Topological Inversion of SecG Is Essential for Cytosolic SecA-dependent Stimulation of Protein Translocation*

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SecG, a subunit of the protein translocon, undergoes a cycle of topology inversion. To further examine the role of this topology inversion, we analyzed the activity of membrane vesicles carrying a SecG-PhoA fusion protein (SecG-PhoA inverted membrane vesicles (IMVs)). In the absence of externally added SecA, SecG-PhoA IMVs were as active in protein translocation as SecG+ IMVs per SecA. Consistent with this observation, insertion of membrane-bound SecA into SecG-PhoA IMVs was normally observed. On the other hand, externally added SecA did not affect the activity of SecG-PhoA IMVs, but it caused >10-fold stimulation of the translocation activity of SecG+ IMVs, indicating that the topology inversion of SecG, which cannot occur in SecG-PhoA IMVs, is essential for cytosolic SecA-dependent stimulation of protein translocation. SecG-PhoA IMVs generated a 46-kDa fragment of SecA upon trypsin treatment. The accumulation of this membrane-inserted SecA in the SecG-PhoA IMVs was responsible for the loss of the soluble SecA-dependent stimulation. Moreover, fixation of the inverted SecG topology was found to be dependent on soluble SecA. The dual functions of SecG in protein translocation will be discussed.

*This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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ture of $^{35}$S Met and Cys, and Na$^{125}$I (14.8 GBq/ml; ICN) were used to label proteins. Trypsin was purchased from Washington Biochemical Corporation. Creatine phosphate and arabinose were from Sigma. Proteinase K was from Merck. ATP, AMP-PNP, and creatine kinase were from Roche Applied Science. Anti-SecA (17), anti-SecE (20), anti-SecG (6), and anti-SecY (20) antisera were raised in rabbits. A Protistoblot system, composed of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Promega), or an ECL system (Amersham Biosciences) was used to detect the immunodecorated proteins.

**Assaying of Protein Translocation**—Translocation activity was determined by using proOmpA as a substrate. Translocation-competent proOmpA was prepared as follows. The in vitro synthesized proOmpA (~1 × 10$^7$ cpm) was denatured with 10% trichloroacetic acid, followed by recovery by centrifugation (10,000 × g, for 5 min at 4 °C). After the pellets had been successively washed with acetone and ether successively, $^{35}$S proOmpA was solubilized in 100 μl of 8 M urea, 50 mM potassium phosphate (pH 7.5). It was then mixed with nonradioactive proOmpA (0.5 mg), followed by dilution in 5 ml of 50 mM potassium phosphate (pH 7.5), 300 mM NaCl containing 1 mg of SecB-CHis to allow the formation of the proOmpA-SecB-CHis complex. After incubation at room temperature for 20 min, 500 μl of a TALON metal affinity resin (Clontech) was added to the mixture, which was incubated at room temperature for another 15 min with mild rotating. The resin was then transferred to a column and subsequently washed with 10 ml of 50 mM potassium phosphate (pH 7.5), 300 mM NaCl, 10 mM imidazole. The proOmpA-SecB-CHis complex was eluted with 50 mM potassium phosphate (pH 7.5), 300 mM NaCl, 250 mM imidazole. The radioactive fractions were collected and dialyzed against 50 mM potassium phosphate (pH 7.5), 10% glycerol. The amount of proOmpA was determined from the density of the proOmpA bands on a Coomassie Brilliant Blue-stained gel, using the purified proOmpA as a standard.

The translocation reaction mixture contained IMVs (0.1 mg/ml as membrane proteins), SecA (0 ~ 60 μg/ml), SecY (20) antisera were raised in rabbits. A Protistoblot system, composed of 10% acrylamide-0.27% $\text{N}_{2}$-methylenebisacrylamide was used. For the detection of SecY, a gel composed of 5 mM creatine phosphate and 10 μg/ml creatine kinase, and 50 mM potassium phosphate (pH 7.5). Where specified, IMVs washed with 4 M urea were recovered with 10% trichloroacetic acid and then analyzed by SDS-PAGE and fluorography, as described (22). The amount of the translocated materials were determined with an ATTO densitograph.

**Analysis of the SecA Insertion-Deinsertion Cycle**—$^{35}$S-labeled SecA or $^{35}$S-labeled SecA was used to analyze the SecA cycle. The radiolabeled SecA was either loaded onto 4 M urea-washed IMVs, followed by the translocation reaction (8), or directly used for the translocation reaction as described above, except that $^{35}$S proOmpA was omitted. Where specified, ATP or proOmpA was omitted. To analyze the SecA deinsertion, cold SecA (40 μg/ml) was added during the reaction. After the specified reaction time at 37 °C, an aliquot (100 μl) was withdrawn and subjected to either PK digestion (1 mg/ml) or trypsin digestion (1 mg/ml) on ice for 30 min. The protease-protected fragments were then analyzed by SDS-PAGE and fluorography.

**Determination of Topologically Inverted SecG after Inhibition of Protein Translocation**—The inverted topology of SecG was evaluated by quantitative immunoblotting after PK digestion of IMVs (7–9), as follows. ProOmpA translocation was carried out as described above, except that $^{35}$S proOmpA was omitted. To the reaction mixture, 20 mM AMP-PNP and 20 mM MgSO$_4$ were added at 5 min after the start of the reaction, followed by a further 5-min incubation at 37 °C. After chilling on ice for 2 min, aliquots (20 μl) were withdrawn from the reaction mixture (120 μl), and each was digested with an equal volume of PK dissolved in 50 mM potassium phosphate (pH 7.5) on ice for 30 min. The proteins were precipitated with trichloroacetic acid (10%), followed by successive acetone and ether washing. The resultant precipitates were dissolved in 20 μl of the SDS-containing buffer, of which 5 μl was analyzed by SDS-PAGE and immunoblotting, using anti-SecG antibodies. After electrophoresis, the proteins were transferred to nitrocellulose membranes by means of a semidy transfer apparatus according to the manufacturer’s instructions. The membranes were blocked with 10% horse serum in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl (TBS) at room temperature for 1 h. Anti-SecG antiserum, diluted at 1:5,000 in TBS containing 3% horse serum, was then reacted with the membranes at room temperature for 45 min with mild shaking. After several washings with TBS containing 0.05% Tween 20 (T-TBS), the membranes were incubated with phosphatase-labeled goat anti-rabbit IgG (KPL) in T-TBS at 0.2 μg/ml for 30 min at room temperature with mild shaking. After several washings with T-TBS, the bands derived from SecG were visualized in AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl$_2$ containing 62.5 μg/ml 5-bromo-4-chloro-3-indolyl phosphate and 125 μg/ml nitro blue tetrazolium. Under these conditions, the linearity of the SecG amount with the SecG band was only observed when less than 1 μg of the IMVs was analyzed. When a more sensitive method such as ECL is used, it is essential to determine the conditions in which the amount of SecG can be analyzed quantitatively.

**Other Methods**—The $K_d$ values for SecA with SecG$^+$ IMVs and SecG-PhoA IMVs were determined as described (23) in the presence of a fixed amount of $^{35}$S SecA and various amounts of SecA (0 ~ 600 nM). Translocation ATPase activity was determined as described (24), and SDS-PAGE was performed as described (25, 26). The latter was performed to analyze the topology inversion of SecG. For the detection of SecA, a gel composed of 10% acrylamide-0.27% $N_{2}$-$N'_{2}$-methylenbisacrylamide was used. For the detection of SecY, a gel composed of 12.5% acrylamide-0.33% $N_{2}$-$N'_{2}$- methylenbisacrylamide was used, and for SecE, SecG, and OmpA, a gel composed of 13.5% acrylamide-0.36% $N_{2}$-$N'_{2}$-methylenbisacrylamide was used. The proteins were determined as described (27), using bovine serum albumin as a standard.
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RESULTS

SecG-PhoA Competes with SecG for the Translocation Machinery—To examine the role of SecG inversion, we examined whether SecG-PhoA, of which topology inversion is blocked (7), retains the ability to interact with the Sec machinery. When K003 (secG+) cells harboring pAGP12 (Para-secG-PhoA) were cultivated in the presence of arabinose, the level of SecG-PhoA linearly increased to ~60 min after the addition of 0.2% arabinose and reached a plateau after ~90 min (Fig. 1 A). A possible truncated SecG-PhoA was also observed (Fig. 1A, asterisk). In marked contrast, SecG that was expressed from the chromosome gradually decreased with an increase in the amount of SecG-PhoA to, eventually, an undetectable level (Fig. 1A). Arabinose induction did not affect the SecG expression, because the SecG level in K003 cells harboring the control vector pKQ2 remained unchanged throughout the cultivation (Fig. 1A). The SecG level also did not change when K003/pAGP12 was cultivated in the absence of arabinose (data not shown). Quantitation of the expression levels of SecG and SecG-PhoA revealed that the total level of SecG and SecG-PhoA expression remained roughly constant (Fig. 1B, triangles). These results strongly suggest that SecG-PhoA expressed at a high level from a plasmid competed with SecG constitutively expressed from the chromosome for the Sec machinery. It is quite likely that uncomplexed SecG or SecG-PhoA was readily degraded by the quality control system in membranes, because the SecG-PhoA level did not exceed that of SecG in the wild-type cells. The degradation of the uncomplexed SecG or SecG-PhoA was further verified by means of an ftsH mutant, in which the quality control system for membrane proteins was impaired (14). SecG-PhoA was severalfold overproduced in the ftsH mutant, compared with in the parent strain (Fig. 1C). Moreover, half of the intact SecG remained even after the SecG-PhoA induction (Fig. 1C). When SecG was similarly induced from this vector in the ΔsecG strain, the SecG level did not exceed that in the wild-type cells (15), although the level of transcription from this vector is much higher than that in the case of the secG-leuU locus (28). We therefore concluded that SecG-PhoA retains the ability to form a complex with SecYE and to replace the preexisting SecG. It is known that newly synthesized SecG can be exchanged with preexisting SecG, but SecY and SecE cannot because they form a stable complex (29).

The Amount of SecA Associated with SecG-PhoA IMVs Increases—IMVs were prepared from ΔsecG cells in which SecG-PhoA had been induced at 37 °C. The growth rate of this strain was essentially the same as that of secG+ strain K003 (data not shown), consistent with the observation that ΔsecG cells expressing SecG-PhoA exhibited cold sensitivity but no growth defect at 37 °C (7). With induction with 0.01% arabinose for 2 h, the level of SecG-PhoA was similar to that of SecG in the wild-type cells, without generation of the truncated form (Fig. 2A). The levels of SecYE and SecA were also examined in cells expressing SecG-PhoA. Essentially, the level of SecYE in the IMV fraction was the same for both types of IMVs. In contrast, the SecA level in SecG-PhoA IMVs was up to 3-fold higher than that in SecG+ IMVs (Fig. 2B). When the total cellular level and the cytosolic pool of SecA were analyzed, the amount of SecA...
was not affected by the SecG-PhoA expression (Fig. 2C). In the IMV fraction, again, the amount of membrane-bound SecA with SecG-PhoA IMVs increased (Fig. 2C, IMV). These results indicate that the SecA level and localization did not change in either type of cell but that a significant fraction of SecA associated with SecG+ IMVs had become dissociated and thus was lost during the IMV preparation, which includes separation through the sucrose gradient, as reported (30). It was thus strongly suggested that the affinity of SecA with SecG-PhoA IMVs specifically increased. The $K_\text{d}$ values for SecA with SecG IMVs and SecG-PhoA IMVs were determined to be ~25 and ~2.1 nM, respectively (data not shown), confirming the higher affinity of SecA with SecG-PhoA IMVs. The former value was similar to the previously reported one (~40 nM) (23). Quantitative immunoblotting revealed that the amount of SecA associated with SecG-PhoA IMVs (150–200 pmol as SecA protein/10^8 cells; data not shown) was similar to that of SecYEG (50–100 pmol/mg IMVs) (31, 32), suggesting that all of the translocos carrying SecG-PhoA are associated with SecA.

**Soluble SecA Does Not Stimulate Protein Translocation into SecG-PhoA IMVs**—To examine the effect of SecG inversion on protein translocation, the translocation activity of SecG-PhoA IMVs was determined and compared with that of SecG+ IMVs. PMF was not imposed, because it greatly reduces the SecG requirement (33). Protein translocation is driven by membrane-associated SecA in the absence of externally added SecA (34). Under these conditions, the activity of SecG+ IMVs was low. Strikingly, however, the activity of SecG-PhoA IMVs was 3–4-fold higher than that of SecG+ IMVs (Fig. 3A, left half). We then examined the effect of external SecA (60 μg/ml) on protein translocation. The translocation activity of SecG+ IMVs was significantly stimulated (Fig. 3A, right half), as observed previously (22, 35). In marked contrast, the addition of SecA caused almost no stimulation of the activity of SecG-PhoA IMVs (Fig. 3A). As a result, the activity of SecG-PhoA IMVs became much lower than that of SecG+ IMVs in the presence of external SecA (Fig. 3A). When PMF was imposed in the presence of soluble SecA, the translocation activity of SecG-PhoA IMVs was as high as that of SecG+ IMVs (data not shown), indicating that SecG-PhoA does not have any inhibitory effect on protein translocation. The initial rates of proOmpA translocation were determined with the two types of IMVs and plotted against the concentration of added SecA (Fig. 3B). The translocation activity of SecG-PhoA IMVs was not affected by
SecA concentrations up to 60 μg/ml, but proOmpA translocation into SecG+ IMVs increased with an increase in the added SecA to more than 10-fold. These results indicate that IMVs bearing SecG-PhoA do not apparently respond soluble (or cytosolic) SecA. We then examined the translocation ATPase activities of the two types of IMVs in the presence and absence of external SecA (Fig. 3C). Considering that the level of the membrane-associated SecA was ~3-fold higher in SecG-PhoA IMVs than in SecG+ IMVs (Fig. 2B), the specific activities of SecA in the two types of IMVs were almost the same in the absence of external SecA, because the ATPase activity of SecG-PhoA IMVs was ~3-4-fold higher than that of SecG+ IMVs (Fig. 3C, left panel). In contrast, the translocation ATPase activities in the presence of soluble SecA were similar for the two types of IMVs (Fig. 3C, right panel). The translocation activity and translocation ATPase activity of SecG+ IMVs were stimulated by the addition of soluble SecA. On the other hand, external SecA stimulated the translocation ATPase of SecG-PhoA IMVs with no stimulation of their proOmpA translocation. Thus the ATPase activity of SecG-PhoA IMVs was uncoupled from proOmpA translocation in the presence of soluble SecA.

The results shown in Fig. 3 appeared to indicate that the proOmpA translocation into SecG-PhoA IMVs is independent of SecA. However, this was not the case. SecG-PhoA IMVs were washed with urea to remove the membrane-associated SecA, followed by determination of the translocation activity (Fig. 4). It is known that the washing of IMVs with 4M urea removes and inactivates the membrane-associated SecA, thereby rendering protein translocation absolutely dependent on external SecA (12, 22, 36). After the urea washing, ~80% of SecA was removed from both types of IMVs (Fig. 4A), as reported (22). When SecA was not added, essentially no proOmpA translocation occurred in either type of IMVs. On the other hand, the translocation activity increased with an increase in the amount of SecA (Fig. 4B), indicating that proOmpA translocation into SecG-PhoA IMVs is also SecA-dependent. However, proOmpA translocation into SecG-PhoA IMVs was saturated with a lower amount (2 μg/ml) of SecA, resulting in three times lower activity compared with that in SecG+ IMVs. These results again demonstrate that SecA associates with SecG-PhoA IMVs with much higher affinity than SecG+ IMVs. The maximum translocation activities of SecG-PhoA IMVs were similar before and after urea washing (Figs. 3 and 4), strongly suggesting that all of the translocons carrying SecG-PhoA are occupied by SecA when SecG-PhoA IMVs are not washed with urea.

The SecA Cycle Occurs Normally on SecG-PhoA IMVs—Because the translocation ATPase activity induced by soluble SecA was not coupled with proOmpA translocation into SecG-PhoA IMVs, we examined whether the SecA cycle, revealed by protease protection assay (37), occurs on SecG-PhoA IMVs normally (Fig. 5). After 125I-labeled SecA had been loaded onto the urea-washed IMVs, proOmpA translocation was allowed to proceed, followed by trypsin digestion (Fig. 5A). The membrane-inserted fragment of 30 kDa similarly increased in both types of IMVs, indicating that the SecA insertion took place similarly in both types. Moreover, when an excess amount of cold SecA was added during the reaction, the inserted and labeled SecA rapidly disappeared in both types of IMVs. These results indicate that the “insertion–deinsertion cycle” of SecA (4, 8, 37) occurs normally on SecG-PhoA IMVs. These results also indicate that membrane-inserted SecA can be exchanged with soluble SecA even in SecG-PhoA IMVs, although this does not result in the stimulation of protein translocation, thereby causing an uncoupled translocation ATPase.

We then examined the 125I-SecA insertion into urea-unwashed SecG-PhoA IMVs (Fig. 5B). The membrane-inserted fragment of 30 kDa was generated in both SecG+ and SecG-PhoA IMVs in ATP- and proOmpA-dependent manners (lanes 5 and 9). These results indicate that externally added SecA could participate in the reaction even in the presence of membrane-bound SecA on SecG-PhoA IMVs.

A 46-kDa Fragment of SecA Is Specifically Generated on Trypsin Treatment of SecG-PhoA IMVs—The structure of membrane-embedded SecA in SecG-PhoA IMVs was analyzed by means of trypsin digestion (Fig. 6). Urea-unwashed SecG+ and SecG-PhoA IMVs were digested with trypsin, followed by immunodetection using anti-SecA antibodies (Fig. 6A). A char-

FIGURE 4. Translocation activity of urea-washed SecG-PhoA IMVs. A, the amounts of SecA in SecG+ (left panel) and SecG-PhoA (right panel) IMVs were washed with 4M urea. Aliquots of IMV protein were analyzed to detect SecA by immunoblotting. The percentage of SecA that remained after urea washing is also indicated. B, translocation activities of urea-washed SecG+ (open circles) and SecG-PhoA (closed circles) were determined as shown for Fig. 3B and plotted against the concentration of externally added SecA.
characteristic 46-kDa fragment was exclusively generated from SecG-PhoA IMVs (lanes 2 and 4). The generation of the 46-kDa fragment was further examined using 35S-labeled SecA and urea-washed IMVs (Fig. 6B). When the translocation reaction was carried out in the presence of proOmpA and ATP, the well-defined fragments of 30 and 65 kDa, both of which are dependent on protein translocation (38), were generated in both types of IMVs on trypsin digestion (Fig. 6B). The levels of the 30- and 65-kDa fragments were found to be similar in both types (Fig. 6C), indicating that the usual insertion-deinsertion cycle of SecA occurs on SecG-PhoA IMVs. It may be noteworthy that the band corresponding to ~30 kDa generated in the absence of proOmpA translocation migrated slightly slower than in its presence, as reported (38). In addition to these fragments, the 46-kDa fragment was specifically generated on SecG-PhoA IMVs, as shown in Fig. 6A. Although a marginal amount of this fragment was observed in SecG- PhoA IMVs (lane 2), this was com-
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Soluble SecA is essential for the topology inversion of SecG—The soluble SecA-dependent stimulation was not observed when the topology inversion of SecG was blocked by C-terminally fused PhoA. We then examined whether inversion of the SecG topology depends on external SecA. When AMP-PNP, a non-hydrolyzable analogue of ATP, was added during the translocation reaction in the presence of external SecA, the inverted topology of SecG could be fixed (7–9). In the absence of protein translocation, a C-terminal 9-kDa fragment of SecG was generated upon PK digestion of IMVs (Fig. 7A, left), and the intact SecG and the 9-kDa fragment both disappeared on PK treatment of inverted SecG because PK was accessible to the C-terminal region of inverted SecG (Fig. 7A, right; Ref. 7). Upon PK digestion of SecG+ IMVs in the absence of protein translocation, the 9-kDa fragment, whose density is ~50% of that of intact SecG, can be detected by quantitative immunoblotting (7, 8). On the other hand, when protein translocation was initiated in the presence of a sufficient amount of SecA and then blocked by AMP-PNP, both intact SecG and its 9-kDa fragment were hardly generated on PK treatment because of the SecG inversion (Fig. 7B, lanes 7–11; Ref. 7). Using this assay system, the amount of the 9-kDa fragment was examined in the presence of various amounts of external SecA (Fig. 7C). When SecA was not added, the 9-kDa fragment, whose density was ~50% of that of intact SecG, was observed (Fig. 7B, lanes 2–6, and C). The protein translocation activity was low but significant under these conditions (Fig. 3). These results indicate that the topology inversion of SecG depends on soluble SecA. In contrast, when topology inversion was inhibited, protein translocation lost the soluble SecA dependence.

**DISCUSSION**

In this study, we re-evaluated the role of the topology inversion of SecG by means of a SecG-PhoA fusion protein, which has the ability to form a complex with SecYE as SecG. Although SecG-PhoA IMVs were as translocation-proficient as SecG+ IMVs in the absence of soluble SecA, SecG-PhoA IMVs were much less active than SecG+ IMVs in the presence of soluble SecA, the closer conditions to the *in vivo* situation, demonstrating that the soluble SecA-dependent stimulation of protein translocation requires the topology inversion of SecG. We also re-examined whether the topology of SecG is really inverted upon the blockage of proOmpA translocation. We could clearly reproduce the topology inversion of SecG, as previously reported (7–9). On the other hand, we found that fixation of the SecG inversion does require the soluble SecA. These findings coincide with the observation that SecG-PhoA IMVs were not stimulated by the soluble SecA. We therefore conclude that the SecG inversion is essential for the cytosolic (soluble) SecA-dependent stimulation of protein translocation.

The role of cytosolic SecA in protein translocation is still a subject of controversy. It has been reported that a high concentration of SecA compensates for defects in the absence of PMF (22, 35). Our results strongly support the involvement of the cytosolic SecA in stimulation of protein translocation. On the other hand, it has been reported that the membrane-associated SecA is sufficient for protein translocation (34). Moreover, it
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has also been reported that only the membrane-integral form of SecA is fully translocation active (30, 39). Although the SecB-proOmpA complex exhibits much higher affinity to membrane-bound SecA (23, 40), a SecA-SecB complex can also be found in the cytosol (41). In vivo, the concentration of SecA in the cytosol is estimated to be 3–5 μM (300–500 μg/ml) as the protomer (31, 42), which is comparable with our experimental conditions. Moreover, SecA is derepressed upon the inhibition of protein export (43–45), especially that of the SecM protein (46), strongly suggesting that overproduced and soluble SecA can overcome the defects in protein translocation.

In the absence of soluble (cytosolic) SecA, the specific activity of SecA for both proOmpA translocation and translocation ATPase was the same in SecG + and SecG-PhoA IMVs. Moreover, the amounts of the membrane-inserted fragments of 30 and 65 kDa, both of which increase upon protein translocation (37, 38), were almost the same. These findings indicate that the SecA cycle ongoing on SecG-PhoA IMVs is the same as that on SecG + IMVs, if soluble SecA is absent. The external addition of SecA to SecG-PhoA IMVs caused no enhancement of the translocation activity, indicating that all of the translocons are associated with SecA in SecG-PhoA IMVs, and some are not in SecG + IMVs. Because externally added SecA stimulated proOmpA translocation in SecG + IMVs to a much higher level than in SecG-PhoA IMVs, the stimulation does not simply reflect a process in which the translocon vacant of SecA is fulfilled by SecA. To achieve such high translocation activity in SecG + IMVs, the translocon associated with SecA should be further stimulated by the soluble SecA. The crystal structure of the translocon suggests that its pore is formed by a single SecY molecule with a plug (47). Therefore it seems unlikely that multiple proOmpA molecules are simultaneously translocated by a single translocon. We speculate that soluble SecA molecules might displace the SecA that is already inserted with proOmpA to cause its deinsertion. Alternatively, multiple SecA molecules might bind with a proOmpA molecule that is being translocated and thus be inserted successively. In both cases, successive SecA insertions can occur without waiting for SecA deinsertion, one of the rate-limiting steps that can be also driven by PMF (22). In the presence of PMF, the translocation activity of SecG-PhoA IMVs was as high as that of SecG + IMVs (data not shown), consistent with this idea. When SecA is deinserted, the backward movement of proOmpA should be prevented. SecG inversion may be responsible for this step, coupling the successive displacements and/or subsequent insertions of SecA molecules with protein translocation. Because the translocation ATPase in SecG-PhoA IMVs was as high as that in SecG + IMVs in the presence of soluble SecA, such displacements or insertions should occur on the SecG-PhoA IMVs; however, they cannot be utilized for constructive translocation. As a consequence, the interaction between SecA and SecG-PhoA IMVs might be modulated, rendering the affinity of SecA significantly higher with an altered structure from which the fragment of 46 kDa arises, even after urea-washed SecG-PhoA IMVs have been incubated with soluble SecA. It is also a subject of controversy as to whether SecA functions as a monomer (42, 48–51) or a dimer (52–54) on membranes. Although our results do not exclude either model, our results strongly suggest that multiple SecA molecules can function at a single translocon in the presence of soluble SecA.

Our results also suggest that SecG possesses dual functions in protein translocation. One is involvement in the soluble SecA-dependent stimulation, which strictly requires topology inversion of SecG, as discussed above. The other is stimulation that does not necessarily require topology inversion as seen in SecG-PhoA IMVs in the absence of soluble SecA. Driessen and coworkers (11) reported that IMVs in which SecG was partially cross-linked to SecY were active. This may reflect that the cross-linked SecG, of which topology inversion is blocked, retains some SecG activity similar to that of SecG-PhoA. Nevertheless, the IMVs prepared from the secG null mutant were much more defective in the absence of PMF irrespective of the presence or absence of soluble SecA (7, 33). It has also been found that the oligomeric structures of detergent-solubilized preparations are quite different between SecYE and SecYE (55). In the absence of soluble SecA, topology inversion may not be required; however, it has been demonstrated that SecG undergoes topology inversion when PhoA is not fused even in the absence of soluble SecA (10), as discussed below.

Topology inversion of SecG is supported by several lines of evidence, with both IMVs and spheroplasts. In spheroplasts, the C-terminal region of SecG is completely sensitive to externally added PK on ice; however, with incubation at 20 °C, where protein translocation occurs, the 9-kDa fragment is generated. More significantly, such a 9-kDa fragment increased upon the inhibition of SecA by adding sodium azide (7). The topology inversion in spheroplasts has been further confirmed by labeling of a SecG mutant that possesses a single Cys residue on the cytoplasmic face with a membrane-impermeable reagent, AMS (10). In an in vitro system, IgG, recognizing the C-terminal region of SecG, specifically inhibits protein translocation even in the absence of externally added SecA (6, 7). The AMS labeling of SecG, which has a single Cys residue in the C-terminal region (exposed inside the IMVs), increases in a translocation-dependent manner (10). The membrane-bound SecA was sufficient in the case of Cys labeling (10). The C-terminal region of SecG can be digested by PK upon the inhibition of protein translocation by AMP-PNP (7, 8, 10), as shown in Fig. 7. In this case, however, the soluble SecA was essential, as discussed above. Genetically, a double mutant carrying both secAcSRI1 and ΔsecG confers synthetic lethality, demonstrating the functional interaction between SecA and SecG (8). This cold-sensitive SecA mutant is unable to invert SecG, especially in the cold (8).

It is known that the overproduction of SecYE restores the growth of SecG and SecDF disruptants (56), indicating that an increased number of translocons bypasses the functions of SecG or SecDF. Therefore, the overproduction of SecYE may also reduce the requirement of some factor(s), causing different mechanisms from ones in the wild-type situation. We could not observe topology inversion of SecG using SecYE-overproduced IMVs or proteoliposomes reconstituted with the purified SecYEG (data not shown), suggesting that another factor(s) such as SecDF is required to invert SecG. Although SecYE overproduction causes significant stimulation of protein translocation, the extent of the stimulation is far lower than that of...
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SecYEG overproduction (56–58). One possible reason is that SecG is unable to be inverted under these conditions, consistent with a previous report (11).

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