Role of the Non-essential Region Encompassing the N-Terminal Two Transmembrane Stretches of *Escherichia coli* SecE

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SecE is an essential component of the protein translocation machinery of *Escherichia coli* and has three transmembrane stretches. An N-terminal region (SecE-N) encompassing the first two transmembrane stretches is dispensable for protein translocation but a SecE derivative (SecE-C) lacking this region is very unstable. We show here that FtsH, the AAA (ATPases associated with diverse cellular activities) family protease, causes the instability of SecE-C. SecE-C became stable when SecE-N was co-expressed. Deletion of the N-terminal region of SecE also rendered the SecE-SecY-SecG complex unstable. In spite of these altertations, the N-terminal region of SecE had little stimulatory effect on protein translocation in vivo or in vitro.

Key words: protein translocation; proteoliposome; FtsH; SecE; *Escherichia coli*

A machinery comprising seven Sec factors (A, B, D, E, F, G, and Y) catalyzes the translocation of presecretory proteins across the *Escherichia coli* cytoplasmic membrane. Only SecA, SecE, and SecY are absolutely essential for the translocation, as the translocation machinery can be reconstituted into proteoliposomes with these three proteins. SecE and SecY form a protein-conducting channel in the membrane, through which SecA delivers presecretory proteins by undergoing the cycle of membrane-insertion and deinsertion. The SecA cycle is dependent on ATP binding and hydrolysis, and modulated by SecG, SecD/F, and a proton motive force.

SecE of *E. coli* spans the membrane three times, and the N-terminal region encompassing the first and second transmembrane stretches is dispensable for protein translocation. Only a subset of SecE homologs found in Gram-negative bacteria have three transmembrane stretches (Fig. 1), while others, including the eukaryotic SecE homolog, Sec15, have a single transmembrane stretch. All prlG (secE) mutations in *E. coli* that suppress a signal sequence defect are located in the third transmembrane stretch and the following periplasmic region. The sequences of the third transmembrane stretch and preceding cytoplasmic region are conserved among SecE homologs but the sequence similarity of other regions is poor (Fig. 1). These observations indicate that the dispensable N-terminal region may have no role in protein translocation. However, SecE becomes highly unstable upon deletion of the N-terminal region.

We examined here the mechanism underlying the instability of a SecE derivative lacking the N-terminal region and the integrity of the translocation machinery containing the derivative.

Materials and Methods

Sequence data on SecE homologs. Preliminary sequence data were obtained from the following sources; the Institute for Genomic Research website at http://www.tigr.org/ (Vibrio cholerae, Shewanella putrefaciens and Thiobacillus ferrooxidans), the Sanger Center website at http://www.sanger.ac.uk (Bordetella pertussis), the OU-ACGT website at http://www.genome.ou.edu/act.html (Actinobacillus actinomycetemcomitans), the Pseudomonas Genome Project website at http://www.pseudomonas.com (P. aeruginosa), the Genome Sequencing Center of Washington University website at http://genome.wustl.edu/gsc/bacterial/salmonella.shtml (S. typhimurium), and the University of Minnesota *P. multocida* genome sequencing website at http://www.cbc.umn.edu/ResearchProjects/AGAC/Pm/index.html (Pasturella multocida PM70).

Bacterial strains and plasmids. *E. coli* strains AR796 (MC4100 zhd-33::Tn10 zhj-3198::Tn10kan), AR797 (AR796 ftsH-1), SM7000 (W3110 ompT secC501 argE::Tn10), PS259 (phoAPAuvII lacAX74 galE galK rpsL).
Fig. 1. Prokaryotic SecE Homologs Having Three Transmembrane Stretches.

The sequences of SecE homologs possessing three membrane spanning regions were aligned with CLUSTALW (http://www.clustalw.genome.ad.jp). Transmembrane regions (TM) 1 to 3 of E. coli SecE are indicated according to the reported results.9 Those of other bacteria were predicted with a program (http://acusa.proteome.bio.tuat.ac.jp/asafl/). White letters on a black background indicate residues conserved in all the homologs, and residues conserved in more than half of the homologs are shaded. The species are as follows: E. coli (Eco), S. typhimurium (Sty), V. alginolyticus (Val), V. cholerae (Vch), S. putrefaciens (Spu), P. aeruginos A (Pae), A. actinomycetemcomitans (Aac), H. influenzae (Hin), P. multicida PM70 (Pmu), B. pertussis (Bpe), and T. fer rooxidans (Tfe). The SeCE sequences of E. coli,9 V. alginolyticus10 and H. influenzae (Accession No. U32754, H10716) were reported.

Those of other organisms were obtained from preliminary sequence data, as described under Materials and Methods. The sequence of H. influenzae SecE may be incorrect and may lack the N-terminal region due to a frame shift, since another frame gave a very similar N-terminal sequence to those of A. actinomycetemcomitans and P. multicida. We therefore assume three membrane spanning regions for H. influenzae SecE.

pcnB80 zadi::Tn1010) and PS274 (PS259 recA::cut secEA19-111)10 were used. The last two strains harbor pJS65, encoding the secE, phoA and kan' genes.10 Plasmid pE615 encodes Ptac-secE-C (SecE74-127). Plasmid pE2 encoding Ptac-secE-N (SecE74-127) had been constructed by the introduction of a stop codon followed by a MluI site.11 However, the downstream region of the stop codon remained intact. To delete the downstream region, pE2 was digested with MluI, treated with Klenow enzyme, and then digested with BamHI, which cut the immediately upstream region of secE-N. A 240 bp BamHI-MluI fragment encoding secE-N was inserted into the BamHI-Smal site of pUS1216 to construct pE19.

To construct pE42 encoding Ptac-secE-C-Ptac-secE-N, pE19 was digested with EcoRI, treated with Klenow enzyme, and then digested with BglII. The EcoRI-BglII fragment encoding Ptac-secE-N was cloned into the Smal-BglII site of pE6, which is downstream of Ptac-secE-C.

Construction of a secE null strain harboring a plasmid encoding SecE-C with or without SecE-N. PS274 harboring pJS65 was transformed with pE6 (SecE-C) or pE42 (SecE-C, SecE-N). PhoA' and kanamycin-sensitive transformants were selected on plates containing 1.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The curing of pJS65 and absence of intact SecE in the transformants were confirmed.

Materials. Antibodies were raised in rabbits against purified SecE,17 OmpA18 and SecGI19 as described. Anti-SecY antibodies were raised against the Met1-Arg22 region of SecY as reported.20 E. coli phospholipids were prepared as described.21 β-Octylglucopyranoside (octylglucoside) was purchased from Dojindo. Protein A Sepharose was from Pharmacia. Restriction endonucleases and DNA-modifying enzymes were from Takara Shuzo Co., Ltd.

Purification of SecE-N. The cytoplasmic membranes (40 mg) prepared from SM7000/pE19 cells, which had been induced with 1.5 mM IPTG, were solubilized with 1.5% octylglucoside, 10 mM potassium phosphate (pH 6.0), and 10 mM magnesium sulfate on ice for 10 min. After centrifugation at 170,000 × g for 30 min, sodium acetate (pH 5.0) was added to 10 mM to the supernatant, followed by fractionation on a cation exchange column (MonoS, 1×10 cm; Pharmacia), which had been equilibrated with 1.5% octylglucoside and 10 mM sodium acetate (pH 5.0). The column was developed at the flow rate of 4 ml/min with a linear gradient of NaCl (0-0.3 M, 40 ml). SecE-N was eluted with about 0.1 M NaCl. The fractions containing SecE-N were collected and dia lyzed against 15 mM Tris·Cl (pH 9.5), 1.5% octylglucoside, 10% glycerol. The sample was then applied to an anion exchange column (MonoP, 0.5×20 cm; Pharmacia), which had been equilibrated with the same buffer. The column was developed at the flow rate of 2 ml/min. SecE-N was eluted in the flow-through fractions, whereas most other proteins were bound to the column. The flow-through fractions were concentrated by means of membrane filtration (Centriflon 10, Amicon). Finally, the sample was applied to a gel filtration column (Superose 12HR, 1×30 cm; Pharmacia), which had been equilibrated with 1.5% octylglucoside, 50 mM potassium phosphate (pH 7.5), 150 mM NaCl, 10% glycerol. The column was developed at the flow rate of 0.5 ml/min, and then the fractions containing SecE-N were collected and concentrated. This preparation (0.12 mg) was used as the purified SecE-N (purity, 95%).

Reconstitution of proteoliposomes and in vitro protein translocation assay. SecA22 and SecY2 were
purified as described. SecE-C was purified from SM7000/pE1 cells as described.\textsuperscript{11} Reconstitution of proteoliposomes was carried out as described.\textsuperscript{2} Briefly, \textit{E. coli} phospholipids (1.25 mg) were added to a mixture of SecY, SecE-C and SecE-N, followed by incubation on ice for 20 min. Proteoliposomes were formed by diluting octylglucoside by the addition of a 40-fold volume of 50 mM potassium phosphate (pH 7.5), 150 mM NaCl, and then collected by centrifugation at 170,000 × g for 2 h at 4°C. They were suspended in 110 µl of 50 mM potassium phosphate (pH 7.5), 150 mM NaCl, frozen, thawed at room temperature, and then sonicated briefly. The proteoliposomes thus reconstituted were used for the translocation assay\textsuperscript{25} using proOmpA D26\textsuperscript{25} as the substrate.

Other methods. Inverted membrane vesicles were prepared as described.\textsuperscript{24} Protein was measured by the method of Lowry \textit{et al.}\textsuperscript{25} SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting were done as described.\textsuperscript{11}

Results

Stabilization of overproduced SecE-C requires simultaneous expression of SecE-N or inactivation of FtsH

FtsH rapidly degrades SecY that is not complexed with SecE.\textsuperscript{14} Overproduction of SecY therefore requires simultaneous overproduction of SecE.\textsuperscript{26} We previously showed that SecE-C is efficiently overproduced only when SecG or SecY is overproduced.\textsuperscript{11,25} In contrast, SecE overproduction does not depend on the overproduction of another Sec factor,\textsuperscript{26} suggesting that an N-terminal region encompassing two transmembrane stretches is important for the protection of overproduced SecE from proteases, although this region is dispensable for protein translocation. To examine the stabilization by the N-terminal region, SecE-C was overproduced with or without simultaneous overproduction of SecE-N in a strain carrying the wild type FtsH or its temperature-sensitive derivative, FtsH-1\textsuperscript{27} (Fig. 2A). When SecE-C alone was overexpressed at 30°C, a permissive temperature for FtsH-1, its expression was hardly detectable in either strain even though a potent \textit{tac} promoter was used to express SecE-C. In marked contrast, when SecE-N was simultaneously expressed, SecE-C was greatly overproduced. We previously showed that the simultaneous expression of SecE-N did not stabilize the overproduction of SecY.\textsuperscript{11} Therefore, the observed effect of SecE-N is specific to SecE-C. When SecE-C was expressed at 42°C, the simultaneous expression of SecE-N was not required for the stabilization of SecE-C in the FtsH-1 mutant but was in the wild type strain. Taken together, these results indicate that SecE-N and SecE-C interact with each other, rendering SecE-C resistant to proteolysis. These results also showed that degradation by FtsH is the main reason for the instability of overproduced SecE-C. Unlike SecE-C, the level of chromosomally encoded SecE was not affected by the FtsH activity (Fig. 2B). Furthermore, in the presence of SecE, overexpression of SecE-N or SecE-C or both did not cause the accumulation of proOmpA (Fig. 2C), indicating that the
membrane integrity is normal under these conditions.

Co-expression of SecE-N was inhibitory to SecE-C dependent protein translocation

The stabilization of SecE-C by SecE-N was examined in SecE+ cells, therefore, it could not be seen whether or not the expression of SecE-N together with SecE-C has any effect on protein translocation. To address this, we constructed strains that lack secE on the chromosome and harbor a plasmid encoding either SecE-C or SecE-C and SecE-N under the control of the tac promoter. The growth and protein translocation of this secE null strain absolutely depend on SecE-C.10 On the other hand, a secE null mutant carrying secE-N on a plasmid could not be constructed, indicating that SecE-N cannot replace SecE. Since the constructed strains also carry the pcnB80 mutation,29 the plasmid copy number was significantly reduced and the level of SecE-C was similar to that of SecE in the SecE+ strain (data not shown). Simultaneous expression of SecE-C caused an increase in the level of SecE-C, as observed with the SecE+ strain, albeit at a lesser extent (Fig. 3A). The accumulation of proOmE in these cells was examined at 37°C by immunoblotting with anti-OmpA antibodies (Fig. 3B). Despite the plots being extensively visible, proOmE was undetectable in the wild-type strain. Only a marginal amount of proOmE was accumulated in the cells expressing SecE-C. In contrast, the accumulation of proOmE was appreciable in the cells expressing both SecE-C and SecE-N (Fig. 3B) even though the level of SecE-C essential for protein translocation was increased by the simultaneous expression of SecE-N (Fig. 3A). These results most likely suggest that the association of SecE-C with SecE-N at the Sec machinery, thereby decreasing the incorporation of SecE-C into the Sec machinery. However, as the expression of SecE-N together with SecE-C had no appreciable effect on growth, the translocation defect observed here seems to be marginal.

Subunit interaction of the Sec machinery containing SecE-C

Since the secE null mutant can grow if SecE-C is expressed,10 SecE-C must be incorporated into the Sec machinery, SecY, SecE, and SecG form a complex that can be precipitated after membrane solubilization by an antibody raised against any one of the three proteins.29 We next examined whether or not SecE-C could be precipitated with SecY or SecG. Inverted membrane vesicles prepared from PS259 (secE+), and PS274 (ΔsecE) possessing SecE-C or SecE-C and SecE-N were solubilized with octylglucoside, followed by immunoprecipitation with anti-SecY or anti-SecG antibodies (Fig. 4). SecE-C was co-precipitated with SecY and SecG, but SecE-C was precipitated with neither protein, suggesting that the Sec-E-SecY-SecG complex is less stable when SecE lacks the N-terminal region. As expected from the results shown in Fig. 3B, co-expression of SecE-N did not improve the recovery of SecE-C.

To confirm that SecE-N has no stimulatory effect on protein translocation, purified Sec-E-C and SecY with or without SecE-N were reconstituted into proteoliposomes. The amounts of SecE-C and SecY reconstituted into proteoliposomes were unaffected by SecE-N. The translocation activity of these proteoliposomes was examined in the presence of SecA and ATP (Fig. 5). When SecE-C or SecY was not

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**Fig. 3.** Effects of SecE-N on SecE-C-dependent Protein Translocation.

PS274 cells harboring pE6 (SecE-C, C) or pE42 (SecE-C and SecE-N, CN) were grown on an LB medium with 1.5 mM IPTG at 37°C. When the turbidity of the culture reached 1.0, the cells were harvested, and then cellular proteins (10 μg) were analyzed by SDS-PAGE and immunoblotting with anti-SecE (A) or anti-OmpA (B) antibodies. In B, PS259 (SecE, WT) was also examined as a control. The positions of SecE-N, SecE-C, proOmpA (p), and mature OmpA (m) are indicated. In order to detect SecE-N in PS274 cells, more extensive visualization of the blots was required.

**Fig. 4.** Sec-E-C is Not Co-precipitated with SecY or SecG.

Inverted membrane vesicles prepared from PS259 (secE+, WT), PS274/pE6 (ΔsecE, secE-C, C), or PS274/pE42 (ΔsecE secE-C secE-N, CN) were solubilized on ice for 15 min in 1.25% octylglucoside, 150 mM NaCl, 10 mM Tris-Cl (pH 8), 25% glycerol, 3.4 mg/ml E. coli phospholipids. Each supernatant (1 ml) obtained on centrifugation at 100,000 × g for 30 min was reacted at 4°C for 2 h with anti-SecY or anti-SecG antibodies. The mixture was then treated with Protein A Sepharose for 1 h at 4°C, followed by centrifugation at 10,000 × g. The precipitate was washed twice with 50 mM Tris-Cl (pH 8) containing 150 mM NaCl, 40% glycerol, 1.5 mg/ml phospholipids, and 1.25% octylglucoside, and then dissolved in the sample buffer for SDS-PAGE analysis. Immunoblotting was done with anti-SecE antibodies. The migration positions of SecE and SecE-C are indicated.
Molecular Dissection of SecE

Fig. 5. Translocation Activities of Proteoliposomes Reconstituted with SecY, SecE-C and SecE-N. Proteoliposomes were reconstituted with 3 μg SecY, 4 μg SecE-C and the indicated amounts of SecE-N as described under Materials and Methods. The translocation of proOmpA D26 was examined at 37°C. SecE-N: 0 μg (open circles), 2 μg (closed circles), 4 μg (open triangles), 8 μg (closed triangles), 12 μg (open squares). Closed squares represent the activity in the absence of SecE-C.

reconstituted, the proteoliposomes had no translocation activity. The translocation activity remained essentially the same when increasing amounts of SecE-N were reconstituted together with SecE-C and SecY, indicating that SecE-N has no stimulatory effect on protein translocation. The association of SecE-C with SecE-N observed in vivo was not apparent in these reconstitution experiments.

Discussion

The protein translocation machinery is an evolutionarily conserved mechanism found in bacteria to animal cells. Nevertheless, SecE homologs with three membrane spanning stretches are only present in a subset of Gram-negative bacteria, while the homologs in other organisms have a single membrane spanning stretch. Since the overproduction of SecE-C is significantly stabilized by the simultaneous expression of SecE-N, we anticipated that the region encompassing the N-terminal two membrane spanning stretches may have some role in protein translocation and/or formation of the translocation machinery. So far as we examined it, the stabilization of SecE-C by SecE-N contributed little to the translocation activity. The stability of the SecE-SecY-SecG complex after solubilization appreciably decreased on deletion of the N-terminal region of SecE. However, co-expression of SecE-N did not stabilize the complex. Taking these results together, it is concluded that the N-terminal region of SecE affects the stability of the SecE-SecY-SecG complex in a detergent solution but has little stimulatory effect on protein translocation.

We found that FtsH, the AAA family protease, causes instability of SecE-C. FtsH has been reported to be involved in the degradation of membrane proteins such as SecY and a cytosolic σ. Protection of SecE-C from the FtsH activity by SecE-N most likely results from the association of SecE-C and SecE-N outside of the translocation machinery. On the other hand, FtsH seems to be inaccessible to SecE-C located in the Sec machinery. Thus, the secE null mutant carrying wild type FtsH could grow if SecE-C was expressed (Fig. 3). It is thought that a multimeric of the SecE-SecY-SecG complex comprises a single unit of translocation machinery. The dimerization of cytosine-containing SecE derivatives indicates that two SecE molecules exist close to each other in the machinery. Since the association of SecE-C with SecE-N decreased the translocation activity, this association does not seem to represent the physiological association of two SecE molecules in the machinery.

It is now increasingly important to characterize the mode of interaction of Sec subunits in the translocation machinery. Co-immunoprecipitation showed that SecY, SecE, and SecG form a complex, while SecD, SecF, and YajC, encoded by the secD operon, form another complex. The synthetic lethal combinations of prlA (secY) and prlG (secE) mutants have shown some details of the SecY-SecE interaction. The formation of disulfide bonds between cysteine-containing mutants of SecY and SecE has also identified the interaction site. A secY24 mutation was reported to impair the SecY-SecE interaction. Although SecE-C can replace SecE in secY+ strains, it may be unable to do so in secY24 mutants. The machinery containing SecE-C instead of SecE seems to be important for the details of the subunit interaction, which is critical for protein translocation.

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