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Topologically Fixed SecG Is Fully Functional

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It has been proposed that the bitopic membrane protein SecG undergoes topology inversion during translocation of (pre)proteins via SecYEG. Here we show that SecG covalently cross-linked to SecY cannot invert its topology while remaining fully functional in protein translocation. Our results strongly disfavor topology inversion of SecG during protein translocation.

The translocation of proteins across the cytoplasmic membrane of bacteria occurs via an integral membrane protein complex composed of the three proteins SecY, SecE, and SecG (3). The functions of SecY and SecE are well established: SecY forms the protein-conducting channel (2, 9), and SecE is required for the stability of SecY (12, 21). SecG stimulates the activity of SecYE but is not required for viability or in vitro protein translocation (8, 16, 17). Although early studies on a *secG* deletion strain suggested that SecG is required for growth at low temperature (16), later studies showed that cold sensitivity is only observed in strains that also carry a mutation in GlpR, the regulator of glycerol phosphate metabolism (6). The molecular basis for this synergistic effect remains to be established, but it explains why all the *secG* deletion strains that have been constructed in a *glpR*⁺ background are not cold sensitive (1, 5, 7, 11; N. Nouwen, unpublished data).

The membrane topology of SecG has been determined with PhoA fusions (18) and cysteine labeling studies (15). SecG consists of two transmembrane segments that are connected by a mildly hydrophobic cytoplasmic loop, while both termini are located in the periplasm. During protein translocation SecG has been proposed to completely invert its membrane topology (14, 18). This is a highly unusual phenomenon for a stably membrane-integrated protein. The topology inversion theory is, however, mostly based on indirect accessibility studies with proteases and chemical reagents. We reasoned that we could critically test the theory with a topologically fixed version of SecG. Disulfide cross-linking seemed the method of choice to fix the SecG topology, since it can efficiently and reversibly generate covalent bonds between proteins.

In a cysteine-directed cross-linking study we have previously identified a cytoplasmic residue in SecY (Thr¹⁷⁹) that can be chemically cross-linked to SecG (23). Mutagenesis of Lys²⁶ of SecG to arginine abolished this cross-link (data not shown) (20). To investigate whether SecY(T179C) could form a disulfide bond with SecG(K26C) we expressed both mutants together with SecE in the *Escherichia coli* SF100-derived *secG*

deletion strain NN104 [$F^- \Delta lacX74 galK thi rpsL strA \Delta phoA(PvuII) \Delta ompT \Delta secG$] (22). When inner membrane vesicles (IMVs) overexpressing SecY(T179C)EG(K26C) complexes were oxidized by a 30-min incubation with 1 mM Na₂S₄O₆ at 37°C, a SecY-SecG cross-link product was formed with more than 75% efficiency (Fig. 1A to C, lane 6). The oxidation could be reversed by the addition of dithiothreitol (data not shown).

It seems unlikely that SecG which is cross-linked to the shortest cytoplasmic loop of SecY can invert its topology, as this would require a major topological inversion of several SecY helices as well. To corroborate this, we subjected the oxidized SecY(T179C)EG(K26C) complexes to the proteolytic topology inversion assay. When wild-type IMVs are incubated with an externally added protease under nontranslocating conditions, SecG is cleaved in its cytoplasmic loop, resulting in a 9-kDa C-terminal fragment that can be detected with antibodies directed against the extreme C terminus of SecG (18). Similarly, cleavage of the 50-kDa SecY-SecG cross-link product is accompanied by the appearance of the 9-kDa C-terminal fragment (Fig. 2A, panels 1 and 2), indicating that cross-linked SecG has the same topology as non-cross-linked SecG.

In the proposed topology inversion model, the inverted SecG is characterized by the complete disappearance of the C-terminal epitope when translocation of a preprotein is blocked by the nonhydrolyzable ATP analog adenylyl imidodiphosphate (AMP-PNP) after initiation with ATP (18). When IMVs containing the cross-linked SecYEG complexes were subjected to these conditions, the 9-kDa C-terminal fragment was still quantitatively generated (Fig. 2B, panels 1 and 2), indicating that cross-linked SecG does not invert its topology. From these data we conclude that SecG cross-linked to SecY has a fixed topology resembling that of wild-type SecG under nontranslocating conditions.

Since the topology inversion assay requires a functional SecYEG complex, it could be argued that cross-linked SecG does not invert its topology because it is inactive. Therefore we determined the activity of SecYEG complexes containing cross-linked SecG with two different activity assays. After oxidation as described above and reharvesting of the membranes to remove the oxidator, we assayed IMVs containing cross-linked SecY(T179C)EG(K26C) complexes under reducing (Fig. 3A) and nonreducing conditions (Fig. 3B) for the in vitro

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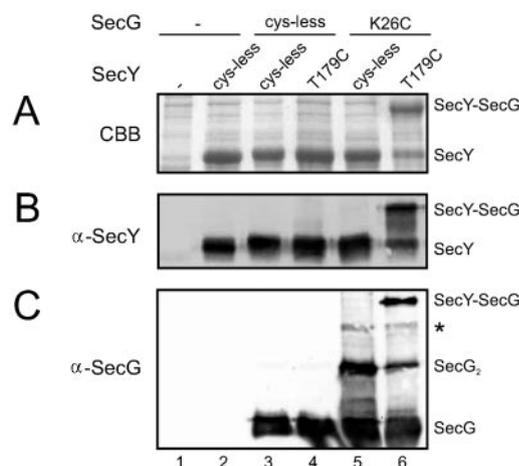


FIG. 1. Highly efficient disulfide cross-linking of SecY(T179C) to SecG(K26C). NN104-derived IMVs overexpressing different combinations of SecY (SecE) and SecG or the empty expression vector (lanes 1) were oxidized with Na₂S₄O₆ and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Coomassie brilliant blue staining (A), anti-SecY (B), or anti-SecG immunodetection (17) (C) according to standard procedures. Bands corresponding to SecY, SecG, dimeric SecG (SecG₂), and the SecY-SecG cross-link product are labeled correspondingly, and the weak band indicated with an asterisk represents SecG cross-linked to the N-terminal proteolytic fragment of SecY (23). For quantitation of the SecY-SecG cross-linking efficiency, the Coomassie brilliant blue-stained gel was imaged and analyzed. Note that no quantitative information can be gained from the Western blots due to the reduced blotting efficiency of the cross-linked adducts.

translocation of fluorescently labeled pro-OmpA as described before (23). Translocation reactions were incubated for 7 min at 37°C and contained limiting amounts of IMVs to ensure that it reflects the activity of the cross-linked SecYEG complexes. The stimulatory effect of SecG can be clearly observed by comparing IMVs containing overexpressed SecYE (Fig. 3, lanes 2) to those with overexpressed SecYEG (lanes 3). Neither the single cysteine mutations in SecY (lanes 4) (20, 23) and SecG (lanes 5) nor the combination of both mutations (lanes 6) influences the translocation activity of SecYEG. Importantly, the cross-linked SecYEG complexes (Fig. 3B, lane 6) show translocation activity similar to that of non-cross-linked SecYEG (Fig. 3A and B, lanes 3 to 5).

We also analyzed the effect of the SecY-SecG cross-link on the pro-OmpA-stimulated SecA ATPase activity as a function of the SecA concentration by assaying oxidized IMVs overexpressing SecY(T179C)EG(K26C) or cysteineless SecYEG under nonreducing conditions. As can be seen in Fig. 3C, both cross-linked (open symbols) and non-cross-linked SecYEG complexes (solid symbols) exhibit identical SecA dependency of the translocation ATPase activity. From these two activity assays we conclude that SecYEG complexes containing cross-linked SecG are fully functional and that the static behavior in the topology inversion assay is not caused by inactivation of the complex. Importantly, this implies that the proposed topology inversion of SecG is not required for the functionality of the SecYEG complex.

The results presented here disfavor the topology inversion theory. However, in previous studies (13, 19), the topology

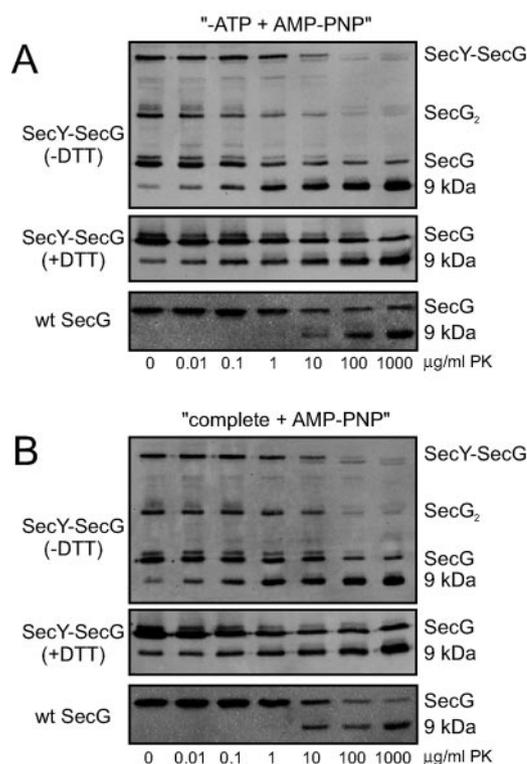


FIG. 2. SecG cross-linked to SecY does not invert its membrane topology. Oxidized IMVs overexpressing SecY(T179C)EG(K26C) (panels 1 and 2) or wild-type SF100 IMVs (panels 3) were subjected to the SecG topology inversion assay (A: -ATP plus AMP-PNP; B: complete plus AMP-PNP; see text for details) as described before (18) under nonreducing conditions. Samples were analyzed by nonreducing (panels 1) or reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (panels 2 and 3) followed by immunodetection with antibodies raised against the extreme C terminus of SecG (17). Bands corresponding to the SecY-SecG cross-link product, SecG, dimeric SecG (SecG₂), and the 9-kDa C-terminal fragment of SecG, are indicated. The applied concentrations of proteinase K (PK) are indicated at the bottom of each panel. Note that a small amount of the 9-kDa fragment is always observed upon overexpression of SecG (18).

inversion assay has also yielded results that differ substantially from those originally described by Nishiyama et al. (14, 18). In both of these studies (13, 19), under conditions in which the C-terminal epitope of SecG was expected to disappear completely, SecG was hardly cleaved by the protease and a small amount of the 9-kDa fragment was generated. Importantly, these results disfavor the topology inversion theory. Our attempts to reproduce the topology inversion with endogenous levels of wild-type SecG in IMVs derived from various *E. coli* strains (K002, K003, DH5α, SF100, and NN100) also failed and resulted in the generation of the 9-kDa fragment, very similar to the results described above for cross-linked SecG (Fig. 2A and B, panels 3). Based on these three independent observations, in particular the finding that topologically fixed SecG is fully functional, we conclude that SecG maintains its original topology during protein translocation via SecYEG. We propose that the reported changes in accessibility for proteases and cysteine-modifying reagents reflect a SecA-induced conformational change of SecG within the SecYEG complex rather than an inversion of the SecG topology.

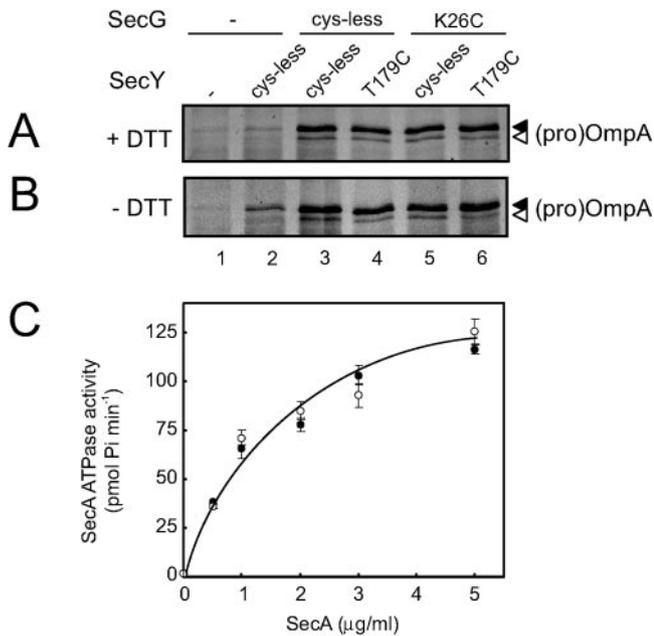


FIG. 3. Cross-linked SecY(T179C)EG(K26C) is as active as wild-type SecYEG. Oxidized IMVs overexpressing the indicated SecYEG complexes (see legend to Fig. 1) were analyzed for *in vitro* translocation of fluorescein maleimide-labeled pro-OmpA(C302S) under non-reducing conditions (A) or in the presence of 5 mM dithiothreitol (DTT) (B) as described (23). Cysteineless SecYEG has previously been shown to be as active as wild-type SecYEG (10). (C) Oxidized IMVs overexpressing SecYEG (open symbols) or SecY(T179C)EG(K26C) (solid symbols) were analyzed for pro-OmpA-stimulated SecA ATPase activity under nonreducing conditions as described (4) with the indicated amounts of SecA.

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