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Dimeric SecA is Essential for Protein Translocation

Don Oliver
Wesleyan University, doliver@wesleyan.edu

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Lucia B. Jilaveanu, Christopher R. Zito, and Donald Oliver*

Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, CT 06459

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SecA facilitates bacterial protein translocation by its association with presecretory or membrane proteins and the SecYEG translocon channel. Once assembled, SecA ATPase undergoes cycles of membrane insertion and retraction at SecYEG that drive protein translocation in a stepwise fashion. SecA exists in equilibrium between a monomer and dimer, and association with its translocation ligands shifts this equilibrium dramatically. Here, we examined the proposal that protein translocation can occur by means of a SecA monomer. We produced a mutant SecA protein lacking residues 2–11, which was found to exist mostly as a monomer, and it was unable to complement a conditional-lethal *secA* mutant, was inactive for *in vitro* protein translocation, and was poorly active for translocation ATPase activity. Furthermore, we developed a technique termed membrane trapping, where wild-type SecA subunits became trapped within the membrane by overproduction of membrane-stuck mutant SecA proteins, and, in one case, a membrane-associated SecA heterodimer was demonstrated. Finally, we examined both endogenous and reconstituted membrane-bound SecA and found a significant level of SecA dimer in both cases, as assessed by chemical crosslinking. Collectively, our results strongly suggest that membrane-bound SecA dimer is critical for the protein translocation cycle, although these results cannot exclude participation of SecA monomer at some stage in the translocation process. Our findings have important implications regarding SecA motor function and translocon assembly and activation.

ATPase | protein oligomerization | protein transport | translocon

Protein translocation across the bacterial plasma membrane by the Sec-dependent pathway is one of the best-characterized cellular protein export pathways. Key components include SecA ATPase, which binds the signal peptide of presecretory proteins or the signal-anchor domains of membrane proteins and associates with the core component of the bacterial translocon, SecYE, the proposed translocation channel (see ref. 1 and references contained within). ATP hydrolytic cycles at SecA are coupled to SecA membrane insertion and retraction cycles at SecYE that have been proposed to be the driving mechanism for translocation of proteins in a stepwise fashion (2, 3). The translocon also contains accessory components: SecG is associated with SecYE and has been implicated in enhancing SecA membrane cycling through its topology inversion, and SecDFyajC is important for efficient protein translocation (4–7).

The oligomeric state of both SecA and SecYEG proteins in their functional forms has been the subject of considerable controversy recently. Low-resolution structures of the SecYEG complex obtained by cryo-electron microscopy, as well as biochemical analysis of this complex in detergent solutions or reconstituted into proteoliposomes, have revealed dimers and tetramers of SecYEG along with the suggestion that the translocation channel may lie at the oligomer interface (see ref. 1 and references contained within). Such findings contradict additional biochemical and structural studies (8–10) indicating that the functional translocation channel may be formed from a SecYEG monomer, where the channel could be located at the interface between the two halves of SecY, which together form a potentially gated, hourglass-shaped structure .

Crystal structures of SecA dimer of *Bacillus subtilis* and *Mycobacterium tuberculosis* have been published previously, along with a recent structure of a *B. subtilis* SecA monomer that may represent an activated state for binding preproteins (11–13). Purified SecA is predominantly a dimer in solution, although the monomer-dimer equilibrium is sensitive to both temperature and salt concentration (14). Initial studies suggested that SecA functions as a dimer based on the maintenance of the dimeric state at the membrane under translocating conditions, as assessed by fluorescence resonance energy transfer and investigation of the *in vitro* protein translocation activity of heterodimers composed of wild-type SecA and 8-azido-ATP-inactivated subunits, where strong dominance effects were observed (15). However, a recent report (16) raised the possibility that SecA may be active as a monomer. In this study, the authors reported that SecA completely dissociated into monomers in the presence of acidic phospholipids or certain detergents and that the protein existed as a monomer when bound to SecYEG. Furthermore, signal peptides were reported to monomerize SecA in solution, although this result has not been subsequently confirmed (17). Finally, these investigators reported a low level of *in vitro* protein translocation activity for a monomeric mutant SecA protein, although they could not rigorously exclude the existence of a low level of SecA dimer. Recent additional studies (9, 17–19) have also reported on the effects of phospholipids and SecYEG binding on altering the SecA monomer-dimer equilibrium.

Given the diverse and sometimes conflicting reports concerning the oligomeric state of SecA *in vivo* and *in vitro*, we set out to study this problem using a combination of genetic and biochemical approaches. Our results show that membrane-bound SecA dimer exists and is required for effective protein translocation.

Materials and Methods

Chemicals. 1-Ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDAC) and Ni^{2+}-conjugated horseradish peroxidase were purchased from Pierce. $Na^{125}I$ was purchased from Amersham Biosciences, whereas the enhanced chemiluminescence kit for Western blotting was purchased from PerkinElmer. Unless otherwise noted, most other chemicals were obtained from Sigma.

Strains and Plasmids. *Escherichia coli* BL21.19 [*secA13(Am) supF(Ts) trp(Am) zch*::*Tn10 recA*::*CAT clpA*::*KAN*] (20) or BL21.20 [*secA13(Am) supF(Ts) trp(Am) zch*::*Tn10 recA*::*KAN*] are derived from BL21(λDE3) (21) and were used as hosts for *secA*-containing plasmids. Plasmids pT7secA2, pT7secA-K108R, and pT7secA-D209N have been described (20). pT7secA-N619-his contains the first 619 codons of *secA* fused in frame with the amino-terminal his-tag in pET28b (Novagen), whereas pT7secA-his or pT7secAΔ11-his contains the *secA* gene or the *secA* gene missing codons 2–11, respectively, fused to a carboxyl-terminal his-tag in pET29b (Novagen). They were constructed by PCR amplification of the appropriate region of *secA* flanked by appropriate restriction sites (NdeI and BamHI for pT7secA-N619-his, and NdeI and XhoI for pT7secA-his and

Abbreviations: EDAC, 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide; IMV, inverted membrane vesicle; IPTG, isopropyl-β-D-thiogalactopyranoside.

*To whom correspondence should be addressed. E-mail: doliver@wesleyan.edu.

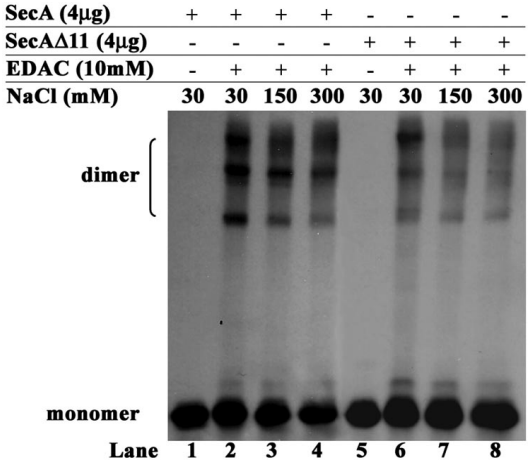

Fig. 1. SecAΔ11-his protein exists primarily as a monomer. Four micrograms (0.8 μM) of purified SecA-his or SecAΔ11-his in 50 μl HKM buffer supplemented with NaCl as indicated was subjected to EDAC crosslinking and analyzed by Western blotting with SecA antiserum.

pT7secAΔ11-his), and, after restriction enzyme digestion, the *secA* fragment was cloned into the corresponding sites of pET28b or pET29b. pT7secAD209N-his and pT7secAΔ11/D209N-his were constructed similarly to pT7secA-his and pT7secAΔ11-his, except using pT7secAD209N as the template for PCR amplification. Plasmid pBB-secA-his is a derivative of the low copy number plasmid pGB2 (22) that contains *secA* with a carboxyl-terminal his-tag under control of the T7 ϕ10 promoter. It was constructed by PCR amplification of the appropriate region of pT7secA-his flanked by XmaI and BamHI sites, and, after restriction enzyme digestion, the *secA* fragment was cloned into the corresponding sites of pGB2. All newly constructed plasmids were verified by DNA sequence analysis. The MC4100 derivative, CK1801.4 [Δ(*uncB-uncC*) *secA13(Am) supF (Ts) trp(Am) zch*::*Tn10*] was used to make inverted membrane vesicles (IMVs).

Preparation of SecA and IMV. *E. coli* SecA protein was purified as described (23). SecA was labeled with $Na^{125}I$ (Amersham Pharmacia) by using the Iodo-gen direct method to a specific activity of 0.5×10^5 to 1.0×10^5 cpm/pmol, as described (24). Urea-treated IMVs were prepared as described (25).

Cell Growth and Subcellular Fractionation. BL21.19 and BL21.20 containing appropriate plasmids were grown in LB supplemented with appropriate antibiotics (100 μg/ml ampicillin, 100 μg/ml spectinomycin, 10 μg/ml chloramphenicol, where needed) at 30°C to an OD_{600} of 0.4 when isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 0.5 mM, where indicated, and the culture was shifted to 40°C for an additional 1 h. Plasmid-bearing BL21 (λDE3) strains were grown similarly with inclusion of 30 μg/ml kanamycin, where needed, but at 37°C without temperature shift. Cells were chilled on ice and harvested by sedimentation at 7,000 $\times$ *g* for 5 min at 4°C. Cell pellets were resuspended in 0.02 vol of ice-cold TKMDP (25 mM TrisOAc, pH 7.5/25 mM KCl/1 mM $Mg(OAc)_2$/1 mM DTT/0.5 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride/100 μM leupeptin/1 μM pepstatin/0.3 μM aprotinin) and broken by two passages at 8,000 lb/in^2 in a French pressure cell. Unbroken cells were removed by two successive sedimentations at 13,000 $\times$ *g* for

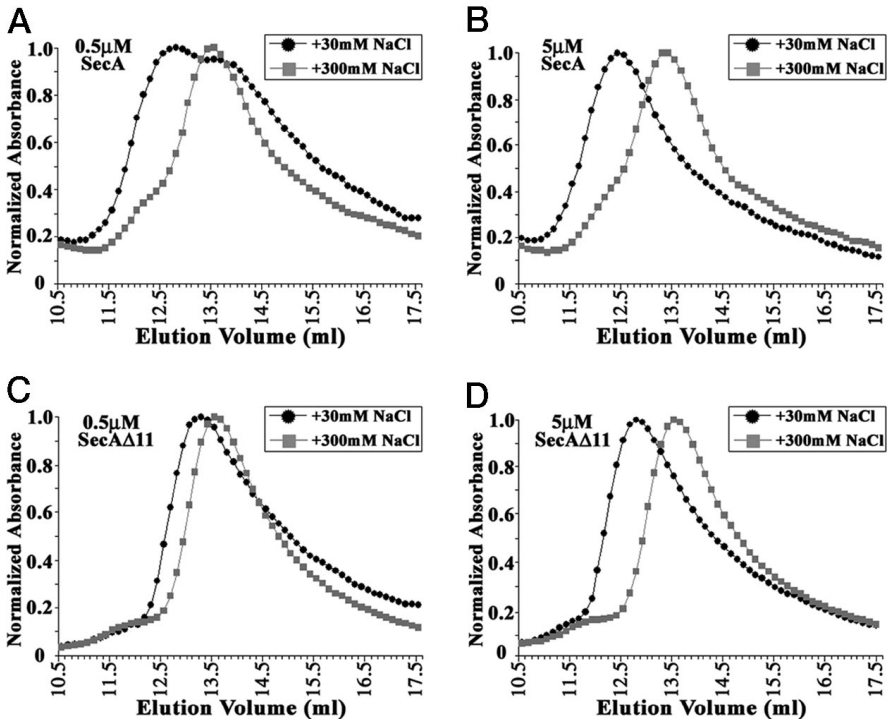

Fig. 2. Size-exclusion chromatographic analysis of SecAΔ11-his. SecA proteins were analyzed by size exclusion chromatography on a Superose 12 10/300 GL column at 4°C in 10 mM Tris, pH 7.5/50 mM KCl/10 mM $Mg(OAc)_2$,/5 mM 2-mercaptoethanol supplemented with NaCl as indicated. Fractions (0.125 ml) were collected at a 0.5 ml/min flow rate and analyzed by absorbance at 280 nm by using a UV detector. Absorbance was normalized for each run to the maximum peak height. (*A*) For 0.5 μM SecA-his, the major peaks correspond to 12.75 ml and 13.5 ml (30 mM NaCl) or 13.5 ml (300 mM NaCl). (*B*) For 5 μM SecA-his, the major peaks correspond to 12.375 ml (30 mM NaCl) or 13.25 ml (300 mM NaCl). (*C*) For 0.5 μM SecAΔ11-his, the major peaks correspond to 13.25 ml (30 mM NaCl) or 13.5 ml (300 mM NaCl). (*D*) For 5 μM SecAΔ11-his, the major peaks correspond to 12.75 ml (30 mM NaCl) or 13.5 ml (300 mM NaCl). Identical peak values were obtained in a duplicate experiment.

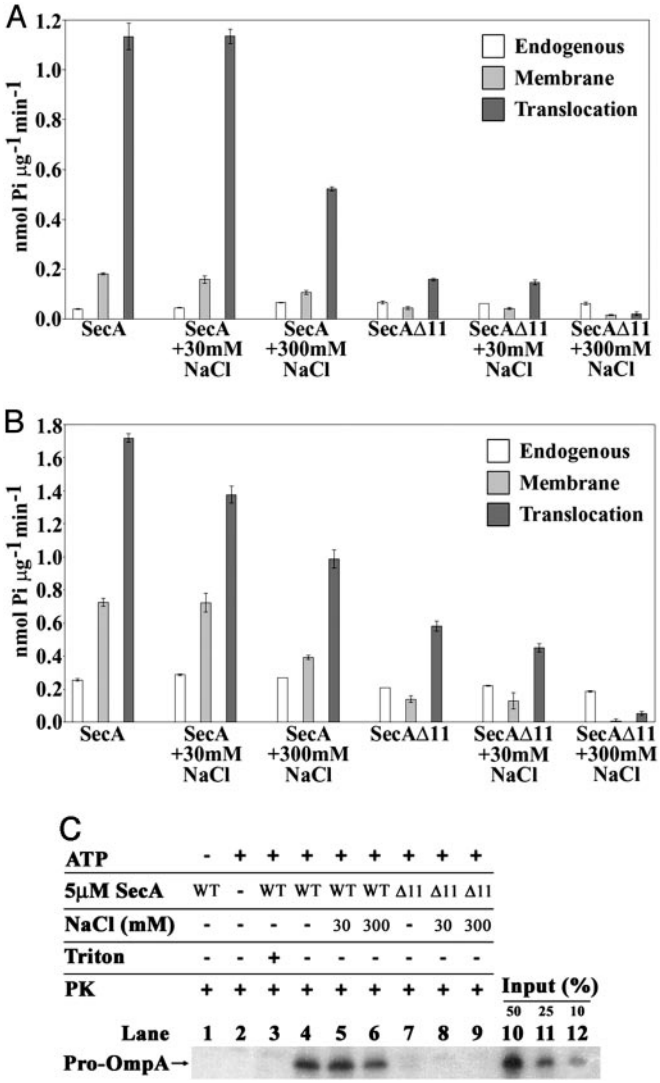

Fig. 3. Translocation ATPase and *in vitro* protein translocation activities of SecAΔ11-his are defective. The endogenous, membrane, and translocation ATPase activities of 0.5 μM (*A*) or 5 μM (*B*) SecA-his or SecAΔ11-his was determined as described except that the incubation time for the 5 μM SecA samples was 2 min (24). (*C*) *In vitro* protein translocation activity of SecA-his or SecAΔ11-his was assessed in the presence of the indicated concentration of NaCl as described (24).

10 min at 4°C, giving rise to the total cleared lysate (Total). Soluble (S300) and membrane (P300) fractions were obtained by sedimentation at 320,000 × *g* for 30 min at 4°C in a Sorvall RC M100 microultracentrifuge. S300 was carefully removed, and the P300 fraction was briefly washed and resuspended in the original volume of TKMD buffer (25 mM TrisOAc, pH 7.5/25 mM KCl/1 mM $Mg(OAc)_2$/1 mM DTT). For analysis of integral membrane proteins, P300 was resuspended in the original volume of 0.2 M sodium carbonate (pH 11.5), or 10 mg/ml heparin or 1 M NaCl and incubated on ice for 30–45 min followed by sedimentation at 320,000 × *g* for 30 min. The supernatant (P300S) was carefully removed, and the pellet (P300P) was resuspended in the original volume of TKMD or HKM (50 mM Hepes-KOH, pH 7.2/50 mM KCl/1 mM $Mg(OAc)_2$).

Crosslinking and Purification on His-Bind Resin. Subcellular fractions, IMV, and purified SecA were subjected to chemical crosslinking with 10 mM or 12 mM EDAC in HKM buffer supplemented with 50 mM KOAc at room temperature for 15 min, and the reactions were quenched on ice by the addition of 50 mM Tris·HCl (pH 7.5), followed by addition of sample buffer (2% SDS/125 mM Tris·HCl, pH 6.8/5% 2-mercaptoethanol/15% glycerol/0.005% bromophenol blue). For isolation of the SecA-SecA-N619-his heterodimer, P300 of BL21(λDE3) (pT7secA-N619-his) (grown with IPTG induction) in HKM buffer supplemented with 50 mM KOAc was crosslinked with 12 mM EDAC, quenched on ice by the addition of 50 mM Tris·HCl (pH 7.5), followed by sedimentation at 320,000 × *g* for 30 min at 4°C. The membrane pellet was solubilized in one-sixth vol of HKM buffer supplemented with 2% digitonin and 10% glycerol by rocking at 4°C for 90 min. Insoluble material was removed by sedimentation at 16, 000 × *g* for 20 min at 4°C, and the supernatant was applied to a 1-ml column of His-Bind resin, and his-tagged protein was isolated as described by the manufacturer (Novagen).

Results

Analysis of a Monomer-Biased SecA Mutant. To directly address the issue of the activity of SecA monomer, we created a *secA* mutant that produced a protein that was shifted substantially in its equilibrium toward the monomer. Examination of the crystal structure of the highly homologous SecA protein from *B. subtilis* showed that the first 10 residues of SecA make substantial intersubunit contacts, contributing to the stability of the dimer. Accordingly, we engineered an *E. coli* his-tagged SecA protein that lacked residues 2–11, SecAΔ11-his, and characterized its oligomeric status.

SecAΔ11-his was overproduced and purified, and its oligomeric status was defined by EDAC crosslinking and gel filtration. Woodbury *et al.* (14) have shown that the SecA monomer-dimer equilibrium is sensitive to both temperature and salt concentration. Comparison of SecAΔ11-his and SecA-his proteins by EDAC crosslinking showed that more of the mutant protein existed as a monomer and that raising the salt concentration shifted the mutant protein largely into a monomer (Fig. 1). EDAC crosslinking has been used previously to monitor for SecA dimer formation (26), and, as noted previously, it produced several distinct species of crosslinked SecA dimer, presumably due to differing crosslinks between carboxyl and amino groups between SecA subunits (16). On Superose 12 chromatography, the two proteins displayed the complex chromatographic behavior of a protein undergoing monomer-dimer transition as described by Woodbury *et al.* (14), where a given SecA peak was often asymmetric and contained a weighted average size of molecules in the population. When 0.5 μM protein was applied to the column, SecAΔ11-his was largely monomeric at either low or high salt, whereas SecA-his was shifted from somewhat more dimer to mostly monomer by increasing salt concentration (Fig. 2 *A* and *C*). When 5 μM protein was applied to the column, the

Table 1. Quantification of SecAΔ11-his growth defect

Strain	Efficiency of plating
BL21.19 (pT7SecAΔ11)	5.5×10^{-5}
BL21.20 (pT7 SecAΔ11/pLysE)	3.5×10^{-6}
BL21.20 (pT7 SecAΔ11/pLysS)	3.4×10^{-6}
BL21.19 (pT7SecA)	1.0
BL21.20 (pT7 SecA/pLysE)	1.1
BL21.20 (pT7 SecA/pLysS)	1.3

Strains were grown overnight in LB supplemented with appropriate antibiotics at 30°C. Each culture was diluted serially, and 100-μl dilutions were plated on pairs of LB plates supplemented with appropriate antibiotics, which were incubated at 30°C or 42°C for 24 h. The efficiency of plating is the ratio of the titer of viable cells at 42°C divided by the titer of viable cells at 30°C for a given strain. All strains gave similar titers of viable cells at 30°C.

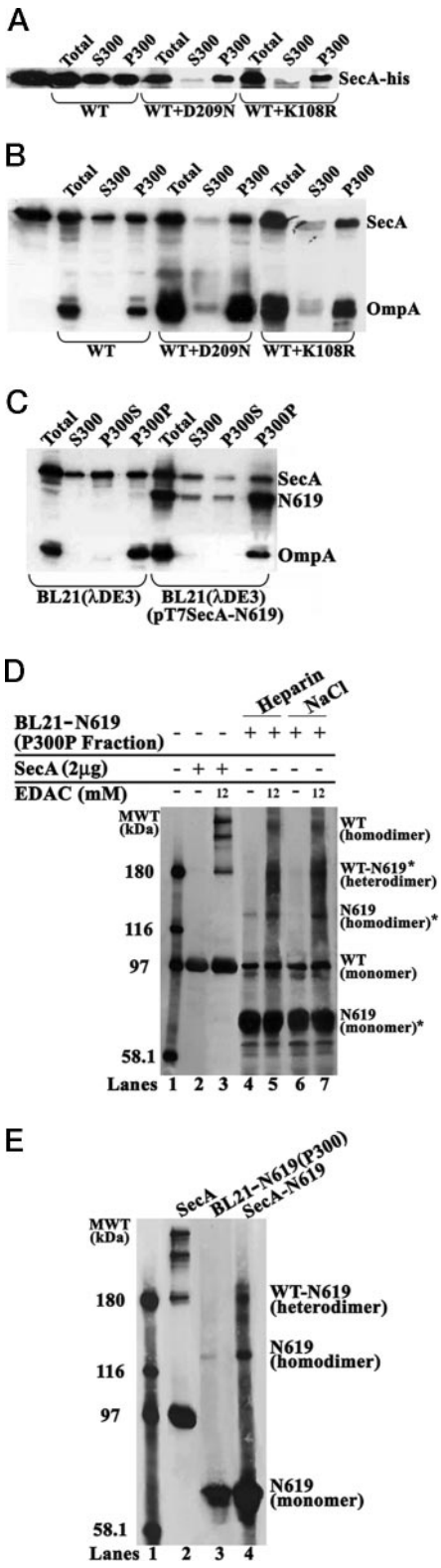

Fig. 4. Membrane trapping of wild-type SecA by overexpression of membrane-stuck mutant SecA. (*A*) Total, S300, and P300 fractions of BL21.19 (pBB-secA-his) (WT) (0.25-ml culture equivalent), BL21.19 (pBB-secA-his, pT7secA-D209N) (WT + D209N) (1.0-ml culture equivalent), or BL21.19 (pBB-secA-his, pT7secA-K108R) (WT + K108R) (1.0-ml culture equivalent) (all grown without IPTG induction) were prepared and subjected to Western blotting with Ni^{2+}-conjugated horseradish peroxidase as described in *Materials and Methods*. Lane 1 contains 1 μg of his-tagged SecA. (*B*) Reprobing of the blot shown in *A* with SecA and OmpA antisera. (*C*) Total, S300, P300S, and P300P fractions of BL21 (λDE3) or BL21 (λDE3) (pT7secA-N619-his) (grown with IPTG induction) were prepared and subjected to Western blotting with SecA and OmpA antisera. A 0.25-ml culture equivalent of each fraction was loaded per lane. (*D*) Two micrograms of his-tagged SecA in 50 μl or a 6-fold concentrated P300P fraction (obtained by heparin or NaCl treatment) of BL21 (λDE3) (pT7secA-N619-his) (grown with IPTG induction) in HKM buffer was subjected to EDAC crosslinking as indicated, followed by Western blotting with SecA antiserum. Nine milliliters of culture equivalent of P300P fraction was loaded per lane. Molecular mass markers are given in lane 1. An asterisk indicates a species that was reactive to Ni^{2+}-conjugated horseradish peroxidase. (*E*) Western blot probed with Ni^{2+}-conjugated horseradish peroxidase. Lane 1, biotinylated molecular mass markers; lane 2, 2 μg of his-tagged SecA crosslinked with 12 mM EDAC; lane 3, 20 μl of 6-fold concentrated P300 fraction of BL21 (λDE3) (pT7secA-N619-his); lane 4, 15 μg of protein (final) from P300 of BL21 (λDE3) (pT7secA-N619-his) that was crosslinked, solubilized, and purified on His-Bind resin as described in *Materials and Methods*.

monomer-dimer equilibrium of both proteins could be manipulated by salt concentration, although the tendency of SecAΔ11-his to exist in a weighted average favoring the monomer was still apparent even at low salt (Fig. 2 *B* and *D*). We conclude that the monomer-dimer equilibrium of SecAΔ11-his has been shifted significantly toward the monomer, and that the protein is largely monomeric at low protein or high salt concentrations.

We next addressed the functionality of SecAΔ11-his both *in vitro* and *in vivo*. Membrane-binding assays showed that the affinity of SecAΔ11-his for SecYEG was normal (Fig. 6, which is published as supporting information on the PNAS web site). ATPase assays of SecAΔ11-his showed that it had normal endogenous (basal) ATPase activity, but its membrane (SecYEG dependent) and translocation (SecYEG and preprotein-dependent) ATPase activities were low: 24% and 14%, respectively, of wild-type values (Fig. 3*A*). To see whether this defect could be suppressed by higher protein concentration (favoring dimerization) or exacerbated at higher salt concentration (favoring monomerization), we conducted ATPase assays at a 10-fold higher protein concentration or in the presence of increasing salt. As expected, increasing the concentration of SecAΔ11-his partially suppressed its membrane and translocation ATPase defects, whereas higher salt exacerbated them (Fig. 3 *A* and *B*). However, even under the optimal conditions, SecAΔ11-his possessed only 33% of the translocation ATPase activity of SecA-his. Furthermore, SecAΔ11-his was defective for pro-OmpA-dependent *in vitro* protein translocation activity even without added salt (Fig. 3*C*). This latter result was consistent with the inability of SecAΔ11-his protein to complement the *secA(Am) supF(Ts)* strain at 42°C whether it was expressed at physiologic or higher levels (Table 1 and Fig. 7*A*, which is published as supporting information on the PNAS web site). The efficiency of plating of the SecAΔ11-his-producing strains was decreased between four and six logs depending on SecAΔ11-his expression level, indicating that the mutant protein was inactive *in vivo*. Finally, fractionation of a SecAΔ11-his-producing strain showed that it contained very little integral membrane SecA protein (Fig. 7*B*). This result is consistent with the presence of SecA dimer in the integral membrane fraction normally (see next two sections below), and it suggests that SecA dimer may be important during the stage of SecA membrane insertion.

Membrane-Trapping of SecA. We sought an additional *in vivo* approach to test for the existence of membrane-bound SecA dimer. We reasoned that, if membrane-bound SecA dimer exists *in vivo*, then certain mutant SecA proteins that are stuck in their membrane-bound state should trap wild-type SecA through heterodimer formation. By contrast, if SecA completely monomerizes when it binds to membranes as has been proposed (16), then no such membrane trapping should occur. Two types of *secA* mutants were used for this purpose. One mutant class

produced SecA proteins defective in ATP-hydrolysis, SecA-K108R or SecA-D209N, which stabilizes SecA in its membrane-inserted state (20, 27). The other mutant produced a truncated SecA protein lacking its C-domain, SecA-N619-his, which remains proficient in SecYEG binding (28, 29). Both mutant SecA proteins have been shown to preferentially accumulate in the integral membrane fraction (28, 30).

To look for SecA membrane trapping, we constructed strains that coexpress his-tagged SecA (from a low copy number plasmid) along with mutant SecA (from a pBR322-derived plasmid) and where chromosomal *secA* expression can be conditionally shut off above 37°C due to the presence of *secA(Am) supF(Ts)* mutations. His-tagged SecA produced alone was found to fractionate normally (Fig. 4*A*). Remarkably, however, when his-tagged SecA was coexpressed with either SecA-D209N or SecA-K108R, it was present almost entirely in the membrane fraction along with mutant SecA (Fig. 4, compare *A* and *B*, which visualize his-tagged SecA and total SecA, respectively). An identical result was obtained when these strains were grown at 30°C, where they are viable (results not shown). To examine whether the amino-terminal dimerization determinant of SecA was required for entry into the SecA membrane pool, we constructed a strain producing solely the SecAΔ11/D209N-his double mutant protein and examined its fractionation properties. Most of this double mutant protein was present in the cytosol with very little peripheral or integral membrane SecA observed (Fig. 7*C*). This result suggests that the amino-terminal dimerization determinant of SecA (and presumably SecA dimer formation) was necessary for membrane binding and integration in this case.

When the strain coexpressing wild-type (chromosomally derived) SecA and SecA-N619-his was examined, membrane trapping was observed as well, where both wild-type SecA and SecA-N619-his were enriched in the integral membrane fraction at the expense of peripheral membrane SecA (Fig. 4*C*, compare P300P and P300S fractions, respectively). We could visualize the putative membrane-bound SecA-SecA-N619-his heterodimer by EDAC crosslinking of the integral membrane fraction that had been treated previously with either heparin or sodium chloride to remove peripheral membrane SecA (31, 32) (Fig. 4*D*). The SecA-SecA-N619-his heterodimer was further identified after solubilization of crosslinked P300 followed by purification on His-Bind resin (Fig. 4*E*). The simplest explanation for the observed membrane trapping of wild-type SecA is through formation of heterodimers with mutant SecA. Whereas the mutant proteins may bias the system regarding the size and stability of the pool of membrane-bound SecA dimer, it seems likely that our results reflect the normal ability of SecA to assume a dimeric state within the membrane. This conclusion is supported also by the need for the amino-terminal dimer determinant of SecA not only for the stability of the soluble pool of SecA dimer, but also for the integral membrane pool of SecA dimer as well.

SecA Dimer Level *in Vivo* and *in Vitro*. Because the results in the two preceding sections ran counter to the conclusions of Or *et al*. (16), who indicated that membrane-bound SecA exists solely as a monomer based in part on EDAC crosslinking data, we decided to reexamine this issue. To avoid artificial results, we used EDAC crosslinking conditions that were less potent than those of Or *et al*. (12 mM versus 20 mM EDAC, respectively, for 15 min at 24°C). When sucrose gradient-purified IMVs were examined, which contain only SecYEG-bound SecA (33, 34), there was an appreciable level of SecA dimer present, although the majority of SecA was probably monomeric as indicated by EDAC titration experiments (Fig. 5*A* and data not shown). SecA dimer was also detected in the integral membrane fraction after treatment of membranes with either heparin or NaCl (Fig. 5*B*),

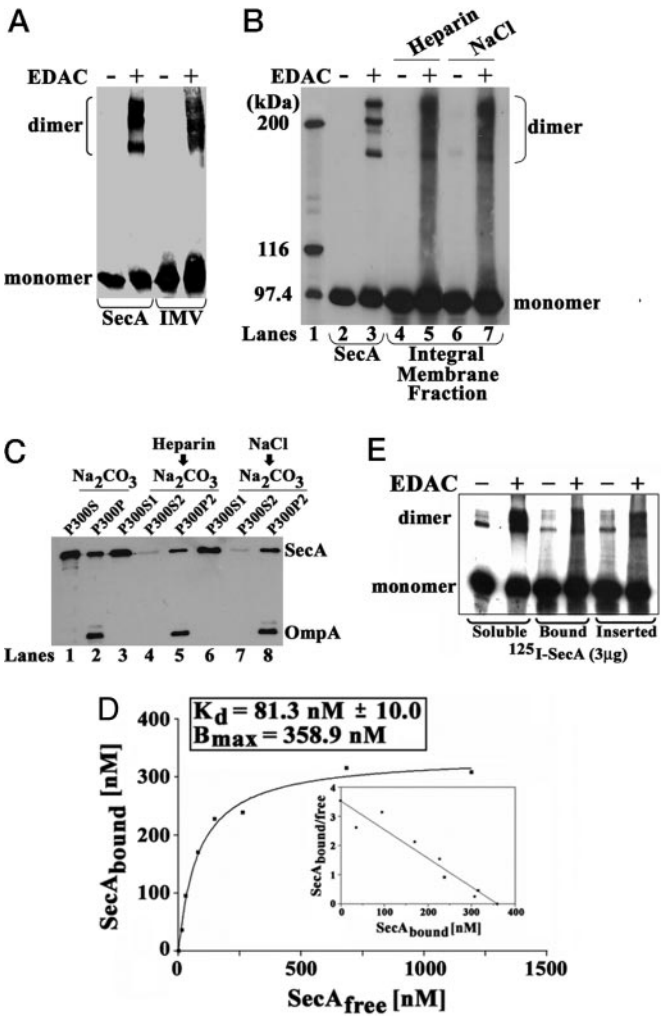

Fig. 5. Detection of membrane-bound SecA dimer. (*A*) Three micrograms of SecA or 80 μg (total protein) of IMV in 50 μl of HKM buffer was subjected to EDAC crosslinking at 12 mM as indicated, followed by Western blotting with SecA antiserum. (*B*) Two micrograms of purified SecA in 50 μl, or a 6-fold concentrated P300P fraction (obtained by heparin or NaCl treatment), of BL21.19 (pT7secA2) in HKM buffer was subjected to EDAC crosslinking at 12 mM as indicated, followed by Western blotting with SecA antiserum. Nine milliliters of culture equivalent of P300P fraction was loaded per lane. (*C*) BL21.19 (pT7secA2) was grown and subjected to subcellular fractionation as described in *Materials and Methods*, and fractions were analyzed by Western blotting with SecA and OmpA antisera. Lanes 1 and 2, peripheral (P300S) and integral membrane (P300P) fractions generated by treatment of P300 with sodium carbonate; lanes 3–8, membrane fractions generated by successive treatment of P300 with either heparin or NaCl (P300S1) followed by sodium carbonate (P300S2), yielding a final integral membrane fraction (P300P2). A 0.25-ml culture equivalent of each fraction was loaded per lane. (*D*) SecA binding curve with Scatchard insert constructed as described (24) except with inclusion of 200-μg membrane protein equivalents of urea-treated IMV in 100 μl that specifically bound up to 3 μg of SecA. SecA concentration is expressed in dimer equivalents. (*E*) Three micrograms of ^{125}I-SecA in solution (Soluble), specifically bound to 200 μg of urea-treated IMV (Bound), or specifically bound and inserted into 200 μg of urea-treated IMV (Inserted) by inclusion of 3 mM adenylyl β,γ-imidodiphosphate (AMP-PNP) and 20 mM sodium azide (24) was subjected to EDAC crosslinking at 12 mM, SDS/PAGE, and autoradiography after drying.

which were as effective as sodium carbonate (pH 11.5) in removal of peripheral membrane SecA (31, 32) (Fig. 5*C*). SecA present in this latter fraction presumably corresponds to molecules that are tightly associated with SecYEG and may have undergone membrane insertion (27, 35).

We also characterized the oligomeric state of SecA that was specifically bound at SecYEG *in vitro*. The amount of SecA needed to get specific binding to urea-treated IMV was first determined (Fig. 5*D*). Next, analysis of specifically bound SecA revealed an appreciable level of SecA dimer (Fig. 5*E*, Bound). However, similar to endogenous SecA in IMV, the majority of membrane-associated SecA was monomeric (Fig. 5, compare *A* and *E*). In this experiment, a minor amount of covalent dimer was present before crosslinking, presumably due to the iodination procedure used. Inclusion of adenylyl β,γ-imidodiphosphate (AMP-PNP) and azide, conditions that promote and stabilize SecA membrane insertion (34), did not significantly change the level of SecA dimer (Fig. 5*E*, Inserted). Whereas Or *et al*. (16) reported that SecYEG-bound SecA was entirely monomeric, their study differs from ours in several important aspects: (*i*) they used SecYEG reconstituted proteoliposomes rather than IMV, (*ii*) they used extremely low SecA concentrations (5 nM) that favor monomerization, and (*iii*) they had a low level of crosslinking under their conditions (16). These differences could account for the inability to detect SecA dimer.

Discussion

Our results indicate that a pool of membrane-bound SecA dimer exists, and that dimeric SecA seems to be required both *in vivo* for cell viability and *in vitro* for efficient protein translocation. These conclusions are supported by analysis of the properties of the monomer-biased SecAΔ11-his protein, by our *in vivo* membrane-trapping studies, and finally by direct demonstration of endogenous and reconstituted membrane-bound SecA dimer by EDAC crosslinking. Although each of these approaches has its limitations, together they make a compelling case for the importance of membrane-bound SecA dimer in protein translocation. Clearly, our results do not exclude the possibility that SecA monomer may participate at some stage in the translocation process as well, or that the monomer and dimer may somehow cycle during the translocation process as has been suggested recently (17). Additional genetic and biochemical approaches that stabilize the SecA dimer and probe its function are certainly warranted. Our findings do have important implications regarding understanding SecA motor function as well as translocon assembly and activation.

Note. While we were writing up this study, three additional papers appeared on the topic. Or *et al*. (36) published an additional paper indicating that SecA may function as a monomer based on analysis of a SecA mutant Δ11/N95 (missing residues 2–11 and 832–901). Although it is difficult to strictly compare our results, given that somewhat different SecA proteins were used and SecA function was often augmented through use of the PrlA4 (SecY) mutant protein, we worry whether the *recA*$^+$ background or the transient depletion conditions used by these investigators may have resulted in the presence of contaminating wild-type SecA. However, both of us found that the Δ11 truncation substantially monomerizes SecA, and that the truncate (either SecAΔ11 or Δ11/N95) has substantially diminished protein translocation activity (e.g., see figures 3*C* and 6*A* of ref. 36), although we clearly differ on the extent of the defect and its interpretation. Our results are more comparable with Randall *et al*. (37), who found that SecAΔ11 is largely monomeric and inactive for *in vitro* protein translocation of either maltose-binding protein or galactose-binding protein precursors. Karamanou *et al*. (38) reported that SecA9-861 (missing residues 2–8 and 862–901) is both dimeric and functional *in vivo* and *in vitro*. From our vantage point, these results suggest that the level of SecA dimer drops significantly in going from SecAΔ8 to SecAΔ11, in agreement with measurements by Randall *et al*. (37) for these two proteins, and they support our conclusion that membrane-integrated SecA dimer plays an important role in the activity of this protein.

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1. Veenendaal, A., van der Does, C. & Driessen, A. (2004) *Biochim. Biophys. Acta* **1694,** 81–95.
2. Economou, A. & Wickner, W. (1994) *Cell* **78,** 835–843.
3. van der Wolk, J. P. W., de Wit, J. G. & Driessen, A. J. M. (1997) *EMBO J.* **16,** 7297–7304.
4. Nagamori, S., Nishiyama, K. & Tokuda, H. (2002) *J. Biochem.* **132,** 629–634.
5. Nishiyama, K.-I., Suzuki, T. & Tokuda, H. (1996) *Cell* **85,** 71–81.
6. Duong, F. & Wickner, W. (1997) *EMBO J.* **16,** 4871–4879.
7. Pogliano, J. A. & Beckwith, J. (1994) *EMBO J.* **13,** 554–561.
8. Yahr, T. L. & Wickner, W. T. (2000) *EMBO J.* **19,** 4393–4401.
9. Duong, F. (2003) *EMBO J.* **22,** 4375–4384.
10. van den Berg, B., Clemons, W. M., Collinson, I., Modls, Y., Hartmann, E., Harrison, S. C. & Rapoport, T. A. (2003) *Nature* **427,** 36–44.
11. Hunt, J. F., Weinkauf, S., Henry, L., Fak, J. J., McNicholas, P., Oliver, D. B. & Deisenhofer, J. (2002) *Science* **297,** 2018–2026.
12. Sharma, V., Arockiasamy, A., Ronning, D. R., Savva, C. G., Holzenburg, A., Braunstein, M., Jacobs, W. R. & Sacchettini, J. C. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 2243–2248.
13. Osborne, A. R., Clemons, W. M. & Rapoport, T. A. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 10937–10942.
14. Woodbury, R. L., Hardy, S. & Randall, L. (2002) *Protein Sci.* **11,** 875–882.
15. Driessen, A. (1993) *Biochemistry* **32,** 13190–13197.
16. Or, E., Navon, A. & Rapoport, T. (2002) *EMBO J.* **21,** 4470–4479.
17. 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. & Hunt, J. F. (2003) *J. Biol. Chem.* **278,** 3628–3638.
18. Bu, Z., Wang, L. & Kendall, D. (2003) *J. Mol. Biol.* **332,** 23–30.
19. Tziatzios, C., Schubert, D., Lotz, M., Gundogan, D., Betz, H., Schagger, H., Hasse, W., Duong, F. & Collinson, I. (2004) *J. Mol. Biol.* **340,** 513–524.
20. Mitchell, C. & Oliver, D. B. (1993) *Mol. Microbiol.* **10,** 483–497.
21. Studier, W. F., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990) *Methods Enzymol.* **185,** 60–89.
22. Churchward, G., Belin, D. & Nagamine, Y. (1984) *Gene* **31,** 165–171.
23. Ding, H., Hunt, J. F., Mukerji, I. & Oliver, D. (2003) *Biochemistry* **42,** 8729–8738.
24. Zito, C. R. & Oliver, D. (2003) *J. Biol. Chem.* **278,** 40640–40646.
25. Rhoads, D., Tai, P. C. & Davis, B. (1984) *J. Bacteriol.* **159,** 63–70.
26. Hirano, M., Matsuyama, S. & Tokuda, H. (1996) *Biochem. Biophys. Res. Commun.* **229,** 90–95.
27. Economou, A., Pogliano, J. A., Beckwith, J., Oliver, D. B. & Wickner, W. (1995) *Cell* **83,** 1171–1181.
28. Dapic, V. & Oliver, D. (2000) *J. Biol. Chem.* **275,** 25000–25007.
29. Vrontou, E., Karamanou, S., Baud, C., Sianidis, G. & Economou, A. (2004) *J. Biol. Chem.* **279,** 22490–22497.
30. Rajapandi, T. & Oliver, D. (1996) *Mol. Microbiol.* **20,** 43–51.
31. Watanabe, M. & Blobel, G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9011–9015.
32. Cabelli, R. J., Dolan, K. M., Qian, L. & Oliver, D. B. (1991) *J. Biol. Chem.* **266,** 24420–24427.
33. Hartl, F.-U., Lecker, S., Schiebel, E., Hendrick, J. P. & Wickner, W. (1990) *Cell* **63,** 269–279.
34. Eichler, J., Rinard, K. & Wickner, W. (1998) *J. Biol. Chem.* **273,** 21675–21681.
35. Ramamurthy, V. & Oliver, D. (1997) *J. Biol. Chem.* **272,** 23239–23246.
36. Or, E., Boyd, D., Gon, S., Beckwith, J. & Rapoport, T. A. (2004) *J. Biol. Chem.* **280,** 9097–9105.
37. Randall, L., Crane, J., Lilly, A., Liu, G., Mao, C. & Hardy, S. (2005) *J. Mol. Biol.* **348,** 479–489.
38. Karamanou, S., Sianidis, G., Gouridis, G., Pozidis, C., Papanikolau, Y., Papanikou, E. & Economou, A. (2005) *FEBS Lett.* **279,** 1267–1271.