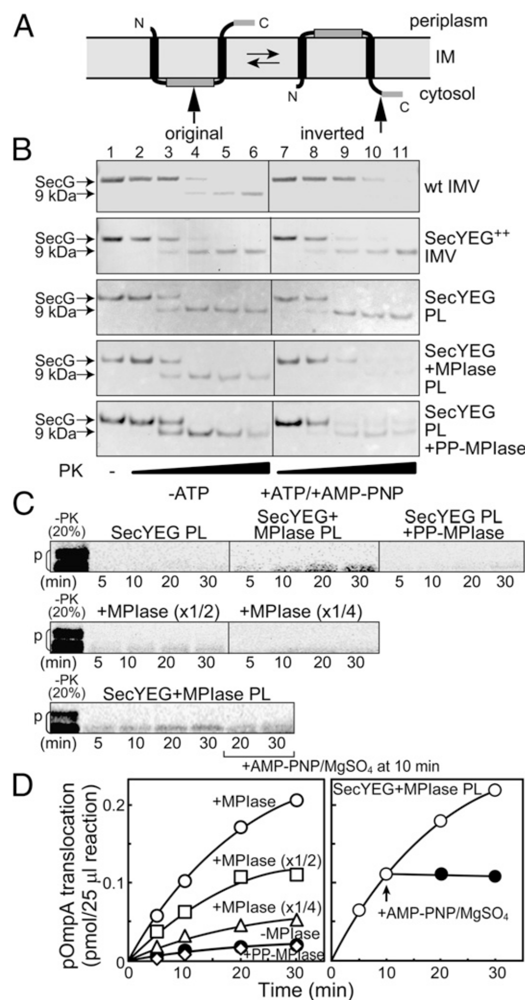

Fig. 1. MPIase is essential for SecG inversion. (*A*) Topological arrangement of SecG. The gray bar and black and gray boxes represent the antigenic region (17) and strongly and weakly hydrophobic regions, respectively. Arrows indicate the sites of PK digestion. (*B*) Topology inversion assay involving the specified vesicles. From top, IMV from K003 (WT IMV), IMV from B351/pEYG (SecYEG^{++} IMV), PL reconstituted with SecYEG (50 µg; SecYEG PL), PL reconstituted with SecYEG (50 µg) and MPIase (150 µg; SecYEG + MPIase PL), and PL reconstituted with SecYEG supplemented with PP-MPIase (added during the assay at 50 µg/mL; SecYEG PL + PP-MPIase) were used. Noninverted (−ATP) and inverted (+ATP/+AMP-PNP) conditions were used, followed by PK-digestion and immunoblotting. The positions of SecG and the 9-kDa fragment are indicated by arrows. (*C*) The translocation of pOmpA into PL was allowed for the indicated periods at 37°C under the same conditions as in *B*, except that the [^{35}S]pOmpA/SecB complex (25 µg/mL as pOmpA) was used as a substrate (*Upper*). The translocation into PL reconstituted with SecYEG (50 µg) and various amounts of MPIase (75 µg for ×1/2 and 37.5 µg for ×1/4) was also examined (*Middle*). AMP-PNP/$MgSO_4$ (20 mM) was added 10 min after the start of the translocation reaction into SecYEG + MPIase PL (*Lower*). The translocated materials were analyzed by SDS/PAGE and autoradiography. As shown at left (-PK), 20% of the input pOmpA was also analyzed. Although pOmpA synthesized in vitro gives several bands at slower-migrating positions, reflecting the unfolded states, presumably by binding with SecB (21), the translocated pOmpA migrated only at the position of its molecular weight (~37 kDa). (*D*) Translocation activities obtained in *C* were determined and plotted against time.

PP-MPIase), although a slight decrease of the 9-kDa fragment was observed after AMP-PNP addition, suggesting that the lipid anchor of MPIase is essential for SecG inversion.

When the same PL were subjected to pOmpA translocation assaying, significant stimulation of pOmpA translocation was observed (Fig. 1*C*), as reported previously (26). The stimulation was dependent on the amount of MPIase used for reconstitution (Fig. 1*C*). Note that signal sequence was not processed because of the lack of leader peptidase. Quantitation of the activity revealed that MPIase stimulates pOmpA translocation by ~10-fold (Fig. 1*D*). PP-MPIase, which did not cause SecG inversion, did not stimulate pOmpA translocation (Fig. 1 *C* and *D*). These results confirm the importance of SecG inversion. The addition of AMP-PNP/$MgSO_4$ (20 mM) during translocation immediately inhibited the reaction, with the translocated amount remaining constant (Fig. 1 *C* and *D*). These results indicate that the vesicle structure of PL was not affected during the topology inversion assay.

SecYEG Missing MPIase Is Less Active. Because the stimulation of pOmpA translocation was clearly observed on the addition of MPIase (Fig. 1*C*), the pOmpA translocation activities of WT and SecYEG^{++} IMV were compared in detail. The activity was enhanced by SecYEG overproduction (Fig. 2 *A* and *B*), as reported (27–29). When the amount of SecYEG was examined by immunoblotting, ~10-fold overproduction of each subunit was observed (Fig. 2*C*). The extent of SecE overproduction was a little larger, presumably because of the position of the *secE* gene on the expression vector (29). However, the amount of MPIase was not affected by SecYEG overproduction (Fig. 2*C*). The expression levels of SecD (another Sec protein) and subunits of the ATP transporter for lipoproteins, LolC and LolD (inner membrane proteins unrelated to Sec proteins) were also examined. The levels of these control proteins were not changed in either type of the IMV (Fig. 2*C*). The Coomassie Brilliant Blue (CBB)-stained profiles were similar except that SecYEG overproduction was observed in SecYEG^{++} IMV (Fig. 2*D*). Considering the extent of SecYEG overproduction, the specific activity as to SecYEG was much lower when SecYEG^{++} IMV were used (1/6~1/7 of WT; Fig. 2*E*). These results are consistent with MPIase-dependent stimulation of preprotein translocation with SecG inversion.

MPIase Modulates the Dimer Orientation of SecYEG. To clarify the mechanism underlying the MPIase effect, SecG 60C (9) and SecE 106C (11) mutants were used (Fig. 3). These mutants carry a cysteine substitution at specified positions that correspond with TM 2 and TM 3 of the respective proteins. SecE 106C is thought to make up the interface of the back-to-back dimer (4, 5). IMV in which either mutant was expressed were subjected to pOmpF-Lpp (a model presecretory protein composed of the leader peptide of pOmpF and the mature region of major lipoprotein Lpp) translocation, which is absolutely SecG-dependent in the absence of a proton motive force (PMF) (9) (Fig. 3*A*, ΔSecG). When SecG 60C IMV in which SecG 60C was expressed at the WT level in the *secG* null background were used, the activity was specifically inhibited by ~1/3 on oxidation (ΔSecG/SecG 60C). In contrast, the overproduced SecYEG with SecG 60C was resistant to oxidation [SecYEG(60C)$^{++}$]. Conversely, when SecE 106C IMV were used in which SecE 106C had been expressed at the WT level in the *secE* null background, the activity did not change on oxidation (ΔSecE/SecE 106C), whereas translocation into SecYE(106C)G^{++} IMV was affected by oxidation [SecYE(106C)G^{++}], which is consistent with a previous report (11). These results strongly suggest that the dimer interface of SecYEG differs between WT SecYEG and overproduced SecYEG. In the former case, the SecG dimer can be formed, whereas SecE is located at the interface in the latter case. This idea was verified on immunoblotting. As expected, the SecG 60C dimer was formed only when SecYEG had been expressed at the WT level (Fig. 3*B*, *Left*). Conversely, the SecE 106C dimer was observed only when SecYEG had been overproduced but was hardly detected at the WT level, even if nonspecific bands were stained (Fig. 3*B*, *Right*). To confirm that these results were caused by a lack of MPIase, dimer formation of SecG or SecE was monitored using PL reconstituted from the purified SecYEG complex with SecG 60C

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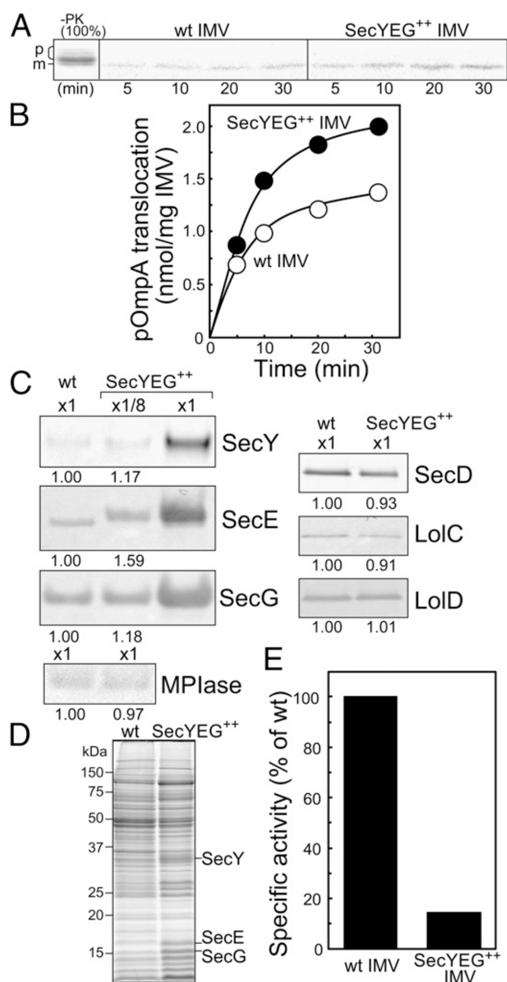

Fig. 2. The specific activity of SecYEG decreases on overproduction. (*A*) The translocation of pOmpA into either WT or SecYEG^{++} IMV was allowed for the indicated periods at 37°C, as described in the legend to Fig. 1*C*. The input pOmpA was also analyzed, as shown at left (-PK). (*B*) The translocation activities obtained in *A* were determined and plotted against time. (*C*) The extent of SecYEG overproduction in IMV was measured. The SecY, SecE, and SecG bands were visualized by immunoblotting. Eightfold diluted samples of SecYEG^{++} IMV were also analyzed (×1/8). The MPIase bands were also visualized by immunoblotting. SecD, LolC, and LolD were used as loading controls. Relative amounts as to WT IMV were determined and are indicated at the bottom of the gel. Note that the slower migration of overproduced SecE is a result of the attachment of a His tag. (*D*) Total IMV protein profiles of WT and SecYEG^{++} IMV. The IMV proteins (20 μg) were analyzed by SDS/PAGE and then stained with CBB. The positions of SecYEG are indicated. (*E*) Specific activity of SecYEG as to pOmpA translocation, determined in *B*, is presented as a percentage of that into WT IMV. SecYEG overproduction of 9.4-fold, determined from SecY and SecG blots in *C*, was taken into account.

or SecE 106C and MPIase. The SecG dimer increased (Fig. 3*C*, *Left*), but the SecE dimer decreased (Fig. 3*C*, *Right*) as MPIase increased. Although the recovery of the SecE 106C monomer after oxidation of PL was less efficient, as reported (11), an MPIase-dependent decrease of the SecE dimer was clearly observed. These results confirm that MPIase modulates the dimer orientation of SecYEG. Next, the SecYEG complex was purified under the WT and overproduction conditions to determine whether or not MPIase can be copurified with SecYEG. It was clearly indicated that MPIase was copurified only when SecYEG had not been overproduced (Fig. 3*D*). Thus, the dimer orientation of the overproduced SecYEG is consistent with the model depicted in Fig. 3*E* (*Left*), designated back-to-back (4, 5), whereas SecYEG with MPIase found under the WT conditions may be the one depicted in Fig. 3*E* (*Right*), designated side-by-side.

TM 2 of SecG Lies in a Relatively Hydrophilic Environment. Because the side-by-side orientation suggests that the two TMs of SecG lie in different environments in membranes, cysteine mutants of SecG (Fig. 4*A*) were used to monitor each environment. *N*-ethylmaleimide (NEM), a membrane-permeable reagent, requires a hydrophilic environment to react with cysteine residues. When WT IMV were used, pOmpF-Lpp translocation, which is highly dependent on SecG, as shown in Fig. 3*A*, was not inhibited, even in the presence of a high concentration of NEM (5 mM; Fig. 4*B*, WT and ΔSecG). When SecG 39C IMV (cytoplasm) or SecG 75C IMV (periplasm) were used, a significant decrease in pOmpF-Lpp translocation was observed in the presence of NEM, indicating that the inhibition by NEM was specific to SecG mutants. Although the activities of TM 1 mutants (4C, 8C, 13C, and 18C) were not affected, those of TM 2 mutants (57C, 60C, 67C, and 71C) were inhibited to less than half by NEM treatment. These results indicate that TM 2 was labeled with NEM, which inhibited the translocation activity. To determine whether the cysteine residues were actually labeled with NEM, NEM-treated IMV were solubilized and then subjected to 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS; molecular weight, 536.44) labeling, which enabled detection of unlabeled SecG on immunoblotting (Fig. 4*C*). All of the mutants examined here except 8C (TM 1) were not affected by AMS labeling when they had been treated with NEM, indicating that even the TM 2 mutants (60C and 67C), but not a TM 1 mutant (8C), were actually labeled with a high concentration of NEM. Note that the cysteine mutants of both TM 1 and TM 2 are resistant to NEM labeling when only radioactive NEM is used at a very low concentration, confirming that they are TMs (9, 32).

SecG Dimer Dissociates on SecG Inversion. We finally examined whether or not a SecG homodimer could be formed on SecG inversion (Fig. 5*A*). When SecG 8C (TM 1) IMV were oxidized, no dimer formation was observed in the presence or absence of pOmpA translocation (SecG 8C). As shown in Fig. 3*B*, the SecG 60C dimer was generated in the absence of translocation (SecG 60C). After pOmpA translocation had occurred, the dimer formation was slightly decreased. Strikingly, under the SecG inversion conditions (+ATP, then +AMP-PNP), such dimer formation was completely abolished (SecG 60C). Under these conditions, topology inversion of SecG was indeed observed (Fig. 5*B*). The decrease in the dimer was translocation-dependent, as omission of ATP, pOmpA, or SecA did not affect the dimer formation (Fig. 5*A*, SecG 60C). Therefore, these results are consistent with the dynamic structural change of SecYEG during pOmpA translocation, and it is possible that the two neighboring SecG molecules at TM 2 dissociate on topology inversion (Fig. 5*C*).

Discussion

In this study, we demonstrated that MPIase, a unique glycolipozyme involved in membrane protein integration (25), is essential for SecG inversion. We studied the significance of SecG inversion by means of IMV and mutant cells in which SecYEG in either the WT or a mutant form had been expressed at the WT level (9, 17–20). Under the conditions used, MPIase could sufficiently interact with SecYEG. In contrast, under the SecYEG-overproduction conditions, most of SecYEG was unable to interact with MPIase, as the SecYEG overproduction did not increase the expression level of MPIase (Fig. 2*C*). Likewise, MPIase was missing in the reconstitution system involving only purified SecYEG. We also demonstrated that MPIase modulates the dimer orientation of SecYEG, with this being the reason MPIase

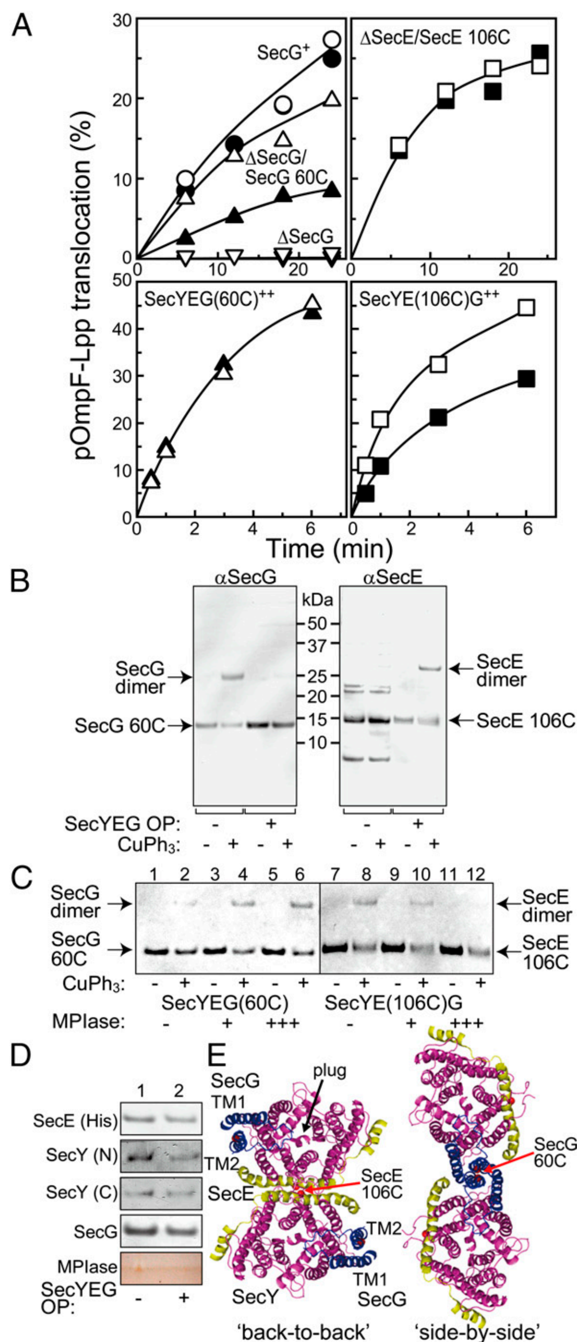

Fig. 3. MPIase modulates dimer orientation of SecYEG. (*A*) pOmpF-Lpp translocation into the specified IMV, which had been treated with 50 μM $CuPh_3$ (closed) or ethanol (open). The translocation reaction was allowed to proceed in the absence of PMF at 37°C for the indicated periods. The translocation activity was expressed as a percentage of the input pOmpF-Lpp. (*B*) Dimer formation of SecG 60C and SecE 106E under the WT and SecYEG overproduction (OP) conditions was monitored. In the left gel, IMV from KN553/pAG60C (-SecYEG OP; 0.5 μg) and B351/pEYG(60C) (+SecYEG OP; 0.05 μg) were oxidized as indicated and then subjected to SecG detection. The positions of SecG 60C and the SecG dimer are indicated by arrows. In the right gel, IMV from PS274/pAE106C (-SecYEG OP; 2.0 μg) and B351/pE(106C)YG (+SecYEG OP; 0.2 μg) were oxidized and subjected to SecE detection. The positions of SecE 106C and the SecE dimer are indicated by arrows. (*C*) PL reconstituted with the purified SecYEG (50 μg) carrying the SecG 60C (*Left*) and SecE 106C (*Right*) mutation supplemented with [50 μg (+) and 150 μg (+++)] or without (−) MPIase were oxidized as indicated. The positions of SecG 60C and the SecG dimer (*Left*), and SecE 106C and the SecE dimer (*Right*) are indicated by arrows. (*D*) SecYEG (His tag at SecE) was expressed in the Δ*secE* cells at the WT (PS274/pAEwt) or overproduction level (PS274/pEYG) and then purified. Sec proteins (immunoblots) and MPIase (TLC) were detected. The same amount of SecE was used. Note that SecY was cleaved on IMV solubilization with the OmpT protease but was still active (30, 31). (*E*) The SecYEG protomer (6) was modeled as back-to-back (*Left*) (4, 5) and side-by-side (*Right*) dimers by means of the PyMOL program.

is essential for SecG inversion. The back-to-back structure, which is predominantly found in crystals (4, 5) and SecYEG-overproducing conditions (11), and the front-to-front structure found on cryo-electron microscopy (8) may be caused by a lack of MPIase. However, SecYEG in WT cells or purified SecYEG supplemented with MPIase exhibited the side-by-side orientation, allowing five- to 10-fold more efficient preprotein translocation with SecG inversion.

According to the side-by-side orientation, TM 2 of SecG lies in the central part of the dimer, surrounded by SecY and TM 1 of SecG. This positioning of TM 2, apart from the lipid bilayer, would allow NEM labeling of the SecG-Cys mutants at a high concentration, which requires a relatively hydrophilic environment in which deprotonation of sulfhydryl residues is possible. Nonetheless, it is obvious that SecYEG undergoes dynamic structural changes during the catalytic cycle of preprotein translocation, including the dissociation of the two neighboring TM 2 on SecG inversion.

SecA drives preprotein translocation in an ATP-dependent manner (33). The mechanisms underlying SecA-dependent translocation have been studied and discussed in many ways. A membrane insertion-deinsertion model has been proposed on the basis of the results of biochemical analyses (34, 35). However, this model seems not to fit the crystal structures, as the pore found in one SecYEG unit is too small for insertion of SecA with a preprotein (5). Alternatively, a model in which SecA pushes a preprotein into the SecYEG pore has been proposed (6, 7). This model is likely to be consistent with the observations made with SecYEG-overproducing IMV and SecYEG-proteoliposomes. Now that MPIase has been found to modulate SecYEG structure and function, the molecular mechanisms underlying preprotein translocation should differ between the WT and SecYEG-overproduction conditions. In this respect, the SecA insertion-deinsertion model should be reexamined in the absence and presence of MPIase. It is possible that the five- to 10-fold more efficient translocation is achieved by MPIase through SecG inversion, and possibly SecA insertion-deinsertion.

MPIase is a membrane protein integrase (24, 25) that drives both Sec-dependent and Sec-independent integration of membrane proteins (26). As the initial step of preprotein translocation is signal sequence insertion into a membrane, MPIase may also assist this step through a mechanism similar to that of the catalysis of membrane integration of Sec-independent substrates (25). Even if this were the case, the side-by-side structure of SecYEG, caused by MPIase, is important for preprotein translocation, as significant stimulation of preprotein translocation is achieved with SecG inversion. In fact, MPIase is also essential for Sec-dependent protein integration into membranes (26). The mechanisms underlying the Sec-dependent integration remain to be clarified; that is, whether MPIase integrates Sec-dependent membrane proteins by directly interacting with them or whether only the side-by-side structure of SecYEG caused by MPIase is integration-competent.

The mode of interaction between MPIase and SecYEG is totally unknown. As MPIase stimulates preprotein translocation into SecYE PL (26), MPIase might interact with SecE or SecY. Considering that purified SecYEG predominantly forms a dimer with SecE as the interface (13), the back-to-back orientation should be thermodynamically stable. Therefore, MPIase might function either as an inhibitor by directly binding with TM 3 of

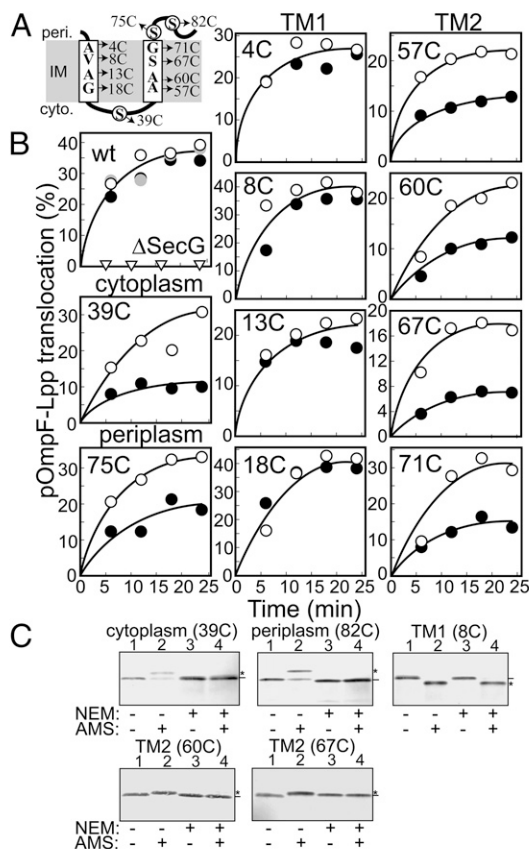

Fig. 4. TM 2 of SecG lies in a relatively hydrophilic environment. (*A*) The positions of cysteine substitutions in SecG are shown. (*B*) Translocation of pOmpF-Lpp into IMV with a mutant SecG is shown. IMV were prepared from K003 (WT), KN553 (ΔSecG; triangles), or KN553 expressing the specified SecG-Cys derivatives. IMV were subjected to pOmpF-Lpp translocation in the presence of NEM at 2 mM (gray circles, WT only) or 5 mM (closed circles) or in its absence (open symbols). The translocation activities were determined and plotted as a percentage of the input pOmpF-Lpp. (*C*) AMS treatment of NEM-treated IMV. IMV (0.9 mg/mL) treated with 5 mM NEM at 37°C for 15 min were 10-fold diluted and solubilized with 2.5% (wt/vol) octyl glucoside in 50 mM potassium phosphate at pH 7.5, followed by AMS (5 mM) labeling at 37°C for 30 min. After quenching of the reaction with 10 mM DTT, SecG was detected by immunoblotting. Asterisks and bars represent the positions of SecG labeled and unlabeled with AMS, respectively. Some mutants including SecG 8C migrate faster on AMS-labeling (9).

SecE or as an enhancer that increases the affinity between two SecY molecules giving the side-by-side orientation. In either case, as a soluble form of MPIase (PP-MPIase) was unable to support SecG inversion, and therefore to stimulate translocation, interaction of MPIase with SecYEG on membranes would be necessary for retention of the side-by-side structure of SecYEG. Analysis of the crystal structure of SecYEG with MPIase is necessary to clarify the molecular mechanisms underlying the highly efficient translocation observed in WT cells.

Materials and Methods

Materials. IMV were prepared from *E. coli* K-12 strains K003 (HfrH *pnp-13 tyr met* RNaseI$^-$ *uncB*-C::Tn*10* Lpp$^-$), KN553 (K003 Δ*secG*) (17), PS274 (PS259 Δ*secE* Δ*recA*) (36), and B351 (BL21 *met*), as described (37). PL were reconstituted as described (26) with slight modifications. Briefly, a mixture (100 μL) of *E. coli* polar phospholipids (1 mg; Avanti Polar Lipids), SecYEG (50 μg), and MPIase, in 50 mM Hepes-KOH at pH 7.5, 20% (vol/vol) glycerol, 150 mM NaCl, and 1.5% (wt/vol) octyl glucoside was incubated on ice for 20 min, followed by dialysis against 50 mM Hepes-KOH at pH 7.5 and 1 mM DTT (500 mL) at 4°C for >2 h. The reconstituted PL suspension was 10-fold diluted with 50 mM Hepes-KOH at pH 7.5, followed by recovery by centrifugation (170,000 × *g*, 1 h, 4°C). SecA (19), the pOmpA/SecB complex (21), SecYEG (26), and MPIase (24) were purified as described. PP-MPIase was prepared by digesting MPIase with pyrophosphatase (25). MPIase was detected on TLC plates (24) or on immunoblots (25). Anti-SecY (30), SecE (38), SecG (17, 22), SecD (39), LolC (40), LolD (40), and MPIase (25) antisera were raised in rabbits. Plasmids pE(106C)YG and pEYG(60C) are SecYEG overproducers with SecE106C and SecG60C, respectively, and were derivatized from pEYG (29) encoding a synthetic operon of His-tagged *secE*, *secY*, and *secG* under the arabinose (*ara*) regulon. To construct them, the corresponding regions in pEYG were replaced with the respective mutations. The WT and mutant forms of SecYEG were overproduced in B351. Plasmids pAEwt and pAE106C are low-copy plasmids encoding His-tagged SecE and SecE 106C, respectively. To yield these plasmids, the BamHI-SalI fragments of pEYG and pE(106C)YG were cloned into pKQ2 (22). Plasmid pAG5 (22) and its mutants with a cysteine substitution (9) are also derivatives of pKQ2. Plasmids pAEwt and pAG5 express WT levels of SecE and SecG, respectively.

Assaying of Preprotein Translocation and Topology Inversion. pOmpA translocation was performed using pOmpA synthesized in vitro and complexed with SecB as described (21). When necessary, the [^{35}S]pOmpA/SecB complex (21) was used. To inhibit pOmpA translocation, 20 mM AMP-PNP/$MgSO_4$ was added 5 min after initiation of the reaction. After the reaction, samples were digested with proteinase K (PK) to determine the translocation activity or the membrane topology of SecG (17). When pOmpF-Lpp translocation was examined, [^{35}S]pOmpF-Lpp, synthesized in vitro (9), was used instead of the pOmpA/SecB complex. When IMV prepared from strains other than K003 and KN553 were used, 20 μM carbonyl cyanide *m*-chlorophenyl hydrazone

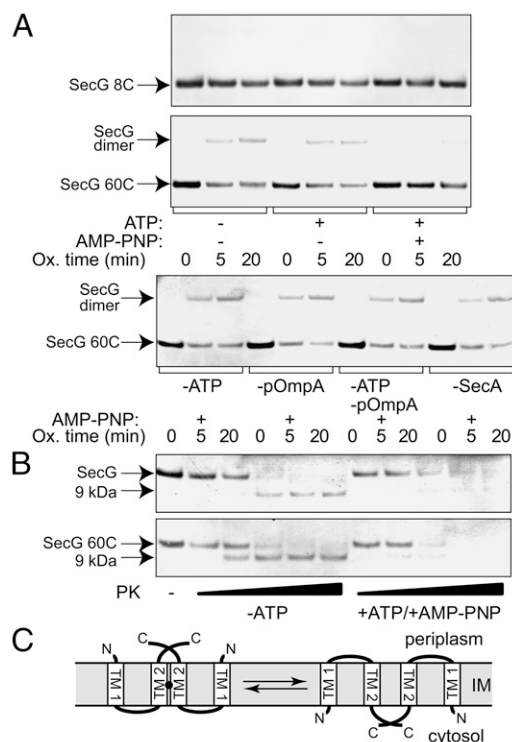

Fig. 5. The SecG dimer dissociates on SecG inversion. (*A*) Dimer formation of SecG. Translocation involving IMV containing SecG 8C (TM 1) or SecG 60C (TM 2) was carried out. Where indicated, AMP-PNP was added 5 min after initiation of the translocation reaction. IMV were then oxidized with 2 mM $CuPh_3$. The SecG dimer was detected by immunoblotting. (*B*) Topology inversion of SecG 60C. IMV from K003 (WT; *Upper*) and KN553/pAG 60C (ΔSecG/SecG 60C; *Lower*) were subjected to the SecG inversion assay. Non-inverted (−ATP) and inverted (+ATP/+AMP-PNP) conditions were used as described in the legend to Fig. 1*B*. The positions of SecG, SecG 60C, and the 9-kDa fragment are indicated by arrows. (*C*) Schematic representation of the structural change of SecG coupled with translocation. Other Sec factors and the precursor proteins are omitted. The formation of the 60C dimer is indicated by a dot.

(CCCP) was added to quench PMF. Oxidation by copper phenanthroline ($CuPh_3$) was carried out with 50 μM for IMV or 100 μM for PL at 37°C for 5 min. Under these conditions, translocation was not inhibited. For maximum oxidation, 2 mM $CuPh_3$ was used. The reaction was terminated by NEM treatment with 10 mM for a further 5 min at 37°C.

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1. Rapoport TA (2007) Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes. *Nature* 450(7170):663–669.
2. Mandon EC, Trueman SF, Gilmore R (2009) Translocation of proteins through the Sec61 and SecYEG channels. *Curr Opin Cell Biol* 21(4):501–507.
3. du Plessis DJ, Nouwen N, Driessen AJ (2011) The Sec translocase. *Biochim Biophys Acta* 1808(3):851–865.
4. Breyton C, Haase W, Rapoport TA, Kühlbrandt W, Collinson I (2002) Three-dimensional structure of the bacterial protein-translocation complex SecYEG. *Nature* 418(6898):662–665.
5. Van den Berg B, et al. (2004) X-ray structure of a protein-conducting channel. *Nature* 427(6969):36–44.
6. Zimmer J, Nam Y, Rapoport TA (2008) Structure of a complex of the ATPase SecA and the protein-translocation channel. *Nature* 455(7215):936–943.
7. Tsukazaki T, et al. (2008) Conformational transition of Sec machinery inferred from bacterial SecYE structures. *Nature* 455(7215):988–991.
8. Mitra K, et al. (2005) Structure of the E. coli protein-conducting channel bound to a translating ribosome. *Nature* 438(7066):318–324.
9. Nagamori S, Nishiyama K, Tokuda H (2000) Two SecG molecules present in a single protein translocation machinery are functional even after crosslinking. *J Biochem* 128(1):129–137.
10. Manting EH, van Der Does C, Remigy H, Engel A, Driessen AJ (2000) SecYEG assembles into a tetramer to form the active protein translocation channel. *EMBO J* 19(5): 852–861.
11. Kaufmann A, Manting EH, Veenendaal AK, Driessen AJ, van der Does C (1999) Cysteine-directed cross-linking demonstrates that helix 3 of SecE is close to helix 2 of SecY and helix 3 of a neighboring SecE. *Biochemistry* 38(28):9115–9125.
12. Duong F (2003) Binding, activation and dissociation of the dimeric SecA ATPase at the dimeric SecYEG translocase. *EMBO J* 22(17):4375–4384.
13. Dalal K, Chan CS, Sligar SG, Duong F (2012) Two copies of the SecY channel and acidic lipids are necessary to activate the SecA translocation ATPase. *Proc Natl Acad Sci USA* 109(11):4104–4109.
14. Osborne AR, Rapoport TA (2007) Protein translocation is mediated by oligomers of the SecY complex with one SecY copy forming the channel. *Cell* 129(1):97–110.
15. Park E, Rapoport TA (2012) Bacterial protein translocation requires only one copy of the SecY complex in vivo. *J Cell Biol* 198(5):881–893.
16. Yahr TL, Wickner WT (2000) Evaluating the oligomeric state of SecYEG in preprotein translocase. *EMBO J* 19(16):4393–4401.
17. Nishiyama K, Suzuki T, Tokuda H (1996) Inversion of the membrane topology of SecG coupled with SecA-dependent preprotein translocation. *Cell* 85(1):71–81.
18. Sugai R, Takemae K, Tokuda H, Nishiyama K (2007) Topology inversion of SecG is essential for cytosolic SecA-dependent stimulation of protein translocation. *J Biol Chem* 282(40):29540–29548.
19. Morita K, Tokuda H, Nishiyama K (2012) Multiple SecA molecules drive protein translocation across a single translocon with SecG inversion. *J Biol Chem* 287(1): 455–464.
20. Nagamori S, Nishiyama K, Tokuda H (2002) Membrane topology inversion of SecG detected by labeling with a membrane-impermeable sulfhydryl reagent that causes a close association of SecG with SecA. *J Biochem* 132(4):629–634.
21. Nishiyama K, Tokuda H (2010) Preparation of a highly translocation-competent proOmpA/SecB complex. *Protein Sci* 19(12):2402–2408.
22. Nishiyama K, Mizushima S, Tokuda H (1993) A novel membrane protein involved in protein translocation across the cytoplasmic membrane of *Escherichia coli*. *EMBO J* 12(9):3409–3415.
23. van der Sluis EO, van der Vries E, Berrelkamp G, Nouwen N, Driessen AJ (2006) Topologically fixed SecG is fully functional. *J Bacteriol* 188(3):1188–1190.
24. Nishiyama K, et al. (2010) A novel complete reconstitution system for membrane integration of the simplest membrane protein. *Biochem Biophys Res Commun* 394(3): 733–736.
25. Nishiyama K, et al. (2012) MPIase is a glycolipozyme essential for membrane protein integration. *Nat Commun* 3:1260.
26. Nishiyama K, et al. (2006) A derivative of lipid A is involved in signal recognition particle/SecYEG-dependent and -independent membrane integrations. *J Biol Chem* 281(47):35667–35676.
27. Douville K, Price A, Eichler J, Economou A, Wickner W (1995) SecYEG and SecA are the stoichiometric components of preprotein translocase. *J Biol Chem* 270(34): 20106–20111.
28. van der Does C, et al. (1996) SecA is an intrinsic subunit of the *Escherichia coli* preprotein translocase and exposes its carboxyl terminus to the periplasm. *Mol Microbiol* 22(4):619–629.
29. Duong F, Wickner W (1997) Distinct catalytic roles of the SecYE, SecG and SecDFyajC subunits of preprotein translocase holoenzyme. *EMBO J* 16(10):2756–2768.
30. Nishiyama K, et al. (1991) SecY is an indispensable component of the protein secretory machinery of *Escherichia coli*. *Biochim Biophys Acta* 1065(1):89–97.
31. Brundage L, Hendrick JP, Schiebel E, Driessen AJ, Wickner W (1990) The purified E. coli integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation. *Cell* 62(4):649–657.
32. Guan L, Kaback HR (2007) Site-directed alkylation of cysteine to test solvent accessibility of membrane proteins. *Nat Protoc* 2(8):2012–2017.
33. Sardis MF, Economou A (2010) SecA: A tale of two protomers. *Mol Microbiol* 76(5): 1070–1081.
34. Economou A, Wickner W (1994) SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. *Cell* 78(5): 835–843.
35. Economou A, Pogliano JA, Beckwith J, Oliver DB, Wickner W (1995) SecA membrane cycling at SecYEG is driven by distinct ATP binding and hydrolysis events and is regulated by SecD and SecF. *Cell* 83(7):1171–1181.
36. Schatz PJ, Bieker KL, Ottemann KM, Silhavy TJ, Beckwith J (1991) One of three transmembrane stretches is sufficient for the functioning of the SecE protein, a membrane component of the *E. coli* secretion machinery. *EMBO J* 10(7):1749–1757.
37. Yamada H, Tokuda H, Mizushima S (1989) Proton motive force-dependent and -independent protein translocation revealed by an efficient *in vitro* assay system of *Escherichia coli*. *J Biol Chem* 264(3):1723–1728.
38. Nishiyama K, Hanada M, Tokuda H (1994) Disruption of the gene encoding p12 (SecG) reveals the direct involvement and important function of SecG in the protein translocation of *Escherichia coli* at low temperature. *EMBO J* 13(14):3272–3277.
39. Matsuyama S, Fujita Y, Mizushima S (1993) SecD is involved in the release of translocated secretory proteins from the cytoplasmic membrane of Escherichia coli. *EMBO J* 12(1):265–270.
40. Narita S, Kanamaru K, Matsuyama S, Tokuda H (2003) A mutation in the membrane subunit of an ABC transporter LolCDE complex causing outer membrane localization of lipoproteins against their inner membrane-specific signals. *Mol Microbiol* 49(1): 167–177.