SecM facilitates translocase function of SecA by localizing its biosynthesis

Hitoshi Nakatogawa,[1] Akiko Murakami, Hiroyuki Mori, and Koreaki Ito[2]

Institute for Virus Research and CREST, Japan Science and Technology Corporation, Kyoto University, Kyoto 606-8507, Japan

"Arrest sequence" of *Escherichia coli* SecM interacts with the ribosomal exit tunnel and arrests its own translation elongation, which is released by cotranslational export of the nascent SecM chain. This property of SecM is essential for the basal and regulated expression of SecA. Here we report that SecM has an additional role of facilitating SecA activities. Systematic determinations of the SecA-abundance-protein export relationships of cells with different SecA contents revealed that SecA was less functional when SecM was absent from the upstream region of the *secM–secA* message, when SecM had the arrest-defective mutation, and also when SecM lacked the signal sequence. These results suggest that cotranslational targeting of nascent SecM to the translocon plays previously unrecognized roles of facilitating the formation of functional SecA molecules. Biosynthesis in the vicinity of the membrane and the Sec translocon will be beneficial for this multiconformation ATPase to adopt ready-to-function conformations.

[*Keywords*: SecM; SecA; *Escherichia coli*; protein secretion; cotranslational targeting; ribosomal tunnel]

Received September 8, 2004; revised version accepted December 15, 2004.

In the major pathway of protein translocation across the *Escherichia coli* plasma membrane, SecA functions as a motor that drives the movement of secretory polypeptides into and through the SecYEG polypeptide-conducting channel (Mori and Ito 2001; van den Berg et al. 2004; Veenendaal et al. 2004). SecA interacts with multiple components of the secretory system, a preprotein, SecB, acidic phospholipids, and the SecYEG complex (Hartl et al. 1990; Vrontou and Economou 2004). According to the three-dimensional structures of SecA from *Bacillus subtilis* and *Mycobacterium tuberculosis* (Hunt et al. 2002; Sharma et al. 2003; Osborne et al. 2004), the SecA protomer consists of several domains including two ATPase-like catalytic and regulatory domains, the C-terminal ATPase-repressing domain, a preprotein-interacting region, and backborn-like long α-helices. The ATPase activity of SecA is normally down-regulated by intramolecular actions of the C-terminal regulatory region and one of the ATPase-like domains (Karamanou et al. 1999; Nakatogawa et al. 2000; Sianidis et al. 2001), but it is stimulated by anionic phospholipids, SecYEG-containing membranes, a preprotein, and most strikingly by the interaction with both SecYEG and preprotein (Lill et al. 1990). While SecA assumes a dimeric structure in solution (Driessen 1993), it undergoes conformational changes referred to as "membrane insertion" when it is functioning upon binding of ATP and a preprotein (Economou and Wickner 1994) at the SecYEG site of the membrane (Eichler et al. 1997; Matsumoto et al. 1997). SecA insertion is then followed by "deinsertion" and release of the preprotein coupled with ATP hydrolysis (Economou and Wickner 1994).

SecA is a versatile protein that adopts multiple conformations (Ding et al. 2003), oligomeric states, and localizations. Its cytosolic soluble form may bind to RNA, possibly as a translational autogenous repressor (Schmidt and Oliver 1989). Recent studies suggest that SecA is in dynamic equilibria between different oligomeric states, including a monomeric form, which is affected by the solution environments and membrane association (Or et al. 2002; Woodbury et al. 2002; Benach et al. 2003; Duong 2003; Osborne et al. 2004; Tziatzios et al. 2004).

The synthesis of SecA, encoded by a polycistronic mRNA together with an upstream gene, *secM*, is up-regulated under conditions of impaired protein secretion (Oliver and Beckwith 1982; Schmidt et al. 1988). This regulation is at the translation level, in which SecM, a peculiar secretory protein, plays an essential role (Oliver et al. 1998; Nakatogawa and Ito 2001). SecM undergoes translation elongation arrest (Nakatogawa and Ito 2001) via its "arrest sequence" (FXXXXWIXXXXGIRAGP), which has the ability to interact with the ribosomal exit tunnel (Nakatogawa and Ito 2002). The elongation arrest is transient in secretion-active cells, presumably because the nascent SecM is "pulled" by the Sec export reaction

[1]Present address: Department of Cell Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan
[2]Corresponding author.
E-MAIL kito@virus.kyoto-u.ac.jp; FAX 81-75-771-5699.
Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.1259505.

(Nakatogawa and Ito 2001; Butkus et al. 2003), whereas it is strikingly prolonged in secretion-blocked cells. We showed that SecM elongation arrest is essential for the basal level expression of SecA as well as its up-regulation in response to a secretion defect (Murakami et al. 2004). Presumably, the stalled ribosome disrupts the mRNA secondary structure formed in the *secM–secA* intergenic region, thereby exposing the translation initiation site of *secA* (McNicholas et al. 1997; Murakami et al. 2004). In this way, SecM monitors protein export activity of the cell and accordingly modulates the translation frequency of *secA*.

During the course of characterization of *secM* mutations, we were intrigued by the observation that SecA synthesized without normal *secM* was less functional in vivo. Tian and Beckwith (2002) noted that certain *secM* mutants were weakly defective in protein secretion although they had apparently normal amounts of SecA. In the present study, we investigated whether SecM has a positive role in the post-translational expression of the translocase activities. Our results show that SecM indeed has a novel role of enhancing the functionality of SecA by virtue of its ability to enable biosynthesis of this multiconformation ATPase in the vicinity of the membrane and the translocon.

Results

Effects of the arrest-compromising secM166 *mutation on the functionality of SecA*

The Pro166Ala substitution in SecM abolishes the elongation arresting property of SecM (Nakatogawa and Ito 2002). The chromosomal *secM166* is lethal unless complemented with excess SecA (Murakami et al. 2004). We used a plasmid expressing SecA conditionally under the *araBAD* promoter control to compare the *secM*⁺ and the *secM166* cells when the SecA supply from the plasmid was shut off by removing arabinose from the medium. We followed cell growth by turbidity measurements (Fig. 1A), SecA contents by anti-SecA immunoblotting (Fig. 1B), and export activity by pulse-labeling OmpA (Fig. 1B). Growth of the mutant cells declined progressively (Fig. 1A, open circles), whereas the *secM*⁺

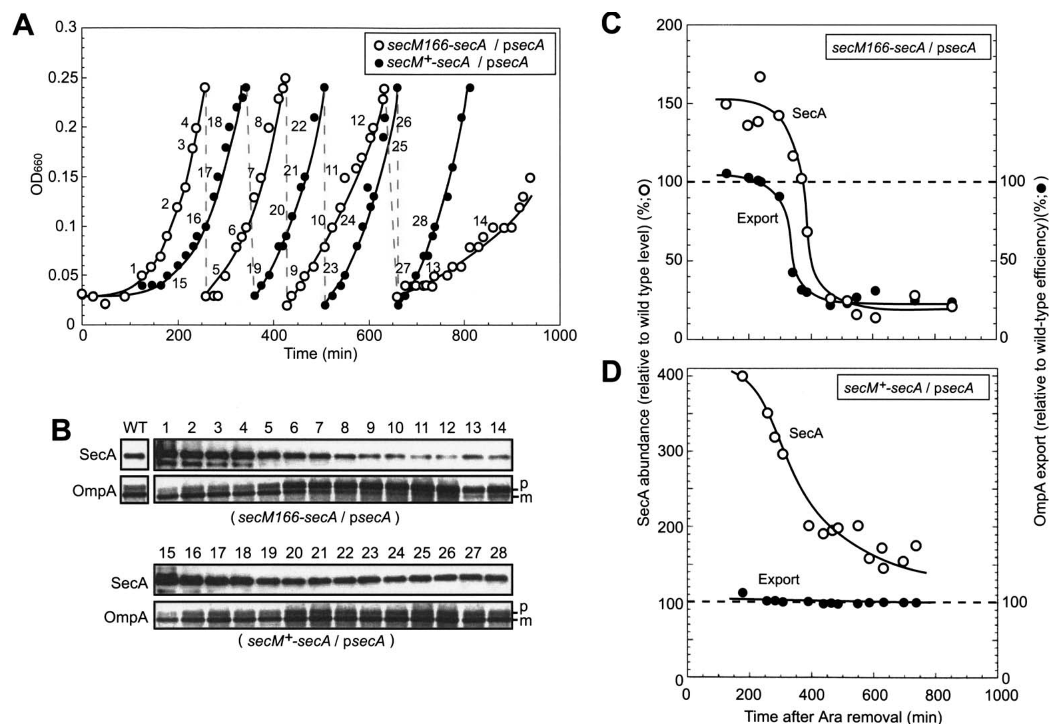

Figure 1. SecA abundance and OmpA export in the *secM166* mutant after shutting off the complementing SecA synthesis. (*A*) Growth curves. Strains AN144 (*secM*⁺/P_{araBAD}–*secA*; solid circles) and AN145 (*secM166*/P_{araBAD}–*secA*; open circles) were grown first in M9–amino acids–arabinose (0.2%) medium at 37°C. Cells were then centrifuged down, washed three times with the same volume of M9 salts, and inoculated into the same medium containing glucose (0.4%) instead of arabinose. Cell growth was followed, with appropriate dilutions as indicated, by turbidity measurements (OD_{660}). (*B*) SecA abundance and OmpA export. At each time point shown in *A* by sampling numbers, portions of AN144 (lanes *1–14*) and AN145 (lanes *15–28*) (the lane numbers correspond to the sampling numbers) cultures were removed for SDS-PAGE and anti-SecA immunoblotting, while other portions were subjected to pulse-labeling with ^{35}S-methionine for 30 sec and OmpA immunoprecipitation. The precursor and the mature forms of OmpA are indicated by "p" and "m," respectively. HM1246 carrying empty vector pBAD33 was examined similarly as a control for the "wild-type" situation (lane WT). (*C*,*D*) Graphical representation of changes in SecA abundance and OmpA export efficiency. Intensities of SecA relative to that in lane WT in *B* are shown by open circles. Proportions of the mature form in pulse-labeled OmpA are shown as relative values to the WT sample, indicated by solid circles. (*C*) AN145 (*secM166*). (*D*) AN144 (*secM*⁺).

cells continued to grow (Fig. 1A, solid circles). SecA abundance in the *secM*⁺ cells was decreasing to the level seen in the wild-type (without plasmid) cells (Fig. 1B [lanes 15–28], D [open circles]), whereas that in the *secM166* mutant rapidly decreased below the wild-type level, down to ~20% (Fig. 1B [lanes 1–14], C [open circles]). It was thus confirmed that SecA is only poorly expressed when the upstream *secM* contains an arrest-impairing mutation (Murakami et al. 2004).

In the *secM*⁺ cells, ~90% of OmpA pulse-labeled for 30 sec was in its mature form irrespective of the sampling time (Fig. 1B [lanes 15–28], D [solid circles]). OmpA export in the *secM166* cells became severely defective after ~300 min of growth in the absence of arabinose (Fig. 1B [lanes 6–14], C [solid circles]). It was noted that the export defect was observable before the decline in the SecA abundance went below the wild-type level. Specifically, this discrepancy was evident between the 300- and 370-min sampling points (Fig. 1C), during which OmpA export efficiency was decreasing from ~90% to ~30% of the wild-type value (Fig. 1C, solid circles), despite the higher than normal SecA contents (Fig. 1C, open circles). Thus, at these time points at least, the translocation machinery in the *secM166* mutant was not fully active. We interpret this to mean that SecA molecules were not fully functional. because the defect in this system was corrected by excess SecA, which should have been the limiting element that determined the export activities. Impaired export in relation to the SecA abundance change was also observed for maltose-binding protein in the *secM166* mutant (data not shown).

We also examined membrane integration of a fusion protein, MalF–PSBT, having a biotin-accepting domain attached to the periplasmic region of MalF (Jander et al. 1996). Its biotinylation by the cytosolic biotin ligase depends on the duration of the cytosolic residence of the normally periplasmic domain. MalF–PSBT was expressed in the *secM166* and *secM*⁺ strains described above. It was biotinylated significantly in the mutant (Fig. 2, lanes 4,5) but not in the *secM*⁺ control cells (Fig. 2, lanes 2,3). The biotinylation was significant even when the mutant cells contained more than normal amount of SecA (Fig. 2, cf. lanes 4 and 1); it was enhanced further when the SecA abundance decreased to nearly the wild-type level (Fig. 2, lane 5). Thus, cells with the arrest-defective *secM* mutation require increased concentrations of SecA to effectively support MalF–PSBT integration into the membrane. In the *secM166* strain used in the above series of experiments, the complementing SecA was synthesized in the absence of *cis*-located *secM*, and the chromosomal SecA was expressed in the absence of the elongation-arresting property of SecM. SecA thus synthesized proved less functional.

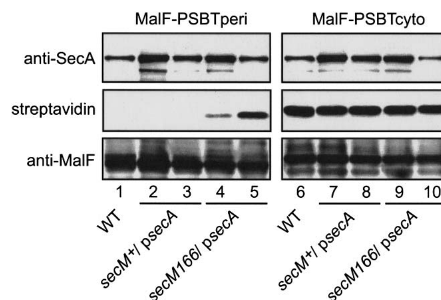

Figure 2. Effects of the *secM166* mutation on membrane integration of MalF–PSBT. Strain W3110 that carried pBAD33 (WT; lanes *1*,*6*), AN225 (*secM*⁺/P_{araBAD}–*secA*; lanes *2*,*3*,*7*,*8*) and AN226 (*secM166*/P_{araBAD}–*secA*; lanes *4*,*5*,*9*,*10*) were transformed further with either pGJ78-J (MalF–PSBTperi; lanes *1–5*) or pGJ78-K (MalF–PSBTcyto; lanes *6–10*). pGJ78-J and pGJ78-K encode MalF–PSBT (a biotin-accepting domain) fusion proteins with a respective fusion joint at the second periplasmic region and the third cytoplasmic region, respectively (Jander et al. 1996). Cells were grown at 37°C in L-medium containing 0.2% arabinose, washed three times with an equal volume of arabinose-free L-medium, and inoculated into L-medium supplemented with 0.4% glucose and 1 mM isopropyl-β-D-thiogalactopyranoside (for MalF–PSBT induction). Samples were withdrawn at earlier (lanes *1*,*2*,*4*,*6*,*7*,*9*) and later (lanes *3*,*5*,*8*,*10*) time points during further grown after the wash, with appropriate dilutions. Sampling times were chosen to give an excess (early) and nearly wild-type (late) levels of SecA (140, 225, 225, and 140 min for lanes *2*,*4*,*7*,*9*; 406, 425, 472, and 225 min for lanes *3*,*5*,*8*,*10*, respectively). After SDS-PAGE and electroblotting, filters were decorated with anti-SecA (*top*), streptavidin (*middle*), and anti-MalF (*bottom*) and detected by horseradish peroxidase reactions.

The SecM effect is cis-*specific*

The *secM* gene forms a single transcription unit with *secA* (Schmidt et al. 1988). This *cis*-configuration is important for SecM to control the *secA* translation (Oliver et al. 1998; Murakami et al. 2004). It is possible, however, that the activity-enhancing role of SecM was executed by the diffusible SecM product, although the periplasmic localization and the extreme instability of SecM (Rajapandi and Oliver 1991; Nakatogawa and Ito 2001) make this possibility unlikely. To address whether SecM only activates SecA molecule encoded from the *cis*-located *secA* gene, we constructed a strain with its chromosomal *secM–secA* region disrupted by a tetracycline-resistance determinant, *tetA*, while the viability was ensured by an arabinose-inducible *secA* plasmid (Materials and Methods). The chromosomal disruption and its lethality were verified, respectively, by PCR and the arabinose-dependent growth (data not shown).

We then constructed a series of Δ*secM–secA* strains that were complemented with different configurations of *secM*/*secA* under arabinose promoter control on plasmids (Fig. 3). Plasmids used were p*secA*, p*secM–secA*, and a combination of compatible plasmids p*secM* and p*secA*. The SecA synthesis directed by p*secA* was directly driven by the arabinose promoter without involving the *secM–secA* intergenic sequence, and its level was higher than that directed by p*secM–secA* (Fig. 3, cf. lanes 4 and 3,5). Cells growing in the presence of arabinose were assayed for OmpA export by pulse-labeling and for SecA abundance by immunoblotting. OmpA export was

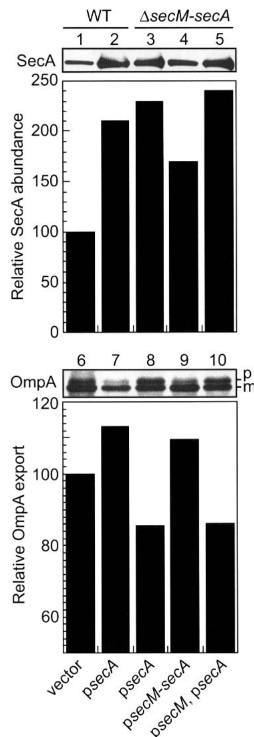

Figure 3. *Cis*-specific positive effect of SecM on SecA translocase function. Cells of strains indicated below were grown at 37°C in M9–amino acids–arabinose (0.2%) medium and subjected to anti-SecA immunoblotting (lanes *1–5*) and pulse-labeling test for OmpA export (lanes *6–10*), as described in Figure 1. Strains used were as follows: (Lanes *1*,*6*) HM1246 (*secM*$^+$) carrying two empty vectors pBAD33 and pBAD18. (Lanes *2*,*7*) HM1246 carrying p*secA* (pAN31) and pBAD18. (Lanes *3*,*8*) NH852 (Δ*secM–secA*/p*secA*) additionally carrying pBAD18 (empty vector). (Lanes *4*,*9*) NH853 (Δ*secM–secA*/p*secM–secA*) additionally carrying pBAD18. (Lanes *5*,*10*) NH852 (Δ*secM–secA*/p*secA*) additionally carrying p*secM* (pAN54). Relative SecA abundance and relative OmpA export efficiency are also graphically represented, with each value in HM1246/pBAD33/pBAD18 (lanes *1*,*6*) set as 100.

suboptimal in the absence of SecM (p*secA*) (Fig. 3, lane 8) even though the SecA content was ~2.3-fold higher than normal (Fig. 3, lane 3). Although the additional presence of p*secM*, and thus a *trans* supply of SecM, did not improve the export efficiency (Fig. 3, lane 10), cells carrying p*secM–secA* had normal export ability (Fig. 3, lane 9). Note that the SecA content in the p*secM–secA* cells was lower than that in the two partially export-defective cells without the *cis-secM* (Fig. 3, cf. lanes 4 and 3,5). The possibility that the lowered export seen in the absence of the *cis*-located *secM* was due to a toxicity of excess SecA in these cells was ruled out because OmpA export in the chromosomal *secM*$^+$–*secA*$^+$ wild-type strain was not interfered with by p*secA* (Fig. 3, lanes 2,7). From these results, we conclude that *secM* must be present in the immediate upstream of *secA* in the *cis* configuration to enhance the functionality of the SecA product.

Translocon targeting of SecM is important for the SecA activation

SecM is subject to transient elongation arrest, which is soon released by the engagement of the nascent chain in the Sec translocation reaction (Nakatogawa and Ito 2001; Sarker and Oliver 2002). This indicates that SecM is targeted to the membrane/Sec translocon cotranslationally. This property could be crucial for SecM's ability to enhance the functionality of SecA, because SecM will bring the *secM–secA* mRNA to the membrane to allow biosynthesis of SecA in a membrane environment. Prompted by this tempting possibility, we studied the importance of the SecM signal sequence in the facilitation of SecA functions. We introduced a signal sequence deletion mutation (Δ*ss*) as well as the *secM166* mutation into the *secM–secA* plasmid. They were induced in the Δ*secM–secA* strain and then shut off to follow SecA abundance and OmpA export subsequently (Fig. 4). The immunoblotting intensities of SecA and the signal sequence processing efficiencies of pulse-labeled OmpA were normalized to the respective values (set as 100) determined for the plasmid-free wild-type cells (Fig. 4A, lane WT). Their mutual relationships are graphically depicted in Figure 4B, in which the steady-state situation in the wild-type cell (100% export and 100 SecA abundance) is indicated by a star.

After repression of the arabinose promoter by the medium switch, all of these strains continued to grow with nearly normal rates within the time scale of the Figure 4 experiments (data not shown). In the p*secM*$^+$–*secA* cells (Fig. 4A, lanes 1–6), relative export efficiencies of OmpA were essentially 100 in the presence of 100% or higher abundance of SecA, whereas they decreased with decreasing SecA contents down to a value of ~20 (Fig. 4B, circles). In the case of the p*secM166–secA* strain (Fig. 4A, lanes 7–12), the OmpA export efficiency was already suboptimal (~70%) when the cellular content of SecA was 100%, below which the export efficiencies decreased further, to much lower levels than the p*secM*$^+$–*secA* cells that had corresponding amounts of SecA (Fig. 4B, triangles). When signal sequence was deleted from SecM, protein export activity was lowered even further (Fig. 4A, lanes 13–18). Thus, the SecA abundance-export curve obtained with the p*secM*Δ*ss*–*secA* cells was shifted further to the right (Fig. 4B, squares). In this case, export efficiency of OmpA was only ~80% and ~45% in the presence of as much as ~150% and ~100% of SecA, respectively. Further reduction in the SecA abundance in the *secM*Δ*ss* mutant resulted in severe export defects (Fig. 4A, lanes 17,18).

These results indicate that SecA synthesized without the signal sequence on SecM is much less active on average than that synthesized in combination with normal SecM. It should be noted that the elongation arrest should have been prolonged by the signal sequence mutation. Therefore, the elongation arrest per se is not sufficient in terms of the SecA-activating ability of SecM. The results obtained suggest that targeting of nascent SecM to the membrane translocon is another crucial fea-

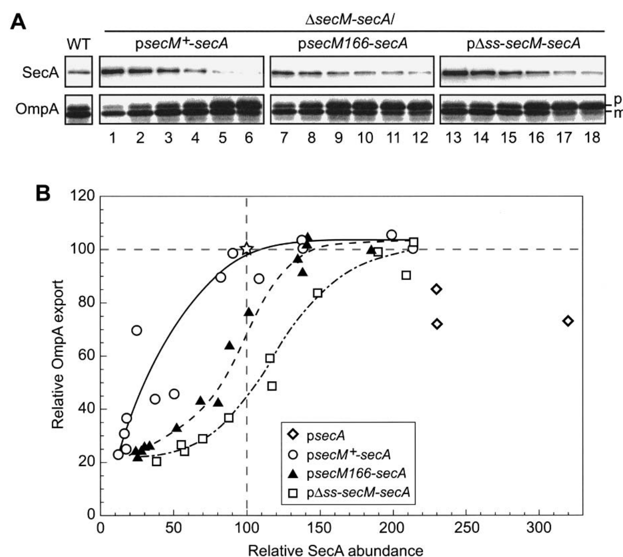

Figure 4. Signal sequence deletion in SecM lowers the functionality of SecA. (*A*) Strains NH853 (Δ*secM–secA*/p*secM*+–*secA*; lanes *1–6*), NH854 (Δ*secM–secA*/p*secM166–secA*; lanes *7–12*), and NH855 (Δ*secM–secA*/p*secM*Δ*ss–secA*; lanes *13–18*) were cultured in the arabinose-free M9–amino acids–glucose medium as described in the legend for Figure 1. At various time points (40–360 min) after the arabinose removal, samples were assessed for the SecA abundance by immunoblotting and for OmpA export by pulse-labeling as described in Figure 1. To obtain steady-state wild-type values, strain HM1246 (*secM*+) carrying an empty vector pBAD33 was examined similarly (lane WT). Three independent experiments were carried out, and results of one particular experiment are shown in *A*. (*B*) OmpA export efficiencies relative to that of the HM1246/pBAD33 control, which is set as 100, are plotted against relative values of SecA abundance with that of HM1246/pBAD33 set as 100. The data points includes those obtained in all three independent experiments, using NH853 (Δ*secM–secA*/p*secM*+–*secA*; circles), NH854 (Δ*secM–secA*/p*secM166–secA*; triangles) and NH855 (Δ*secM–secA*/p*secM*Δ*ss–secA*; squares). The star indicates the wild-type position. Data points with the Δ*secM–secA*/p*secA* strain growing in the presence of arabinose (see Fig. 3) are shown by diamonds.

ture for SecM to enhance the SecA activities. Finally, we examined cells carrying p*secA* only under the arabinose-induced conditions due to their poor growth upon repression. The data points shown by diamonds in Figure 4B (one of which was from the experiment shown in Fig. 3, lanes 3,8) indicate that cells completely lacking SecM did not gain the full translocase activity.

Discussion

In this work, we revealed that SecM has a new regulatory role of enhancing the functionality of the specific target protein, SecA, in a manner tightly coupled with its biosynthesis and translocation, adding another peculiarity to this already peculiar protein. Although SecM is eventually exported to the periplasm, it is unlikely that it functions in this compartment, where it is rapidly degraded by Tsp and some other proteases (Nakatogawa and Ito 2001). We believe that the biological role of SecM is executed during the course of its biosynthesis and translocation. The most remarkable peculiarity of SecM is that it contains an arrest sequence interacting with the ribosomal exit tunnel and halting translation elongation beyond this segment. It is also remarkable that this elongation arrest is subject to modulation by the engagement of the N-terminal region of the nascent SecM polypeptide in the Sec-dependent transport across the membrane. Thus, the duration of the arrest is inversely correlated with the activity of the translocation machinery to pull the growing chain out of the ribosome.

The export of SecM seems to follow an atypical route. SecM has an unusually long signal sequence for an exported protein (Sarker et al. 2000), and its export is SRP-dependent (Nakatogawa and Ito 2001). It is one of the few secretory proteins that depend on the SRP, a targeting factor that is considered more or less specific for membrane protein integration in *E. coli* (Luirink and Sinning 2004). Its export also depends on SecA, SecYEG, and SecD, but not on SecB (Rollo and Oliver 1988; Nakatogawa and Ito 2001). Clearly, SecM is exported cotranslationally as translation resumes from the elongation-arrested state. Our deletion analysis has indicated that effective release of the arrest requires signal sequence and the following mature part of ~80 amino acids (A. Murakami, H. Nakatogawa, and K. Ito, unpubl.) and Butkus et al. (2003) showed that the placement of a hydrophobic (stop-transfer) sequence after the signal sequence abolishes the release, being consistent with the "pulling model" of elongation resumption (Nakatogawa et al. 2004).

SecM can control translation of *secA* by virtue of its cotranscription with *secA* into a contiguous mRNA (Schmidt et al. 1988; McNicholas et al. 1997; Nakatogawa and Ito 2002). The *secM166* (Pro166Ala) mutation abolishes the elongation arrest and lowers the SecA translation level by about fivefold, whereas weaker *secM* mutations and an arrest-alleviating rRNA mutation impair the secretion defect-responses of SecA translation (Murakami et al. 2004). Thus, the cellular importance of SecM in ensuring proper levels of SecA translation has been established by our genetic analyses.

In the present study, we revealed that SecM plays a role not only in the maintenance of the proper translation levels but also in the establishment of the fully active states of SecA being synthesized. We carried out systematic comparisons between the cellular abundance of SecA and the cellular ability to export proteins, and we found that the *secM* mutants required higher SecA levels for the full export activity. The fact that the simple absence of *secM* gave the strongest defect in the SecA functionality indicates that SecM has a positive role in the biogenesis of SecA. However, SecM cannot fulfill this role when it is encoded by a separate mRNA; it only assists SecA molecules that are directed by the *cis*- and downstream-located *secA* gene. This unusual facilitator of protein biogenesis could be called a SecA-specific "*cis*-chaperone." In agreement with the *cis*-

chaperone activity of SecM, some partially defective *secA* alleles showed genetic complementation when they were present in the *secM–secA* configuration but not when they were present in *secA* itself isolated from *secM* on plasmids (H. Mori, unpubl.).

We have identified two elements in SecM that are important for its *cis*-chaperone activity. First, the signal sequence is essential, since its deletion results in a severe impairment of SecA activity. The cotranslational targeting of the nascent SecM polypeptide to the Sec translocation machinery is crucial for the SecA activating function of SecM. SecM will tether the *secM–secA* mRNA to a translocon through its cotranslational engagement in export, which in turn allows the synthesis of SecA in the vicinity of the translocon or the membrane. The second element important for the *cis*-chaperone activity of SecM is its elongation-arresting property, as demonstrated by the negative effect of the *secM166* mutation. Although the elongation arrest is released quite rapidly (half-life, ~1 min) (Nakatogawa and Ito 2001) in normal cells, this brief period of arrested elongation will contribute to the localized biosynthesis of SecA.

The concept of *cis*-chaperone can be challenged by recent reports that *secM–secA* mRNA is subject to endonucleolytic cleavages around the intergenic region (Hayes and Sauer 2003; Collier et al. 2004; Sunohara et al. 2004). We believe, however, that detailed kinetic studies will be required to determine whether such mRNA cleavage takes place as rapidly and as intensively as to affect the validity of our model of polycistronic mRNA localization. We must stress again that kinetics of the events will have major effects as discussed above regarding the transient nature of the elongation arrest. Tian and Beckwith (2002) first noted that some nonsense and frame-shift *secM* mutants were weakly defective in secretion, although they had normal levels of SecA, and that these defects were complemented by excess SecA. Since these mutations will prevent the arrest sequence region from being translated, the defects could now be explained by the lack of elongation arrest.

What might be the advantage for SecA to be synthesized in the vicinity of the translocon or the membrane? Because SecA can assume multiple conformations and multiple oligomeric states, its folding pathways could also be divergent. It is conceivable that biosynthesis in the vicinity of the membrane guides SecA to fold into the functional form that is ready to interact with the SecYEG translocon (Eichler et al. 1998). In this connection, it should be noted that recent studies suggest that SecA may undergo a dimer to monomer transition when it is in membranous environments (Or et al. 2002; Woodbury et al. 2002; Benach et al. 2003; Duong 2003; Tziatzios et al. 2004), and such transition could produce a working conformation of this protein (Osborne et al. 2004). It is possible then that biosynthesis of SecA in the vicinity of the membrane allows it to bypass the monomerization step. It is of vital importance to determine structural differences between the newly synthesized and steady-state SecA molecules, as well as between those synthesized in the presence and absence of upstream SecM.

Although it is possible to discuss the action of SecM in terms of its ability to localize the site of biosynthesis of SecA, it is also important to know whether SecM has any special feature besides its signal and the arrest sequences. Even the *cis*-specific nature could be explained by the extreme instability coupled with a localized function (Derbyshire and Grindley 1996). To address whether any other cotranslationally targeted secretory or membrane protein can substitute for SecM with respect to the *cis*-chaperone activity for SecA, we constructed a chimeric sequence encoding the N-terminal region of SecY up to its third periplasmic domain followed by the *secM–secA* intergenic segment (H. Mori, unpubl.). Placement of this chimeric sequence in front of *secA* allowed significantly enhanced SecA activity compared to the p*secA* construct shown in Figure 4B (diamonds), although drastic changes in the expression level made a rigorous conclusion difficult. At any event, it is conceivable that SecM has in fact been optimized for the *cis*-chaperone function. For instance, such a guide protein may be designed to not interact with cellular components other than the translocon and to be free from steric constraints. The composite nature of SecM, having a transmembrane sequence-like signal sequence (Sarker et al. 2000) and unstable periplasmic domain (Nakatogawa and Ito 2001) may make it possible not only to monitor the membrane protein integration activity of the cell but also to serve as the *cis*-chaperone for SecA.

Our results show that SecM is required for full activity of SecA in normally growing wild-type cells. Under the secretion-defective conditions, SecM elongation arrest is prolonged and the SecA level increases. This secretion-defect response might also be accompanied by further up-regulated SecA functions due to the prolonged cotranslational targeting of SecM to the translocon, assuming that the targeting reaction itself may be preserved under physiological secretion-compromising conditions such as low temperatures (Pogliano and Beckwith 1993; Murakami et al. 2004; Nakatogawa and Ito 2004; Nakatogawa et al. 2004). Our results predict that newly synthesized SecA molecules might preferentially be recruited for the translocation reaction. Functional heterogeneity of the intracellular SecA molecules is a challenging but interesting subject left for future analyses, along with the conformational differences among these molecules.

Materials and methods

Media

L-medium contained 1% bacto-tryptone, 0.5% bacto-yeast extract, 0.5% NaCl, and 1.7 mM NaOH. For agar plates, 1.2% agar was added. M9 minimal medium (Miller 1972) supplemented with arabinose (0.2%) or glucose (0.4%), thiamine (2 µg/mL), and 18 amino acids (20 µg/mL each, except Met and Cys) was used for the pulse-labeling experiments. For growth of plasmid-harboring strains, ampicillin (50 µg/mL), chloramphenicol (20 µg/mL), and/or kanamycin (25 µg/mL) were included.

Plasmid constructions

Plasmid pAN31 (Murakami et al. 2004) carried *secA* cloned under the arabinose promoter on pBAD33 (a pACYC184-based and chloramphenicol-resistant vector; Guzman et al. 1995). Plasmid pAN54 carrying *secM* was constructed by inserting an ~550-bp SacI–SphI fragment of pNH26 (Nakatogawa and Ito 2001) into pBAD18 (a pBR322-based and ampicillin-resistant vector; Guzman et al. 1995). For construction of pNH196 encoding *secM*+–*secA*, a *secM–secA* region was PCR-amplified from pAN1 (Murakami et al. 2004) using primers 5′-AAAGAGCTCTTG AATATGATCGGGATGGC-3′ (the SacI recognition site is underlined) and 5′-TTCGATGGAGATAGTACCC-3′, and ligated with pAN31 after digestion with SacI and BglII, a unique site within the *secA* ORF. pNH197 (*secM166–secA*) was similar to pNH196 except that the fragment was amplified from pAN3 such that it contained the *secM166* mutation (Murakami et al. 2004). pNH198 carried *secMΔss–secA* and was constructed similarly except that an oligonucleotide, 5′-AAAGAGCTCTT GAATATGATCGGGATGGCAATAACGTGGCCGAACCAA ACGCGCCCGC-3′, was used as the upstream primer. All the regions that experienced in vitro DNA synthesis reactions were confirmed by nucleotide sequencing to lack any unwanted mutation.

Bacterial strains

Bacterial strains used in this study were mostly derived from either MC4100 [*araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*] (Silhavy et al. 1984) or W3110 [F–l–IN(*rrnD-rrnE*)*1*] (Bachmann 1987). HM1246 (MC4100, *ompT*::*kan ara*+; constructed by appropriate P1 transductions) was used as a host to express genes under the control of the *araBAD* promoter. AN144 and AN145 were a pair of HM1246-derived *secM*+ and *secM166* strains carrying plasmid pAN31 (P_{araBAD}–*secA*) and described previously (Murakami et al. 2004). AN225 and AN226 were W3110-derived *secM*+/pAN31 and *secM166*/pAN31 strains, respectively, constructed by P1 transduction (Murakami et al. 2004).

The *secM–secA* segment of the chromosome was disrupted as follows. First, *tetA* (tetracycline resistance determinant) in Tn*10* was PCR-amplified from the chromosome of strain AN26 (Murakami et al. 2004) using primers 5′-TGCGCTAAATACG TTTGAATATGATCGGGATGGCAATAACCCGACCTCATT AAGCAGCTC-3′ and 5′-AGGCGCAGAATCCTGCGCCTT TTACTTCAACAGTTAGCTTAACTAAGCACTTGTCTCCT G-3′ (the chromosomal nucleotide sequences in the upstream of *secM* and in the downstream of *secA* are underlined, respectively). The product was then electroporated into a recombination-enhanced strain, AB1157 (Murphy 1998) that harbored pKM201 (λ recombinase) as well as pAN31 for transformant selection on L-agar containing tetracycline (12.5 µg/mL), chloramphenicol (20 µg/mL), and arabinose (0.2%). Tetracycline-resistant transformants were purified by single-colony isolation and subjected to PCR screening using primers (5′-GATGAT CAAACAAAGGACAC-3′ and 5′-CTACAGACGTTTAAGCC TTGTCTCCTG-3′) designed to amplify the *secM–secA* segment. One that gave an ~3-kb (instead of normal ~4.9-kb) PCR product was confirmed to exhibit arabinose-dependent growth and was named AN209 (Δ*secM–secA*::*tetA*). P1 transduction (Miller 1972) was carried out to transfer the Δ*secM–secA*::Tn*10* marker from AN209 to HM1246 that carried either pAN3 (*secA*), pNH196 (*secM*+–*secA*), pNH197 (*secM166–secA*), or pNH198 (*secMΔss–secA*). Strains thus constructed were named NH852, NH853, NH854, and NH855, respectively.

Immunoblotting and protein detection after SDS-PAGE

Immunoblotting detection of proteins was carried out essentially as described by Shimoike et al. (1995). Whole-cell proteins were precipitated by direct treatment of culture with 5% trichloroacetic acid and then dissolved in SDS. Samples from a fixed cellular mass (corresponding to 0.04 mL culture of turbidity, A_{660} = 0.1) were used for SDS-PAGE separation of soluble proteins (Laemmli 1970) and membrane proteins (Ito 1984), which were then electroblotted onto an Immobilon PVDF membrane filter (Millipore), decorated with appropriate rabbit antisera, and visualized by horseradish peroxidase-conjugated second antibodies (Bio-Rad). The above amount of cells gave SecA intensities that were within an approximate range of linearity. Biotinylated MalF–PSBT was detected directly with streptavidin-conjugated horseradish peroxidase (Amersham Pharmacia Biotech). Chemiluminescence images were recorded and quantified with a Fuji LAS1000 lumino-imager.

Pulse-labeling analysis of protein export

Pulse-labeling and immunoprecipitation experiments were carried out as described (Matsumoto et al. 1997). To evaluate protein export activity, cells were pulse-labeled with ^{35}S-methionine for 30 sec, and immediately mixed with an equal volume of ice-cold 10% trichloroacetic acid. Protein precipitates were sedimented, washed with acetone, and dissolved in 1% SDS–50 mM Tris-HCl (pH 8.1)–1 mM EDTA, followed by immunoprecipitation with anti-OmpA or anti-MBP and separation by SDS-PAGE (10% acrylamide) into precursor (intracellular) and mature (exported) forms of these proteins. Radioactive proteins were visualized and quantified with a Fuji BAS1800 phosphor imager. Proportions of the mature form, after correction for the methionine contents, were taken as the efficiencies of protein export.

Acknowledgments

We thank Hironori Niki and Yoshinori Akiyama for materials and useful discussion; Jon Beckwith for generous gifts of anti-MalF and pGJ78; and Kiyoko Mochizuki, Michiyo Sano, and Kunihito Yoshikaie for their technical support. This work was supported by CREST, JST (Japan Science and Technology Corporation), grants from the Japan Society for the Promotion of Science, and the National Project on Protein Structural and Functional Analyses of the Ministry of Education, Culture, Sports, Science and Technology, Japan. H.N. was supported by a JSPS post-doctoral fellowship for young Japanese Scientists.

References

Bachmann, B.J. 1987. Escherichia coli *and* Salmonella typhimurium*: Cellular and molecular biology.* American Society for Microbiology Press, Washington, D.C.

Benach, J., Chou, Y.T., Fak, J.J., Itkin, A., Nicolae, D.D., Smith, P.C., Wittrock, G., Floyd, D.L., Golsaz, C.M., Gierasch, L.M., et al. 2003. Phospholipid-induced monomerization and signal-peptide-induced oligomerization of SecA. *J. Biol. Chem.* **278:** 3628–3638.

Butkus, M.E., Prundeanu, L.B., and Oliver, D.B. 2003. Translocon 'pulling' of nascent SecM controls the duration of its translational pause and secretion-responsive *secA* regulation. *J. Bacteriol.* **185:** 6719–6722.

Collier, J., Bohn, C., and Bouloc, P. 2004. SsrA-tagging of *Escherichia coli* SecM at its translational arrest sequence. *J. Biol. Chem.* **279:** 54193–54201.

Derbyshire, K.M. and Grindley, N.D. 1996. Cis preference of the IS903 transposase is mediated by a combination of transposase instability and inefficient translation. *Mol. Microbiol.* **21:** 1261–1272.

Ding, H., Mukerji, I., and Oliver, D. 2003. Nucleotide and phospholipid-dependent control of PPXD and C-domain association for SecA ATPase. *Biochemistry* **42:** 13468–13475.

Driessen, A.J. 1993. SecA, the peripheral subunit of the *Escherichia coli* precursor protein translocase, is functional as a dimer. *Biochemistry* **32:** 13190–13197.

Duong, F. 2003. Binding, activation and dissociation of the dimeric SecA ATPase at the dimeric SecYEG translocase. *EMBO J.* **22:** 4375–4384.

Economou, A. and Wickner, W. 1994. SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. *Cell* **78:** 835–843.

Eichler, J., Brunner, J., and Wickner, W. 1997. The protease-protected 30 kDa domain of SecA is largely inaccessible to the membrane lipid phase. *EMBO J.* **16:** 2188–2196.

Eichler, J., Rinard, K., and Wickner, W. 1998. Endogenous SecA catalyzes preprotein translocation at SecYEG. *J. Biol. Chem.* **273:** 21675–21681.

Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.* **177:** 4121–4130.

Hartl, F.U., Lecker, S., Schiebel, E., Hendrick, J.P., and Wickner, W. 1990. The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the *E. coli* plasma membrane. *Cell* **63:** 269–279.

Hayes, C.S. and Sauer, R.T. 2003. Cleavage of the A site mRNA codon during ribosome pausing provides a mechanism for translational quality control. *Mol. Cell* **12:** 903–911.

Hunt, J.F., Weinkauf, S., Henry, L., Fak, J.J., McNicholas, P., Oliver, D.B., and Deisenhofer, J. 2002. Nucleotide control of interdomain interactions in the conformational reaction cycle of SecA. *Science* **297:** 2018–2026.

Ito, K. 1984. Identification of the *secY* (*prlA*) gene product involved in protein export in *Escherichia coli. Mol. Gen. Genet.* **197:** 204–208.

Jander, G., Cronan Jr., J.E., and Beckwith, J. 1996. Biotinylation in vivo as a sensitive indicator of protein secretion and membrane protein insertion. *J. Bacteriol.* **178:** 3049–3058.

Karamanou, S., Vrontou, E., Sianidis, G., Baud, C., Roos, T., Kuhn, A., Politou, A.S., and Economou, A. 1999. A molecular switch in SecA protein couples ATP hydrolysis to protein translocation. *Mol. Microbiol.* **34:** 1133–1145.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Lill, R., Dowhan, W., and Wickner, W. 1990. The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins. *Cell* **60:** 271–280.

Luirink, J. and Sinning, I. 2004. SRP-mediated protein targeting: Structure and function revisited. *Biochim. Biophys. Acta* **1694:** 17–35.

Matsumoto, G., Yoshihisa, T., and Ito, K. 1997. SecY and SecA interact to allow SecA insertion and protein translocation across the *Escherichia coli* plasma membrane. *EMBO J.* **16:** 6384–6393.

McNicholas, P., Salavati, R., and Oliver, D. 1997. Dual regulation of *Escherichia coli secA* translation by distinct upstream elements. *J. Mol. Biol.* **265:** 128–141.

Miller, J.H. 1972. *Experiments in molecular genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Mori, H. and Ito, K. 2001. The Sec protein-translocation pathway. *Trends Microbiol.* **9:** 494–500.

Murakami, A., Nakatogawa, H., and Ito, K. 2004. Translation arrest of SecM is essential for the basal and regulated expression of SecA. *Proc. Natl. Acad. Sci.* **101:** 12330–12335.

Murphy, K.C. 1998. Use of bacteriophage λ recombination functions to promote gene replacement in *Escherichia coli*. *J. Bacteriol.* **180:** 2063–2071.

Nakatogawa, H. and Ito, K. 2001. Secretion monitor, SecM, undergoes self-translation arrest in the cytosol. *Mol. Cell* **7:** 185–192.

———. 2002. The ribosomal exit tunnel functions as a discriminating gate. *Cell* **108:** 629–636.

———. 2004. Intraribosomal regulation of expression and fate of proteins. *Chembiochem.* **5:** 48–51.

Nakatogawa, H., Mori, H., and Ito K. 2000. Two independent mechanisms down-regulate the intrinsic SecA ATPase activity. *J. Biol. Chem.* **275:** 33209–33212.

Nakatogawa, H., Murakami, A., and Ito, K. 2004. Control of SecA and SecM translation by protein secretion. *Curr. Opin. Microbiol.* **7:** 145–150.

Oliver, D.B. and Beckwith, J. 1982. Regulation of a membrane component required for protein secretion in *Escherichia coli*. *Cell* **30:** 311–319.

Oliver, D., Norman, J., and Sarker, S. 1998. Regulation of *Escherichia coli secA* by cellular protein secretion proficiency requires an intact gene X signal sequence and an active translocon. *J. Bacteriol.* **180:** 5240–5242.

Or, E., Navon, A., and Rapoport, T. 2002. Dissociation of the dimeric SecA ATPase during protein translocation across the bacterial membrane. *EMBO J.* **21:** 4470–4479.

Osborne, A.R., Clemons Jr., W.M., and Rapoport, T.A. 2004. A large conformational change of the translocation ATPase SecA. *Proc. Natl. Acad. Sci.* **101:** 10937–10942.

Pogliano, K.J. and Beckwith, J. 1993. The Cs *sec* mutants of *Escherichia coli* reflect the cold sensitivity of protein export itself. *Genetics* **133:** 763–773.

Rajapandi, T. and Oliver, D. 1991. The first gene in the *Escherichia coli secA* operon, gene X, encodes a nonessential secretory protein. *J. Bacteriol.* **173:** 7092–7097.

Rollo, E.E. and Oliver, D.B. 1988. Regulation of the *Escherichia coli secA* gene by protein secretion defects: Analysis of *secA*, *secB*, *secD*, and *secY* mutants. *J. Bacteriol.* **170:** 3281–3282.

Sarker, S. and Oliver, D. 2002. Critical regions of *secM* that control its translation and secretion and promote secretion-specific *secA* regulation. *J. Bacteriol.* **184:** 2360–2369.

Sarker, S., Rudd, K.E., and Oliver, D. 2000. Revised translation start site for *secM* defines an atypical signal peptide that regulates *Escherichia coli secA* expression. *J. Bacteriol.* **182:** 5592–5595.

Schmidt, M.G. and Oliver, D.B. 1989. SecA protein autogenously represses its own translation during normal protein secretion in *Escherichia coli. J. Bacteriol.* **171:** 643–649.

Schmidt, M.G., Rollo, E.E., Grodberg, J., and Oliver, D.B. 1988. Nucleotide sequence of the *secA* gene and *secA*(Ts) mutations preventing protein export in *Escherichia coli. J. Bacteriol.* **170:** 3404–3414.

Sharma, V., Arockiasamy, A., Ronning, D.R., Savva, C.G., Holzenburg, A., Braunstein, M., Jacobs Jr., W.R., and Sacchettini, J.C. 2003. Crystal structure of *Mycobacterium tuberculosis* SecA, a preprotein translocating ATPase. *Proc. Natl. Acad. Sci.* **100:** 2243–2248.

Shimoike, T., Taura, T., Kihara, A., Yoshihisa, T., Akiyama, Y., Cannon, K., and Ito, K. 1995. Product of a new gene, *syd*, functionally interacts with SecY when overproduced in *Escherichia coli. J. Biol. Chem.* **270:** 5519–5526.

Sianidis, G., Karamanou, S., Vrontou, E., Boulias, K., Repanas, K., Kyrpides, N., Politou, A.S., and Economou, A. 2001. Cross-talk between catalytic and regulatory elements in a DEAD motor domain is essential for SecA function. *EMBO J.* **20:** 961–970.

Silhavy, T.J., Berman, M.L., and Enquist, L.W. 1984. *Experiments with gene fusions.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sunohara, T., Jojima, K., Tagami, H., Inada, T., and Aiba, H. 2004. Ribosome stalling during translation elongation induces cleavage of mRNA being translated in *Escherichia coli. J. Biol. Chem.* **279:** 15368–15375.

Tian, H. and Beckwith, J. 2002. Genetic screen yields mutations in genes encoding all known components of the *Escherichia coli* signal recognition particle pathway. *J. Bacteriol.* **184:** 111–118.

Tziatzios, C., Schubert, D., Lotz, M., Gundogan, D., Betz, H., Schagger, H., Haase, W., Duong, F., and Collinson, I. 2004. The bacterial protein-translocation complex: SecYEG dimers associate with one or two SecA molecules. *J. Mol. Biol.* **340:** 513–524.

van den Berg, B., Clemons Jr., W.M., Collinson, I., Modis, Y., Hartmann, E., Harrison, S.C., and Rapoport, T.A. 2004. X-ray structure of a protein-conducting channel. *Nature* **427:** 36–44.

Veenendaal, A.K.J., van der Does, C., and Driessen, A.J.M. 2004. The protein-conducting channel SecYEG. *Biochim. Biophys. Acta* **1694:** 81–95.

Vrontou, E. and Economou, A. 2004. Structure and function of SecA, the preprotein translocase nanomotor. *Biochim. Biophys. Acta* **1694:** 67–80.

Woodbury, R.L., Hardy, S.J., and Randall, L.L. 2002. Complex behavior in solution of homodimeric SecA. *Protein Sci.* **11:** 875–882.