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Membrane Topology Inversion of SecG Detected by Labeling with a Membrane-Impermeable Sulfhydryl Reagent that Causes a Close Association of SecG with SecA[1]

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SecG stimulates protein translocation in *Escherichia coli* by facilitating the membrane insertion–deinsertion cycle of SecA. SecG was previously shown to undergo membrane topology inversion, since SecA-dependent protein translocation renders the membrane-protected region of SecG sensitive to external proteases. To examine this topology inversion in more detail without protease-treatment, SecG derivatives with a single cysteine residue at various positions were labeled in the presence and absence of protein translocation with a membrane impermeable SH reagent, 4-acetamido-4′-maleimidylstilbene-2-2′-disulfonic acid (AMS). Treatment of spheroplasts with AMS revealed that a cysteine residue in the cytoplasmic region of SecG could be labeled from the periplasm side only in the presence of protein translocation, whereas a cytoplasmic protein, elongation factor, Tu, remained unlabeled. Treatment of inverted membrane vesicles with AMS also revealed that cysteine residues in the periplasmic region were labeled from the cytoplasmic side of membranes only when protein translocation was in progress. This labeling required ATP, SecA and a precursor protein, and became more efficient as the position of the cysteine residue became closer to the C-terminus. Crosslinking analyses revealed that the interaction between SecG and SecA in membranes markedly increases when SecA and SecG undergo membrane-insertion and topology inversion, respectively. Thus, the two most dynamic components of the translocation machinery were found for the first time to interact with each other when both undergo conformational changes.

Key words: Cys-specific labeling, membrane protein topology, protein translocation, SecA, SecG.

Protein translocation across the *Escherichia coli* cytoplasmic membrane is mediated by a complex comprising Sec factors, and is energized by ATP and a proton motive force (*1–6*). The direct driving force for translocation is thought to be the membrane-insertion and deinsertion cycle of SecA coupled to ATP binding and hydrolysis. Each SecA cycle is proposed to translocate 20 to 30 aminoacyl residues of a precursor protein through a putative protein-conducting channel comprising SecY and SecE. The proton motive force accelerates the SecA cycle, making the protein translocation efficient (*7*). SecD and SecF modulate the SecA cycle (*8, 9*). SecG stimulates the SecA cycle and thereby allows efficient protein translocation, especially at low temperature, at low ATP concentration, and/or in the absence of the proton motive force (*10–12*). The membrane topology inversion of SecG, which is coupled to the SecA cycle, is assumed to be responsible for the SecG-dependent stimulation of the SecA cycle (*13*).

SecG spans the membrane twice, its N- and C-termini both being exposed to the periplasm, which leaves the weakly hydrophobic region on the cytoplasmic side of the membrane (*13, 14*). When inverted membrane vesicles (IMVs) are treated with proteinase K, the weakly hydrophobic region is cleaved, yielding a C-terminal 9 kDa fragment containing an epitopic sequence for anti-SecG antibodies (*13*). In contrast, when spheroplasts are treated with proteinase K, these antibodies detect no SecG fragment since the epitopic C-terminal region has been digested. Strikingly, however, the membrane topology of SecG observed in the absence of protein translocation undergoes inversion upon initiation and subsequent blockage of protein translocation (*13*). Proteinase K-treatment under such conditions generates the above 9 kDa SecG fragment in spheroplasts but not in IMVs (*13*). However, detailed analyses of the topology inversion and structure of the Sec machinery involving topology-inverted SecG is not possible after proteinase K-treatment.

We previously constructed SecG derivatives possessing a single cysteine residue at various positions (*14*). We report here the topology inversion of these derivatives, as examined by labeling of the cysteine residues with a membrane-

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Abbreviations: AMS, 4-acetamido-4′-maleimidylstilbene-2-2′-disulfonic acid; IMVs, inverted membrane vesicles; TCEP, tris-(2-carboxyethyl)phosphine hydrochloride; EF-Tu, elongation factor Tu; AMP-PNP, β,γ-imido adenosine 5′-triphosphate; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; DSS, disuccinimidyl suberate; BS^3, bis[sulfosuccnimidyl]suberate.

impermeable SH reagent from the trans-side of the membrane. Furthermore, it is shown for the first time that interaction between SecA and SecG, the two most dynamic components of the translocation machinery, significantly increases in the membrane upon protein translocation.

MATERIALS AND METHODS

Materials—Tran^{35}S-label (37 TBq/mmol as [^{35}S]Met) was obtained from ICN. AMS and TCEP [tris-(2-carboxyethyl)-phosphine hydrochloride] were purchased from Molecular Probes. ATP was from Boehringer Mannheim. DSS (disuccinimidyl suberate) and BS^3 [bis (sulfosuccinimidyl)suberate] were from Pierce. Proteinase K was from Merck. Anti-SecG antibodies were raised in rabbits against a synthetic peptide corresponding to the C-terminal 16 amino acid residues of SecG (*15*). Anti-EF-Tu antibodies were raised in rabbits against the purified protein (*15*).

Bacterial Strains and Plasmids—*E. coli* EK414 (MC4100 *ara*$^+$ *ΔsecG::kan*) (*13*), K003 (HfrH *pnp-13 tyr met* RNaseI$^-$ Lpp$^-$ *ΔuncB-C::Tn10*) (*16*), and KN553 (K003 *ΔsecG::kan*) (*13*) were used. Plasmids encoding SecG-Cys derivatives under the control of the *ara* regulon were constructed as reported previously (*14*). These derivatives were expressed in KN553 or EK414 with the addition of 0.2% arabinose.

Preparation of Spheroplasts and IMVs—Spheroplasts were prepared from EK414 cells expressing the indicated SecG-Cys derivatives according to a reported method (*17*). Stable spheroplasts were obtained with this strain (*13*). K003 and KN553 cells expressing the specified SecG-Cys derivatives were used to prepare IMVs according to a reported method (*18*). IMVs were kept frozen in 50 mM potassium phosphate (pH 7.5), 10% glycerol, and 8.5% sucrose at 30–40 mg/ml. Where specified, IMVs were washed with 6 M urea to remove endogenous SecA as described (*19*).

AMS Labeling of Spheroplasts—Spheroplasts (0.3 mg/ml) were suspended in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM KCl, and 1 mM TCEP, and then preincubated for 3 min on ice or at 20°C. The spheroplast suspension was then treated with 2 mM AMS for 10 min at 20°C or 20 min on ice. The labeling reaction was stopped by the addition of 9 volumes of a buffer comprising 10 mM DTT, 0.38 M sucrose, 10 mM Tris-HCl (pH 7.5), and 150 mM KCl. Spheroplasts were recovered by centrifugation, and then analyzed by SDS-PAGE and immunoblotting with anti-SecG antibodies (*13*). AMS-labeled SecG migrated more slowly than the unlabeled form on SDS-PAGE (*14*). Immunoblots were quantitated with an ATTO Densitograph.

AMS Labeling of IMVs—Prior to AMS-labeling, IMVs (10 mg/ml) were treated with 10 mM DTT for 10 min at 37°C to reduce disulfide bonds. The IMVs were then diluted and subjected to proOmpA translocation in a reaction mixture comprising 0.1 mg/ml IMVs, 60 μg/ml SecA, 50 μg/ml SecB, 25 μg/ml proOmpA, and 50 mM potassium phosphate (pH 7.5). After preincubation for 3 min at 37°C, 1 mM ATP, an ATP generating system comprising 2.5 mM creatine phosphate and 5 μg/ml creatine kinase, and 1 mM $MgSO_4$ were added to start the reaction. Where indicated, 1 mM AMS was added at the specified time. AMS labeling was performed for 5 min and stopped by the addition of Laemmli sample buffer. AMS-labeled SecG was analyzed and quantitated as described above.

In Vitro Protein Translocation—[^{35}S]ProOmpA was synthesized *in vitro* as described (*20*). The reaction mixture (25 μl), comprising 0.2 mg/ml IMVs, 1 mM ATP, 1 mM $MgSO_4$, [^{35}S]proOmpA, 25 μg/ml cold proOmpA, 50 μg/ml SecB, 60 μg/ml SecA, the ATP generating system described above, and 50 mM potassium phosphate (pH 7.5), was incubated at 37°C for the indicated times. Where specified, 2 mM AMS was added at 0 or 6 min. The reaction mixture was placed on ice and then treated with 1 mg/ml proteinase K for 30 min to terminate the reaction. OmpA was precipitated with 10% TCA, successively washed with acetone, and then analyzed by SDS-PAGE and fluorography. The translocation activity was determined by densitometric quantification of proteinase K–resistant OmpA with an ATTO Densitograph.

Preparation of [^{35}S]SecA—[^{35}S]SecA was synthesized *in vitro* in the presence of Tran^{35}S-label using an EcoPro transcription/translation system (Novagen) and pMAN400 (*21*), which carries *secA* under the *tac* promoter. The transcription/translation reaction was allowed to proceed for 1 h at 37°C, after which insoluble materials were removed by centrifugation (170,000 ×*g* for 30 min at 4°C). The supernatant was then applied to a Sephadex G75 column (Pharmacia), which had been equilibrated with 50 mM potassium phosphate (pH7.5) and 10% glycerol, to remove small molecules. The fractions containing [^{35}S]SecA were collected and concentrated by means of membrane filtration (Amicon). Typically, ~60 ng of [^{35}S]SecA (780 kBq) was obtained.

RESULTS

Structures of SecG and Its Cys-Containing Derivatives—SecG comprises 110 amino acid residues and has no Cys residue. We previously constructed 18 SecG derivatives having a single Cys at various positions (*14*). All the derivatives retained the SecG function and stimulated protein translocation both *in vivo* and *in vitro*. Of these derivatives, the eight shown in Fig. 1 were used in this study. Previous AMS labeling experiments carried out with IMVs estab-

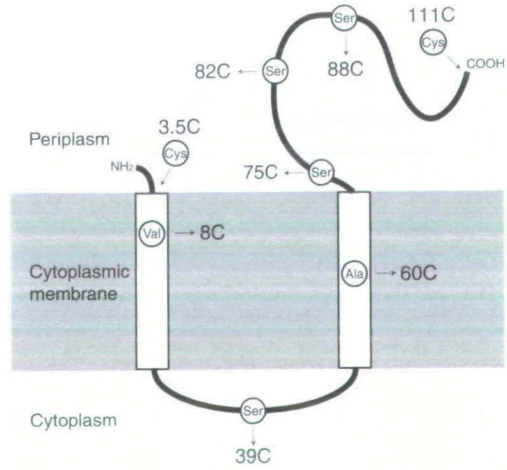

Fig. 1. **Eight SecG derivatives with a single cysteine residue at various positions.** The Cys residue was added after residue 3 in 3.5C and at the C-terminus in 111C. Other derivatives had Cys in place of the indicated residues.

lished that 39C is located in the cytoplasmic loop whereas 75C to 111C are in the periplasmic region, i.e. solubilization of IMVs with a detergent significantly increased the labeling of 75C to 111C, while 39C was labeled irrespective of the presence or absence of detergent (*14*). AMS labeling caused slower migration of these derivatives on SDS-PAGE, thereby enabling quantification of the labeled derivatives (*14*).

Cys in the Cytoplasmic Loop of SecG Can Be Labeled with AMS from the Periplasmic Side—Spheroplasts containing 39C, 75C, 82C, or 88C were treated with AMS on ice or at 20°C, and then analyzed by SDS-PAGE and immunoblotting (Fig. 2). AMS efficiently labeled 75C (lanes 9 and 10), 82C and 88C (data not shown) both on ice and at 20°C, since these derivatives have Cys in the periplasmic region. The Cys of 39C is exposed to the cytoplasm and not labeled with AMS on ice, where protein translocation does not occur (lane 5). On the other hand, a significant portion (22%) of 39C was labeled with AMS at 20°C (lane 6), where protein translocation takes place. EF-Tu, a cytoplasmic protein, was not labeled with AMS even at 20°C (lanes 6 and 10) unless the spheroplasts had been solubilized (lane 2) or sonicated (lanes 3 and 7). The AMS labeling of 39C observed at 20°C is therefore not caused by non-specific leakiness of the spheroplast membranes. Instead, the results indicate that the cytoplasmic loop of SecG is exposed to the periplasm when protein translocation takes place.

Cys in the Periplasmic Region of SecG Can Be Labeled with AMS from the Cytoplasmic Side—We next examined AMS labeling with IMVs containing a SecG-Cys derivative in the presence and absence of protein translocation (Fig. 3). The extent of labeling varied slightly with the preparation of IMVs. We, therefore, repeated the experiments three times with different IMV preparations. Typical results are shown in Fig. 3A, and the averages of the three experiments are shown in Fig. 3B. When protein translocation was prevented by the omission of ATP, about 10% of 75C to 111C was labeled. As reported previously (*14*), this labeling is most likely caused by rightside out membrane vesicles contaminating the IMV preparation, since the Cys residues of these derivatives are located in the periplasmic region (Fig. 1) and more intensely labeled in spheroplasts (Fig. 2). The addition of ATP significantly increased the AMS labeling of 88C and 111C. This ATP-dependent increase in AMS labeling was also appreciable, but less, for 82C. In contrast to the results with spheroplasts (Fig. 2), AMS labeling of 75C remained low in both the presence and absence of ATP. Taken together, these results indicate that the ATP-dependent increase in the labeling of a periplasmic Cys becomes greater as the position of the Cys becomes closer to the C-terminus. Furthermore, since ATP had no effect on the AMS-labeling of 75C, neither non-specific leakiness of the membranes nor permeation through the translocation channel can be the reason for the ATP-dependent labeling of the periplasmic Cys. We previously showed that 3.5C, 8C, and 60C were labeled with AMS only after solubilization of the IMVs with detergents (*14*). These derivatives, which have Cys in the membrane-spanning region (8C and 60C) or near the membrane surface (3.5C), were not labeled with AMS irrespective of the presence or absence of ATP. The Cys residue of 39C located in the cytoplasmic loop (Fig. 1) was labeled with AMS in both the presence and

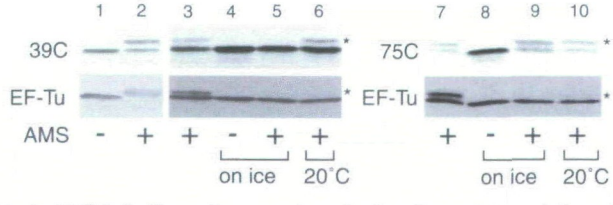

Fig. 2. **AMS labeling of a cytoplasmic Cys from the periplasmic side of membranes.** Spheroplasts were prepared from EK414 (*ΔsecG::kan*) cells expressing 39C (lanes 1 to 6) or 75C (lanes 7 to 10), and then subjected to AMS labeling for 20 min on ice or 10 min at 20°C as described under "MATERIALS AND METHODS." To generate both AMS-labeled and -unlabeled proteins, spheroplasts were briefly sonicated and then treated with AMS at 20°C for 5 min (lanes 3 and 7). Spheroplasts bearing 39C were also solubilized with 2% β-D-octylglucopyranoside and then treated with (lane 2) or without (lane 1) AMS at 20°C for 5 min. SecG derivatives and EF-Tu labeled with AMS were analyzed by SDS-PAGE and immunoblotting with anti-SecG and anti–EF-Tu antibodies, respectively. Note that the AMS labeling of EF-Tu was complete in the detergent and near 50% with sonicated spheroplasts. Spheroplasts containing 4 and 1 μg protein were analyzed for SecG and EF-Tu, respectively. Asterisks indicate the AMS-labeled-proteins.

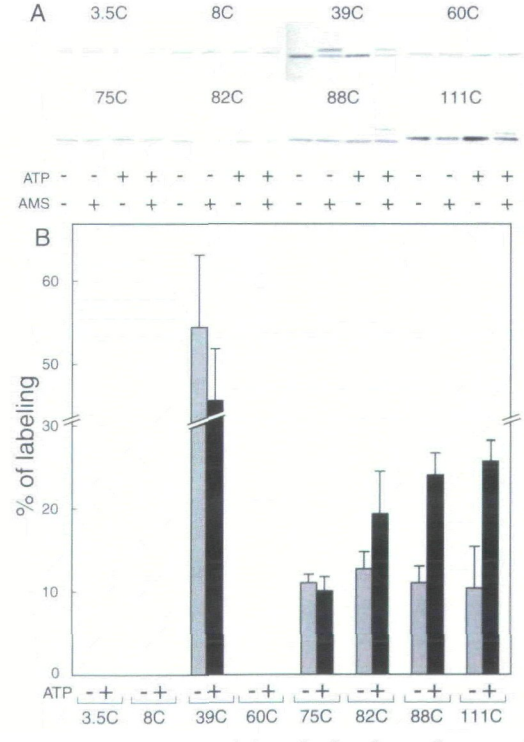

Fig. 3. **AMS labeling of a periplasmic Cys from the cytoplasmic side of membranes.** (A) IMVs containing the specified SecG derivatives were subjected to proOmpA translocation at 37°C in the presence and absence of ATP as described under "MATERIALS AND METHODS." AMS (1 mM) was added at 6 min after the start of proOmpA translocation, followed by 5 min incubation at 37°C. AMS labeling was stopped by the addition of Laemmli sample buffer. Aliquots of the mixture containing 0.7 μg IMVs were then analyzed by SDS-PAGE and immunoblotting with anti-SecG antibodies. AMS-labeled derivatives migrated more slowly than unlabeled ones on SDS-PAGE. (B) The amounts of AMS-labeled and unlabeled derivatives in the presence and absence of ATP were determined as described under "MATERIALS AND METHODS." The averages of three experiments are shown, with error bars, as percentages of the total amounts of derivatives.

absence of ATP. The extent of AMS labeling of 39C appeared to decrease when ATP was present. This suggests that protein translocation may make the Cys of 39C less accessible to external AMS.

Labeling of a Periplasmic Cys Requires proOmpA and SecA—The ATP-dependent labeling of a periplasmic Cys with AMS was further examined with 88C (Fig. 4). Omission of proOmpA prevented the ATP-dependent labeling (Fig. 4A). When native IMVs possessing endogenous SecA were used, omission of external SecA only marginally decreased the extent of ATP-dependent labeling (Fig. 4A). In marked contrast, when endogenous SecA was removed from IMVs by washing with 6 M urea, the external addition of SecA was essential for ATP-dependent labeling, although the level of AMS labeling decreased with urea-washing for an unknown reason (Fig. 4B). Taken together, these results indicate that the labeling of a periplasmic Cys with AMS added on the cytoplasmic side of the membrane absolutely requires proOmpA and SecA as well as ATP.

IMVs containing 111C were subjected to proOmpA translocation and then treated with AMS at various times. The amount of AMS-labeled 111C in the absence of ATP did not vary with the time of AMS addition (Fig. 5A). An ATP-dependent increase in AMS labeling was only observed when AMS was added at 6 min after the start of proOmpA translocation. ATP did not enhance labeling when AMS was added at 0 or 30 min. We examined proOmpA translocation with IMVs containing wild-type SecG, and found that AMS is a potent inhibitor of protein translocation (Fig. 5B). No proOmpA translocation occurred after the addition of AMS. Moreover, proOmpA translocation leveled off at about 15 min even when AMS was not added, indicating that essentially no protein translocation was in progress when AMS was added at 30 min. Taken together, these results indicate that protein translocation must be in progress for the ATP-dependent labeling of a periplasmic Cys. Immediate inhibition of proOmpA translocation by AMS may, therefore, further inhibit labeling of the periplasmic Cys.

It was reported that SH reagents modify Cys residues in the C-terminal region of proOmpA and thereby cause the accumulation of translocation intermediates (*22*). However, AMS did not cause the accumulation of translocation intermediates (data not shown). Moreover, AMS also inhibited the translocation of a Cys-less precursor, suggesting that the modification of proOmpA with AMS is not the reason for the immediate inhibition of proOmpA translocation.

Taken together, the above results indicate that the membrane topology inversion of SecG enables the AMS-labeling of SecG-Cys derivatives from the trans-side of membranes.

Crosslinking between Membrane-Inserted SecA and Topology-Inverted SecG—To examine a possible alteration in the SecA-SecG interaction during protein translocation, IMVs containing wild-type SecG were incubated with proOmpA and ^{35}S-labeled SecA in the presence of ATP. AMP-PNP was then added to stabilize membrane-inserted SecA (*9*) and topology-inverted SecG (*13*). As a control, the translocation was examined in the absence of a nucleotide. IMVs were then isolated by centrifugation and treated with a hydrophobic (DSS) or hydrophilic (BS^3) crosslinker. [^{35}S]-SecA recovered with the IMVs was directly analyzed by SDS-PAGE (Fig. 6A, upper panel) or immunoprecipitated with anti-SecG antibodies prior to SDS-PAGE analysis (Fig. 6A, lower panel). Smeared bands were detected above

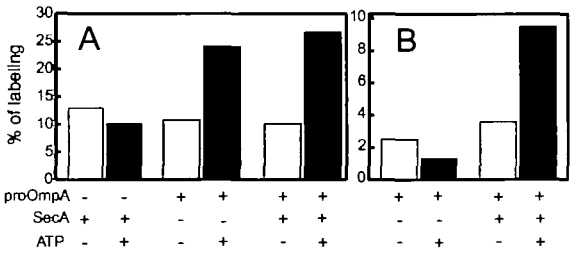

Fig. 4. **ATP-dependent increase in the AMS labeling of a periplasmic Cys requires proOmpA and SecA.** IMVs containing 88C were subjected to AMS labeling in the presence of proOmpA translocation as described in the legend to Fig. 3. AMS labeling was examined under the specified conditions with native IMVs (A) or urea-washed IMVs (B).

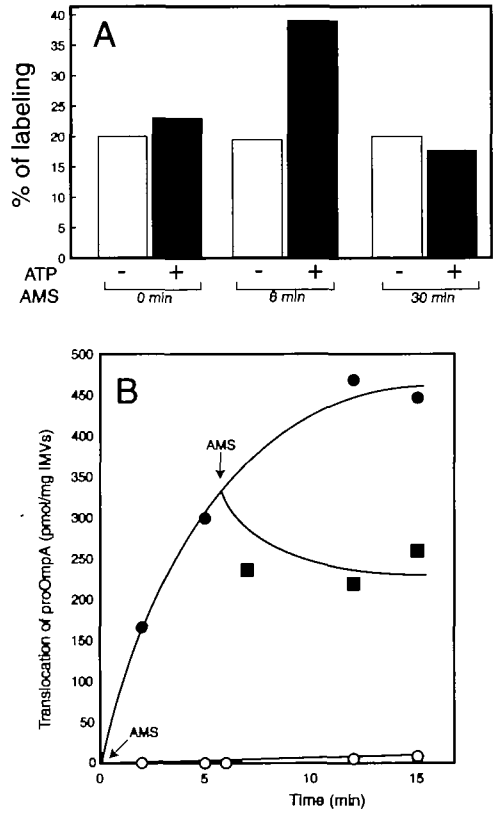

Fig. 5. **Ongoing protein translocation is required for the AMS labeling of a periplasmic Cys.** (A) IMVs containing 111C were subjected to proOmpA translocation in the presence and absence of ATP as described in the legend to Fig. 3. AMS was added at 0, 6, and 30 min. After 5 min incubation at 37°C, AMS labeling was stopped by the addition of Laemmli sample buffer. Aliquots of the mixture containing 0.7 μg IMVs were then analyzed by SDS-PAGE and immunoblotting with anti-SecG antibodies, followed by densitometric quantitation as described under "MATERIALS AND METHODS." The amounts of AMS-labeled 111C in the presence and absence of ATP are expressed as percentages of the total amount of 111C. (B) The translocation of proOmpA into IMVs containing the wild-type SecG was examined as described under "MATERIALS AND METHODS." AMS was added at 0 (open circles) or 6 (squares) min. Closed circles represent the translocation in the absence of AMS.

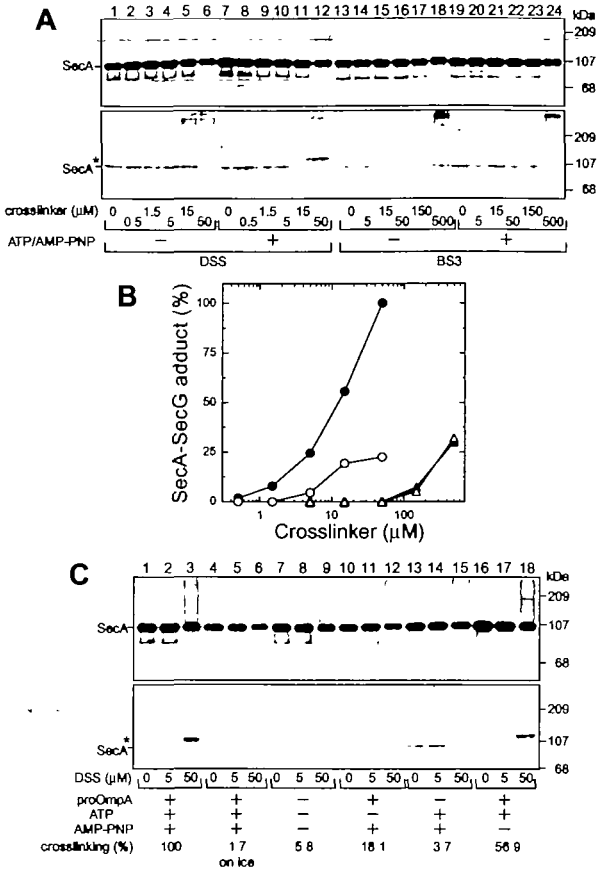

Fig. 6. **SecA-SecG interaction increases upon protein translocation.** (A) *In vitro* proOmpA translocation was performed with IMVs prepared from K003 cells in the presence and absence of ATP as described under "MATERIALS AND METHODS" except that non-radioactive proOmpA and ^{35}S-labeled SecA (~200 kBq/ml) were used. AMP-PNP and $MgSO_4$, each at 20 mM, were added to the reaction mixture containing ATP at 5 min after the start of the reaction, followed by 5 min incubation. As a control, the reaction mixture without ATP was incubated for 10 min. The reaction mixtures (800 μl) were each overlaid on an equal volume of 50 mM potassium phosphate (pH 7.5) containing 0.2 M sucrose and then centrifuged at 170,000 ×*g* for 30 min. IMVs in the pellet fraction were resuspended in 500 μl of 50 mM potassium phosphate (pH 7.5). Aliquots (40 μl) of the suspension were treated with 2 μl of DSS or BS^3 dissolved in dimethyl sulfoxide or 50 mM potassium phosphate (pH 7.5), respectively, to give the indicated final concentrations. The crosslinking reaction was performed at 37°C for 10 min and terminated by the addition of Tris-HCl (pH 7.5) at 100 mM. After 5-min incubation, aliquots (5 μl) of the mixture were treated with Laemmli sample buffer and analyzed by SDS-PAGE (upper panel). The rest of the mixture was treated with an equal volume of 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and 1% SDS, followed by immunoprecipitation with anti-SecG antibodies and analysis by SDS-PAGE (lower panel). Asterisks indicate the SecA-SecG adducts. (B) The amounts of the ~120 kDa band materials in A were determined and expressed as percentages taking that of the densest band (lane 12) as 100%. AMP-PNP was added (closed symbols) or not added (open symbols) to the translocation mixture, and the crosslinking reaction was performed with DSS (circles) or BS^3 (triangles). (C) The translocation reaction was performed as in A except that proOmpA, ATP, or AMP-PNP was omitted, as specified. Where indicated, proOmpA translocation was performed on ice (lanes 4–6). The crosslinking reaction was performed as in A with DSS at the indicated concentrations. SDS-PAGE analysis before (upper panel) or after immunoprecipitation (lower panel) was performed as in A. The relative amounts of the crosslinked products at 50 μM DSS are expressed as in B and shown below the gel.

the position of 102 kDa SecA with the highest crosslinker concentration. After immunoprecipitation, SecA-SecG adducts with molecular masses of ~120 kDa were detected. These bands were most prominent for samples treated with AMP-PNP and crosslinked with hydrophobic DSS. On the other hand, crosslinking with hydrophilic BS^3 was only marginal and not affected by treatment with AMP-PNP. Some non-crosslinked SecA molecules were non-specifically precipitated in this particular experiment (Fig. 6A, lower panel), whereas they were hardly precipitated in another experiment (Fig. 6C, lower panel). The amounts of the ~120 kDa band materials were determined with various crosslinker concentrations (Fig. 6B). The results indicate that the interaction between SecA and SecG in a hydrophobic environment significantly increases when membrane-insertion of SecA and topology inversion of SecG take place.

Crosslinking between SecA and SecG was examined with DSS under various conditions (Fig. 6C). When proOmpA translocation was assayed on ice, subsequent crosslinking generated a marginal amount of the ~120 kDa band materials (lanes 4–6), despite the similar recovery of SecA with IMVs (upper panel). Omission of ATP (lanes 10–12) or proOmpA (lanes 13–15), or both (lanes 7–9), significantly reduced the amounts of the ~120 kDa band materials. In contrast, the addition of AMP-PNP was not essential but produced a ~2-fold increase in the amount of cross-linked product (compare lanes 1–3 and 16–18). Taken together, these results indicate that SecG interacts closely with SecA in the membrane when its topology is inverted.

DISCUSSION

Integral membrane proteins are believed to exist in a fixed topology, which principally follows the positive-inside rule (*23*). In contrast, we previously revealed by means of proteinase K digestion that the membrane topology of SecG undergoes inversion when protein translocation is initiated and then blocked by AMP-PNP (*13*). The membrane topology inversion of SecG is therefore remarkable and worth examining by another method. Here we established a second method for detecting the topology inversion of SecG.

Labeling of Cys residues introduced into SecG with membrane-impermeable AMS from the *trans*-side of membranes requires ATP, proOmpA, SecA, and a physiological temperature. Moreover, protein translocation must be in progress for this labeling. Since the position of the Cys is critical for the ATP-dependent labeling of a periplasmic Cys, and since AMS added on the periplasmic side did not label cytoplasmic EF-Tu, it is clear that the labeling of Cys from the trans-side of membranes reflects the topology inversion of SecG, *i.e.*, it is not caused by leakiness of the membranes. The proportion of SecG derivatives labeled with AMS from the trans-side of membranes was at most about 20%. The following are possible reasons why limited portions of SecG derivatives are labeled from the trans-side of membranes. (i) AMS does not instantaneously label Cys even when AMS and Cys are present on the same side of membranes (*14*). Therefore, some SecG molecules may recover the original topology before AMS labels the inverted. (ii) Only a certain portion of SecG may exist in an inverted topology at the moment of AMS addition even when protein translocation is in progress. (iii) Since AMS inhibits protein translocation, the translocation-dependent

labeling occurs only for a short period. Despite the less efficient labeling, the method reported here allows more detailed analysis of topology inversion.

In order to detect topology inversion by the previous method, the addition of external SecA was required (unpublished observation). Essentially all SecG molecules were assumed to be fixed in the inverted topology under these conditions since proteinase K generated no 9 kDa SecG fragment in IMVs. On the other hand, Cys-specific labeling does not require the external addition of SecA for the detection of topology inversion, indicating that Cys labeling is more sensitive than the previous method. AMS was found to label most of the C-terminal periplasmic region of SecG when protein translocation into IMVs was initiated. This is consistent with our previous observation that antibodies raised against the C-terminal 16 residues of SecG inhibit protein translocation into IMVs (*13, 15*). However, the Cys-specific labeling revealed that the periplasmic region immediately following the second transmembrane segment of inverted SecG is inaccessible from an aqueous environment. Furthermore, we also found that when protein translocation was blocked by AMP-PNP, the ATP-dependent labeling of a periplasmic Cys was prevented (data not shown), suggesting that the C-terminal region of inverted SecG is entirely concealed after stabilization of membrane-inserted SecA. Since these results suggest a close interaction between inverted SecG and inserted SecA, we carried out crosslinking experiments and found an intimate interaction between SecG and SecA (Fig. 6). This interaction is dependent on protein translocation and can be revealed by a hydrophobic, but not a hydrophilic, crosslinker. Moreover, stabilization of membrane-inserted SecA and topology-inverted SecG by AMP-PNP significantly increased the amount of the crosslinked product. Although protein translocation is likely to increase the interaction between SecA and SecYEG, the results shown here suggest that the interaction between SecG and SecA depends on the topology inversion of SecG. It is known that membrane-insertion of SecA is induced by AMP-PNP even in the absence of a precursor protein (*9*). This is, therefore, an idling insertion (*11*). On the other hand, AMP-PNP alone increases neither the SecA-SecG interaction (Fig. 6) nor the topology inversion of SecG (*13*). These results suggest that the topology inversion of SecG is coupled not only to SecA insertion but also to proOmpA insertion. It has been proposed that SecG cooperates with SecA to facilitate efficient precursor insertion into membranes (*11*).

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REFERENCES

1. Duong, F., Eichler, J., Price, A., Leonard, M.R., and Wickner, W. (1997) Biogenesis of the gram-negative bacterial envelope. *Cell* **91**, 567–573
2. Economou, A. (2000) Bacterial protein translocase: a unique molecular machine with an army of substrates. *FEBS Lett.* **476**, 18–21
3. Manting, E.H. and Driessen, A.J. (2000) Escherichia coli translocase: the unravelling of a molecular machine. *Mol. Microbiol.* **37**, 226–238
4. Mori, H. and Ito, K. (2001) The Sec protein-translocation pathway. *Trends Microbiol.* **9**, 494–500
5. Müller, M., Koch, H.G., Beck, K., and Schäfer, U. (2000) Protein traffic in bacteria: multiple routes from the ribosome to and across the membrane. *Prog. Nucleic Acid Res. Mol. Biol.* **66**, 107–157
6. van Voorst, F. and de Kruijff, B. (2000) Role of lipids in the translocation of proteins across membranes. *Biochem. J.* **347**, 601–612
7. Nishiyama, K., Fukuda, A., Morita, K., and Tokuda, H. (1999) Membrane deinsertion of SecA underlying proton motive force-dependent stimulation of protein translocation. *EMBO J.* **18**, 1049–1058
8. Duong, F. and Wickner, W. (1997) Distinct catalytic roles of the SecYE, SecG and SecDFyajC subunits of preprotein translocase holoenzyme. *EMBO J.* **16**, 4871–4879
9. Economou, A., Pogliano, J.A., Beckwith, J., Oliver, D.B., and 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**, 1171–1181
10. Hanada, M., Nishiyama, K., and Tokuda, H. (1996) SecG plays a critical role in protein translocation in the absence of the proton motive force as well as at low temperature. *FEBS Lett.* **381**, 25–28
11. Matsumoto, G., Mori, H., and Ito, K. (1998) Roles of SecG in ATP- and SecA-dependent protein translocation. *Proc. Natl. Acad. Sci. USA* **95**, 13567–13572
12. Suzuki, H., Nishiyama, K., and Tokuda, H. (1998) Coupled structure changes of SecA and SecG revealed by the synthetic lethality of the *secAcsR11* and *ΔsecG::kan* double mutant. *Mol. Microbiol.* **29**, 331–341
13. Nishiyama, K., Suzuki, T., and Tokuda, H. (1996) Inversion of the membrane topology of SecG coupled with SecA-dependent preprotein translocation. *Cell* **85**, 71–81
14. Nagamori, S., Nishiyama, K., and Tokuda, H. (2000) Two SecG molecules present in a single protein translocation machinery are functional even after crosslinking. *J. Biochem.* **128**, 129–137
15. Nishiyama, K., Mizushima, S., and Tokuda, H. (1993) A novel membrane protein involved in protein translocation across the cytoplasmic membrane of *Escherichia coli*. *EMBO J.* **12**, 3409–3415
16. Yamane, K., Ichihara, S., and Mizushima, S. (1987) *In vitro* translocation of protein across *Escherichia coli* membrane vesicles requires both the proton motive force and ATP. *J. Biol. Chem.* **262**, 2358–2362
17. Osborn, M.J., Gander, J.E., Parisi, E., and Carson, J. (1972) Mechanism of assembly of the outer membrane of *Salmonella tyhimurium*. Isolation and characterization of cytoplasmic and outer membrane. *J. Biol. Chem.* **247**, 3962–3972
18. Yamada, H., Tokuda, H., and 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**, 1723–1728
19. Hanada, M., Nishiyama, K., Mizushima, S., and Tokuda, H. (1994) Reconstitution of an efficient protein translocation machinery comprising SecA and the three membrane proteins, SecY, SecE, and SecG (p12). *J. Biol. Chem.* **269**, 23625–23631
20. Tani, K., Shiozuka, K., Tokuda, H., and Mizushima, S. (1989) In vitro analysis of the process of translocation of OmpA across the *Escherichia coli* cytoplasmic membrane. A translocation intermediate accumulates transiently in the absence of the proton motive force. *J. Biol. Chem.* **264**, 18582–18588
21. Kawasaki, H., Matsuyama, S., Sasaki, S., Akita, M., and Mizushima, S. (1989) SecA protein is directly involved in protein secretion in *Escherichia coli*. *FEBS Lett.* **242**, 431–434
22. Tani, K. and Mizushima, S. (1991) A chemically cross-linked nonlinear proOmpA molecule can be translocated into everted membrane vesicles of *Escherichia coli* in the presence of the proton motive force. *FEBS Lett.* **285**, 127–131
23. von Heijne, G. (1992) Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. *J. Mol. Biol.* **225**, 487–494