

SecY and SecA interact to allow SecA insertion and protein translocation across the *Escherichia coli* plasma membrane

Gen Matsumoto, Tohru Yoshihisa¹ and Koreaki Ito²

Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan

¹Present address: Department of Chemistry, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan

²Corresponding author
e-mail: kito@virus.kyoto-u.ac.jp

SecA, the preprotein-driving ATPase in *Escherichia coli*, was shown previously to insert deeply into the plasma membrane in the presence of ATP and a preprotein; this movement of SecA was proposed to be mechanistically coupled with preprotein translocation. We now address the role played by SecY, the central subunit of the membrane-embedded heterotrimeric complex, in the SecA insertion reaction. We identified a *secY* mutation (*secY205*), affecting the most carboxy-terminal cytoplasmic domain, that did not allow ATP and preprotein-dependent productive SecA insertion, while allowing idling insertion without the preprotein. Thus, the *secY205* mutation might affect the SecYEG 'channel' structure in accepting the preprotein–SecA complex or its opening by the complex. We isolated *secA* mutations that allele-specifically suppressed the *secY205* translocation defect *in vivo*. One mutant protein, SecA36, with an amino acid alteration near the high-affinity ATP-binding site, was purified and suppressed the *in vitro* translocation defect of the inverted membrane vesicles carrying the SecY205 protein. The SecA36 protein could also insert into the mutant membrane vesicles *in vitro*. These results provide genetic evidence that SecA and SecY specifically interact, and show that SecY plays an essential role in insertion of SecA in response to a preprotein and ATP and suggest that SecA drives protein translocation by inserting into the membrane *in vivo*.

Keywords: protein translocation/SecA insertion/SecY/suppressor mutation/translocation channel

Introduction

Selective translocation of newly synthesized secretory proteins across the membrane is accomplished by a series of protein–protein interactions. Such protein interactions should be very dynamic since they involve a newly synthesized presecretory protein, which is moving across the membrane. In addition, recent studies on *Escherichia coli* protein translocase revealed that some components of the translocation machinery also undergo remarkable transmembrane movements (Economou and Wickner, 1994; Economou *et al.*, 1995; Nishiyama *et al.*, 1996).

In vitro studies on preprotein translocation using inverted membrane vesicles or reconstituted proteoliposomes suggest the following mechanisms responsible for protein translocation across the *E.coli* plasma membrane (Wickner and Leonard, 1996). Newly synthesized preproteins are first recognized by the secretion-specific chaperone, SecB, and maintained in a translocation-competent conformation. They are then transferred to SecA, an ATPase residing both in the cytosol and on the plasma membrane. Binding of ATP to SecA drives insertion of a ~20–30-residue segment of the preprotein into the membrane, and ATP hydrolysis is accompanied by a release of the bound precursor (Schiebel *et al.*, 1991). Thus, repeated cycles of ATP binding and hydrolysis will result in stepwise movements of the preprotein, although the released preprotein may also be driven for further translocation by the proton-motive force across the membrane (Schiebel *et al.*, 1991; Driessen, 1992). A breakthrough in our understanding of how SecA drives protein translocation was provided by Economou and Wickner (Economou and Wickner, 1994; Economou *et al.*, 1995), who showed that in the presence of ATP, a preprotein, and functional membrane vesicles, a 30 kDa portion of SecA inserts deeply into the membrane such that it is protected from proteolytic digestion. SecA is also accessible from the periplasmic side under certain *in vivo* conditions (Kim *et al.*, 1994). The inserted SecA segment comes back (deinserts) upon hydrolysis of ATP. More recently, it was shown that membrane insertion of SecA is not confined to the carboxy-terminally located 30 kDa domain (Price *et al.*, 1996); the amino-terminally located 65 kDa ATPase domain also inserts into the membrane under the conditions that allow productive translocation (Eichler and Wickner, 1997).

The membrane-embedded portion of protein translocase includes a complex of three integral membrane proteins, SecY, SecE and SecG, with SecY serving as a central subunit with which SecE and SecG associate independently (Ito, 1996; Homma *et al.*, 1997). It is generally believed that the SecYEG complex forms a channel-like pathway for a preprotein. Integral membrane proteins SecD, SecF and YajC also have some roles in translocation (Pogliano *et al.*, 1994; Duong and Wickner, 1997). Nishiyama *et al.* (1996) reported that the SecG protein changes its orientation in the membrane during preprotein translocation. They proposed that the topology inversion of SecG has a role of assisting in the SecA/preprotein insertion reaction (especially at low temperature where the *secG*-disrupted mutant fails to grow).

The unexpected transmembrane dynamism of SecA and SecG raises an immediate question of how directly their movements are coupled with the preprotein translocation. If the movement of SecA is indeed mechanistically coupled with the movement of the SecA-bound preprotein, it

becomes essential to elucidate the mechanisms for membrane insertion of a preprotein–SecA complex, rather than those for insertion of a free polypeptide. In this regard, it is particularly important to clarify the roles of the SecYEG ‘channel’ proteins in the insertion of the SecA–preprotein complex and the nature of interaction between SecA and SecYEG, which allows the transmembrane delivery of such a large protein complex without affecting general permeability barrier.

In the work presented here, we addressed these questions by genetic and biochemical approaches. We report on a *secY* mutation that specifically impairs the productive mode of SecA insertion, as well as on isolation and characterization of *secA* mutations that suppress the *secY* defect in allele-specific manners. A mutant SecA was purified and suppression was demonstrated *in vitro* with respect to the translocation and SecA insertion defects. These results provide the first genetic evidence for the specific SecY–SecA interaction, which is relevant to the SecA insertion reaction. Good correlations were observed between *in vivo* protein export phenotypes, *in vitro* protein translocation activities and *in vitro* SecA insertion abilities for different combinations of SecY and SecA variants. It was thus shown that SecY plays active and direct roles in the SecA insertion reaction, which proved important for translocation facilitation.

Results

Screening of the cold-sensitive *secY* mutants for an *in vitro* defect in SecA insertion reaction

We previously isolated and characterized a number of cold-sensitive *secY* mutants with defects in protein export (Baba *et al.*, 1990; Taura *et al.*, 1994). The mutations, a total of 10, were introduced into a genetic background that included the *ompT::kan* and Δunc mutations. The latter mutations were included to prevent artificial cleavage of SecY *in vitro* (Akiyama and Ito, 1990) and to avoid the interchange between ATP and proton motive force in subsequent analyses. Inverted membrane vesicles (IMVs) were prepared from each of the mutant strains grown at 37°C and they were treated with 6 M urea to remove fractions of membrane-bound SecA and to inactivate remaining SecA. SecY contents of these IMVs, as determined by immunoblotting experiments, were not significantly different from that of the wild-type IMV (data not shown). To assess their abilities to support the SecA insertion reaction, purified SecA was iodinated with ^{125}I and incubated with IMVs in the presence or absence of purified proOmpA protein and ATP (or its non-hydrolysable analogue, AMP–PNP). SecA insertion was observed as a labelled 30 kDa fragment that was protected by IMVs from trypsin digestion (Economou and Wickner, 1994). Using wild-type IMV, we were able to reproduce the essential features of the results reported by Economou and Wickner (1994); the insertion occurred either when both ATP and proOmpA were present (Figure 1A, upper panel, lane 2) or when AMP–PNP was present (Figure 1A, upper panel, lanes 3 and 6). The SecA insertion that was observed in the presence of AMP–PNP was independent of proOmpA (Figure 1A, lane 6; Economou *et al.*, 1995), suggesting that SecA insertion occurs at certain frequency without being accompanied by a prepro-

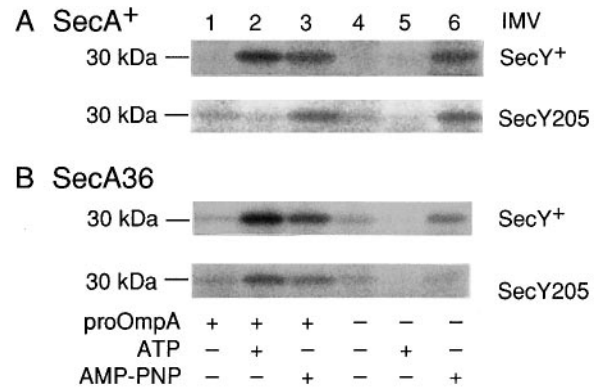


Fig. 1. Mutational alterations of SecA insertion reactions. (A) ^{125}I -labelled SecA (4 µg) and urea-treated IMV (35 µg protein) from wild-type (upper panel) or *secY205* (lower panel) cells were preincubated at 0°C (200 µl), and IMV–SecA complex was isolated by centrifugation and subjected to SecA insertion reaction (50 µl) containing the ^{125}I -SecA-bound IMV (5 µg) in the presence or absence of proOmpA (3.3 µg), ATP (2 mM) and AMP–PNP (2 mM), as indicated, at 37°C for 15 min. After trypsin digestion, the 30 kDa fragment was visualized by SDS–PAGE and phosphorimager exposure. (B) SecA insertion reactions with ^{125}I -SecA36 were carried out as in (A).

tein, and such an ‘idling’ mode of insertion becomes apparent when deinsertion is inhibited by the non-hydrolysable analogue of ATP. In contrast, the ATP-driven SecA insertion was only observed in the presence of a preprotein and can be referred to as the ‘productive’ mode of insertion.

We screened IMVs prepared from the 10 different mutant strains for any abnormality in the SecA insertion reaction and found two mutant IMVs that behaved anomalously in reproducible manners. The IMV prepared from a mutant, *secY125*, with an amino acid alteration in the most amino-terminal periplasmic domain gave an enhanced SecA insertion (data not shown). Characterization of this mutant will be described elsewhere. Another mutant, *secY205* with an amino acid alteration, Tyr429 to Asp, in the most carboxy-terminal cytoplasmic domain gave IMV that did not effectively support SecA insertion in the presence of ATP and proOmpA (Figure 1A, lower panel, lane 2).

The *secY205* alteration in SecY impairs the productive SecA insertion

The SecY205 IMV did not effectively support the productive mode of SecA insertion. To examine whether SecA binding to the high-affinity sites was abolished by the mutation, SecA binding assays were carried out. Scatchard analysis of the results showed that the SecY205 IMV possessed near-normal numbers of the high-affinity binding sites for SecA, with a K_d value ~2.5-fold the wild-type value (Figure 2). This decrease in the binding affinity explains the defective SecA insertion only partly, since the concentrations of SecA in the insertion assays were above the K_d values, and we actually pre-isolated similar amounts of IMV-bound SecA before the insertion assays (see the legend to Figure 4). Thus, the defective insertion should have been due largely to a defect after the SecA binding.

The apparent defect could have been due to an abnormally enhanced deinsertion, while insertion *per se* was

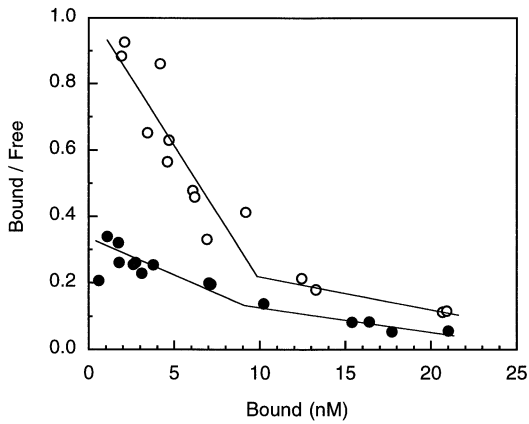


Fig. 2. Scatchard plots of SecA binding. Four to four-hundred nM of SecA, in which ^{125}I -SecA occupied 4 nM, was mixed with urea-washed IMV (100 $\mu\text{g}/\text{ml}$) in a 50 μl reaction at 0°C for 30 min, followed by isolation of IMV–SecA complex by centrifugation. Radioactivities of the sediment and supernatant were determined by an LKB γ -ray counter. \circ , wild-type IMV; \bullet , SecY205 IMV. K_d values estimated were 16 nM for wild-type IMV and 61 nM for SecY205 IMV, and the numbers of the high-affinity binding sites were estimated to be 3.3×10^{12} and 4×10^{12} per μg (protein) IMVs from wild-type and *secY205* cells, respectively. The lines were drawn by applying the least squares method to manually selected data segments.

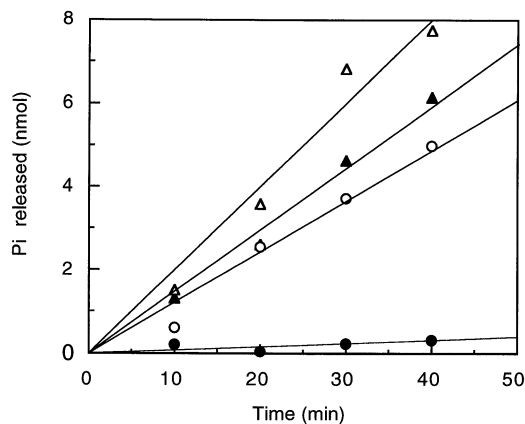


Fig. 3. Mutational alterations of SecA translocation ATPase activity. SecA translocation ATPase activities of wild-type SecA protein (\bullet, \circ) and the SecA36 mutant protein ($\blacktriangle, \triangle$) were assayed using urea-washed IMV from the wild-type strain (\circ, \triangle) or the *secY205* mutant (\bullet, \blacktriangle). Reaction in the absence of proOmpA (membrane ATPase) was subtracted from that in the presence of proOmpA for each point. Membrane ATPase activity was slightly higher for the SecA36 protein than the wild-type protein.

affected only to a small extent. We measured SecA translocation ATPase activities using wild-type and SecY205 IMVs. It was found that the mutant IMV supported very low level of activity (Figure 3, closed circles) as compared with the wild-type IMV (Figure 3, open circles). The low ATPase activity indicates that the SecA cycle involving hydrolysis of ATP does not operate effectively with the mutant IMV; SecA stalls either before insertion or after insertion. Our failure to detect the inserted form of SecA suggests a defect before insertion. In other words, it is difficult to explain the results shown in Figure 1A in terms of a near-normal insertion that is negatively compensated by an enhancement in ATP hydrolysis-dependent deinsertion

In contrast to the results obtained in the presence of

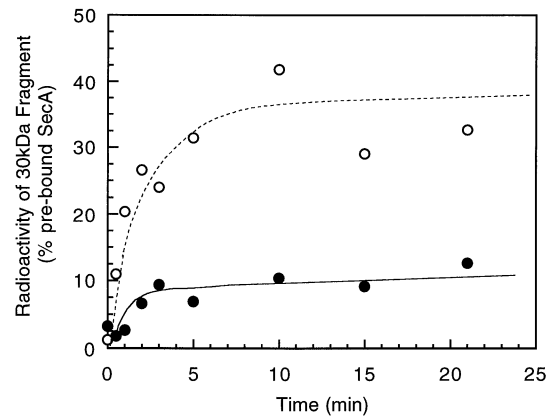


Fig. 4. Time courses of preprotein-independent 'idling' SecA insertion in the presence of AMP–PNP. Insertion of ^{125}I -labelled SecA was allowed in the absence of proOmpA and in the presence of AMP–PNP, as described under Figure 1, and inserted 30 kDa fragment was quantified after trypsin digestion and SDS–polyacrylamide gel electrophoresis. Values were presented as % pre-bound SecA radioactivity. About 21% of the input SecA was pre-bound to both SecY⁺ IMV and SecY205 IMV. Thus, ~ 0.5 pmol of SecA per μg (protein) IMV was brought into the final reaction in both cases. \circ , reaction using wild-type IMV; \bullet , reaction using SecY205 IMV.

ATP and proOmpA, the SecY205 mutant IMV supported a significant level of SecA insertion when AMP–PNP was included instead of ATP (Figure 1A, lower panel, lanes 3 and 6). Similar results were obtained when ATP γ S, another non-hydrolysable analogue, was used (data not shown). It might be argued, however, that these results were misleading, since the data only represented SecA insertion at a single (15 min) point; even if the SecY205 IMV is uniformly inactive, a combination of a residual activity and inhibition of deinsertion by the ATP analogue might lead to a gradual accumulation of inserted SecA during the 15 min incubation period. We thus compared the initial speeds of 'idling' insertion between the reaction using the SecY205 IMV and that using the SecY⁺ IMV. The two reactions occurred in similar time courses (Figure 4). It was thus shown that the initial rate of SecA insertion into the SecY205 IMV was indeed of a significant level. These results rule out the gradual accumulation as the cause of the different results obtained with different nucleotides. Thus, the SecY205 IMV allows the idling mode of SecA insertion, albeit at a lower than normal efficiency.

All the results so far obtained suggest that the SecY205 IMV has differential defects in accepting SecA itself and the proOmpA–SecA complex. It is tempting to speculate that this is due either to a structural alteration in the SecYEG channel or an alteration in the gate opening processes.

Mutations in *secA* that suppress *secY205*

If the *secY205* mutation alters some specific aspect in interaction between SecY and SecA, then it will be possible to isolate its suppressor mutations in *secA*. Pools of revertants capable of growing at 20°C were selected from a strain carrying both *secY205* and *leu::Tn10*. The *leu* and *secA* genes are located closely such that they are co-transducible in P1-mediated generalized transduction. After enrichment of *Tn10*-linked mutations by P1 transduction, we were able to isolate suppressor mutations that were co-transducible with *leu::Tn10* at frequencies ($\sim 40\%$)

expected for a *secA* mutation. Some of the mutants exhibited increased resistance to azide, a trait characteristic of *secA* mutations (Oliver *et al.*, 1990; Huie and Silhavy, 1995). We cloned a *secA* segment of the chromosome from three representative suppressor strains and determined the *secA* nucleotide sequences. We found a single nucleotide substitution in *secA* for each of the suppressor clones (Figure 5).

Mutation *secA31*, which did not confer an azide resistance, was expected to cause an amino acid replacement (Ala116 → Val) near the nucleotide-binding site (NBS) I (Mitchell and Oliver, 1993) of the SecA protein. Mutation *secA36*, which conferred an increased azide resistance, was also mapped near NBS I, causing an Ala112 → Val change. Mutation *secA81* slightly increased azide resistance and, more interestingly, conferred temperature-sensitivity for cell viability when combined with the *secY205* mutation (see below). This mutation was mapped near NBS II, causing a Gly516 → Asp change in the protein product.

Allele-specific suppression of the *secY205* defect by the *secA* mutations

The mutations *secA31*, *secA36* and *secA81* in the suppressor strains were introduced into various *sec* strains by P1 transduction using *leu::Tn10* as the selective marker (Table I). Upon re-transduction into a *secY205* background, each suppressed donor yielded cold-resistant transductants at a frequency of ~40%. However, no cold-resistant transductants were obtained when other cold-sensitive *secY* mutants were used as recipients (Table I). That the



Fig. 5. Amino acid substitutions predicted for the *secY205*-suppressing *secA* mutations. The results of the sequence determinations for *secA31*, *secA36* and *secA81* mutations are summarized. The nucleotide changes identified were C1168 to T, C1156 to T, and G2368 to A, for *secA31*, *secA36* and *secA81*, respectively. The nucleotide residue numbers were as defined by Schmidt *et al.* (1988).

secA mutations had indeed been introduced was confirmed for some of the transductants of each combination by further transduction into the *secY205* background. These results indicated that the suppression by each of the *secA* mutations was specific for the *secY205* allele of *secY*.

These *secA* mutations, when present in the *secY*⁺ background, did not themselves show any growth abnormalities under several experimental conditions examined, except in the presence of azide (Table I). On agar plates, the *secY*⁺ strain grew in the presence of up to 0.5 mM NaN₃. The *secA36* and the *secA81* transductants resisted up to 2.0 mM NaN₃ and 1.0 mM NaN₃, respectively, at 37°C, whereas the *secA31* transductant did not appreciably affect the azide resistance (Table I). We found that many *sec* mutant strains had somewhat increased sensitivities to azide; they did not grow in the presence 0.2 mM NaN₃ (Table I, see numbers in parentheses). The *secA36* mutation increased the azide resistance of many, but not all, of these strains (Table I). Thus, the increased azide resistance was a more general property of the *secA36* mutation than the suppression of cold-sensitivity, which was observed only for the specific *secA36*–*secY205* combination. The partial azide resistance and the suppression of the cold-sensitivity did not segregate in transduction into the *secY205* mutant.

We examined whether the *secA* mutations indeed suppressed the protein export defect associated with the *secY205* mutation. Pulse-chase experiments at 20°C showed that maltose-binding protein (MBP, a periplasmic protein) was labelled almost exclusively as the precursor form in the *secY205* mutant (Figure 6A, lane 4). The precursor was processed slowly to the mature form (Figure 6A, lane 6). The same protein was labelled almost exclusively as the mature form in wild-type cells (Figure 6A, lane 1). When the *secY205* mutation was combined with either *secA31*, *secA36* or *secA81*, processing of MBP at 20°C was restored to near wild-type levels (Figure 6A, lanes 7–15), although the effect of *secA31* was somewhat weaker than those of the other *secA* mutations. These results establish that the three suppressor mutations in *secA* suppress specifically the protein export defect of the *secY205* mutant.

Table I. Growth properties of various strains that received the *secA* mutations

1st mutation in <i>secA</i>	2nd mutation combined							
	<i>secY205</i>	<i>secY39</i>	<i>secY104</i>	<i>secY125</i>	<i>secY129</i>	Δ <i>secG</i>	<i>secE501</i>	<i>sec</i> ⁺
<i>secA</i> ⁺	Cs (<0.2)	Cs (0.5)	Cs (<0.2)	Cs (0.5)	Cs (0.2)	Cs (<0.2)	Cs (<0.2)	+ (0.5)
<i>secA31</i>	+ (<0.2)	Cs (nt)	Cs (nt)	Cs (nt)	Cs (nt)	Cs (nt)	Cs (nt)	+ (0.5)
<i>secA36</i>	+ (1.0)	Cs (1.0)	Cs (0.2)	Cs (1.0)	Cs (0.2)	+ (0.5)	Cs (0.5)	+ (2.0)
<i>secA81</i>	Ts (0.5)	Cs (0.5)	Cs (<0.2)	Cs (0.5)	Cs (0.2)	Cs (0.2)	nt	+ (1.0)

P1 transduction experiments were carried out using a donor strain carrying one of the *secA* mutations and *leu::Tn10* and a recipient strain bearing the *sec* mutation as indicated. Growth properties of the transductants that were confirmed to have received the *secA* mutation by further transduction into the *secY205* mutant are indicated: +, Cs and Ts indicate, respectively, growth at any temperature between 20–42°C, inability to grow at 20°C and inability to grow at 42°C. The numbers in parentheses indicate the highest NaN₃ concentration (mM) that allowed bacterial growth on L-agar medium. nt, not tested.

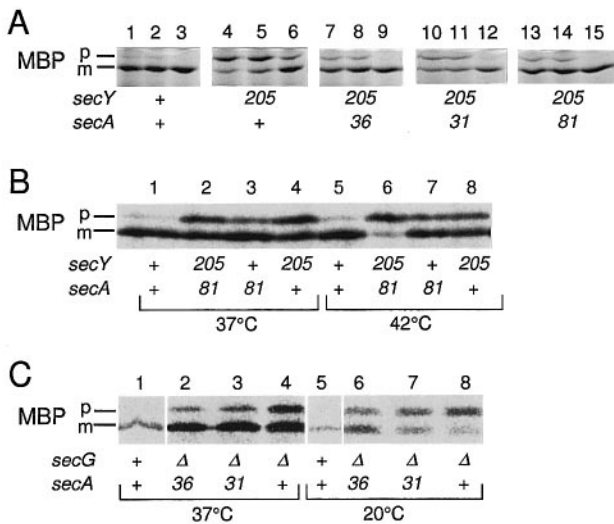


Fig. 6. Protein export phenotypes of strains carrying various combinations of the *sec* mutations. **(A)** Wild-type strain (GN40; lanes 1–3), *secY205* strain (GN17; lanes 4–6), *secY205 secA36* transductant (lanes 7–9), *secY205 secA31* transductant (lanes 10–12), and *secY205 secA81* transductant (lanes 13–15) were grown first at 37°C, and then at 20°C for 20 min, where cells were pulse-labelled with [³⁵S]methionine for 1 min. Samples were withdrawn after chase with unlabelled excess methionine for 0.5 (lanes 1, 4, 7, 10 and 13), 1 (lanes 2, 5, 8, 11 and 14) or 5 (lanes 3, 6, 9, 12 and 15) min. MBP was immunoprecipitated and separated into the precursor (p) and mature (m) forms, as indicated, which were visualized by autoradiography. **(B)** Wild-type cells (GN40, lanes 1 and 5), *secY205 secA81* transductant (lanes 2 and 6), *secY⁺ secA81* transductant (lanes 3 and 7), and *secY205* strain (GN17; lanes 4 and 8) were grown first at 37°C (lanes 1–4) and then shifted to 42°C for 20 min (lanes 5–8). Cells were pulse-labelled with [³⁵S]methionine for 30 s (lanes 1–4) or 40 s (lanes 5–8), and MBP was immunoprecipitated. **(C)** Wild-type cells (GN40, lanes 1 and 5), Δ *secG secA36* transductant (lanes 2 and 6), Δ *secG secA31* transductant (lanes 3 and 7) and the Δ *secG* mutant (KN370, lanes 4 and 8) were grown first at 37°C (lanes 1–4) and shifted to 20°C for 20 min (lanes 5–8). Cells were pulse-labelled with [³⁵S]methionine for 30 s (lanes 1–4) or 60 s (lanes 5–8), and MBP was immunoprecipitated.

Synthetic temperature sensitivity caused by the *secA81* and the *secY205* mutations

The original *secA81* isolate (in combination with *secY205*) was able to grow at 20°C but not at 42°C. In retransduction into the *secY205* background, cold-resistance and the temperature sensitivity were inseparable, indicating that the same mutation was responsible for the suppression of the *secY205* defect at low temperature and the inability to grow at high temperature. This notion was further supported by reversion studies; some of temperature-resistant revertants regained the cold-sensitivity. The *secA81* mutation did not itself cause a temperature-sensitive phenotype, nor did it in combination with other *secY*, *secE* or *secG* mutations (Table I). These results suggest that, the specific combination of the SecA81 form of SecA and the SecY205 form of SecY renders the protein export system non-functional at 42°C. In support of this notion, export of MBP in the *secA81–secY205* double mutant strain was found to be severely impaired at 42°C (Figure 6B, lane 6). The *secA81* single mutant and the *secY205* single mutant exhibited much less pronounced defect in MBP export at 42°C (Figure 6B, lanes 7 and 8).

The allele-specific suppression of the *secY205* defect by the *secA* mutations as well as the allele-specific

synthetic temperature sensitivity observed for the *secA81–secY205* combination provide the first genetic evidence for the notion that SecY and SecA interact specifically in mediating protein translocation.

Suppression of the *secG* deletion phenotypes by the *secA36* mutation

None of the *secA* mutants suppressed the *secE501* mutation (Riggs *et al.*, 1988; Schatz *et al.*, 1991) with a decreased expression level of *secE* (Table I). Deletion of *secG* causes cold-sensitive cell growth and protein export (Nishiyama *et al.*, 1994). This cold-sensitivity was found to be suppressed by the *secA36* mutation but not by the other two *secA* mutations (Table I). The Δ *secG* mutant exhibited a severe defect of MBP export at 20°C as demonstrated by the preferential labelling of the precursor form of MBP (Figure 6C, lane 8). At 37°C, the mutant was much less defective (Figure 6C, lane 4). Export of MBP at 20°C was markedly restored in the strain that carried *secA36* in addition to Δ *secG* (Figure 6C, lane 6). The *secA31* mutation had a slight positive effect on the MBP export (Figure 6C, lane 7), but much less effective than the *secA36* mutation. Thus, the *secA36* mutation alleviates the requirement for the SecG function normally seen in this strain background at low temperatures.

The SecA36 protein can drive preprotein translocation into the SecY205 IMV

We purified the SecA36 form of the SecA protein. Translocation of ³⁵S-labelled proOmpA was assayed using urea-washed IMVs from either the wild-type cells or the *secY205* mutant cells, in combination with either the SecA protein or the SecA36 protein. Translocated proOmpA molecules were detected after treatment of the reaction mixture with trypsin followed by SDS-PAGE and phosphorimager quantification of the OmpA species. Reactions using the wild-type SecA protein clearly discriminated between the wild-type IMV, which gave ~50% translocation in 5 min (Figure 7, open circles), and the SecY205 IMV, which gave only ~8% translocation (Figure 7, closed circles). The translocation facilitating activity of SecA36 mutant protein was similar to or slightly higher than the wild-type SecA protein under the standard reaction conditions using wild-type IMV (Figure 7, open triangles). Strikingly, SecA36 protein was able to drive proOmpA translocation into the SecY205 IMV at a normal efficiency (Figure 7, closed triangles). Thus, the mutational inactivation of the SecY205 IMV was effectively alleviated by the SecA36 form of SecA *in vitro*.

The SecA36 protein can insert into the SecY205 IMV

To examine whether the SecA36 protein gained the ability to insert into the SecY205 IMV, it was iodinated and subjected to the insertion assay. A combination of the iodinated mutant protein and the wild-type IMV gave essentially normal results of insertion (Figure 1B, upper panel), except that the mutant SecA protein exhibited an increased insertion in response to ATP and proOmpA (Figure 1B, upper panel, lane 2). In contrast to the wild-type SecA that did not insert into the SecY205 IMV in response to ATP and proOmpA (Figure 1A), the SecA36 mutant protein was able to insert into the SecY205 IMV,

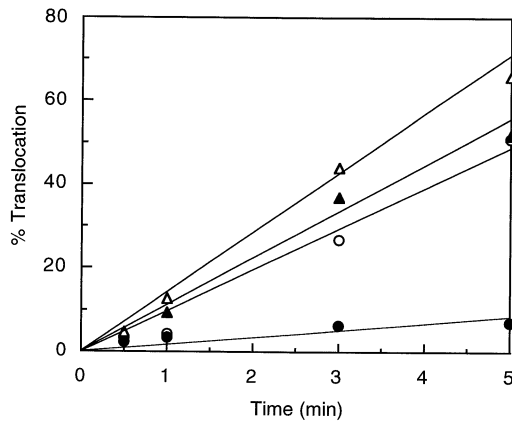


Fig. 7. *In vitro* translocation of proOmpA with different combinations of SecY and SecA variants. Wild-type SecA protein (●,○) and SecA36 mutant protein (▲,△) were combined with urea-washed IMV from either wild-type strain (○,△) or the *secY205* mutant (●,▲) for translocation of ³⁵S-labelled proOmpA. Samples were withdrawn at time points as indicated and trypsinized before SDS–PAGE and quantification of radioactive OmpA bands. Values presented are the sum of the precursor and mature forms that resisted trypsin and represented in % input radioactivities. Basic characteristics of the translocation assay system such as the ATP-dependence of the reaction and the Triton X-100 sensitivity of trypsinization had been confirmed.

although the efficiency of insertion was still lower than normal (Figure 1B, lower panel, lane 2). SecA36 exhibited normal or somewhat enhanced translocation ATPase activity both in combination with the wild-type IMV (Figure 3, open triangles) and SecY205 IMV (Figure 3, closed triangles).

Although the extent of suppression at the level of SecA insertion was lower than the extent of suppression at the level of protein translocation, the *secA36* mutation suppressed the *secY205* mutant phenotypes at the levels of cellular growth, protein export *in vivo*, protein translocation *in vitro*, translocation ATPase activity and membrane insertion of the 30 kDa SecA segment.

Discussion

SecYEG-like trimeric complexes of membrane proteins are conserved throughout living organisms, with the eukaryotic counterpart called the Sec61 complex (Stirling *et al.*, 1992; Jungnickel *et al.*, 1994; Ito, 1996). They presumably function as a translocation channel (Hanein *et al.*, 1996) for secretory proteins, although the nature of the ‘channel’ is largely unknown. It is remarkable that similar integral membrane components function in conjunction with diverse protein-driving systems (Economou and Wickner, 1994; Panzner *et al.*, 1995; Corsi and Schekman, 1996; Ito, 1996). In the eukaryotic co-translational system, ribosomes bind to Sec61 α (Görlich *et al.*, 1992; Kalies *et al.*, 1994), forming a tight seal that maintains the topological segregation of the translocating chain from the cytosol (Crowley *et al.*, 1993, 1994) and that enables the translocation to be driven through polypeptide chain elongation. The dimension of the translocation channel seems to be as large as 40–60 Å (Hamman *et al.*, 1997). By analogy to the ribosome, SecA, the ATPase providing driving forces for post-translational translocation in *E.coli*, may also act as a gatekeeper. It is crucially important to understand how SecA interacts

with the putative channel-forming proteins and how such protein interactions lead to the preprotein translocation and also the membrane insertion of the SecA molecule.

A central question here will be whether SecA inserts directly into the SecYEG channel, and whether the movement of a preprotein is accompanied by an obligatory insertion of SecA. It should be noted that such an insertion complex should be of substantial dimension since almost the entire molecule of SecA now seems to insert into the membrane (Eichler and Wickner, 1997). Insertion of a preprotein–SecA complex is consistent with the results of crosslinking experiments showing that a translocating chain was adjacent to SecA and SecY but not to the lipid environment (Joly and Wickner, 1993). More recently, the 30 kDa membrane-inserted fragment of SecA has been shown to be inaccessible by a lipid-soluble photo-crosslinking reagent, indicating that it is shielded from the lipid phase and possibly residing in some proteinaceous environment (Eichler *et al.*, 1997). Although these observations suggest the attractive model that ATP-driven insertion of a SecA–preprotein complex into the SecYEG channel initiates translocation (Economou and Wickner, 1994; Economou *et al.*, 1995), many aspects of SecY–SecA interaction and its relationship to the SecA and preprotein insertion remain to be studied *in vivo* and *in vitro*.

Biochemical evidence for specific SecA–SecY interaction includes the previous observations that proteoliposomes containing SecY and SecE bind SecA and activate SecA translocation ATPase (Hanada *et al.*, 1994). The high-affinity SecA-binding sites on IMV as well as the capacity to support the translocation ATPase activity of SecA increase in proportion to overproduction of the SecYE complex (Duong and Wickner, 1997). SecA insertion reaction is also enhanced when SecYE is overproduced together with SecG and/or SecDF–YajC (Duong and Wickner, 1997). The SecA binding to IMV protects SecY from trypsin digestion, and anti-SecY inhibits SecA binding to IMV (Hartl *et al.*, 1990). Recently, SecY (or its N-terminal part) after gel electrophoretic separation was shown to be decorated with SecA in ligand blotting experiments (Snyders *et al.*, 1997). However, the physiological significance of this observation is unclear. Although the *E.coli* translocation system has been subject to extensive genetic analysis (Schatz and Beckwith, 1990), genetic evidence for the SecY–SecA interaction has almost been lacking. The Sec titration experiments of Bieker-Brady and Silhavy (1992) suggested some functional interaction between SecA and SecY/SecE, but the experimental system was indirect.

We now demonstrated that a specific aspect of SecY–SecA interaction has been impaired by the *secY205* mutation affecting the most carboxy-terminal cytoplasmic domain of SecY. *In vivo*, this mutation impairs export of periplasmic and outer membrane proteins (Taura *et al.*, 1994). The IMV containing this mutant form of SecY does not allow any detectable insertion of the 30 kDa segment of SecA in response to a preprotein and ATP. However, the mutant IMV allows significant levels of insertion of SecA in the presence of AMP–PNP and in the absence of proOmpA. This observation, together with the results of binding assays, indicates that the mutant IMV allows significant SecA binding to the high-affinity

sites, and therefore that the defect should be in an event after the binding.

The fact that the AMP-PNP-driven SecA insertion occurs independent of the preprotein indicates that SecA itself can insert at a certain frequency upon binding of this ATP analogue. This idling insertion occurred even into the SecY205 IMV with a significant level of initial rate. Thus, the idling mode of insertion was only partially lowered by the *secY205* mutation. The more severe defect with ATP and the milder defect with AMP-PNP might be explained by a mutational enhancement of deinsertion. However, the low translocation ATPase activity with the mutant IMV argues against the possibility that ATP hydrolysis-dependent deinsertion reaction was enhanced. These results, taken together, suggest that the SecY205 IMV is specifically defective in accepting a SecA-pro-OmpA complex in response to ATP-binding. Probably, a specific SecA-SecY interaction that triggers the subsequent initiation of insertion has been impaired by the mutation. The existence of a distinct 'idling' mode of SecA insertion raises a possibility that such a reaction has some functional significance, such as that of keeping the unused translocation channel closed.

Our assays of SecA translocation ATPase using IMVs prepared from the cold-sensitive mutant collection of *secY* showed that a number of mutants that map in the cytoplasmic domain 5 or 6 supported very low activities (Taura *et al.*, 1997). They include the *secY205* mutant characterized here. These results suggest that these cytoplasmic domains of SecY are important for SecY-SecA interaction. Our earlier studies on dominant negative *secY* variants suggested that the carboxy-terminal region of SecY is important for the translocation facilitation (Shimoike *et al.*, 1992; Taura *et al.*, 1994). Our present results agree with this notion. It is noteworthy that IMVs from mutants such as *secY39* (Baba *et al.*, 1990) supported only very low translocation as well as translocation ATPase activities, but they were only marginally defective in the SecA insertion assays (unpublished data). Probably, these mutants allow ATP-dependent insertion but do not allow subsequent processes in which ATP hydrolysis-dependent recycling occurs. In any case, *secY205* is unique in that it does not allow the ATP-dependent SecA insertion.

The notion that the *secY205* mutation affects a specific process of SecA-SecY interaction was supported strongly by our successful isolation of allele-specific suppressors in *secA*. The three mutations we characterized all mapped near NBS I or NBS II, and some of them exhibited partial azide resistance. The allele-specific suppression suggests an alteration in protein-protein interaction (Jarvik and Botstein, 1975).

The purified SecA36 protein had slightly higher activities of translocation facilitation and translocation ATPase facilitation in combination with the wild-type IMV. Although the wild-type SecA protein had a greatly diminished ability to drive proOmpA translocation into the SecY205 IMV, the SecA36 protein was able to stimulate the translocation into the mutant IMV at a normal rate. The mutant SecA protein enhanced protein translocation into the SecY39 mutant IMV only to a small extent (unpublished data). Thus, we were able to demonstrate allele-specific suppression of the translocation defect *in vitro*. Translocation ATPase activity was also recovered

for the SecA36-SecY205 combination. SecA insertion assays showed that SecA36 had indeed a recovered ability to insert into the SecY205 IMV, although the extent of insertion was lower than the wild-type combination. Although the recovery was observed more effectively for translocation than for *in vitro* SecA insertion, we demonstrated qualitative correlations among suppression at the level of cell growth, suppression at the level of *in vivo* protein export, suppression at the level of *in vitro* activity of translocation, and suppression at the level of the ATP and precursor-dependent SecA insertion. These results nicely bridge the SecA insertion and translocation facilitation functions of protein translocase as well as its *in vivo* and *in vitro* functions. They strongly support the view that SecA insertion reaction indeed drives preprotein insertion into the membrane and that SecA specifically interacts with SecY in doing so.

We obtained additional evidence for the specific SecA-SecY interaction. The *secA81* and the *secY205* mutation are incompatible with each other to support cell viability and protein export at 42°C, while the defective SecY205 function is effectively suppressed at low temperature. The double-mutant strain shows a very clear temperature sensitivity, and it may provide a useful system to investigate the nature of the protein translocation channel and the SecA functions.

The *secA* mutations we isolated affect either the high-affinity or the low-affinity ATP binding region in the 65 kDa ATPase domain of SecA (Price *et al.*, 1996). They may induce a conformational change in SecA such that it is now able to insert, with a preprotein, into the altered SecY205 channel. The 30 kDa inserted SecA segment has been identified as the carboxy-terminal region of SecA (Price *et al.*, 1996), which is outside the region where the suppressor mutations mapped. It is possible that the mutations somehow change the conformation of the 30 kDa segment or that the mutations effect a 'gate-opening' process in which preprotein-bearing SecA somehow transmits a signal to the SecYEG channel. However, given the recent finding that the amino-terminally located ATPase domain (65 kDa) also inserts into the membrane (Eichler *et al.*, 1997), it becomes equally possible that the suppressor mutations affect conformation of the 65 kDa domain with respect to its competence in insertion, and the insertion of the 65 kDa domain determines whether or not the 30 kDa segment inserts as well. Huie and Silhavy (1995) showed that many *secA* mutations that suppress defective export of signal sequence-impaired preproteins confer azide resistance. They proposed that the mutant SecA stays longer in the membrane, giving the preprotein an increased probability of acceptance by the SecYEG component. Since SecA36 corrects more pronouncedly the SecY205 translocation defect than its insertion defect, it may enhance a post-insertion event, in which the preprotein is forwarded for further translocation.

Nishiyama *et al.* (1996) proposed that SecG assists in SecA insertion by inverting its own topology in the membrane. Duong and Wickner (1997) indeed showed that SecG enhances SecA insertion. In this connection, it is particularly interesting that the *secA36* mutation suppresses the protein export defects associated with the deletion of *secG*. It is highly possible that the SecA36 protein no longer requires the assistance from SecG in its

insertion into the membrane. Whether this is due to an alteration in its interaction with SecY or due simply to its hyperactivity remains to be investigated.

We have reported an initial success in a combined genetic and biochemical approach to the central question of protein translocation; the interaction between the translocation-driving protein and the core integral membrane proteins. The genetically altered translocase subunits described in this paper will prove useful for further studies at the molecular and cellular levels. For instance, the SecY205 structural change in the SecYEG complex might be an interesting target of structural biology, if it is indeed refractory to a preprotein–SecA complex. The suppressor variants of SecA might then give a deeper insight into the nature of the translocation channel.

Materials and methods

Escherichia coli strains

Cold-sensitive *secY* mutants were described previously [Baba *et al.* (1990) for *secY39* and Taura *et al.* (1994) for other *secY* mutants]. AD202 was a derivative of MC4100 (Silhavy *et al.*, 1984) carrying *ompT::kan* (Akiyama and Ito, 1990). All the mutations were transferred to a genetic background of strain TW155, a derivative of AD202 carrying $\Delta(\text{uncB-uncC})$ (Klionsky *et al.*, 1984), by P1 transduction using *zhd-33::Tn10* as a selective marker. The one that received *secY205* was named GN5 and its isogenic *secY*⁺ strain was named TW156. They were used for preparation of IMVs. The *unc*⁺ *secY* mutant strains, TY1 (*secY205*), TY8 (*secY125*), TY15 (*secY104*) and TY24 (*secY129*), originally described by Taura *et al.* (1994), as well as a similar strain carrying the *secY39* mutation were cured of the *zhd-33::Tn10* insertion by isolating tetracycline-sensitive derivatives (Maloy and Nunn, 1981). Strains PR520 (*secE501*) (Riggs *et al.*, 1988) was also cured of the Tet^R marker. GN15 was a Tet^S derivative of TY1, and GN17 was a *leu::Tn10* transductant of GN15.

The *secY205*-suppressing *secA* mutations were isolated as follows. GN17 cells, after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Miller, 1972), were plated on L-tetracycline agar and colonies appeared after a 3-day incubation at 20°C (10²–10³ colonies, at frequencies of 1–5 × 10^{−3}) were pooled, from which P1vir lysates were prepared. These P1 lysates were used to select Tet^R transductants of GN15 at 20°C. The putative *secA* mutations thus obtained were P1-transduced into the Tet^S derivatives of *secY* and *secE* mutants to examine allele-specificity of suppression. KN370 ($\Delta\text{secG}::\text{kan}$) (Nishiyama *et al.*, 1994) was also used as a recipient. GN40, GN41, GN42 and GN43 were *leu::Tn10* transductants of AD202 that received *secA*⁺, *secA31*, *secA36* or *secA81*, respectively.

Biological experiments

L-medium contained 10 g tryptone, 5 g yeast extract, 5 g NaCl and 1.7 mmol NaOH per litre. Minimal medium M9 was as described (Silhavy *et al.*, 1984). P1 transduction was carried out according to the standard procedures (Miller, 1972). We always purified transductants on L-medium without the selection pressure, and single colonies were examined for the selected as well as unselected growth properties after making suspensions in 0.9% NaCl in microtitre wells and transferring them onto different agar plates using multiple metal sticks that were fixed to fit the microtitre wells.

Determination of nucleotide sequence of *secA* from the mutants

2.5 kb and 0.8 kb *EcoRI* fragments of chromosomal DNA from a mutant were recovered after agarose gel electrophoresis, and cloned into pUC118. Clones containing the *secA* sequence were screened by restriction pattern analyses. Sequencing by ABI Model 373A sequencer was carried out using appropriate primers of known *secA* sequences. We consistently found, even for wild-type clones, two deviations from the published sequence of *secA* (Schmidt *et al.*, 1988); residue 3032 and 3033 were G and C, respectively, instead of C and G as published. Thus, we assume that the correct assignments for amino acid residues 737 and 738 of SecA are Glu and Arg, respectively. All the deviations from the wild-type or the published sequence were confirmed by reading both strands.

Purification of the SecA and the SecA36 proteins

A SecA-overproducing plasmid pKY173 (Yoshihisa and Ito, 1996) was constructed by C. Ueguchi, by cloning a 3.3 kb *secA* fragment generated by partial *EcoRI* digestion of a *secA* plasmid (pMA34 provided by T. Horiuchi) into a *lac* promoter vector pNO1575 (Ito *et al.*, 1983). Cells of KI269 (Akiyama and Ito, 1985) carrying pKY173 were grown on L-glucose (0.4%) at 37°C, induced with 1 mM isopropyl- β -D-thiogalactoside and 0.5 mM cyclic AMP for 2 h, and harvested. They were disrupted by sonication in 10 mM Tris–HCl, pH 8.0, containing 12 mM Mg-acetate, 60 mM KCl, 1 mM dithiothreitol and 0.2 mM phenylmethyl sulfonyl fluoride. Supernatant after high-speed centrifugation was fractionated by Red A dye-binding chromatography with a 0.06–2 M KCl gradient. Fractions of highest SecA purity were pooled and further purified by DEAE–Sepharose Fast Flow column chromatography. The SecA36 protein was purified similarly using its overproducer pGN101 in strain GN47 (a F'*lacI*^R *lacPL8 lacZ*⁺ *Y*⁺ *A*⁺ derivative of GN42). The possibility of the formation of a SecA36/SecA⁺ heterodimer was thus eliminated. pGN101 was constructed by replacing the 0.8 kb *EcoRI* fragment (for the amino-terminal region of SecA) by the corresponding fragment of the *secA36* clone used for sequencing, using appropriate partial digestions. Protein concentrations were determined using the Bio-Rad protein assay solution with bovine γ -globulin as a standard.

SecA insertion and SecA binding assays

SecA was iodinated with Na¹²⁵I as described by Economou and Wickner (1994), except that the incubation at 0°C was extended to 20 min. SecA insertion assay was essentially as described by the above authors. We used urea-washed IMVs from different mutant strains, and pro-3His-OmpA' and SecB that were purified as described previously (Yoshihisa and Ito, 1996). After preincubation of urea-washed IMV and ¹²⁵I-labelled SecA at 0°C for 30 min, IMV was re-isolated together with bound SecA by centrifugation before incubation at 37°C for 15 min with the ProOmpA derivative, SecB, ATP and the ATP generating system as required. AMP–PNP substituted for the last two components in some experiments. The inserted 30 kDa fragment of SecA was detected after trypsinization (1 mg/ml, at 0°C for 15 min) by SDS–PAGE and phosphorimager visualization/quantification (Fuji BAS2000 system in combination with PDI image analysis software).

Quantitative assay for SecA binding to IMV was carried out using different concentrations of SecA that contained a fixed amount of ¹²⁵I-labelled SecA, essentially as described by Hartl *et al.* (1990).

SecA translocation ATPase assay

A reaction mixture (50 μ l) contained urea-washed IMV (2 μ g protein), SecA (2 μ g), and ATP (2 mM) in buffer containing 50 mM Tris–HCl, pH 7.2, 30 mM KCl, 30 mM NH₄Cl, 5 mM Mg-acetate. It was further supplemented with or without 2 μ l of proOmpA (1.82 μ g in 8 M urea) that had been purified according to Crooke *et al.* (1988) and provided by H. Mori (Sato *et al.*, 1997). Results were similar with pro-3His-OmpA'. After incubation at 37°C, inorganic phosphate liberated was quantified by the malachite green method (Lanzetta *et al.*, 1979; Lill *et al.*, 1990). To obtain translocation ATPase activities, values in the absence of proOmpA were subtracted from those in its presence.

In vitro translocation assay

An ³⁵S-labelled proOmpA preparation, provided by H. Mori, had been prepared as follows. *In vitro* translation of proOmpA was programmed in the presence of [³⁵S]methionine as described by Uchida *et al.* (1995), and the translation mixture was precipitated by 7.5% trichloroacetic acid (TCA) followed by washing with 7.5% TCA and with acetone, and solubilization with 6 M urea–50 mM K-phosphate buffer, pH 7.5. Translocation reactions contained IMV (150 μ g protein/ml), SecA (100 μ g/ml), SecB (25 μ g/ml), ATP (2 mM), phosphocreatine (50 mM) and creatine kinase (100 μ g/ml) in buffer consisting of 50 mM Tris–HCl, pH 8.0, 50 mM KCl, 5 mM MgCl₂, 200 μ g/ml bovine serum albumin and 10 mM β -mercaptoethanol. After preincubation at 37°C for 2 min, ³⁵S-labelled proOmpA preparation (1/25 the total volume) was added to start the reaction. Samples were chilled on ice and treated with 1 mg/ml trypsin at 0°C for 15 min followed by TCA precipitation and SDS–PAGE for quantification of translocated OmpA species.

In vivo protein export assay

Cells were grown on M9-glycerol (0.4%) medium supplemented with 0.4% maltose, 5 mM cyclic AMP and 18 amino acids (20 μ g/ml each, other than methionine and cysteine) and pulse-labelled with [³⁵S]methionine (5–13 μ Ci/ml, 1175 Ci/mmol; American Radiolabeled Chemicals, Inc.) for indicated periods. Chase with unlabelled methionine

and immunoprecipitation with anti-MBP serum were as described previously (Baba *et al.*, 1990), except that protein A-Sepharose was used as an immunoabsorbent. Immunoprecipitates were separated by SDS-PAGE and precursor and mature forms of MBP were visualized by exposure to a phosphorimager plate or an X-ray film.

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