J. Biochem. 124, 122-129 (1998)

Amino-Terminal Region of SecA Is Involved in the Function of SecG for Protein Translocation into *Escherichia coli* Membrane Vesicles[1]

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Received for publication, February 9, 1998

Protein translocation across the cytoplasmic membrane of *Escherichia coli* is accomplished by concerted actions of the translocation ATPase SecA and the membrane-embedded SecE/Y/G complex. SecA interacts with preproteins and undergoes ATP-driven cycles of membrane insertion-deinsertion. To address how SecA interacts functionally with other components in the translocation machinery, we characterized a SecA mutant lacking amino-terminal 8 amino acid residues (SecA N-8). Although the absence of the 8 residues did not grossly affect the interaction of SecA with a preprotein, ATP, or phospholipids, nor did it affect the intrinsic ATPase activity, it gave differential effects on the translocation of different preproteins. It also affected the translocation ATPase activity, the ability of membrane insertion, and the topology inversion of SecG coupled with the membrane insertion-deinsertion of SecA. Most noteworthy, SecA N-8 was pronouncedly defective in the translocation of proton motive force-dependent preproteins, in which SecG might have a role. We propose that the amino-terminal region of SecA is important for the functional interaction with SecG.

Key words: *Escherichia coli*, protein translocation, proton motive force, SecA, SecG.

Translocation of secretory proteins across the cytoplasmic membrane of *Escherichia coli* is driven by two energy sources, ATP and the PMF (*1-3*). While ATP is absolutely required, PMF is stimulatory (*1-3*). Reconstitution studies established that the *E. coli* protein translocase consists of a peripheral membrane protein, SecA, and a heterotrimeric membrane-embedded complex consisting of the SecE, SecY, and SecG subunits (*3-5*).

SecA possesses ATPase activity (*6*) and binds to preproteins (*6, 7*). The ATPase activity of SecA is stimulated by preproteins, acidic phospholipids, and SecE/Y/G (*8, 9*). Protease digestion experiments showed that SecA undergoes a conformational change upon the binding of ATP, preproteins, and phospholipids (*10*). Similar conformational changes may be accompanied by the translocation reaction. In the presence of ATP and a preprotein, a segment of SecA is inserted deep into the membrane (*11, 12*) such that it is accessible from the periplasmic side (*12*). The inserted state of SecA can be detected by the appearance of a ~30-kDa fragment that is protected from proteolysis by the membrane (*11*). ATP hydrolysis then causes deinsertion of the SecA. Economou and Wickner (*11*) postulated that the preprotein is accompanied by SecA during insertion and is released from SecA upon ATP-hydrolysis-dependent deinsertion. Thus SecA drives protein translocation by repeated insertion-deinsertion cycles.

SecG was discovered as a factor that stimulates *in vitro* translocation of preproteins (*4*). The requirement of SecG for translocation varies with preproteins. For instance, proOmpF-Lpp absolutely requires SecG for translocation across reconstituted proteoliposomes (*4*). This precursor protein also strongly needs the PMF for translocation across IMVs (*13, 14*). In contrast, the translocation of proOmpA and proOmpA D-26 occurs in a reconstituted system without SecG (*15*), and less depends on the PMF (*14*). Thus, it seems that SecG plays a critical role in the PMF-dependent mode of translocation.

Several lines of evidence suggest that SecG and SecA interact with each other. SecG, in conjunction with SecE/Y, stimulates the ATPase activity of SecA (*9*). Coupled with the insertion-deinsertion cycle of SecA, SecG changes its membrane topology, and this topology change was suggested to be important for translocation (*16*). In the present study, we characterized a mutant SecA that lacks 8 amino acid residues at the N-terminus (SecA N-8) with special reference to its functional alterations in the PMF-depen-

[1] This work was supported in part by Grant No. 0740815 from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; C-terminal and -terminus, carboxyl-terminal and -terminus; IMVs, inverted membrane vesicles; N-terminal and -terminus, amino-terminal and -terminus; PMF, proton motive force; SecA N-8, a SecA mutant lacking 8 amino acid residues at the amino-terminus; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

dent preprotein translocation as well as in the topology inversion of SecG. Our results suggest that the N-terminal region of SecA is important for the functional interaction with SecG.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, DNA-modifying enzymes, *Staphylococcus aureus* V8 protease, and SP6 RNA polymerase were purchased from Takara Shuzo. Proteinase K was from Merck. CCCP was from Sigma. EXPRE^{35}S^{35}S Protein Labeling Mix, a mixture of 80% [^{35}S]-methionine and 20% [^{35}S]cysteine (1,000 Ci/mmol; 1 Ci = 37 GBq), was obtained from Du Pont-New England Nuclear. Na^{125}I (100 mCi/ml) was purchased from ICN. *E. coli* SecA and SecB proteins were purified from SecA- and SecB-overproducing cells, respectively, as described (*7, 17*). ProOmpA for ATPase activity measurements was prepared from *E. coli* JM103Lpp-cells harboring pTac-OmpA, a proOmpA-overproducing strain (*18*), according to the published procedure (*19*). Anti-SecG and anti-SecE antibodies were raised against synthetic peptides corresponding to the C-terminal 16 amino acids of SecG (*4*) and the Lys64-Lys81 region of SecE (*20*), respectively. Anti-SecY antibodies, SecY-1 and SecY-5, were raised against synthetic peptides corresponding to the Met1-Arg16 and Ser426-Arg443 regions of SecY, respectively (*21*).

Bacterial Strains—The *E. coli* strains used were K003 (Lpp$^-$ *ΔuncB-C*-Tn10) for the preparation of IMVs and S100 (*13*), JM83 (*20*) for the preparation of SecE/Y/G-overproducing membrane vesicles, KN370 (FS1576, *ΔSecG:: kan*) for the preparation of SecG-depleted IMVs (*22*), MM66 (F$^-$, *Δlac169, araD139, rpsL, relA, thi, secAam, Tn19, su3ts, trpam*) (*23*) for the preparation of SecA-depleted S100, CK4706 (F$^-$, *araD, ΔlacU, relA, rpsL, thi, SecB+zab:: Tn10, secA853-128*) (*24*) for the expression of SecA N-8, and JM109 (*25*) for DNA manipulation.

Plasmids—pTG1 harboring the *tac-secG* gene (*26*) was a generous gift from Dr. H. Tokuda at the University of Tokyo. pMAN809 and pMAN510 harbor the *tac-secE* and *tac-secY* genes, respectively (*20*). pK125 encodes the gene for proOmpF-Lpp, a model preprotein (*13*). pOAD26 encodes the gene for proOmpA-D26, which is a derivative of proOmpA lacking about 250 amino acid residues at its C-terminus (*27*). pSI053 carries the gene coding for wild-type proOmpA (*28*). pMAN400 carries the *secA* gene under the control of *tac* promoter-operator (*29*).

Construction of a Plasmid Carrying the tac-secA N-8 Gene and Expression of the SecA N-8 Protein—pTA1, a plasmid carrying the gene coding for secA N-8, was constructed as follows. pSAM101 (*29*) carrying the *secA* gene was cleaved with *Pvu*I and *Bam*HI. A 3-kbp *Pvu*I-*Bam*HI fragment containing codons 15-901 of SecA was isolated, mixed with synthetic oligonucleotides (5′-GATCCTAGGA-GGTTTAAATTT*ATG*GTTTTCGGTAGTCGTAACGAT-3′ and 3′-GATCCTCCAAATTTAAATACCAAAAGCCATC-AGCATTGC-5′; the initiation codon is italicized) that harbor an ideal SD sequence followed by an initiation codon and codons 9-14 of SecA, then cloned into the *Bam*HI site of pUS12 (*30*), a high copy vector carrying the *tac* promoter-*lac* operator and *lacI*. The deleted portion of the *secA* gene was confirmed by DNA sequencing.

SecA N-8 protein was purified from *E. coli* strain CK4706 harboring plasmid pTA1 as described (*7*). About 60 mg of SecA N-8 was purified from a 3-liter culture.

In vitro Transcription and Translation—*In vitro* transcription of genes encoding preproteins was carried out with SP6 RNA polymerase as described (*31*), and the translation reaction was performed as described (*32*).

Translocation Reaction—The translocation reaction was assayed as described (*33*). The reaction was initiated by adding 1 volume of the translation mixture to 9 volumes of the translocation mixture [50 mM potassium phosphate (pH 7.6), 5 mM $MgSO_4$, 1 mM ATP, 10 μM CCCP, 100 μg/ml creatine kinase, 30 mM creatine phosphate, 20 μg/ml purified SecA, and 0.25 mg/ml IMVs] which had been preincubated at 37°C for 5 min. After the indicated times, the mixture was treated with 1 mg/ml proteinase K at 0°C for 10 min, and the translocated proteins were analyzed by SDS-PAGE and fluorography. The amount of protein band on the gel was determined with a Fujix bioimage analyzer BAS-2000II. The efficiency of translocation was expressed as the ratio of the intensity of protein bands on the gel before and after the proteinase K treatment.

Removal of SecA from IMVs—IMVs prepared from *E. coli* K003 cells were suspended in 50 mM potassium phosphate (pH 7.6), mixed with 3 volumes of 8 M urea, then placed on ice water for 1 h. The mixture was then centrifuged at 150,000 × *g* for 30 min at 4°C, and the pelleted membrane vesicles were suspended in 50 mM potassium phosphate (pH 7.6) (*34*). More than 80% of the SecA was removed from the membranes through this treatment (*35*).

Construction of an Overexpression Plasmid for Both SecE and SecG—pTG1, an expression plasmid for SecG, was digested with *Eco*RI and *Kpn*I. A 900-bp *Eco*RI-*Kpn*I fragment containing the *tac-secG* gene was isolated, then blunted at both sites with a DNA blunting kit (Takara Shuzo). The fragment was ligated into the pMAN809 in which a *Kpn*I site had been cleaved and converted to blunt ends, to construct pMON414 carrying both the *tac-secG* and *tac-secE* genes.

Determination of ATPase Activity—A coupled spectrophotometric assay involving pyruvate kinase and lactate dehydrogenase was performed as described (*36*). The cuvette contained, in 1 ml, 50 mM potassium phosphate (pH 7.6), 2 mM $MgSO_4$, 3 mM phosphoenolpyruvate, 0.25 mM NADH, 1 mM ATP, 10 units of pyruvate kinase, 15 units of lactate dehydrogenase, and 10 μg of SecA, at 37°C. The endogenous ATPase activity was assayed in the presence of 150 mM NaCl. For measuring the translocation ATPase activity, the assay mixture contained, in addition to above, 5 mM KCN, 20 μg/ml of SecB, and specified amounts of urea-washed IMVs prepared from cells overproducing SecE/Y proteins or SecE/Y/G proteins. ProOmpA was subsequently added at a final concentration of 0.45 mM. Oxidation of NADH was continuously monitored at 340 nm with a Shimadzu UV-3000 spectrophotometer. The amounts of ATP hydrolyzed were calculated using a value of 6,220 for the molar absorption coefficient of NADH.

Proteolytic Digestion of SecA—Proteolytic experiments using *S. aureus* V8 protease were performed as described (*10*).

SecA Iodination and Membrane Insertion Assay—SecA

was iodinated using Iodogen (Pierce) as described (*11*), except that the incubation time for iodination was 20 min and iodinated SecA was passed through a Sephadex-G 50 column to remove free iodine. Thus [^{125}I]SecA ($\sim 5 \times 10^6$ cpm/mg) was obtained. Binding of [^{125}I]SecA to urea-treated IMVs was performed in the reaction mixture comprising 0.2 mg/ml urea-washed membrane vesicles and 4 nM labeled SecA in TL buffer [50 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, and 0.2 mg/ml bovine serum albumin] on ice for 15 min. The membrane vesicles were sedimented by centrifugation at 170,000 $\times g$ for 30 min at 4°C. The precipitate obtained was resuspended in TL buffer. The insertion assay solution containing [^{125}I]SecA-bound membrane vesicles, 1 mM ATP, 10 ng/ml creatine kinase, 5 mM creatine phosphate, 50 μg/ml SecB, 25 μg/ml proOmpA, in TL buffer was incubated at 37°C for 15 min, then treated with proteinase K at a final concentration of 0.1 mg/ml on ice for 15 min. After TCA treatment, the precipitate was analyzed by SDS-PAGE and autoradiography.

Inversion of the Membrane Topology of SecA—All procedures were carried out according to Nishiyama *et al.* (*16*). A solution containing IMVs prepared from *E. coli* K003 cells was treated on ice for 10 min with an equal volume of 8 M urea. The membrane vesicles were sedimented by centrifugation at 170,000 $\times g$ for 30 min at 4°C, then suspended in 50 mM potassium phosphate (pH 7.6). The complete translocation mixture (110 μl) comprised 0.1 mg of protein per milliliter of membrane vesicles, 60 μg/ml SecA, 50 μg/ml SecB, 25 μg/ml proOmpA, 1 mM ATP, 5 mM $MgSO_4$, and 5 mM succinate in 50 mM potassium phosphate (pH 7.6). After incubation at 37°C for 10 min with or without SecA or SecA N-8, 10 mM AMP-PNP, and 10 mM $MgSO_4$ were added to block inversion, and the mixture was further incubated for 5 min. The reaction was terminated by chilling the mixture on ice for 2 min, and aliquots (25 μl) of the mixture were treated with 5 μl of a proteinase K solution on ice for 30 min. After TCA treatment, the precipitate containing 1 μg of protein of membrane vesicles was analyzed by SDS-PAGE and Western blotting with an anti-SecG antibody.

RESULTS

Construction of SecA Lacking 8 Amino Acid Residues at the N-Terminus—SecA has been purified from a SecA-overproducing strain (RR1/pMAN400) by the combination of hydroxylapatite and Superose 12 chromatographies (*7*). We found that SecA was eluted as two peaks from the hydroxylapatite column (data not shown). This separation depended on the lot of hydroxylapatite columns. Sequence analyses revealed that the first peak contains a degradation product of SecA, which lacks 8 amino acid residues at the N-terminus, and the latter peak contains intact SecA (data not shown). Preliminary kinetic analyses showed that the degraded SecA has lower translocation ATPase activity and is less efficient for the translocation assay using urea-treated IMVs (data not shown). To achieve the large-scale purification of the SecA mutant lacking 8 amino acid residues at the N-terminus (termed SecA N-8), we constructed a plasmid carrying the *secA* gene with the corresponding deletion, and the protein was expressed in *E. coli* CK4706 cells. *E. coli* CK4706 encodes, instead of SecA, a SecA mutant in which two SecA molecules are tandemly connected (*24*). Therefore, SecA N-8 could be easily and completely separated from the covalent SecA dimer encoded by the chromosomal gene. Sequence determination showed that the N-terminus of the purified SecA N-8, Val-Phe-Gly-Ser, is identical to residues 9–12 of SecA. The first methionine residue may be cleaved off during the biosynthesis or purification of SecA N-8.

SecA N-8 Is Defective in Translocation of a Class of Preproteins That Strongly Require the PMF—An authentic *E. coli* secretory protein, proOmpA, and two other model

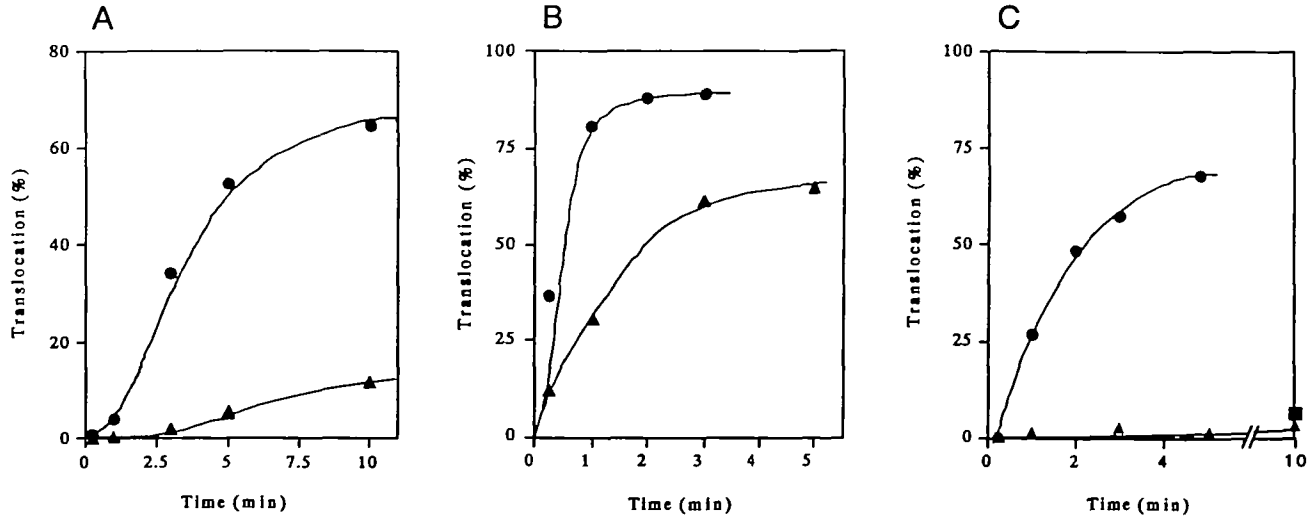

Fig. 1. **PMF-independent *in vitro* translocation of preproteins mediated by wild-type SecA and SecA N-8.** ^{35}S-labeled proOmpA (A), proOmpA D26 (B), and proOmpF-Lpp (C) were subjected to *in vitro* translocation assays with urea-treated IMVs prepared from K003 cells. The translocation reaction was carried out in the presence of 10 μM CCCP using wild-type SecA (closed circles) or SecA N-8 (closed triangles). As a control, translocation was conducted in the absence of SecA (closed square in panel C). After the indicated times, samples were treated with proteinase K (final 1 mg/ml) for 10 min on ice, precipitated with TCA, washed with acetone, then analyzed by SDS-PAGE and fluorography.

secretory proteins, proOmpF-Lpp (*13*) and proOmpA D26 (*27*), were examined in *in vitro* protein translocation assays using wild-type SecA and SecA N-8. Although the *in vitro* translocation of the proOmpA and proOmpA D26 less depends on the PMF (*14*), that of the proOmpF-Lpp strongly requires the PMF (*13, 14, 37*). To evaluate the translocation activity of SecA and SecA N-8 in the absence of the PMF, the translocation reaction was performed in the presence of 10 μM CCCP, so that ATP was the sole energy source (*32*). With wild-type SecA, all the three precursors were effectively translocated (Fig. 1, A-C). With SecA N-8, a significant translocation was observed for proOmpA and proOmpA D26, although the efficiency for proOmpA was lower than that for its derivative (Fig. 1, A and B). In contrast, no appreciable translocation was observed for proOmpF-Lpp with SecA N-8 (Fig. 1C). SecA N-8 also failed to mediate translocation of a derivative of proOmpF whose translocation was strongly dependent on the PMF (data not shown). These results suggest that SecA N-8 has little, if any, translocation activity for the PMF-dependent precursors.

We wanted to know whether SecA N-8 can mediate translocation of proOmpF-Lpp in the presence of the PMF. However, since 6 M urea-treatment of IMVs to remove and/or inactivate membrane-bound SecA greatly reduced the PMF generated with 5 mM succinate (Mori, H. and Ito, K., unpublished observation), we could not perform this experiment.

Translocation ATPase Activity of SecA N-8 Is Not Enhanced by SecG—SecA exhibits low endogenous ATPase activity, which is markedly enhanced by IMVs (referred to as membrane ATPase activity), and IMVs plus a preprotein (referred to as translocation ATPase activity) (*6*). The stimulation of the ATPase activities by IMVs is due to the presence of phospholipids and SecE/Y and SecG in the vesicle membranes (*6, 8*). We were interested in whether SecE/Y and SecE/Y/G activate the ATPase activities of SecA N-8. For this purpose, both pMAN510 and pMON414, carrying the *tac-secY* gene and the *tac-secE/tac-secG/lacI* genes, respectively, were introduced into JM83 cells. All three proteins were overproduced and accumulated in the cytoplasmic membrane (data not shown). Densitometric analyses of a series of dilutions of SecE/Y/G-overproducing membranes showed that SecE, SecY, and SecG were overproduced at least 120-, 80-, and 100-fold, respectively, over the wild-type (*E. coli* K003) levels (data not shown). A 9-kDa band that was recognized by anti-SecG probably represents a proteolytic fragment of SecG (*26*).

We examined the effects of urea-washed IMVs containing excess SecE/Y (called SecEY IMVs hereafter) and excess SecE/Y/G (called SecEYG IMVs hereafter) on the ATPase activities of SecA and SecA N-8. The endogenous ATPase activity of SecA N-8 (0.23 unit/mg) was compa-

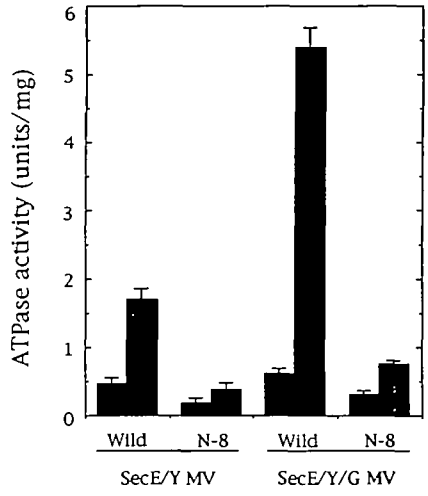

Fig. 2. **ATPase activities of wild-type SecA and SecA N-8.** The ATPase activities of purified wild-type SecA and SecA N-8 were determined: membrane ATPase activity (solid bars) and translocation ATPase activity (hatched bars). Values represent the means of triplicate samples with standard deviations indicated by bars.

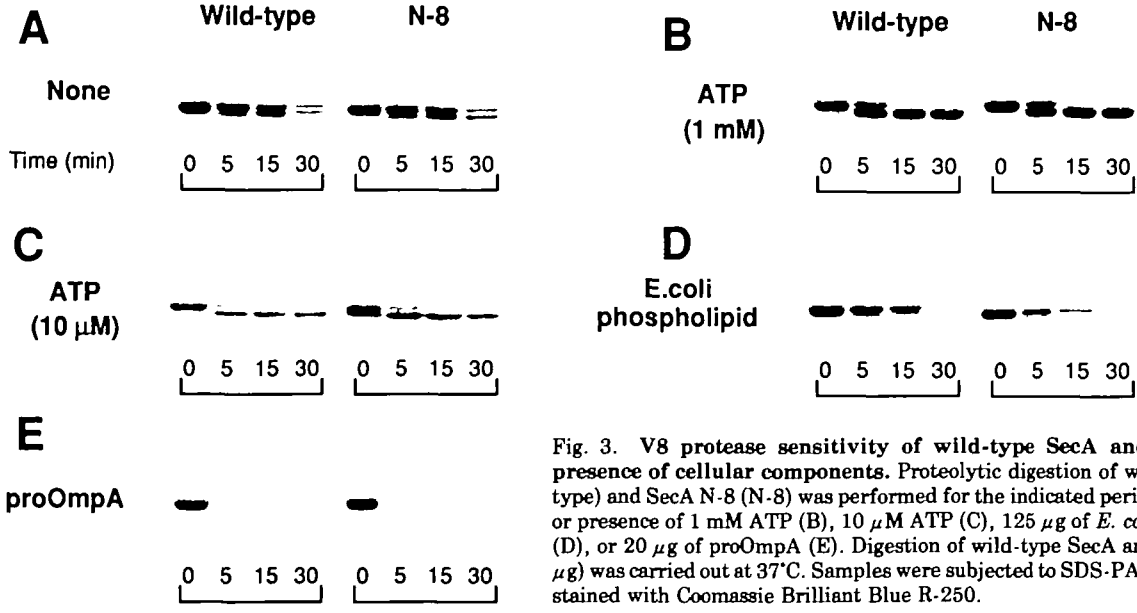

Fig. 3. **V8 protease sensitivity of wild-type SecA and SecA N-8 in the presence of cellular components.** Proteolytic digestion of wild-type SecA (Wild-type) and SecA N-8 (N-8) was performed for the indicated periods in the absence (A) or presence of 1 mM ATP (B), 10 μM ATP (C), 125 μg of *E. coli* total phospholipids (D), or 20 μg of proOmpA (E). Digestion of wild-type SecA and SecA N-8 (each 10 μg) was carried out at 37°C. Samples were subjected to SDS-PAGE, and the gels were stained with Coomassie Brilliant Blue R-250.

rable to that of wild-type SecA (0.27 unit/mg). In the presence of SecEY IMVs or SecEYG IMVs, SecA N-8 exhibited about half the membrane ATPase activity of wild-type SecA (Fig. 2). The degree of stimulation of the ATPase activity of SecA N-8 by a preprotein, proOmpA, in the presence of SecEY IMVs was significantly lower than that of wild-type SecA (2.0 *vs.* 3.4 times). Furthermore, in the presence of SecEYG IMVs, the degree of stimulation of the ATPase activity of SecA N-8 by proOmpA was much lower than that of wild-type SecA (2.5 *vs.* 8.7 times). These results imply that the N-terminal region of SecA is important for the stimulation of the ATPase activity induced by SecG as well as SecE/Y.

SecA N-8 Interacts with ATP, a Preprotein, and Phospholipids—The sensitivity of SecA to *S. aureus* V8 protease was affected by ATP, preproteins, and phospholipids (*10*). In the presence of ATP, the N-terminal 95-kDa fragment was resistant to V8 protease. On the other hand, SecA becomes more sensitive to V8 digestion in the presence of proOmpA, IMVs, and *E. coli* phospholipids. To determine whether or not SecA N-8 can interact with these components, the sensitivity of SecA N-8 to V8 protease was examined. As shown in Fig. 3, the sensitivity of SecA N-8 to V8 protease in the presence or absence of these components was essentially the same as that of wild-type SecA, suggesting that SecA N-8 can interact with ATP, proOmpA, and *E. coli* phospholipids. The interaction of SecA N-8 with the preprotein was also confirmed by means of chemical cross-linking using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and proOmpF-Lpp (data not shown).

The N-Terminal Region of SecA and SecG Are Important for the Efficient Insertion of SecA into the Mem-

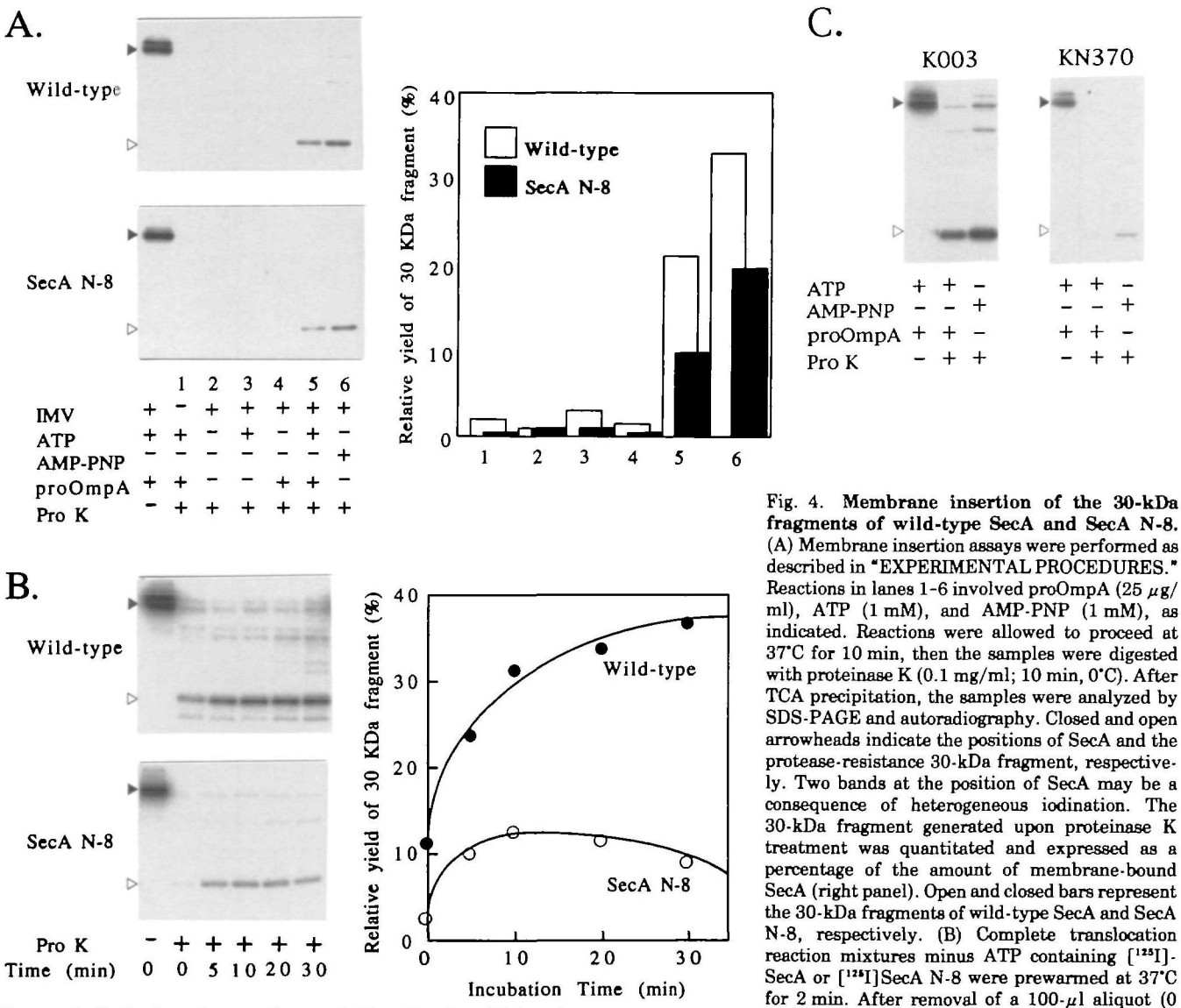

Fig. 4. **Membrane insertion of the 30-kDa fragments of wild-type SecA and SecA N-8.** (A) Membrane insertion assays were performed as described in "EXPERIMENTAL PROCEDURES." Reactions in lanes 1-6 involved proOmpA (25 μg/ml), ATP (1 mM), and AMP-PNP (1 mM), as indicated. Reactions were allowed to proceed at 37°C for 10 min, then the samples were digested with proteinase K (0.1 mg/ml; 10 min, 0°C). After TCA precipitation, the samples were analyzed by SDS-PAGE and autoradiography. Closed and open arrowheads indicate the positions of SecA and the protease-resistance 30-kDa fragment, respectively. Two bands at the position of SecA may be a consequence of heterogeneous iodination. The 30-kDa fragment generated upon proteinase K treatment was quantitated and expressed as a percentage of the amount of membrane-bound SecA (right panel). Open and closed bars represent the 30-kDa fragments of wild-type SecA and SecA N-8, respectively. (B) Complete translocation reaction mixtures minus ATP containing [^{125}I]-SecA or [^{125}I]SecA N-8 were prewarmed at 37°C for 2 min. After removal of a 100-μl aliquot (0 time control), the insertion reaction was initiated by the addition of 1 mM ATP, and 100-μl aliquots were removed at the indicated times. The aliquots were digested with proteinase K (0.1 mg/ml; 10 min, 0°C), TCA-precipitated, then analyzed by SDS-PAGE and autoradiography. The 30-kDa fragment generated was quantitated as described in (A). Closed and open circles represent the 30-kDa fragments of wild-type SecA and SecA N-8, respectively. (C) The insertion assay of wild-type SecA was performed with K003 IMVs containing SecE/Y/G (left panel) or KN370 IMVs containing only SecE/Y (right panel). Other procedures were the same as described in (A).

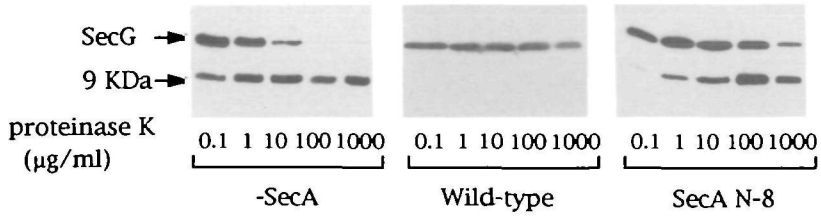

Fig. 5. **Topology inversion of SecG with wild-type SecA and SecA N-8.** Urea-washed IMVs were used. The translocation of proOmpA was started in the complete translocation mixture in the absence (−SecA) or presence of wild-type SecA (Wild-type), or the presence of SecA N-8 (SecA N-8), then blocked with AMP-PNP. Aliquots of the mixtures were treated with proteinase K at the indicated concentrations on ice for 30 min. After proteinase K digestion, SDS-PAGE and immunoblotting were performed.

brane—SecA seems to mediate protein translocation by repeated membrane insertion-deinsertion cycles (*11*). The membrane-inserted form of SecA is partly resistant to protease and yields a ~30-kDa fragment (*11*). To determine whether SecA N-8 is inserted into the membrane, the amount of the ~30-kDa fragment appearing upon protease digestion was determined. In the case of wild-type SecA, the protease-resistant 30-kDa fragment was observed in the presence of ATP and proOmpA (Fig. 4A, lane 5) or AMP-PNP without proOmpA (lane 6) in a time-dependent manner (Fig. 4B). These results were the same as those reported previously (*11*). In the case of SecA N-8, the 30-kDa fragment was similarly formed, but the amount formed in the presence of ATP and proOmpA was 2-3-fold lower than that observed for wild-type SecA (Fig. 4B). It is noteworthy that the extent of binding of SecA N-8 to IMVs was roughly the same as that of wild-type SecA (Fig. 4B, lane 1, time 0 without protease). These results suggest that the N-terminal region of SecA is important for the insertion of the 30-kDa domain of SecA into the membrane, but not for the binding of SecA to the membrane.

When IMVs lacking SecG protein were used, the yield of the 30-kDa fragment of wild-type SecA was one-fourth that observed for SecG-containing IMVs (Fig. 4C). When SecA N-8 and SecG-depleted IMVs were used, little of the 30-kDa fragment appeared (data not shown). These results suggest that SecG is also important for the efficient insertion of SecA.

Inversion of the Membrane Topology of SecG Occurs Less Efficiently in SecA N-8—SecG possesses two strong hydrophobic regions with a weakly hydrophobic one between them (*4*). Nishiyama *et al.* (*16*), using a SecG-PhoA fusion protein, showed that the C-terminal region of SecG is exposed to the periplasmic side in a resting state, but, under certain conditions, SecG undergoes topology inversion so that the C-terminal region faces the cytosol. This inversion seems to be coupled with the insertion of SecA into the membrane (*16*). Since the results described above suggest that the N-terminal region of SecA is important for the function of SecG, we examined whether SecA N-8 can promote the inversion of the membrane topology of SecG. The inversion assay depends on the change in proteinase K-sensitivity coupled with the inversion of the membrane topology of SecG (*16*). In a resting state (normal topology), the middle of SecG in IMVs is cleaved by proteinase K to produce a 9-kDa fragment. When inversion occurs, SecG is cleaved near the C-terminus. Since the antibody used in the inversion assay recognizes the C-terminus of SecG, the cleaved SecG is not recognized by the antibody. As shown in Fig. 5, in the absence of SecA, the 9-kDa fragment was formed upon proteinase K treatment, implying that SecG has the normal topology. In the presence of wild-type SecA, on the other hand, only a trivial amount of the 9-kDa fragment was formed, suggesting that the membrane topology of SecG was inverted. Although Nishiyama *et al.* (*16*) reported that the intact SecG band disappears upon proteinase K treatment when inversion occurs, we found that the intact band was reduced only slightly at the highest proteinase K concentration (1 mg/ml). The reason for this is not clear at present. When SecA N-8 was present, a significant amount of the 9-kDa fragment was formed, suggesting that SecA N-8 promotes the inversion of SecG less efficiently than wild-type SecA.

DISCUSSION

The protein translocase in *E. coli* consists of the translocation ATPase SecA and the membrane-embedded complex comprising SecE/Y/G (*3-5*). SecA and SecE/Y/G are the stoichiometric components of the translocase (*38*). SecG is important for translocation under certain conditions. In the absence of the PMF, SecG plays a critical role in protein translocation into IMVs (*39*). In a system reconstituted from isolated Sec components, SecG is essential for the translocation of PMF-dependent preproteins such as proOmpF-Lpp (*4*). Several lines of evidence suggest the interaction of SecG with SecA. Firstly, SecG stimulates the ATPase activity of SecA (*9*). Secondly, inversion of the membrane topology of SecG is coupled with SecA-dependent protein translocation (*16*). Thirdly, cold-sensitive cell growth due to the deletion of secG is suppressed by a SecA mutant (SecA36) (*40*).

In the present study, we constructed and extensively characterized a SecA mutant lacking 8 amino acid residues at the N-terminus. Like wild-type SecA, SecA N-8 bound to ATP, a preprotein and phospholipids (Fig. 3). This excludes the possibility that a large conformational change occurred as a consequence of the deletion of the N-terminal 8 amino acid residues. Our results suggest that the N-terminal region of SecA is involved in the function of SecG for *in vitro* protein translocation. This conclusion is based on the following observations: (i) SecA N-8 mediated translocation of proOmpA and proOmpA D26, but not proOmpF-Lpp, in the presence of ATP as the energy source (Fig. 1). Similar specificity for preproteins was observed in translocation across proteoliposomes containing SecE/Y but not SecG (*4*). (ii) Compared with wild-type SecA, the translocation ATPase activity of SecA N-8 was not remarkably stimulated by SecG (Fig. 2). Since the degree of stimulation of the translocation ATPase by SecE/Y-overproducing IMVs was also lower in SecA N-8, the N-terminal region of SecA is also important for the interaction of SecE and/or SecY. The low SecE/Y-dependent translocation ATPase activity of SecA N-8 may explain its low translocation

efficiencies for proOmpA and proOmpA D26. (iii) The yield of membrane insertion of SecA N-8 was lower than that of wild-type SecA (Fig. 4). In addition, the extent of the penetration of SecA into the SecG-depleted membrane was lower than that into the wild-type membrane. (iv) SecA N-8 was much less efficient than wild-type SecA in promoting the inversion of the membrane topology of SecG (Fig. 5).

It is not clear how the N-terminal region of SecA interacts with SecG. One possibility is that the N-terminal region of SecA interacts indirectly with SecG *via* SecE/Y or phospholipids. This is suggested by the finding that the membrane-inserted 30-kDa fragment of SecA, which is localized to its C-terminal domain (*41*), is largely inaccessible to the membrane lipid phase (*42*). However, it is also possible that the N-terminal region of SecA directly interacts with SecG as well as SecE/Y. A recent finding showed that, in addition to the C-terminal 30-kDa domain, an N-terminal 65-kDa domain of SecA which contains both high-affinity and low-affinity ATP-binding regions can also insert into the membrane (*43*). Further study is necessary to determine which possibility is correct.

The role of the PMF in translocation remains unknown. The late step of protein translocation is driven by the PMF in the absence of ATP hydrolysis (*18, 44*), suggesting the PMF and ATP play distinct roles in protein translocation and independently enhance the translocation efficiency (*45*). Consistent with this idea, the PMF has no effect on the inversion of the membrane topology of SecG, which is coupled with the ATP hydrolysis-mediated insertion-deinsertion of SecA (*16*). However, several reports have suggested the linkage between the PMF and the hydrolysis of ATP by SecA. The PMF lowers the requirement of ATP for protein translocation (*46*), suggesting that it facilitates the ATP hydrolysis by SecA. The membrane insertion of SecA is regulated by SecD and SecF (*47*), both of which are important for maintaining the PMF gradient (*48*). The idea that the PMF enhances the cycle of ATP hydrolysis by SecA can explain why SecA N-8, which is uncoupled with SecG function due to the loss of the N-terminal 8 amino acid residues, cannot mediate translocation of PMF-dependent preproteins. The translocation of PMF-dependent preproteins may need higher energy, which can be generated only through the SecA-mediated ATP hydrolysis coupled with the SecG inversion cycle.

The results of previous studies also suggested that the N-terminal region of SecA is important for translocation. The high-affinity ATP-binding site (*49*) and preprotein-binding site (*50*) are both located in the N-terminal region of SecA. This region includes the catalytic residue for ATP hydrolysis, Asp133 (*33*), the Mg^{2+}-binding residue, Arg209 (*51*), and the ATP-binding residue, Lys108 (*52*). A part of the ATP-binding region is also located between amino acid residues 75 and 97 (*53*). *secA*^{ts} mutations causing protein export defects were localized to residues 43 to 170 (*1*). It was suggested that the N-terminal region of SecA interacts with acidic phospholipids (*54*). The present study strongly suggests that the N-terminal 8 residues, Met-Leu-Ile-Lys-Leu-Leu-Thr-Lys, are important for the efficient *in vitro* translocation of preproteins, especially ones that require the PMF and SecG. The deleted sequence has two net positive charges and four hydrophobic residues. The strong hydrophobicity of the N-terminus is well conserved among SecA proteins from other species such as *Bacillus subtilis* (*55*) and pea (*56*). Therefore, the hydrophobic interaction of the N-terminus of SecA may be important for the efficient translocation of preproteins.

We thank Drs. Hajime Tokuda and Ken-ichi Nishiyama for generous gifts of plasmid (pTG1) and strain (KN370), and Drs. Koreaki Ito and Yoshinori Akiyama for critical reading of the manuscript and valuable suggestions. We are grateful to Ms. Mie Okazaki and Mr. Shingo Kikuchi for technical assistance.

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