A Significant Fraction of Functional SecA Is Permanently Embedded in the Membrane

SecA CYCLING ON AND OFF THE MEMBRANE IS NOT ESSENTIAL DURING PROTEIN TRANSLOCATION*

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SecA has been suggested to cycle on and off the cytoplasmic membrane of Escherichia coli during protein translocation. We have reconstituted 35S-SecA onto SecA-depleted membrane vesicles and followed the fate of the membrane-associated 35S-SecA during protein translocation. Some 35S-SecA was released from the membranes in a translocation-independent manner. However, a significant fraction of 35S-SecA remained on the membranes even after incubation with excess SecA. This fraction of 35S-SecA was shown to be integrated into the membrane and was active in protein translocation, indicating that SecA cycling on and off membrane is not required for protein translocation. Proteolysis experiments did not support the model of SecA insertion and deinsertion during protein translocation; instead, a major 48-kDa domain was found persistently embedded in the membrane regardless of translocation status. Thus, in addition to catalyzing ATP hydrolysis, certain domains of SecA probably play an important structural role in the translocation machinery, perhaps forming part of the protein-conducting channels.

Translocation of most periplasmic and outer membrane proteins across the cytoplasmic membrane of Escherichia coli occurs through the Sec-dependent pathway (1, 2), which consists of a distinct set of Sec proteins (3, 4). SecA plays a pivotal role in protein translocation. It couples the energy of essential ATP hydrolysis to protein translocation (5–8) and has been shown to be essential for protein translocation both in vivo (9, 10) and in vitro (11–14). SecA interacts with most components of the translocation machinery. It is believed that SecA binds to the membrane through interactions with SecYEG (15, 16) and acidic phospholipids (17–21), and interacts with precursor proteins by recognizing the positive charge at the NH2 terminus of the signal peptides (22, 23). Binding of precursor proteins to SecA is a subunit of the translocase stimulates the translocation ATPase activity of SecA (18). Recently, it has been reported that SecA promotes protein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion (24) at SecYEG, and that this cycle is regulated by SecD/F (25) and the ATP-binding amino terminus of SecA (26).

While SecYEG are integral membrane proteins (27, 28), SecA is considered a peripheral membrane protein since it lacks any predicted transmembrane domains (29), and was found in both cytosolic and membrane fractions (9, 30). It is generally believed, therefore, that SecA cycles between the cytoplasm and the membrane during protein translocation (24, 31). Consistent with this notion, soluble SecA can restore translocation activity of membranes with depleted or inactivated SecA (11–14) or impaired SecY (32). On the other hand, extraction experiments with urea or Na2CO3 suggested that a significant fraction of SecA behaves like an integral membrane protein (30).1 Recently, Kim et al. (33) reported that a strain harboring a plasmid containing the secD secF locus possesses SecA almost entirely as an integral membrane form and displays normal protein translocation as measured by rapid processing of preproteins in vivo (although it is possible that only a small fraction of SecA is active which accounts for the apparent “normal” translocation). Kim et al. (33) suggested that the integral SecA is the catalytically active form in vivo and that, in this state, it might form a part of the reported protein-conducting channels (34). However, it is not clear what fraction of SecA is active and whether this integral SecA remains active and cycles off the membranes as suggested by Economou and Wickner (24).

Here, we present evidence that the integral SecA under the conditions used is indeed active and does not cycle off the membrane during protein translocation. We have determined the fate of the membrane-associated SecA during protein translocation, using membranes reconstituted from 35S-SecA and SecA-depleted inverted membrane vesicles. There were two forms of 35S-SecA on the reconstituted membranes, loosely associated and integral. The loosely associated 35S-SecA can be replaced by excess nonradioactive SecA, and can be extracted by Na2CO3 or heparin. However, the release of this membrane-associated 35S-SecA is translocation-independent. The other hand, a significant fraction of 35S-SecA remains on the membranes even after incubation with excess nonradioactive SecA. This fraction of 35S-SecA is integral, since it was found associated with membranes after flotation centrifugation and was also resistant to Na2CO3 extraction. After extraction with Na2CO3 or heparin, the 35S-SecA remaining on the reconstituted membranes was as active as the SecA remaining on native membranes. Proteolysis revealed that a 48-kDa domain is constantly embedded in the membranes regardless of protein translocation status. Together, these results indicate that a significant fraction of SecA is persistently embedded in the membrane and, as such, is active in protein translocation. Therefore, SecA cycling on and off membrane is not essential for protein translocation.

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This paper is dedicated to the memory of Henry Wu.

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**EXPERIMENTAL PROCEDURES**

*Bacteria Strains—* CK1801 (35), an unc- mutant of MC4100 (F
lacU169 araD136 relA rpsL150 [FbsB301 deoC7 ppsF25 thi] ) was from C.
Kumamoto. BeU1 and BA13 were from D. Oliver. CK1801,1 and BA13 was a secA13(am) supF(ts) mutant of MC4100 (11). In both CK1801 and CK1801.4, the structural genes encoding the F.F.P.
ATPase are deleted. BA13/pM103 was obtained by transforming the BA13 strain with an OmpA plasmid pM103 from M. Nowy.

Buffers and Media—

- **DTK buffer (10 mM Tris-HCl, pH 7.6, 50 mM KCl, and 10 mM Mg(OAc)2):**
- **DTKM buffer:** The mixture first, and then distributed into individual tubes (100 μl each). Translocation assays were carried out at 37 °C for 15 min unless otherwise indicated. After incubation, the reaction mixtures were chilled on ice, treated with 0.2 mg/ml proteinase K on ice for 15 min (no difference of translocated OmpA was observed with up to 1 mg/ml proteinase K). Stop solution (7.7 ml) was added to stop the protein digestion. The membranes were recovered by sedimentation at 95,000 rpm for 20 min through a 0.2-ml sucrose cushion, and were analyzed by SDS-PAGE and autoradiography. Translocation efficiency was calculated from the amount of protease-resistant pOmpA plus OmpA, or OmpA alone when the protease treatment was omitted. The radioactivity of pOmpA was corrected for the loss of one [35S]Met residue in OmpA. For translocation with purified pOmpA, 2 μg (1–2 μl) of purified [35S]-pOmpA in 8 μl area was diluted into 85 μl of translocation buffer containing 5 μg of SecB (and SecA or other factors where indicated); then, 30 μg of membrane vesicles were added. Translocation reactions were performed as above. [35S]-SecA and [35S]-OmpA bands on gel were quantified either by a Phosphorimage (Fuji BAS-1000) or by scanning densitometry using a PDI Image Analysis System (Perkin-Elmer Cetus, Norwalk, CT). Proteins in solution or in membranes were quantitated by Bradford (40) reagent from Bio-Rad using γ-globulin as a standard.

Analysis of SecA Release by Filtration Method—The release of SecA from the membrane was also determined with a MultiScreen assay system (Millipore), which consists of a standard 90-μm microporous filter plate with a sealed microporous membrane bottom and a vacuum manifold. After preliminary tests, we chose a 0.22-μm hydrophilic polyvinylidene difluoride, low protein-binding Durapore plate in the experiments. More than 80% of the membrane was retained by the polyvinylidene difluoride filter membrane, whereas less than 5% of free SecA was retained. Reconstituted membrane vesicles were incubated in 50 μl of reaction mixture containing 3 μg of membrane vesicles and various factors. At the end of incubation, free [35S]-SecA was separated from membrane-associated [35S]-SecA within 1 min by applying vacuum to the system. The filter bottoms containing the membrane-associated [35S]-SecA were then punched out, and counted for radioactivity in a Beckman LS 6500 scintillation counter. All samples were assayed in triplicate.

Biochemicals—

- **DTT, dithiothreitol; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; IPTG, isopropyl-1-thio-
β-n-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; AMP-PCP, adenylyl-(β,γ-methylene)diphosphonate; TK buffer, Tris-
KCl buffer.**

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2 The abbreviations used are: DTT, dithiothreitol; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; IPTG, isopropyl-1-thio-
β-n-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; AMP-PCP, adenylyl-(β,γ-methylene)diphosphonate; TK buffer, Tris-
KCl buffer.

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purified \(^{35}\)S-SecA (50 \(\mu\)g) with SecA-depleted membranes (0.5 mg) at 30 °C for 5 min. About 15 \(\mu\)g of \(^{35}\)S-SecA was associated with the recovered membrane vesicles. We used an unc-CK1801.4 strain in our experiments to eliminate the potential effects of F\(_{1}\)-ATPase that has been reported to restore translocation activity of urea-inactivated membranes (14). Membranes from SecA-depleted CK1801.4 strain contained little SecA (data not shown) and were inactive in translocation of nascent proOmpA without exogenous SecA (Fig. 1, lanes 1 and 2). On the other hand, the reconstituted membranes reached maximum translocation efficiency without additional SecA (Fig. 1, lanes 3 and 4).

To examine the effects of translocation components on the release of \(^{35}\)S-SecA from the membranes, reconstituted membranes were incubated either in a post-translational supernatant containing nascent pOmpA or in translocation buffer under various conditions (Fig. 2A). No significant release of \(^{35}\)S-SecA was observed when the reconstituted membranes were incubated without nonradioactive SecA (Fig. 2A, lanes 1–3 and 7–11). Inclusion of 10 \(\mu\)g of nonradioactive SecA in the reaction mixture “chased” 50–60% of \(^{35}\)S-SecA from the membranes (Fig. 2A, lanes 4 and 5 and lanes 12 and 13). Such replacement by nonradioactive SecA occurred even on ice, where no translocation took place (Fig. 2A, lanes 4 and 12). The post-translational supernatant containing nascent precursor proteins had little effects on \(^{35}\)S-SecA release (compare lanes 1–5 with lanes 8–13), suggesting that the release process is independent of protein translocation.

The conventional sedimentation method used above includes a dilution step (to reduce the nonspecific binding) and a 15-min centrifugation (to collect the membrane vesicles). The dilution and the centrifugation time may have affected the \(^{35}\)S-SecA binding and release. To avoid such concerns, a vacuum filtration method was used for rapid separation of the free \(^{35}\)S-SecA and the membrane-associated \(^{35}\)S-SecA. After incubation with translocation buffer containing the energy sources in a MultiScreen assay system (see “Experimental Procedures”), the membranes were quickly collected by applying vacuum to the system. \(^{35}\)S-SecA remains on the filter only if it binds to the membrane vesicles. First, the kinetics of \(^{35}\)S-SecA binding and release were examined. Both \(^{35}\)S-SecA binding and \(^{35}\)S-SecA release occurred rapidly at 1 min and reached equilibrium within 5 min at 30 °C (Fig. 2B). The dissociation constant and the maximum binding for \(^{35}\)S-SecA were estimated as 72 nM and 82 pmol (as dimer)/1 mg of membrane protein, respectively, close to those obtained with the sedimentation method (data not shown; Ref. 15). Next, the effects of translocation components on the \(^{35}\)S-SecA release were examined with this filtration method (Fig. 2C). The results were similar with those obtained with the centrifugation method (Fig. 2A). These data demonstrate that both the centrifugation and the filtration methods can be used to quantitate the amount of membrane-associated SecA. However, to study the release of SecA during protein translocation, both translocation and SecA release must be monitored simultaneously. This can only be achieved with centrifugation followed by SDS-PAGE. Thus, centrifugation was used to collect membranes after translocation in most of the following experiments, repeating some experiments with the filtration method, as needed.

If cycling of SecA between membrane and cytoplasm is an obligatory step for protein translocation, the release of \(^{35}\)S-SecA from reconstituted membranes would be expected to show some kind of correlation to the protein translocation. However, the results in Fig. 2 suggest that release of SecA is not related to protein translocation. To investigate further the relations between SecA release and protein translocation, we examined the kinetics of release of the membrane-associated \(^{35}\)S-SecA.
during translocation of pOmpA using our standard in vitro translation/translocation assay system (37). The reconstituted membranes were incubated at 37 °C for different periods in post-translational supernatant containing nascent pOmpA. While the amount of translocated OmpA increased with time, the amount of membrane-associated 35S-SecA remained essentially unchanged throughout the same period (Fig. 3). A similar release curve was observed even when the incubations were carried out in translocation buffer without pOmpA. These data reinforce the notion that SecA release is independent of protein translocation.

It is possible that the amount of nascent pOmpA in the reaction mixture might be too little to bring a significant amount of 35S-SecA off the membranes. To amplify the effects of translocation on SecA release, we employed an in vitro translocation system using chemical amount of purified 35S-pOmpA renaturing from urea solution (41). This in vitro assay system used 2 μg of purified 35S-pOmpA, 5 μg of SecB, and 30 μg of membranes containing 1 μg of 35S-SecA. If one 35S-SecA molecule/translocated 35S-pOmpA molecule cycles off the membranes and the translocation efficiency is 50%, the amount of translocated 35S-SecA (1 μg or 28 pmol) should be sufficient to bring all 35S-SecA (1 μg or 5 pmol as a dimer) off the membranes. This may be an underestimate, however, since it has been proposed that one cycle of SecA insertion and deinsertion, therefore, on and off the membranes, moves only 20–30 amino acid residues (42, 43). If this is the case, translocation of one molecule of OmpA would require 12–18 SecA molecules to cycle on and off the membranes. Fig. 4 shows the kinetics of release of the membrane-associated 35S-SecA with chemical amount of purified 35S-pOmpA. A, the reconstituted membranes were added to translocation buffer containing the energy source with (+ pOmpA, lanes 1–6) or without (− pOmpA, lanes 8–13) 35S-pOmpA. After incubation at 37 °C for the times indicated, the membranes were isolated immediately by centrifugation without proteinase K treatment (lanes 1–4 and 8–11) or after proteinase K (0.5 mg/ml) treatment in the absence (lanes 5 and 12) or presence (lanes 6 and 13) of 1% Triton X-100 (TX-100). The isolated membranes were analyzed by SDS-PAGE and autoradiography. Lane 7 contains 1 μg of 35S-SecA and 0.4 μg of 35S-pOmpA. The positions of the precursor and mature forms of OmpA as well as SecA were indicated. B, same as A except that 10 μg of nonradioactive SecA was added to the translocation reaction mixtures to chase the membranes-associated 35S-SecA. C, quantitation of 35S-SecA and 35S-pOmpA bands in A (open symbols) and B (closed symbols). The amount of 35S-SecA and 35S-OmpA was calculated using those in lane 7 as a standard.
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These data suggest that a significant fraction of the membrane-associated $^{35}$S-SecA in the reconstituted membranes cannot be chased. To avoid a possible deleterious effect that high concentrations of SecA (up to 500 μg/ml) may have on the translocation assay, we took advantage of the filtration method that allows an efficient recovery of even a small amount of membranes. The amount of membranes was reduced to 1/10 to examine the $^{35}$S-SecA release during protein translocation in the presence of 50-fold excess nonradioactive SecA. As with the centrifugation method (Fig. 4C), similar release curves were obtained in the presence or absence of pOmpA (Fig. 5B). We conclude that release of the membrane-associated SecA is indeed independent of protein translocation. More importantly, a significant fraction of the membrane-associated SecA does not cycle off the membranes.

Since F$_1$F$_0$-ATPase has been reported to restore the translocation activity of urea-washed membranes (14), we have performed similar experiments with $^{35}$S-SecA-membranes reconstituted from $^{3}$H-SecA and SecA-depleted membranes from BA13 strain, which contains F$_1$F$_0$-ATPase. Results were similar to those obtained with the reconstituted $^{35}$S-SecA-CK1801.4 membranes (data not shown), indicating that the presence or absence of F$_1$F$_0$-ATPase has little effect on the preceding observations. Collectively, these data showed that there are two different populations of SecA on the membranes, loosely associated and tightly associated. The former set can be released and replaced by cytosolic SecA in a translocation-independent manner. It is likely that the latter set is permanently integrated into the membranes. Alternatively, this observation could be due to denatured aggregates that co-sediment with the membranes.

Integral SecA Does Not Cycle Off the Membrane during Protein Translocation—To examine the possibility that the tightly associated $^{35}$S-SecA is simply denatured aggregates that co-sediment with the membranes, a flotation experiment was performed. After translocation reaction in the presence of excess nonradioactive SecA, reconstituted membranes were isolated, resuspended in TK buffer, and subjected to the flotation centrifugation. Under the conditions described previously (44), the membranes float, while the soluble proteins and protein aggregates remain at the bottom of the metrizamide gradient according to their densities. Translocated $^{35}$S-OmpA was used to probe the position of membranes. As shown in Fig. 6A, more than 90% of $^{3}$H-SecA and $^{35}$S-OmpA (thus, membranes) were found in the top three fractions, whereas the soluble $^{35}$S-SecA or denatured $^{35}$S-pOmpA aggregates were recovered in the bottom three fractions. These
data showed that the $^{35}$S-SecA remaining on the membranes after the chase was truly associated with the membranes, since it was retained with the membrane fractions, while soluble proteins and protein aggregates were not.

It is most likely that the $^{35}$S-SecA remaining in the membrane fraction is integrated into the membrane. One criterion for such an integral membrane protein is the resistance to Na$_2$CO$_3$ extraction (45), which was therefore undertaken using treatment with TK buffer as a control. Reconstituted membranes were incubated with excess nonradioactive SecA, reisolated by centrifugation, and then incubated in TK or 0.1 M Na$_2$CO$_3$ on ice for 30 min. After the incubation, membranes were isolated and analyzed by SDS-PAGE. Most of the $^{35}$S-SecA that remained on the membranes after the chase was resistant to extraction with 0.1 M Na$_2$CO$_3$ (Fig. 6B). Similar results were obtained with higher concentrations of Na$_2$CO$_3$ (0.15 and 0.2 M). When the reconstituted membranes were extracted prior to the chase, most of the $^{35}$S-SecA on the Na$_2$CO$_3$ extraction (45), which was therefore undertaken using TK extraction as a control. The reconstituted membranes and the native membranes was similar, indicating that the SecA remaining on the membranes after Na$_2$CO$_3$ extraction is resistant to chase and vice versa. Thus, this fraction of SecA is integrated into the membranes and does not cycle off the membrane during protein translocation.

**Integral SecA Is Functionally Active in Protein Translocation**—Since reconstituted membranes washed with Na$_2$CO$_3$ leaves only integral SecA in the membranes, these membranes were used in translocation assays to examine the translocation activity of the integral SecA. The translocation activities of SecA (molar ratio of translocated pOmpA over SecA dimer) on the Na$_2$CO$_3$- or TK-treated membranes were compared (Fig. 7A). $^{35}$S-SecA on the reconstituted membranes was found as active as SecA on native membranes derived from the parental CK1801 strain. After Na$_2$CO$_3$ extraction, SecA on both membranes lost about two-thirds of their original activities. Nevertheless, each integral SecA dimer was still capable of translocating, on average, up to 4 molecules of OmpA. These data show that though Na$_2$CO$_3$ treatment does partially inactivate SecA, the SecA retained on the membranes remains active in promoting protein translocation.

Watanabe and Blobel (14) reported that peripheral SecA could be specifically stripped from membrane vesicles by poly-acanionic heparin, and such heparin-extracted membrane vesicles were as active as control membrane vesicles. The heparin-extraction was performed with our reconstituted membranes using TK extraction as a control. The reconstituted membranes lost half the activity and a corresponding amount of SecA by the heparin extraction, such that the translocation activity per SecA molecule on the membranes after TK or heparin extraction was similar (Fig. 7B). Less SecA was extracted by heparin from native membranes derived from CK1801, and so was the loss of the translocation activity of these membranes. Thus, the translocation activities of the native membranes after TK or heparin extraction were also similar when normalized to the amount of SecA remaining on the membranes (Fig. 7B). These data indicate that, unlike Na$_2$CO$_3$ treatment, heparin treatment does not inactivate SecA. Extraction experiment showed that SecA on heparin-extracted membranes was resistant to further Na$_2$CO$_3$ extraction, as were SecY and SecE (Fig. 7C), indicating that SecA in the heparin-extracted membranes is integrated into the membranes. After heparin extraction, the translocation activity per SecA molecule on both the reconstituted membranes and the native membranes was similar, indicating that the integrated $^{35}$S-SecA was as active as the integral SecA in the native membranes. On the other hand, the integral SecA appears to be less active compared with SecA on untreated membranes where 8 molecules of pOmpA were translocated per SecA dimer in 15 min (compare Figs. 7 and 4). This apparent loss of translocation activity is probably due to repeated resuspensions and centrifugations. The translocation activity per molecule of SecA is similar for both heparin- and TK-treated membranes (Fig. 7B), suggesting the integral SecA is at least as active as the whole SecA populations on the membranes. Thus, we conclude that integral SecA is functionally active in protein translocation.
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FIG. 8. Protease accessibility of membrane-associated SecA. A, reconstituted membranes (20 μg) were incubated in translocation buffer under the conditions indicated. After 15 min of incubation at 37 °C, samples were chilled in ice bath, digested with freshly made proteinase K at 1 mg/ml for 15 min, and were stopped by adding 0.7 ml of stop solution. The membranes were collected by centrifugation at 95,000 rpm for 20 min in a Beckman TLA 100.2 rotor through a 0.2-ml cushion, and analyzed by SDS-PAGE and autoradiography (lanes 1–6). The upper two-thirds of the centrifuge supernatant were precipitated with 8% trichloroacetic acid, rinsed with 1 ml of ice-cold acetone, dried, dissolved in 30 μl of sample buffer, and analyzed by SDS-PAGE and autoradiography (lanes 7–12). BSA were omitted in translocation assays because it has significant protective effects. The positions of molecular size markers as well as the intact SecA are indicated. The 36-kDa fragment in the supernatant fraction appeared as an diffused band because it overlapped with the huge band of proteinase K.

DISCUSSION

We have shown that there are two populations of SecA present on reconstituted membranes, loosely associated and tightly associated. Loosely associated SecA probably binds to membrane through ionic interaction between SecA and phospholipids. As a result, exchange of this loosely associated SecA is translocation-independent and such SecA could be stripped away from the membranes by the polyanionic heparin. Binding of SecA to the membrane is a spontaneous process, which can occur even on ice without ATP (Fig. 2A, and Ref. 15). The tightly associated SecA might become inserted into the membrane. This possibly occurs through interactions with phospholipids, SecYEG (15, 17–21), or other proteins so that it becomes resistant to extraction by heparin or Na₂CO₃. Our data are consistent with evidence reported by Cabelli et al. (30), who showed that in membranes isolated by chromatography in buffer, one-third of the membrane-associated SecA could not be extracted by Na₂CO₃. In the current studies, the integral nature of this Na₂CO₃-resistant SecA was further confirmed and extended by both its resistance to replacement with excess nonradioactive SecA under translocation condition, and its association with membrane vesicles during flotation centrifugation. The amount of integral SecA is about 10 μg (50 pmol of dimers)/1 mg of membrane protein, similar to the amount of SecA present on membranes from the SecA parental CK1801 and wild type D10 strains. Most of SecA on these native membranes is also resistant to Na₂CO₃ and urea extraction, presumably because loosely associated SecA had previously been removed by the membrane preparation procedures, which consist of a series of density centrifugation in buffer containing EDTA (38). It has been suggested that SecA cycles on and off the membrane during protein translocation (24, 31). However, this cycling model lacks direct experimental evidence. Our data show that a significant fraction of functional SecA is embedded in the membrane during protein translocation; therefore, SecA cycling on and off membrane is not essential in protein translocation.

It appears highly likely that soluble SecA can bind to membranes and a fraction becomes integrated, but that, once integrated, SecA cannot be released into a soluble form. This integral SecA might be modified or rearranged to become permanently embedded in membranes. Several different forms of SecA in the cell have been reported (46). It has been reported (24, 25) that a 30-kDa domain of SecA becomes “protease-inaccessible” during protein translocation and that this 30-kDa fragment could become “protease-accessible” again by chasing it with excess nonradioactive SecA. Based on these data, the...
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authors proposed a model in which membrane-associated SecA undergoes cycles of membrane insertion and disinsertion during protein translocation. However, our data do not support this model in our system. Instead, we found more than six SecA fragments after proteolysis of the reconstituted membranes (Fig. 8A). Similar proteolytic patterns were also observed when native membranes (detected by immunoblot) or membranes reconstituted from urea-washed membranes and 35S-SecA were used.3 The 28-kDa fragment observed here may correspond to the 30-kDa fragment documented by Economou and Wickner (24), since it increased in the presence of proOmpA, although the increase was much smaller (<3-fold) than reported. However, the 28-kDa fragment only accounts for a small fraction of total proteinase K-resistant SecA fragments on the membranes, and the majority of the 28-kDa fragment is in the supernatant regardless of translocation status (Fig. 8A). The differences in the proteolytic patterns might come from the different experimental conditions. Economou and Wickner (24) reconstituted their membranes by incubating 125I-SecA with urea-washed membranes on ice in the absence of ATP, and collected membranes after proteolysis by trichloroacetic acid precipitation, which may include soluble protease-resistant SecA fragments. On the other hand, we reconstituted membranes by incubating 35S-SecA with SecA-depleted membranes at 30°C in the presence of ATP, and collected the membranes by centrifugation. However, even when the reconstitution, translocation, proteolysis, and membrane collection were performed following the described procedures (24), all seven SecA fragments were still observed although the 28- and 25-kDa fragments became more prominent.3 The other difference is in the labeling of SecA. We used a uniform [35S]Met labeling in vivo, whereas Economou and Wickner (24) labeled their SecA with 125I in vitro, which labels only surface-accessible tyrosine residues. Since most tyrosine residues will be embedded in the interior of soluble SecA, and the labeling efficiency is calculated as about 1% from the published data (24), it is most likely that only 1 out of the total 25 tyrosine residues in the SecA was labeled. Therefore, only fragments containing this 125I-labeled tyrosine residue became detectable. The 66- and 48-kDa fragments may not contain the labeled tyrosine residues, thus, missing from their proteolytic patterns. However, we have no satisfactory explanation for the different observation on the 28-kDa/30-kDa fragments. It should be noted that in our hand, the 28- and 25-kDa fragments appear to be intrinsically resistant to proteinase K (1 mg/ml) upon the incubation of SecA with ATP in the presence of membranes.

SecA has been proposed to function as a dimer (47, 48). It is possible that integral SecA exists as a dimer in the membranes, since it is active in protein translocation. The presence of the constant 90-kDa and the 48-kDa SecA fragments (Fig. 8A) suggests that integral SecA is deeply embedded in the membrane. Thus, it is likely that there is a fraction of SecA dimer deeply embedded in the membranes that give rise to 90- and 48-kDa fragments upon protease digestion. These domains of SecA may form a part of the protein-conducting channels (33, 34). Indeed, SecA has been shown to be the nearest neighbor of preproteins during translocation (49). Therefore, SecA on the membranes may play a structural role as well as a catalytic role for protein translocation across the inner membrane. The structural role of the SecA may also provide an explanation for the findings that SecY-deficient membranes and SecE-deficient membranes are active in protein translocation in vivo (14, 50, 51).4,5 Watanabe and Blobel (14) have reported that the urea- or heparin-extracted membrane vesicles, which contained only membrane-integral SecA, were fully active in protein translocation in the presence of F1-ATPase. In addition, they showed that reconstituted proteoliposomes from detergent extracts of heparin-extracted membrane vesicles containing only integral SecA but no F1-ATPase were fully active in protein translocation. The significance of this finding, however, has been questioned (33), since the proteoliposomes also lack SecY, an "indispensable" component of the translocation machinery (52). To address these concerns, we carried out heparin extraction with SecA reconstituted membranes and native membranes and confirmed that the extracted membranes are active (Fig. 7). The amount of SecY is not affected by the extraction and there is no F1-ATPase in the membranes. Thus, the translocation activity of these membranes is indeed due to the integral SecA. F1 forms complex with F0 on the wild-type membrane, blocking the proton flow through the F0 channel (53). When F1 is removed, the membranes become permeable to protons (54), possibly resulting in the collapse of proton motive force and loss of translocation activity (55). Restoration of translocation activity of urea-treated membranes by adding back soluble F1-ATPase (14) may occur through restoration of the proton motive force, which helps protein translocation under certain conditions (35, 42, 56, 57). Since we used unc- membranes, the Na+,CO3- and the heparin treatments do not disrupt the proton motive force due to the F1,F0,ATPase. Therefore, F1-ATPase is not required for the translocation activity of the reconstituted membranes in our system. Watanabe and Blobel (14) also found that heparin-treated membranes containing less SecA are as active as the control membranes. However, we found that the translocation activity levels of heparin-treated membranes are lower than the control membranes, but are the same when normalized to the remaining SecA. This difference probably comes from the amount of precursor proteins used in the in vitro systems. The amount of SecA remaining on the membranes after heparin extraction may be sufficient for maximum translocation of the nascent precursor proteins whose amount is usually small. On the other hand, when purified precursors were used, there are usually more precursor molecules than SecA in the in vitro system. As a result, although adding soluble SecA to our reconstituted membranes did not enhance translocation of the nascent proOmpA (Fig. 1), it did increase the translocation efficiency when purified proOmpA was used (Fig. 4). Thus, our data support and extend these previous findings that integral SecA is active. Moreover, since each integral SecA molecule can support several cycles of OmpA translocation, SecA cycling on and off the membrane is clearly not an obligatory step for protein translocation.

Whether or not cytosolic SecA plays an essential role in protein translocation is controversial. SecA has been reported to interact with the precursor proteins (22, 23, 58). Additionally, soluble SecA/SecB complex has been detected in vivo and was shown to be involved in targeting precursor proteins to the membrane (59). On the other hand, proOmpA or proOmpA/SecB complex has been shown to bind to SecA at membranes (15) and the SecA/SecB cascade is not essential for protein translocation, since SecB only binds to a subset of preproteins.

It is also known that soluble SecA is not required for translocation if sufficient SecA is bound to the membranes (11, 14). Recently, Kim et al. (33) have shown that a strain with all its SecA on the membrane displayed normal translocation of preproteins in vivo. Our in vitro data shows that soluble SecA exchanges with the loosely bound SecA regardless of protein translocation status (Figs. 3 and 4). What then, is the function of the cytosolic or free SecA? One possible function of cytosolic

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3 X. Chen and Tai, unpublished observations.
4 J. P. Lian, Y. Yang, and P. C. Tai, manuscript in preparation.
5 Y. Yang, N. Yu, and P. C. Tai, manuscript in preparation.
SecA to shuttle the SecB/precursor from cytosol to membranes. Another known function for the cytosolic SecA is in the repression of its own synthesis (60). SecA has been shown to compete with ribosomes for the ribosome binding site of its own mRNA (61). It has been suggested that the accumulation of preproteins when translocation is impaired could be sensed by soluble SecA, leading to the derepression of SecA synthesis (62, 63). The increased soluble SecA levels, in turn, could compensate for the impaired Sec components, such as mutant SecY (32, 62). Furthermore, SecA shares sequence homology with RNA helicase (64) and binds to a variety of preprotein mRNAs (32, 62). SecA is to shuttle the SecB/precursor from cytosol to membrane and provide BA13, CK1801.4, and CK1801 strains; M. Inouye for the pM103 vector for the impaired Sec components, such as mutant SecY.

Therefore, soluble SecA may play a more important role in the regulation of protein expression.

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