CHARACTERIZATION OF MEMBRANE-ASSOCIATED SECA

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Master of Science

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by
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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

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and hereby certify that in their opinion it is worthy of acceptance.

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CHARACTERIZATION OF MEMBRANE-ASSOCIATED SECA

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ABSTRACT

Proteins are synthesized in the cytoplasm of bacterial cells and approximately 30 percent of these are exported across the inner membrane and are integrated into the inner membrane or reside in the periplasm or the outer membrane. One system of protein export is the general secretory system which exports precursor proteins through the transmembrane protein complex SecYEG that acts as the selective pore in the inner membrane through which the ATPase SecA translocates the substrate proteins. We have developed an *in vitro* translocation assay where purified SecYEG and SecA are reconstituted simultaneously into proteoliposomes. These proteoliposomes have six times as many active translocons as proteoliposomes that are reconstituted with only SecYEG and have an equivalent number of active translocons as are found in inverted membrane vesicles. This indicates that the active state of SecYEG is preserved during reconstitution into proteoliposomes in the presence of SecA. Quantifying the number of active translocons in both the proteoliposomes and inverted membrane vesicles with two different precursor proteins revealed that an active translocon with precursor galactose-binding protein has twice as many SecYEG trimers as when the precursor protein is the precursor of outer membrane protein A. In our studies we have described for the first time that the stoichiometry of SecYEG in translocation is dependent on the precursor species; twice as many SecYEG heterotrimers are required to translocate pGBP than are required for proOmpA.

We investigated the nature of the interaction between SecA and the membrane using mutant SecA species that lacked the N-terminal ten residues, the C-terminal twenty-one residues, or that lacked the coordinated zinc in the C-terminal twenty-one residues. We observed that SecA species that have the N-terminal ten residues had a higher affinity for the membrane than the SecA deletion mutant SecAdN10 that lacks the ten N-terminal residues. We also identified a role for the N-terminal ten residues of SecA in the translocation process. SecAdN10 is deficient in a step of the translocation process where a 2-kDa segment of the precursor protein is translocated through SecYEG. This deficiency is abolished if a SecA species that contains the N-terminal ten residues is added, and when SecA species that contain the N-terminal ten residues are added to assays with wild-type SecA the rate of the 2-kDa translocation step increases.

Chapter 1

Introduction

The General Secretory System

Transport of proteins across biological membranes is a ubiquitous process that occurs in all domains of life. Proteins may be transported across membranes in their folded or unfolded forms through various translocon pores or in secretory vesicles in and out of different cellular compartments. In eubacteria all proteins are synthesized by ribosomes in the cytoplasm of the cell, yet the final destination of certain proteins can be the inner or outer membrane, or the periplasmic space between the two. To reach these destinations a protein must be inserted into or transported across the inner membrane—a highly unfavorable event energetically. To minimize the amount of energy required for export across the membrane in the secretory (Sec) system, proteins are translocated in unfolded form. Translocating proteins in unfolded form allows for an export pathway that can be used for both membrane and soluble proteins, and proteins of different size and shape. There are homologues of SecYEG in eukaryotes and archaea and these have similar interactions with the motor during translocation—be it the ribosome or SecA (Mandon et al. 2009).

Protein Translocation

Certain proteins, referred to here as precursor proteins, contain a leader peptide sequence that retards folding of the protein which is to be exported by the secretory system. This signal sequence is cleaved by the inner membrane protease leader peptidase after translocation and then the exported protein is integrated into the inner or outer membrane or the periplasm. The secretory system is composed of three main components that accomplish the work of exporting entire precursor proteins across the inner membrane of a bacterial cell. SecYEG is a transmembrane protein complex that resides in

the bacterial inner membrane and functions as a pore through which the precursor protein is exported. SecA binds SecYEG, the precursor, and SecB, and utilizes ATP hydrolysis to power the translocation of the precursor through SecYEG. SecB is a small cytosolic protein that binds the unfolded precursor and SecA (with or without bound precursor), stimulates SecA-dependent precursor translocation and is required for the translocation of certain precursors both *in vivo* and *in vitro* (Powers and Randall 1995). A cartoon of the secretory system is found in Figure 1.

SecYEG

SecYEG is a heterotrimeric transmembrane protein complex. SecY, the largest protein in the complex, has a molecular weight of 48 kDa and SecE and SecG have molecular weights of 14 and 12 kDa respectively. SecY has long cytosolic loops that interact with SecA (Zimmer et al. 2008) and a cross-linking study has shown that SecG interacts through its cytosolic loops with SecA (Das and Oliver 2011). One of the more controversial questions in the secretory system is the oligomeric state of SecYEG. There is evidence for a dimer of SecYEG heterotrimers in the presence of cardiolipin by Blue Native Gel Electrophoresis (Gold et al. 2010) and column chromatography of detergent-solubilized SecYEG (Randall, L.L. unpublished observation), but opinions differ on whether or not oligomeric states of SecYEG are involved in translocation across a lipid bilayer. In one study dimerization-defective SecYEG mutants had no deficiencies in translocation activity or cell phenotype, although it is possible that short-lived oligomeric states of SecYEG could have been present (Park and Rapoport 2012). In another study a SecY mutant that does not dimerize has been shown to be inactive for translocation; thus dimerization may be necessary to induce a conformational change to

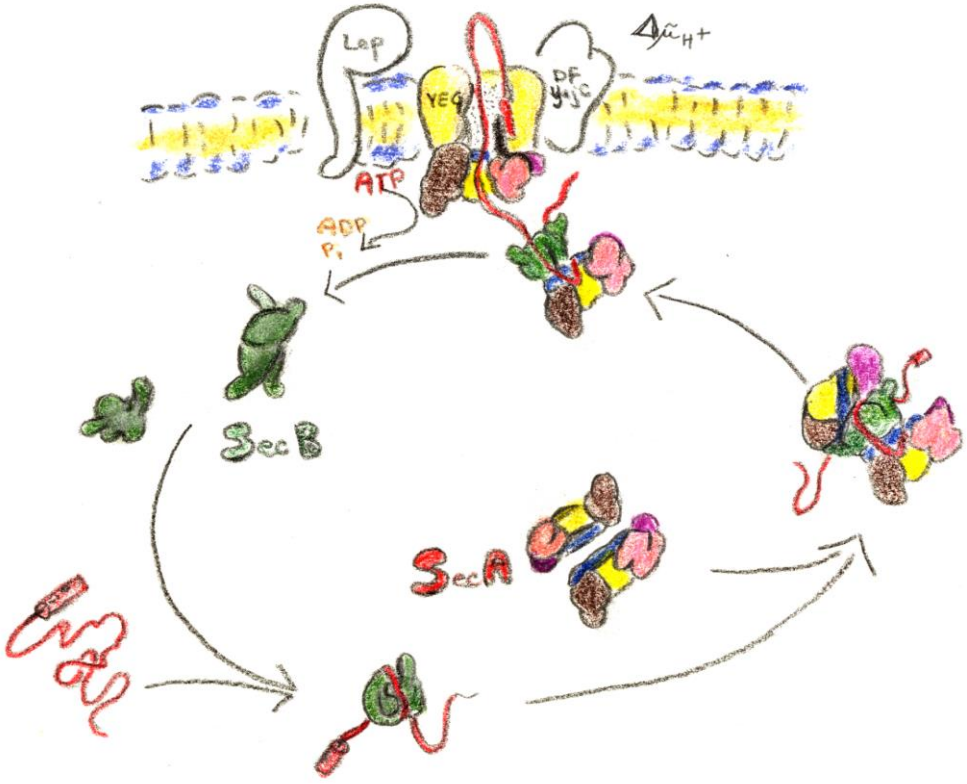


Figure 1. Cartoon schematic of the secretory system. Cytoplasmic SecB binds unfolded precursor (red) and two cytoplasmic SecA protomers bind the SecB:precursor complex forming an A_2:B_4 complex. SecA uses ATP hydrolysis to translocate the precursor through the transmembrane protein complex SecYEG which is found in the bacterial inner membrane and SecDFYajC couples proton motive force to translocation. Leader peptidase (Lep) cleaves the leader sequence from the precursor in the periplasm of the cell (top of figure). Cartoon drawn by Lin L. Randall.

activate SecYEG for translocation (Tam et al. 2005). Disulfide cross-linking studies have been done that show that SecE will cross-link to an adjacent SecE according to the back-to-back arrangement of two heterotrimeric SecYEGs but the activity of this cross-linked species is very poor (Kaufmann et al. 1999; Deville et al. 2011). In one study, in both proteoliposomes and inner membrane vesicles (IMVs), no translocation activity was observed for the E-E cross-linked dimer (Kaufmann et al. 1999). In a different study the E-E cross-linked dimer was incorporated into proteoliposomes and showed similar translocation to wild type; however, less than ten percent of input precursor was translocated (Deville et al. 2011). Alternatively, computer modeling of the results of an *in vivo* cross-linking study supports the front-to-front arrangement of a SecYEG dimer (Das and Oliver 2011). In our studies we have described for the first time that the stoichiometry of SecYEG in translocation is dependent on the precursor species; twice as many SecYEG heterotrimers are required to translocate pGBP than are required for proOmpA (Mao et al. 2013).

SecA

SecA is a 102-kDa soluble protein with several domains. Two nucleotide binding domains, NBD1 and NBD2, form the site where ATP is bound and hydrolyzed. There is a preprotein cross-linking domain (PPXD) that interacts with precursor, SecYEG and SecB (Cooper et al. 2008). There is a helical scaffold domain (HSD) made of a long alpha helix that interacts with precursor, lipids and SecYEG. A short (10 amino acid) linker helix that connects NBD2 and the HSD has been shown to interact with precursor, lipids, SecYEG and SecB (Cooper et al. 2008). The C-terminal domain (CTD) is composed of a helical wing domain (HWD), an intramolecular regulator (IRA1) domain and a long C-terminal

tail. The N-terminal ten residues of SecA are important in dimerization of SecA (Jilaveanu et al. 2005) and also bind to the C-terminal tails of SecB (Randall and Henzl 2010). N-terminal truncations of SecA showed not only impaired dimerization but also reduced ability to form a membrane-integral form *in vivo* (Das et al. 2008), but it is not clear if the reduction in membrane interaction is due to the lack of the N-terminus itself or to the impaired dimerization of SecA. Unpublished studies using circular dichroism and electron paramagnetic resonance (EPR) have provided evidence that these ten residues penetrate the lipid bilayer in a helical structure (Virginia Smith, Bahar Tuba Findik, unpublished). IRA1 contains a 'two-helix finger' involved in the mechanical process of moving the precursor through SecYEG (Bauer and Rapoport 2009). The C-terminus of SecA contains a zinc ion coordinated by histidine and cysteine residues. These C-terminal zinc-containing 21 residues have been shown to be important in binding SecB (Randall et al. 2005) and also in interacting with acidic phospholipids in the membrane (Breukink et al. 1995).

SecA exists at equilibrium between monomer and dimer in solution (Woodbury et al. 2002), and there is evidence for its dissociation in the presence of acidic phospholipids (Or et al. 2002), but whether or not it functions as a dimer in translocation is still under debate. One study showed dimeric SecA is necessary for cell viability and *in vitro* translocation (Jilaveanu et al. 2005). Another study showed that maximal efficiency of translocation is found in SecA species that can form complexes of two SecA protomers to one SecB tetramer (A_2:B_4) *in vitro* (Mao et al. 2009). A recent study's conclusion was that there are two different SecA-SecYEG binding modes; one salt-sensitive and one salt-insensitive (Kusters et al. 2011) and one study showed that SecA forms two different

membrane-embedded forms but the relevance of each form has not been determined (Chen et al. 1998). A model of SecA is found in Figure 2.

SecB

SecB is a small (69 kDa) homotetrameric cytosolic chaperone that binds unfolded precursor proteins as well as SecA. Two SecA protomers bind SecB in an asymmetric manner; deletion of the 21 C-terminal zinc-containing residues or addition of a mimicking peptide abolishes the second SecA protomer's ability to bind SecB (Randall et al. 2005).

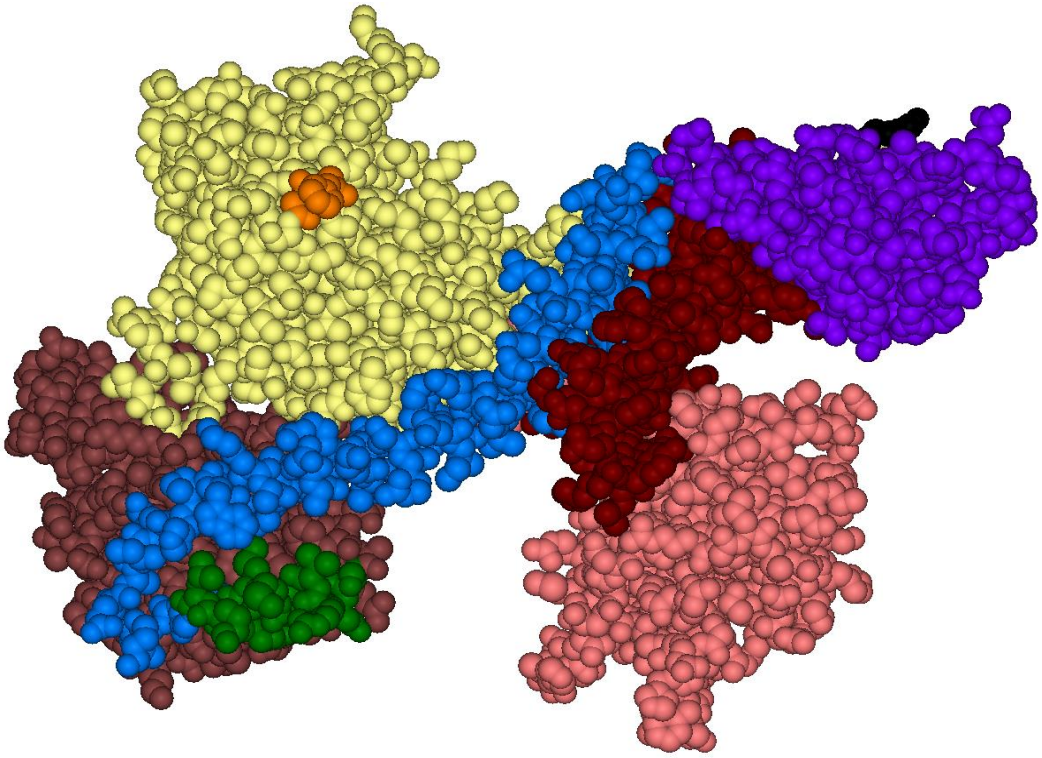


Figure 2. Model of SecA. NBD1 in yellow, and NBD2 in light brown, PPXD in pink, linker helix in green, HSD in blue, HWD in purple, IRA1 in dark brown. The N-terminal ten residues protrude from NBD1 at the gold residue but are not shown. The long C-terminal tail protrudes from the HWD at the black residue but is not shown.

Chapter 2

Activation of SecYEG by co-assembly in proteoliposomes with SecA and stoichiometry of SecYEG in the active translocase

Published as:

Stoichiometry of SecYEG in the active translocase of Escherichia coli varies with precursor species (Proceedings of the National Academy of Sciences of the United States of America, 2013, 110, 29, 11815-11820) Chunfeng Mao, Carl E. Cheadle, Simon J.S. Hardy, Angela A. Lilly, Yuying Suo, Raghavendar Reddy Sanganna Gari, Gavin M. King, Linda L. Randall.

Summary

In vitro assays are powerful for studying biological systems as researchers can replicate cellular processes under strictly defined conditions. Specified mutant proteins can be used in *in vitro* assays to study the roles of individual amino acids or domains in a biological process. Care must be taken, however, to ensure that the *in vitro* assay replicates as closely as possible the cellular process as it occurs *in vivo*. While *in vitro* protein translocation studies in reconstituted proteoliposomes have been published by many laboratories, the extent of translocation has never been demonstrated to be comparable to the extent of translocation observed *in vivo* or in inverted membrane vesicles. In the work we recently published (*Proceedings of the National Academy of Sciences of the United States of America*, 2013, 110, 29, 11815-11820) we described the development of a reconstitution of SecYEG into liposomes in the presence of SecA. This system, referred to as PLYEG•A, showed a five-fold increase of translocation of proOmpA over that seen when SecA was added to proteoliposomes assembled with SecYEG only (referred to as PLYEG). We demonstrated that the number of SecYEG heterotrimers in an active translocon depends upon the precursor species being translocated. Further, we showed that the number of active translocons in PLYEG•A is the same as in vesicles derived from *E. coli* cells. Thus there is an interaction between SecA and SecYEG during the fabrication of co-assembled proteoliposomes that is critical for preserving the active state of SecYEG in its native environment—the bacterial inner membrane.

Results

To develop a robust system that we could use to carry out the research described in the PNAS paper, we optimized the system and determined the conditions in which SecYEG was limiting in translocation in PLYEG•A. This was necessary since we were interested in effects on the activity of SecYEG. In both PLYEG + A and PLYEG•A, with SecYEG at 1 μM, no increase in translocation was observed upon increased concentrations of externally added SecA or precursor. When we increased the concentration of SecYEG, more precursor was translocated. In this way we determined that SecYEG was the limiting component of the translocase under these conditions.

For both precursor proteins assayed for translocation (proOmpA and pGBP), more protein is translocated in PLYEG•A than in PLYEG + A. One possible explanation for the increased activity in co-assembled proteoliposomes is that the number of accessible translocons is greater in PLYEG•A than in PLYEG. To determine the number of accessible translocons (the cytoplasmic face of SecYEG oriented to the outside of the proteoliposomes) spin-labeled SecYEG (from a single-cysteine SecY mutant) was incorporated into PLYEG•A proteoliposomes as well as proteoliposomes containing only SecYEG. The spin label is covalently bonded to cysteine and a reducing agent such as DTT or TCEP can release it. We added the reducing agent TCEP (50 μM) to PLYEG, PLYEG + A, and PLYEG•A, releasing 55 percent of the spin label in each. When the concentration of TCEP was increased to 1 mM, the TCEP permeated the lipid bilayer, releasing all the spin labels on the interior of the proteoliposomes. The increase in the number of active translocons cannot be attributed to a difference in the percentage of accessible translocons in PLYEG•A.

Other possibilities are that there are more active translocons in PLYEG•A than in PLYEG + A or alternatively that each translocon in PLYEG•A can undergo many cycles of translocation whereas translocons in PLYEG + A cannot. To distinguish between the two possibilities we adapted a strategy from Uchida et. al (Uchida et al. 1995) which allowed us to count the number of active translocons in each system. We introduced cysteine point mutations into the precursor proteins proOmpA and pGBP such that there were two cysteines that could be oxidized to form an intramolecular disulfide bond in each. In the proOmpA species the loop size is 59 amino acids (proOmpA L59) and in pGBP the loop size is 34 amino acids. These 'loop' precursors are translocation-competent but their translocation is arrested when the loop encounters ('jams') the translocon. We labeled these 'loop' precursors with radioactive amino acids to quantify the amount of precursor translocated and assumed a stoichiometry of one 'jammed' SecYEG to one 'loop' precursor thereby allowing us to count the number of active translocons in PLYEG + A and PLYEG•A.

In both PLYEG + A and PLYEG•A the 'loop' precursors proOmpA L59 and pGBP loop were competent for translocation. We quantified the translocated precursor from an input of 1 μM (1000 nM) and expressed the value in nM quantities to provide an understandable measure of the activity which is not conveyed when expressed as percentage or molar values. Translocation of the proOmpA L59 loop in PLYEG•A yielded a small amount (approximately 30 nM) of protected full length proOmpA and several partially protected intermediate bands (300 nM) which showed that the loop precursors are not translocated fully into the proteoliposomes. It was critical to determine which bands were translocation-dependent and therefore representing 'jammed'

translocons to accurately count the number of active translocons. Bands with molecular weights in the range of 23.8–30.5 kDa (indicated in Figure 3 by bracket) could only be generated under translocation conditions, increasing in a time-dependent manner. Furthermore addition of DTT after all the translocons were jammed reduced the disulfide bond and the linear precursor was then translocated into the proteoliposomes with a concomitant decrease in the quantity of partially translocated precursor (Figure 4). We conclude that these 23.8–30.5 kDa bands are authentic intermediates in transfer of proOmpA that are jammed in the translocon.

Proteolytic bands of the proOmpA L59 loop with lower molecular weights were observed at the t = 0 time point and their quantities did not change during the time course. These bands were generated when SecYEG was omitted from the translocation assay mix and are not translocation-dependent (Figure 3 indicated by asterisk). Thus these bands were not considered to be translocation intermediates of the precursor jammed in the translocon and were omitted from quantification. The pGBP loop precursor yielded proteolytic fragments (intermediates) of 30.4 and 28.8 kDa that could only be generated under translocation conditions (Figure 5). From this we conclude that the proteolytic 23.8–30.5 kDa fragments of the proOmpA L59 loop and the 28.8-30.4 kDa fragments of the pGBP loop are intermediates of the translocation process and as little full length precursor is protected, the loop precursors are jamming the translocons.

The number of active SecYEG in PLYEG•A with the proOmpA and pGBP loops was determined to be 300 nM and 180 nM for the two species, respectively (Figure 6). Since the same PLYEG•A preparation was used for both precursors these results suggest

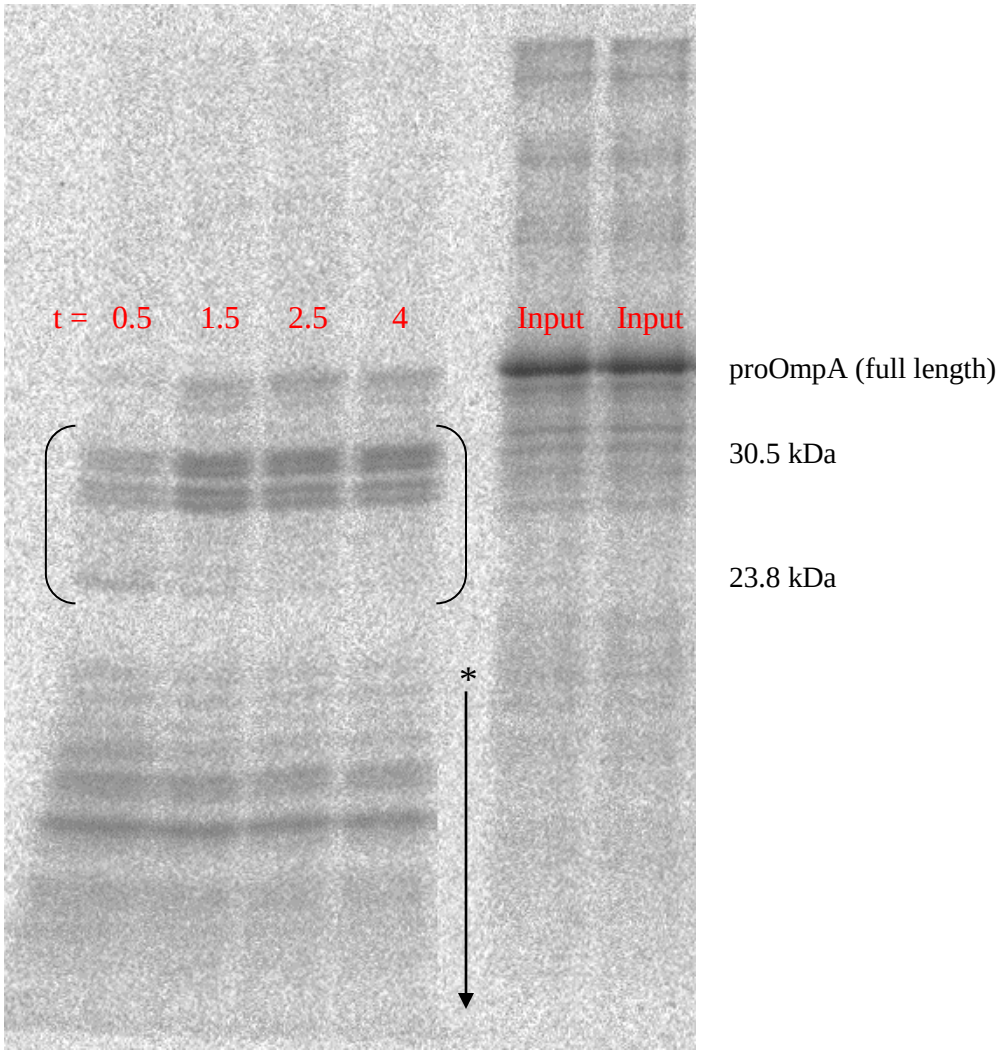


Figure 3. Image of radioactive bands from an SDS-PAGE gel of a time course *in vitro* translocation assay of the proOmpA L59 loop with PLYEG•A. The first four lanes are time points: t = 0.5, 1.5, 2.5, 4 minutes. Quantification of intermediate partially translocated proOmpA gives 36, 227, 267, 283 nM intermediates for times 0.5-4 minutes and a total of 283 nM active translocons. The right two lanes are the input. Translocation-dependent intermediates (I) are bracketed and the translocation-independent bands, which are omitted from quantification, are indicated by the asterisk and arrow.

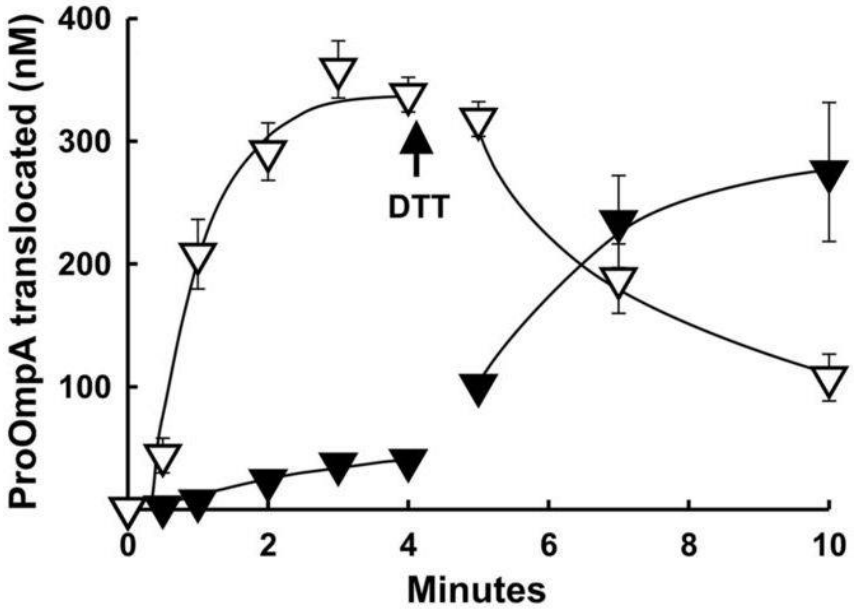


Figure 4. Translocation of the proOmpA L59 loop precursor in PLYEG•A, with DTT added to reduce the disulfide bond at 4 minutes 15 seconds. As published in *Proceedings of the National Academy of Sciences of the United States of America*, 110, 29, 11815-11820.

Release of proOmpA intermediates (open triangles) by the addition of DTT (4 min 15 s) to allow elongation to full-length precursors (closed triangles).

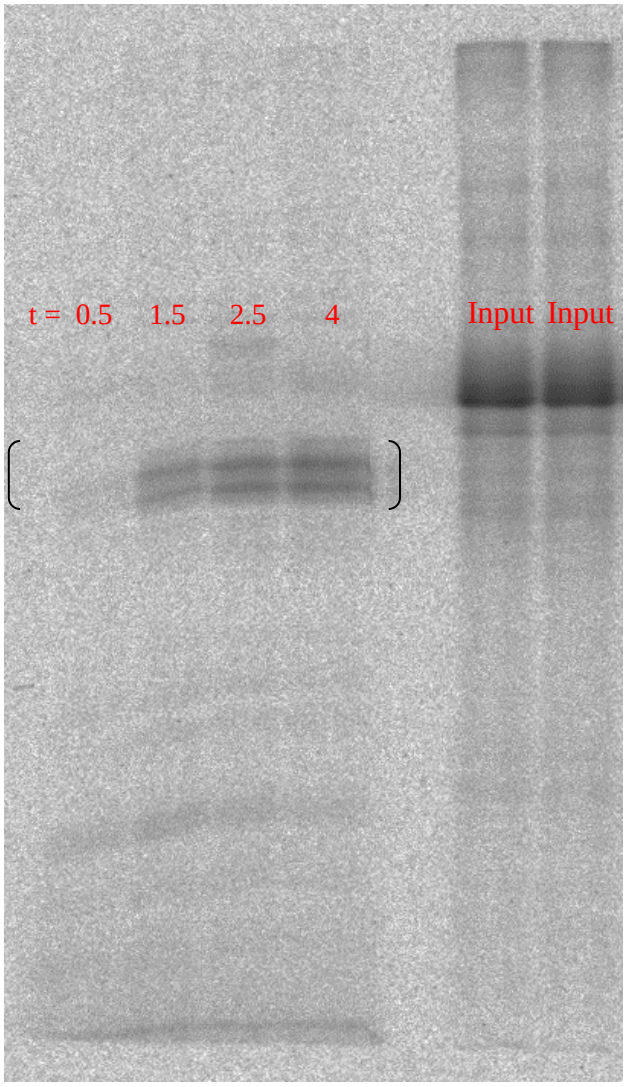


Figure 5. Image of radioactive bands from an SDS-PAGE gel of a time course *in vitro* translocation assay of the pGBP loop with PLYEG•A. The first four lanes are time points: t = 0.5, 1.5, 2.5, 4 minutes. Quantification of intermediate partially translocated pGBP gives 12, 106, 181, 228 nM intermediates for a total of 228 nM active translocons. The right two lanes are the input. Translocation-dependent intermediate (I) bands are bracketed.

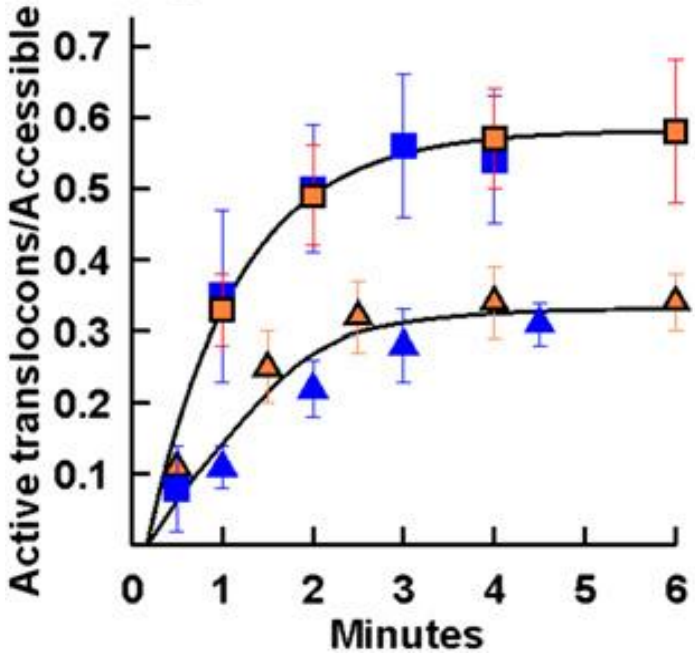


Figure 6. Counting the number of active translocons in PLYEG•A and in IMVs with the proOmpA L59 and pGBP loop precursors shows that translocation of pGBP requires twice as many translocons as does proOmpA and that the active state of SecYEG in the inner membrane is preserved in PLYEG•A. As published in *Proceedings of the National Academy of Sciences of the United States of America*, 110, 29, 11815-11820.

Twofold difference between translocon units used by proOmpA and pGBP. Comparison of inverted inner membrane vesicles (orange symbols) with reconstituted PLYEG•A (blue symbols), using proOmpA (orange □, n =4; blue □, n = 7) and pGBP (orange ∆, n = 7; blue ∆, n = 4). Concentration of intermediates was normalized to that of accessible SecY. Error bars are SD.

that pGBP occupies twice the number of SecYEG units to form an active translocase complex. To demonstrate that this is the case we began the *in vitro* translocation reaction with the pGBP loop (non-radioactive) and added radiolabeled proOmpA after one minute. This yielded only 53 nM intermediates for the proOmpA species (Figure 7). If pGBP used only one SecYEG for translocation 183 nM translocon should have been available for proOmpA. When the concentration of SecYEG in the translocation assay with PLYEG•A was increased the ratio of active translocons for proOmpA : pGBP was consistently 2 : 1. We also observed the same 2 : 1 ratio of active translocons for proOmpA : pGBP in the translocation with IMVs (Figure 6). When we corrected the number of active translocons in the assays with PLYEG•A and IMVs for the sidedness of SecYEG we found that an equivalent number of active translocons for each respective precursor was observed in both systems (Figure 6). From this we concluded that translocation of pGBP requires twice as many translocons as does the translocation of proOmpA and that co-assembly of SecA and SecYEG in proteoliposomes preserves the active state of SecYEG in its native environment—the bacterial inner membrane.

PLYEG•A with mutant SecA species

We wanted to determine how SecA converts SecYEG to an active state during co-assembly in proteoliposomes. It is unclear whether the region of SecA crucial for the ability to convert SecYEG to an active state is separate from the ability to function in translocation, which involves interactions with the chaperone SecB and precursor polypeptides, as well as the hydrolysis of ATP. To separate these functions, we tested defective variants of SecA: SecA880, which is truncated to remove the C-terminal 21 aminoacyl residues that coordinate zinc; SecAC4, which lacks zinc because the

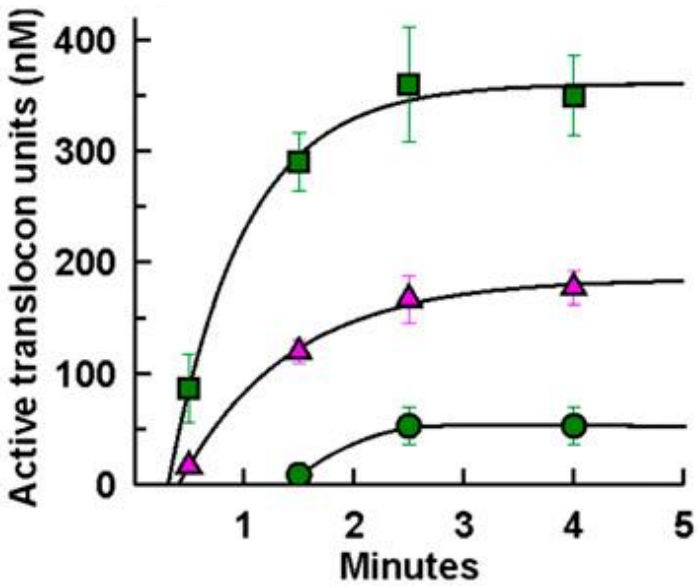


Figure 7. Translocation of proOmpA after jamming the translocons with the pGBP loop. As published in *Proceedings of the National Academy of Sciences of the United States of America*, 110, 29, 11815-11820.

Translocons saturated with pGBP are unable to translocate proOmpA. Translocation of precursors containing oxidized loops assayed with PLYEG•A: ^{14}C-pGBP added at t = 0 (pink ∆), ^{14}C-proOmpA added at t = 0 (green □), ^{14}C-proOmpA added at t = 1 min following addition of nonradiolabeled pGBP at t = 0 (green ○). These data (green ○) were corrected to take into account loss of activity of the system with time. Error bars are SD.

coordinating cysteines are replaced by serine; SecAdN10, which has aminoacyl residues 2–11 deleted; SecAN664, which is truncated after the 664th residue; SecAC619, which begins at the 619th residue, and SecAC662, which begins at the 662nd residue. A diagram of the mutant SecA species is found in Figure 8.

Co-assembly of variant SecA species in PLYEG•A showed that species containing the N-terminal ten residues (SecA880, SecAC4, SecAN664) had a similar number of active translocons to PLYEG•wtSecA (250-300 nM for proOmpA). PLYEG•SecAdN10 had an intermediate number of active translocons (150 nM) and the C-terminal fragments SecAC619 and SecAC662 (mutants that start with SecA residues 619 and 662 as the N-terminal residue) in PLYEG•A showed no activation of SecYEG as these had only 50 nM active translocons (Figure 9). The intermediate value of active translocons in PLYEG•SecAdN10 may result from the fact that there is generally less SecAdN10 than other SecA species in the membrane in co-assembled proteoliposomes. This indicates that the N-terminal residues of SecA are important in the interaction between SecA and SecYEG and this is examined in Chapter 4. We conclude that full activity in translocation is not required for the ability to activate SecYEG.

This work, published in *Proceedings of the National Academy of Sciences of the United States of America*,110, 29, 11815-11820, establishes a strong foundation for future studies of the secretory system *in vitro* as SecYEG can now be reconstituted into proteoliposomes in an active conformation, providing biologically relevant SecA-SecYEG interactions. Furthermore we will be able to determine what part of SecA is

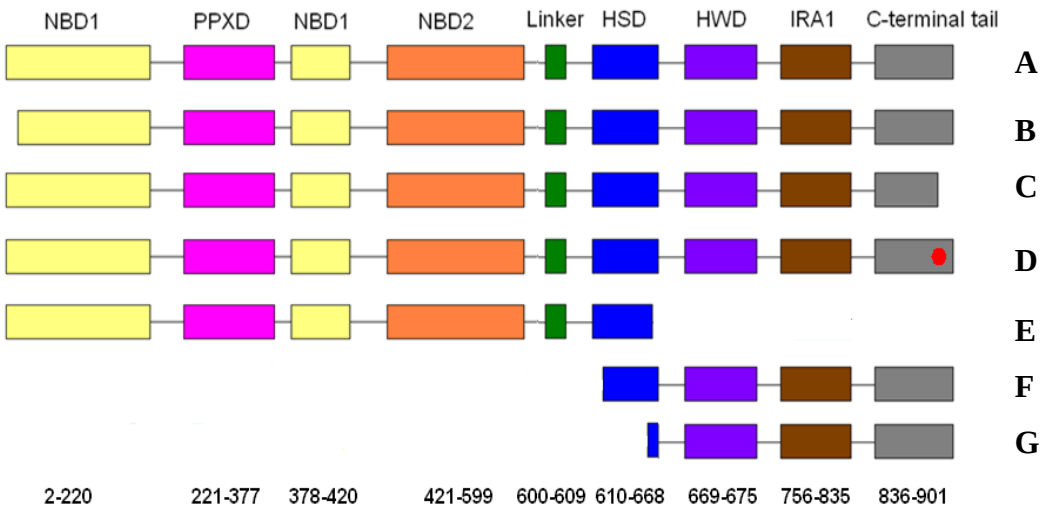


Figure 8. Diagram of SecA species used in these studies with domains (top) and amino acid residues (bottom). In order: wild-type SecA (**A**), SecAdN10 (**B**), SecA880 (**C**), SecAC4 (**D**), SecAN664 (**E**), SecAC619 (**F**), SecAC662 (**G**). For descriptions of domains and a figure of SecA see pages 5-8.

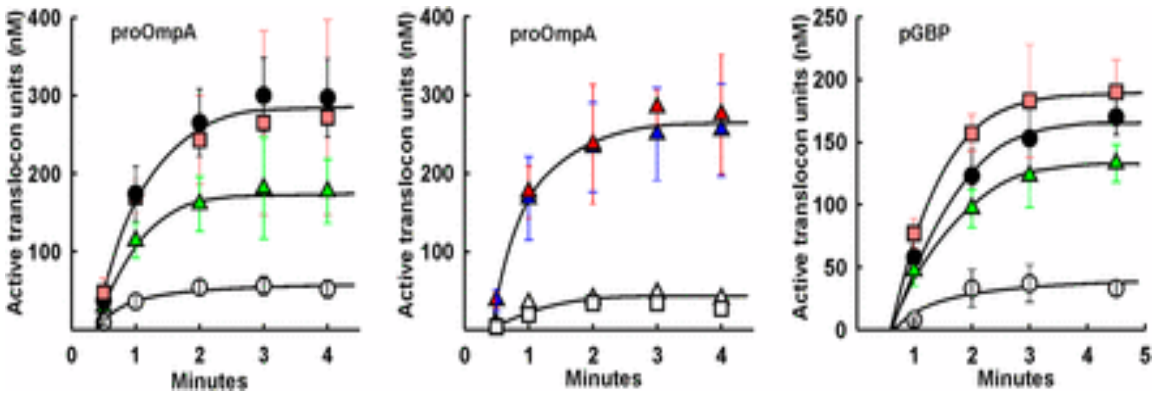


Figure 9. Ability of SecA variants to render SecYEG active. As published in *Proceedings of the National Academy of Sciences of the United States of America*,110, 29, 11815-11820.

Ability of SecA variants to render SecYEG active. Proteoliposomes were made by coassembly of SecYEG and the variant SecA species as follows: SecA880, pink □, *n* = 3; SecAC4, blue ∆, *n* = 6; SecAdN10, green ∆, *n* = 5; SecAN664, red ∆, *n* = 3; SecAC619, ∆; and SecAC662, □. The activity of each PLYEG•A species was assessed with oxidized proOmpA or oxidized pGBP, as indicated. PLYEG•wild-type SecA (●) and PLYEG + wild-type SecA (○). Error bars are SD.

important for preserving the active state of SecYEG using different SecA mutants. Future structural studies will be more accurate as well now that SecA and SecYEG can be studied in conformations that are more true to their *in vivo* states.

Chapter 3

Characterization of the interaction between SecA and SecYEG

Unpublished Results

Summary

The protein translocation activity observed in PLYEG•A is equal to that in native inner membrane vesicles (IMVs) and five-fold greater than in PLYEG + A. Because there are more active translocons in PLYEG•A than in PLYEG + A we set out to characterize the interaction between SecA and SecYEG in both systems. We assumed that the SecA-SecYEG interaction in PLYEG•A is true to the interaction *in vivo* and that their interaction in PLYEG + A is not. We observed that SecA binds the membrane in a specific membrane-peripheral manner in PLYEG•A; that SecA binds the membrane in a non-specific membrane-integral manner in PLYEG + A; and that the N-terminus of SecA is important for membrane association and exchange of membrane-associated SecA.

Results

To determine which parts of SecA are membrane-associated we used region-specific antibodies (a gift from D. Oliver, Wesleyan University) that were generated against fusion proteins of maltose-binding protein and regions of SecA. These antibodies (αA1-αA6) react with the following portions of SecA; αA1 reacts with residues 1-209, αA2 reacts with residues 211-350, αA3 reacts with residues 351-509, αA4 reacts with residues 519-664, αA5 reacts with residues 665-820, and αA6 reacts with residues 822-901 (Figure 10). Digestion of PLYEG + A, PLYEG•A, and liposomes•A (proteoliposomes made with SecA but no SecYEG) with Proteinase K to generate stable SecA fragments was followed by centrifugation to isolate the membrane-bound fragments. These fragments were then analyzed by Western blotting using the region-specific anti-SecA antibodies. By analyzing these fragments with the region-specific antibodies we can learn

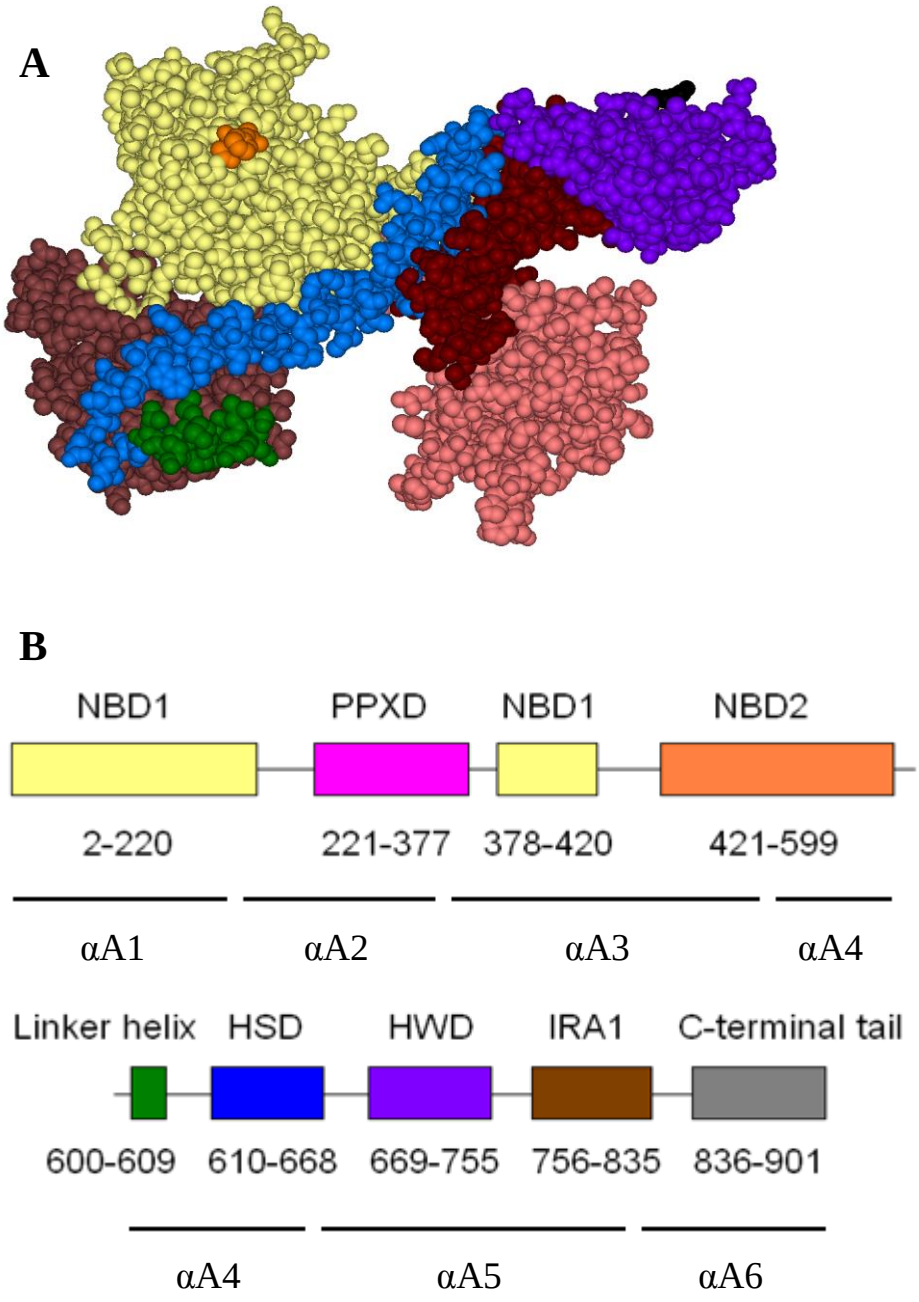


Figure 10. The domains of SecA (**A**); their amino acid residues and where the region-specific antibodies react with SecA (**B**).

what parts of SecA bind the membrane and future researchers may be able to determine what parts of SecA are integral to the lipid bilayer.

Proteolysis experiments showed that there are several proteolytic SecA fragments found in digests of PLYEG + A that are not present in digests of PLYEG•A (Figure 11). These are likely degradation products of various biologically non-relevant SecA conformations that we observed by atomic force microscopy (See Mao et. al 2013 Figure 7) in PLYEG + A but not in PLYEG•A. PLYEG + A generates two fragments of 68 kDa (68a and 68b) of which one remains associated with the membrane and one does not (Figure 11). In contrast PLYEG•A generates one 68-kDa fragment that remains mostly associated with the membrane and three faint smaller fragments that are entirely membrane-associated.

PLYEG + A also generates a proteolytic 44-kDa fragment which is mostly associated with the membrane as it is in PLYEG•A as well as 43.5- and 40-kDa fragments which are not observed in PLYEG•A. In liposomes•A the 68-kDa and 44-kDa fragments are approximately seventy percent in the supernatant fraction. Both the 68-kDa and 44- kDa fragments in PLYEG•A react with the αA4 antibody but only the 44-kDa fragment reacts with the αA5 antibody (Figure 12). This indicates that the 44-kDa fragment cannot be a proteolytic digestion product of the 68-kDa fragment because it has a region not present in the parent fragment. Under translocation conditions the amount of the 44-kDa fragment plateaus as the concentration of SecA increases but the amount of the 68-kDa fragment continues to increase (Figure 13). These data are consistent with the literature (Chen et al. 1998) and suggests that there are at least two distinct

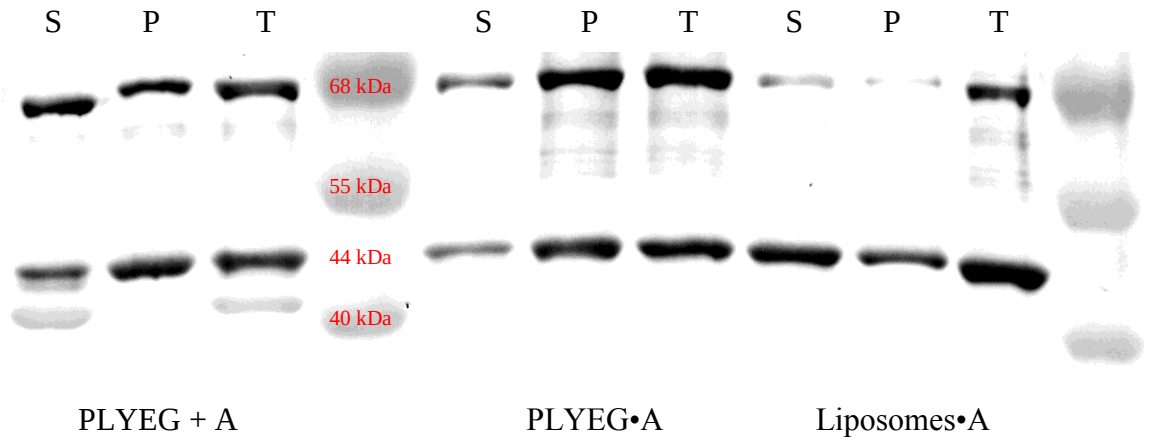


Figure 11. Different proteolytic SecA fragments in PLYEG + A and PLYEG•A by Western blot against the αA3 antibody. Lanes from left to right are PLYEG + A: supernatant, pellet, total; prestained molecular weight markers, PLYEG•A: supernatant, pellet, total, liposomes•A: supernatant, pellet, total. The ~68- and ~44-kDa fragments are most prevalent in the pellet in PLYEG•A. There are also 43.5-kDa and 40-kDa fragments unique to the PLYEG + A supernatant fraction. All lanes have an equivalent volume (i.e. 10 µl of supernatant, pellet and total) loaded.

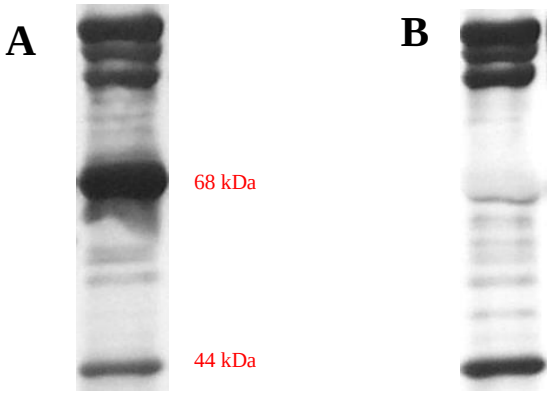
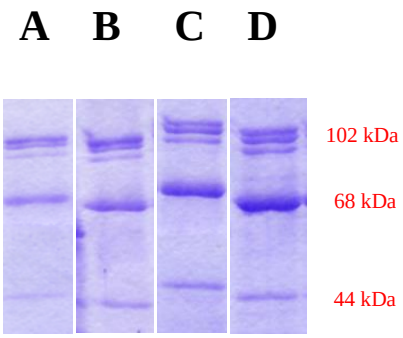


Figure 12. Proteolytic digests of SecA in PLYEG•A analyzed by Western blotting with region-specific antibodies. In **A**, the digest is treated with the αA4 antibody (SecA residues 519-664) and in **B**, the digest is treated with the αA5 antibody (SecA residues 665-820). The 68-kDa fragment in **A** cannot be the parent of the 44-kDa fragment.



SecA880	0.9	0.7	0.9	0.7	μM A_1
SecAwt	0	1.2	1.9	3.1	μM A_1

Figure 13. SDS-PAGE of Proteinase K-treated time points (t = 1.5 minutes) from *in vitro* translocation assays with PLYEG•SecA880 without (**A**) and with wild type SecA added (**B**-**D**). Total SecA concentrations are (**A**) 0.9 μM A_1; (**B**) 1.9 μM A_1; (**C**) 2.8 μM A_1;(**D**) 3.8 μM A_1. As the amount of SecA increases the amount of the 68-kDa fragment increases; however, the amount of the 44-kDa fragment does not increase.

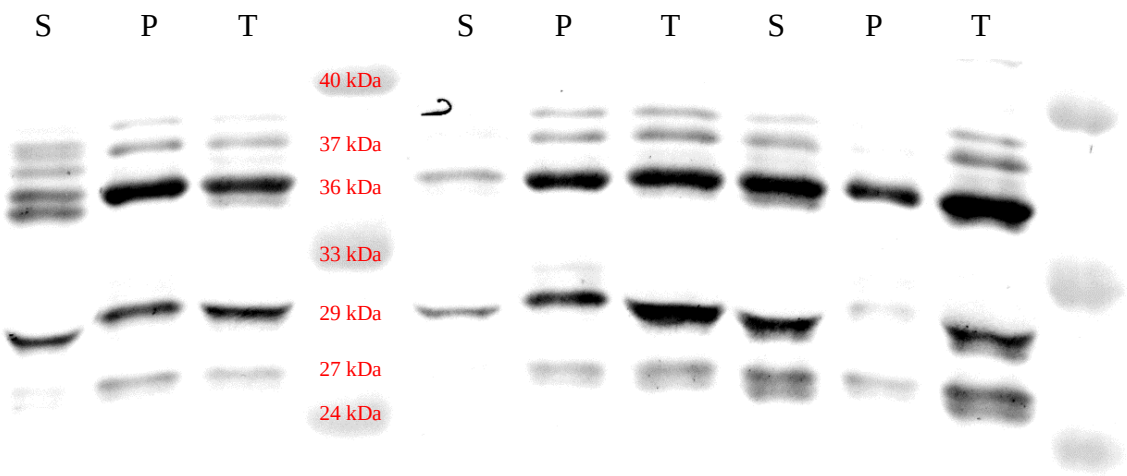


Figure 14. Different proteolytic SecA fragments in PLYEG + A and PLYEG•A by Western blot against the αA2 antibody. Lanes from left to right are PLYEG + A: supernatant, pellet, total; prestained molecular weight markers, PLYEG•A: supernatant, pellet, total, liposomes•A: supernatant, pellet, total. There are fragments of ~37-34 kDa unique to PLYEG + A in the supernatant fraction due to non-specific binding with the membrane and the 36-kDa and 29-kDa fragments are most prevalent in the pellet in PLYEG•A. All lanes have an equivalent volume (i.e. 10 µl of supernatant, pellet and total) loaded.

Fragment	PLYEG + A	PLYEG•A	Liposomes•A
68a	P	N/A	N/A
68b	S	P	S
44	P	P	S
43.5	S	N/A	N/A
41	S	N/A	N/A
38	P	P	S
37a	E	P	S
37b	S	N/A	N/A
36a	P	P	S
36b	S	N/A	S
29	E	P	S
27	P	P	S
26	N/A	N/A	S

Table 1. Localization of proteolytic fragments of SecA. E = evenly dispersed between supernatant and pellet, S = mostly in supernatant, P = mostly in pellet, N/A = not applicable.

conformations of SecA associated with the membrane. Fragments of 38, 37, 36, 29, and 27 kDa react with αA1 and αA2 and are more membrane-associated in PLYEG•A than in liposomes•A or PLYEG + A (Figure 14). For example the 37-kDa fragment is present in all three conditions but in PLYEG•A it is entirely in the pellet fraction, divided between supernatant and the pellet in PLYEG + A, and entirely in the supernatant in liposomes•A (Figure 14). The relative affinities of these fragments for the membrane are summarized in Table 1.

Characterization of the interaction of SecA with the membrane

We used two approaches to see if SecA is more membrane-integral in co-assembled proteoliposomes than in PLYEG. We subjected PLYEG + A, PLYEG•A and liposomes•A to conditions that would remove membrane-peripheral proteins and centrifuged them to separate SecA from the pelleted proteoliposomes. The conditions used were high salt concentrations with both sodium and potassium, high pH (Na_2CO_3), the denaturant urea, a positively charged species (hydroxylamine) and a negatively charged species (heparin). These conditions removed more SecA from PLYEG•A and liposomes•A than from PLYEG + A (Figure 15) which indicates that SecA is more membrane-peripheral in PLYEG•A and liposomes•A than in PLYEG + A.

To further probe the nature of SecA's interaction with the membrane we used the membrane-partitioning reagent ^{125}I-TIDBE (Figure 16). This is a hydrophobic small molecule that partitions into lipid bilayers and has a photo-activatable functional group that when activated covalently bonds to proteins and phospholipids in the membrane. We used this probe to label membrane-integral SecA in PLYEG +A, PLYEG•A, and liposomes•A. No difference was observed in the amount of wild type SecA labeled

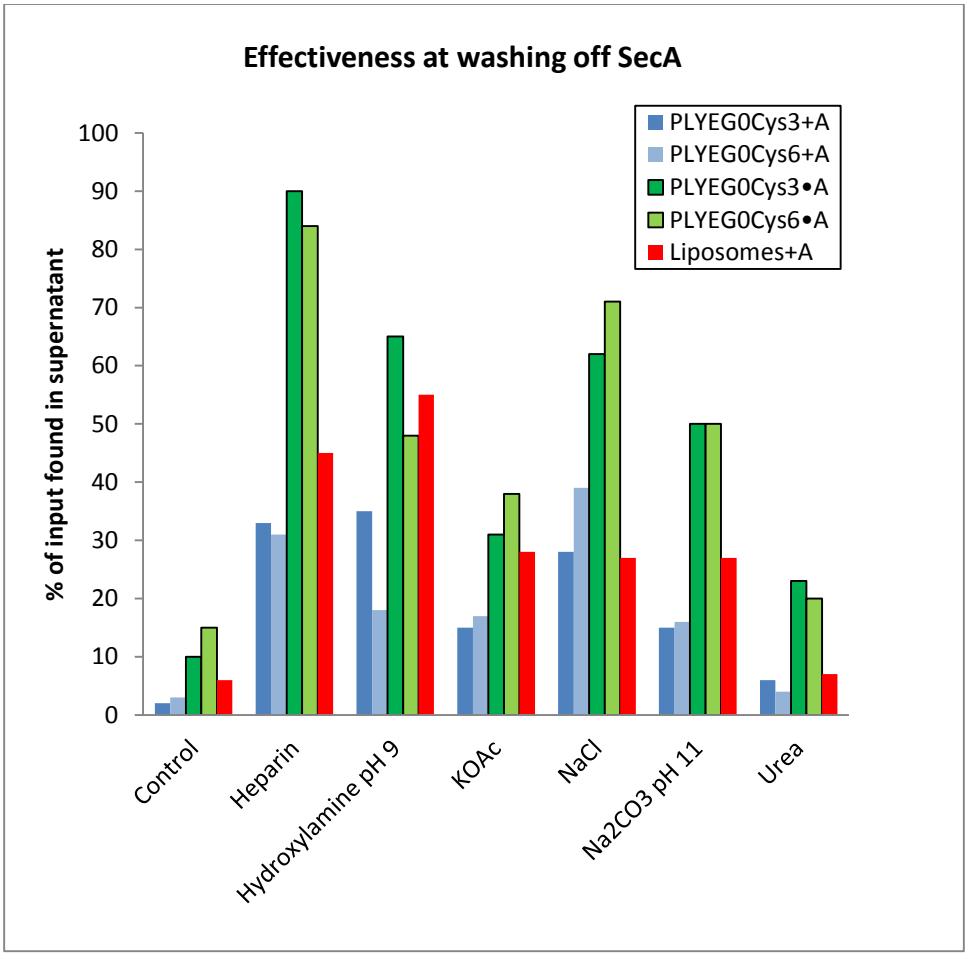


Figure 15. PLYEG•A, PLYEG + A and liposomes•A were subjected to various treatments to remove SecA from the membrane. Control is 10 mM HEPES pH 7.6 250 mM KOAc 1 mM $Mg(OAc)_2$. Heparin, KOAc, NaCl and urea are buffered with 10 mM HEPES pH 7.6.

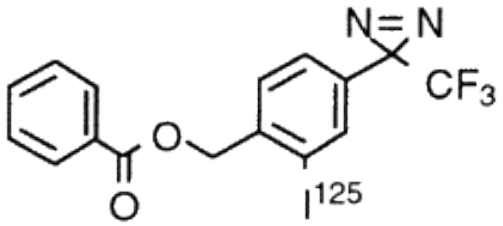


Figure 16. Structure of the hydrophobic membrane-partitioning reagent ^{125}I TIDBE. The diazirine (N=N) group leaves as nitrogen gas upon irradiation with a high intensity lamp generating a reactive carbene species that covalently bonds to proteins and phospholipids in a lipid bilayer.

between PLYEG + A and PLYEG•A (Figure 17). SecY, however, was labeled less in PLYEG•A than in PLYEG + A which indicates that SecYEG may be shielded from the phospholipids in PLYEG•A. The SecA species in PLYEG•A880 and PLYEG•AC4 were labeled more than wild type SecA perhaps due to improper seating of SecA on the translocon. In PLYEG + A with the variant SecA species we observed that species lacking the N-terminal ten residues were labeled half as much as those with the N-terminal ten residues. In PLYEG•SecAdN10, however, SecAdN10 was labeled as much as wild type SecA in PLYEG•A. We attempted to determine what part of SecA was labeled in the lipid bilayer by using Proteinase K to digest ^{125}I TIDBE-labeled SecA species from PLYEG•A but this was not possible because proteolytic SecY fragments overlapped with some of the SecA fragments and we could not quantify the SecA fragments because we did not know if they were in the linear range or not. From this we conclude that SecA in PLYEG•A is more membrane-peripheral than in PLYEG + A. The N-terminal residues may be important in forming a membrane-integral SecA state that is induced in our proteoliposomes by SecYEG. P.C. Tai and colleagues report that the membrane-integral form of SecA is induced by membrane proteins unrelated to the general secretory system (You et al. 2013). There is evidence (Bahar Tuba Findik, unpublished) that the N-terminal ten residues form a helical structure in the lipid bilayer in PLYEG•A. However, if only these ten residues are membrane-integral then SecA in PLYEG•A is unlikely to be labeled more by ^{125}I TIDBE. More work must be done to define what parts of SecA are membrane-integral and whether it is more membrane-integral in PLYEG•A or PLYEG + A.

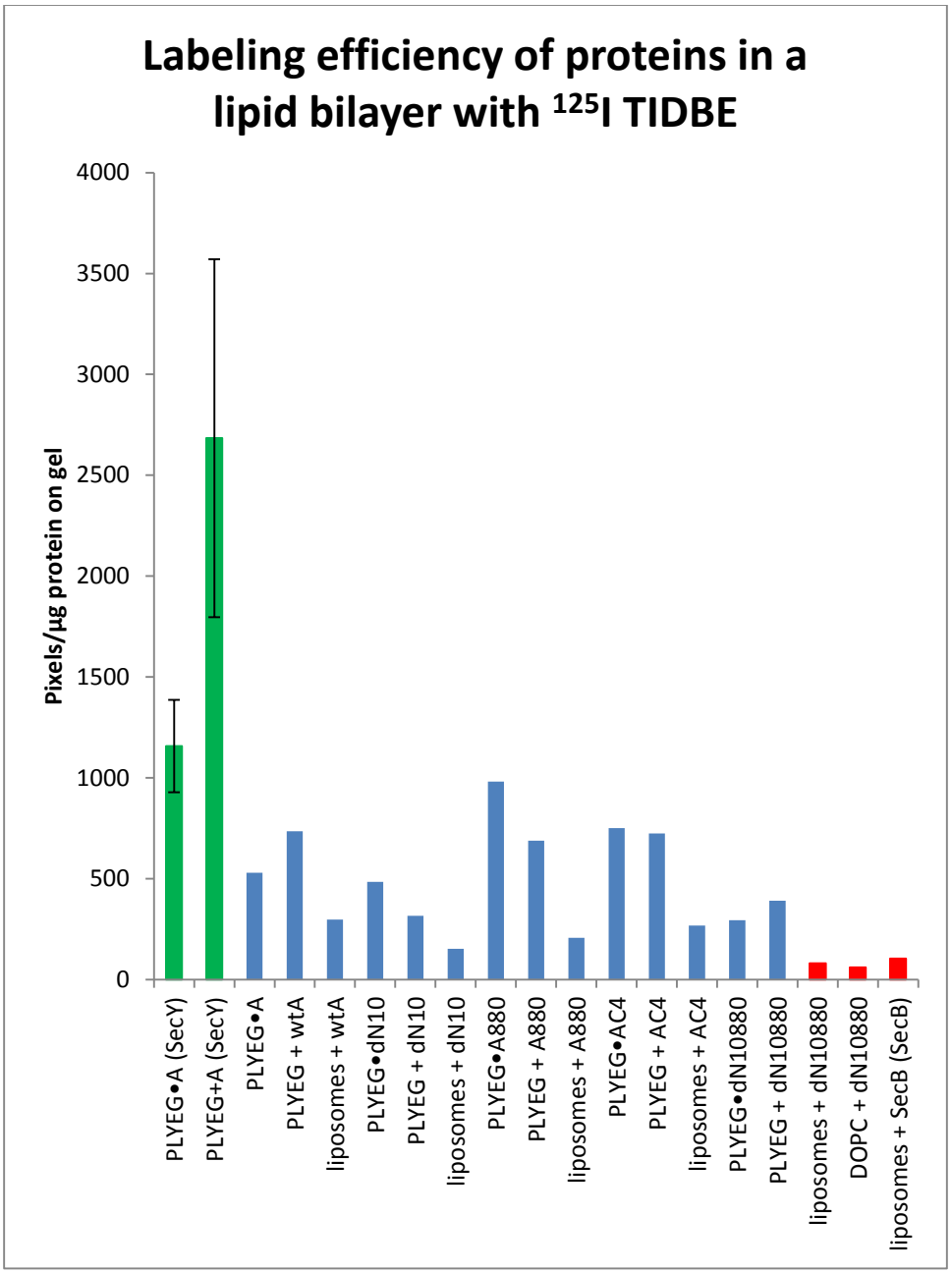


Figure 17. Results of experiments using the bilayer-partitioning photoactivatable probe ^{125}I TIDBE to label membrane-integral proteins. Negative controls (red columns) are liposomes + SecAdN10880, DOPC liposomes + SecAdN10880, and liposomes + SecB. SecA (blue columns) is labeled above background (negative controls) but less than the membrane-integral protein SecY (green columns).

Exchange experiments

To determine the relative affinities of each built-in SecA species for the membrane, we added competing SecA species to PLYEG•A to exchange the accessible built-in SecA species from the membrane. PLYEG•A, PLYEG•AdN10, PLYEG•A880, and PLYEG•AC4 (with ^{14}C- or ^{3}H-labelled SecA species) were subjected to externally added wild-type SecA, SecAdN10, SecA880 and SecAC4. The exchange series data are consistent with AFM data, translocation assay data and literature (Das et. al 2008) that indicate that the N-terminal ten residues of SecA are important in binding the membrane.

SecAdN10 has very little affinity for the membrane as even in the absence of competitor 72 percent of this species came off the membrane (Figure 18). Wild type SecA showed the greatest affinity for the membrane as even at a 20-fold excess of competitor only 60% of the accessible wild type SecA could be displaced (Figure 19). SecA880 and SecAC4 can be displaced completely by wild type SecA, SecA880 or SecAC4 (Figure 20, Figure 21). SecAdN10 can displace SecA880 (30 percent of accessible) more effectively than it can SecAC4 or wild type SecA (15 percent of accessible displaced). SecAdN10 has little affinity for the bacterial membrane but is able to displace SecA880 from PLYEG•SecA880. Since SecA880 is missing the C-terminal 21 amino acids this implies that there is a binding mode between SecA and SecYEG where the C-terminal region of SecA contributes more than the N-terminal ten residues.

These data, together, indicate that the N-terminal ten residues of SecA are important for SecA to bind the membrane and for cytoplasmic SecA to exchange membrane-associated SecA and that there are two membrane-associated states of SecA with different affinities for the membrane.

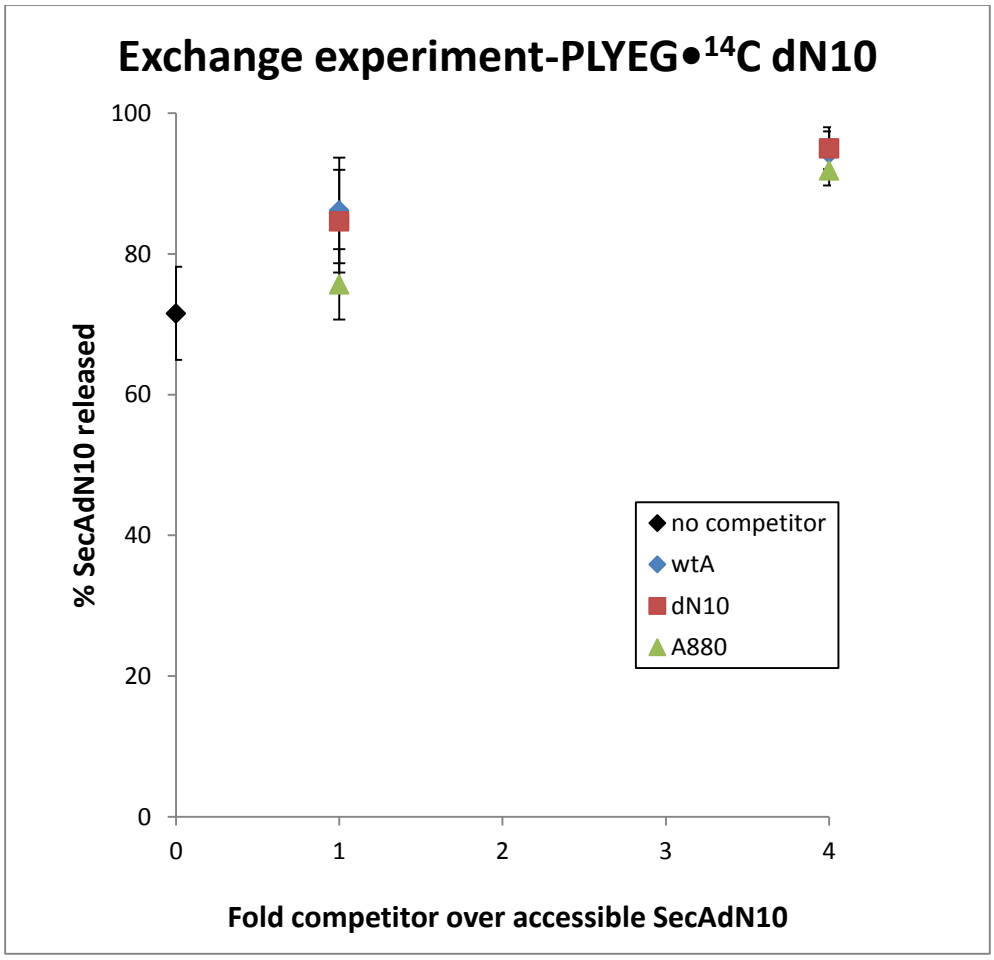


Figure 18. Exchange experiments for PLYEG•AdN10. Seventy-two percent of the accessible SecAdN10 is displaced in the absence of competitor indicating that the ten N-terminal residues of SecA are important for membrane association of SecA.

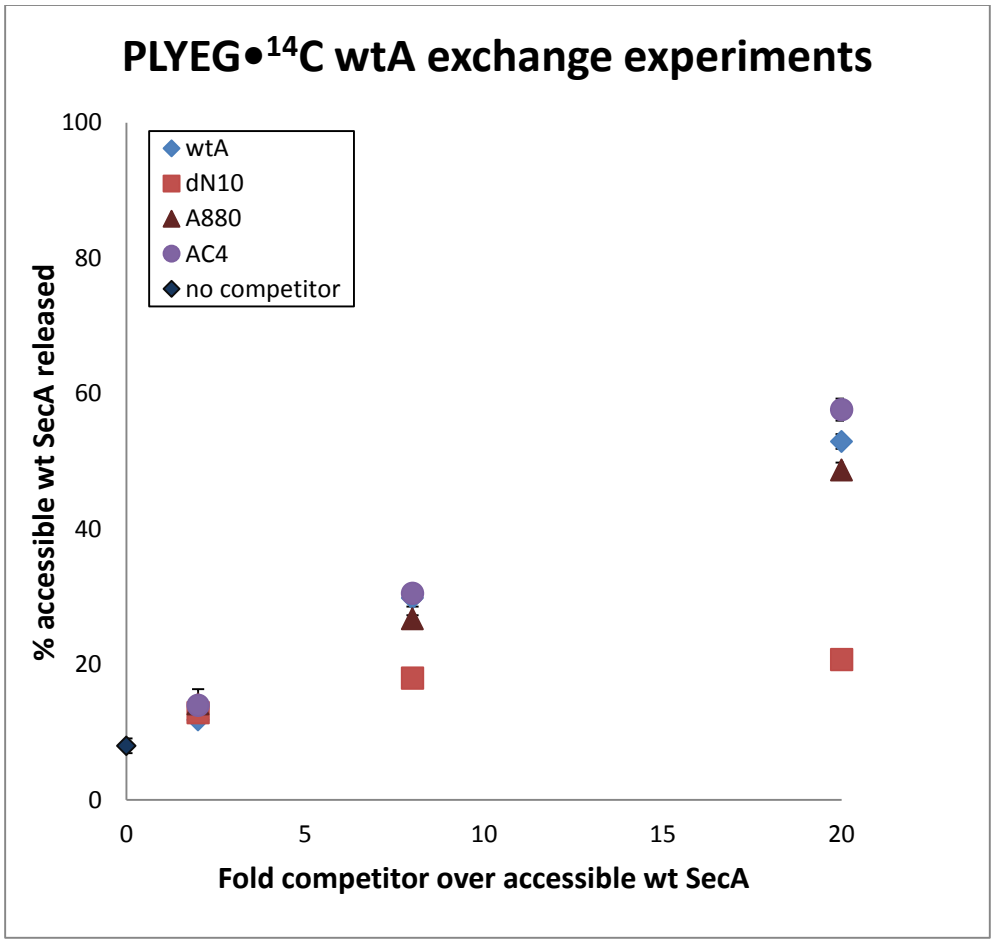


Figure 19. Exchange series for PLYEG•A. SecAdN10 has very little ability to displace built-in wild type SecA.

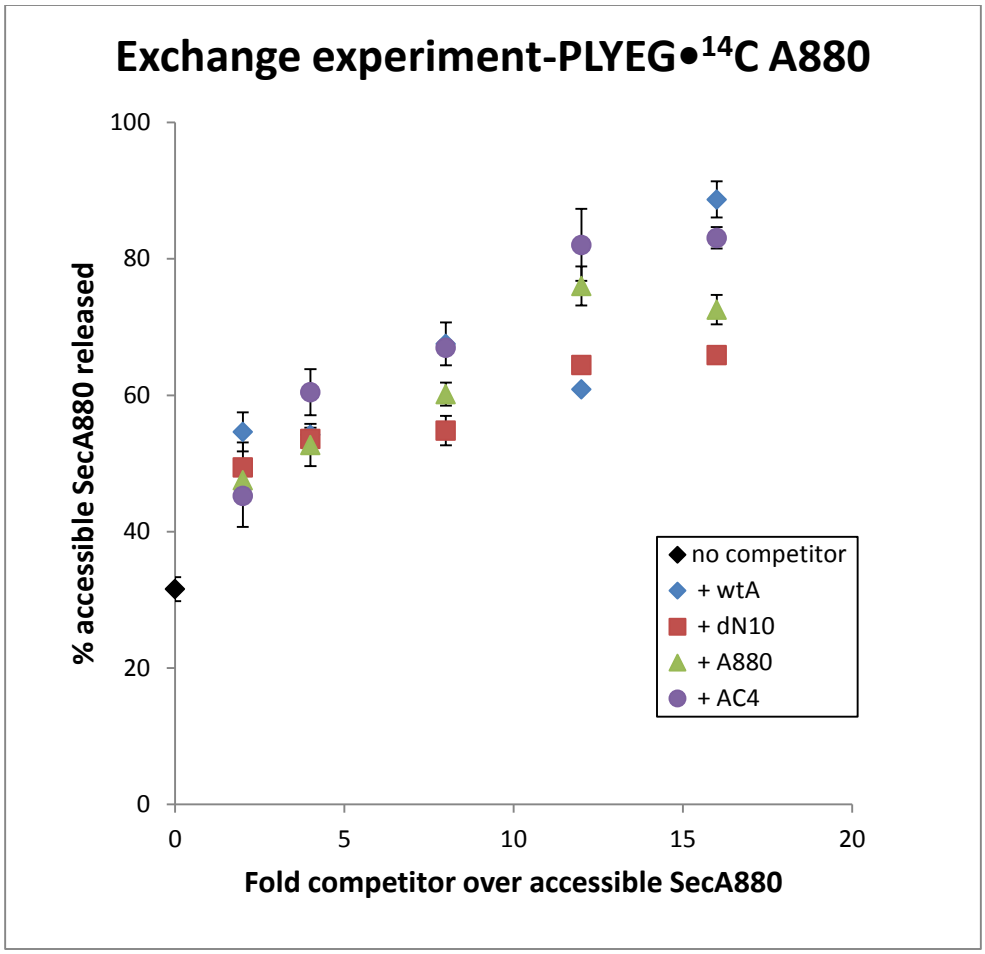


Figure 20. Exchange series for PLYEG•A880. SecAdN10 is able to displace built-in SecA880.

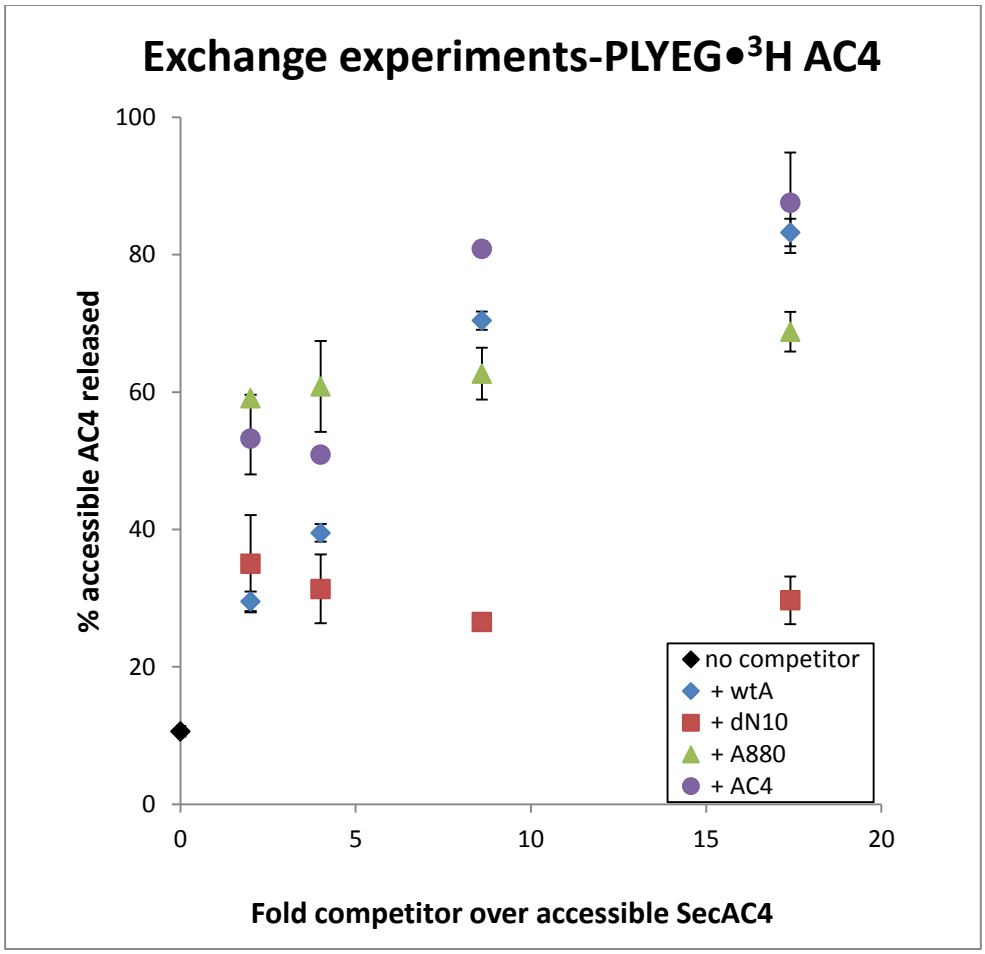


Figure 21. Exchange series for PLYEG•AC4. SecAdN10 has very little ability to displace built-in SecAC4 but wild type SecA, SecA880 and SecAC4 can displace it readily.

Chapter 4

The N-terminal ten residues of SecA in translocation

Unpublished Results

Summary

The ten N-terminal residues of SecA have been shown to be important in binding the membrane, binding SecB, exchanging membrane-associated SecA, and in dimerization of soluble SecA. We have used our powerful *in vitro* translocation system with PLYEG•A and the loop precursors of proOmpA and pGBP to obtain novel data concerning the role of these N-terminal ten residues in translocation. Careful analysis of the proteolytic fragments of the loop precursor species (partially translocated intermediates) from *in vitro* translocation assays has revealed two separate steps in the translocation process. The first step, partial translocation of the precursor, does not require the N-terminal ten residues of SecA. The second step, further translocation of the partially translocated precursor by ~2 kDa, does require these ten residues.

Results

In Uchida et al (1995), the researchers used proOmpA species with disulfide-bonded loops of various sizes between 59 and 25 amino acids that had the first cysteine residue between 244 and 278. These had the same second cysteine residue at 302. When these mutants were translocated *in vitro*, those with loop sizes between 29-59 amino acids generated translocation intermediates of the same molecular weight (approximately 25.8 kDa), and those with loop sizes between 10-25 amino acids generated intermediates of the same molecular weight (approximately 28.7 kDa). From this the researchers proposed that SecA translocates the precursor in a ~3-kDa segment.

When the proOmpA L59 loop is translocated *in vitro* two proteolytic doublets are generated as the major intermediate species of translocation. One doublet contains a 30.5-kDa fragment and a 29.5-kDa fragment and the other contains a 28.5-kDa fragment and a

27.8-kDa fragment (Figure 22). When 2.4 μM $SecA_1$ is added externally to PLYEG•A in the *in vitro* translocation assay no change is observed in the number of active translocons, but the ratio between the higher-MW doublet and the lower-MW doublet increases once all the translocons are occupied. This ratio continues to increase after 2.5 minutes when all the active translocons have been occupied, suggesting that translocation is still occurring after the initial step (Figure 23). The same phenomena is observed if SecA880 is added externally to PLYEG•A but not if SecAdN10 is added (Figures 24, 25). Furthermore PLYEG•SecAdN10 is deficient in the elongation step as the ratio does not change over time and additional SecAdN10 does not cause the ratio to increase (Figure 26), but the deficiency observed in PLYEG•SecAdN10 can be abolished if wild type SecA or SecA880 is added. PLYEG•SecA880 is not deficient in elongation (Figure 27) but translocates protein more slowly than does PLYEG•SecAdN10.

In vitro translocation of the pGBP loop precursor gives two protease-protected fragments of ~30.4 and ~28.8 kDa (Figure 22). The ratio of these pGBP translocation intermediates increased when 2.4 μM A_1 wild type SecA was added to PLYEG•A (Figure 28). Translocation of the pGBP loop in PLYEG•SecAdN10 is also similar to translocation of the proOmpA L59 loop. SecAdN10 is deficient in the elongation step of translocation of pGBP and this deficiency is abolished by adding wild type SecA or SecA880, but adding SecAdN10 only increases the rate of translocation (Figure 29). PLYEG•SecA880 is not deficient in elongation of pGBP but has a slower rate of translocation than does SecAdN10 (Figure 30). From this we conclude that the 2-kDa translocation step requires the N-terminal ten residues of SecA for both precursor species proOmpA and pGBP.

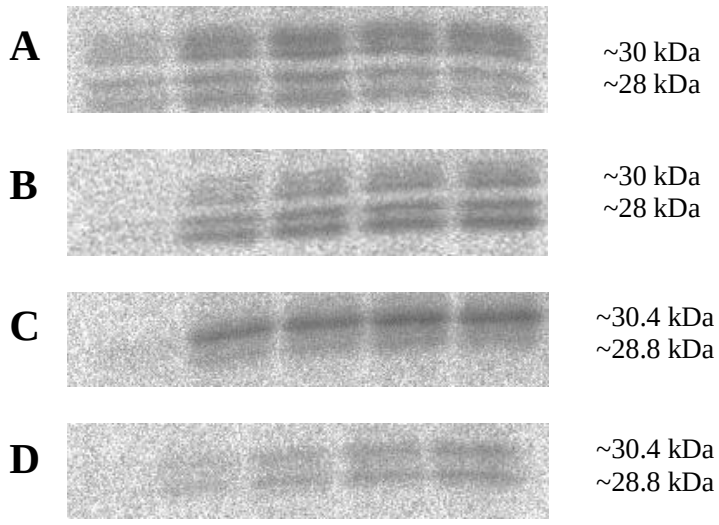


Figure 22. Translocation intermediates of the proOmpA L59 loop in (**A**) PLYEG•A; (**B**) PLYEG•SecAdN10 and of the pGBP loop in (**C**) PLYEG•A; (**D**) PLYEG•SecAdN10.

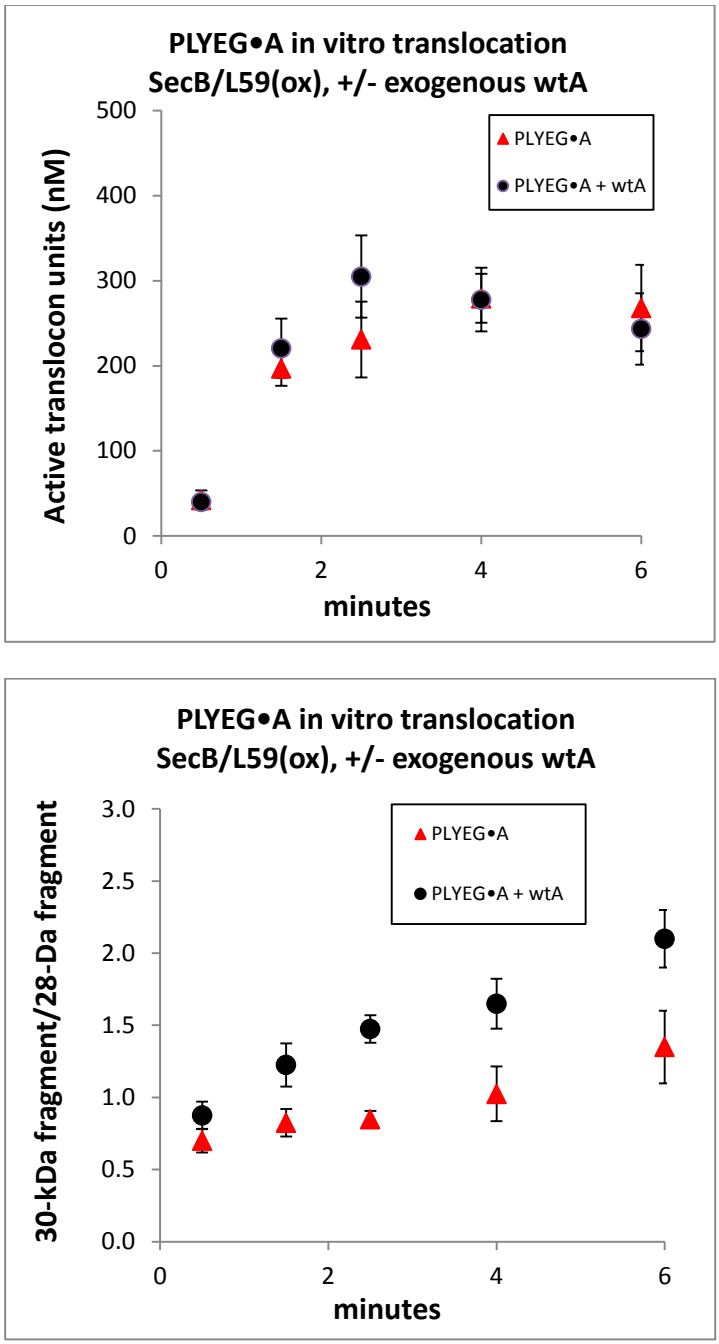


Figure 23. Translocation assay of PLYEG•A with the proOmpA L59 loop. All translocons are jammed after 2.5 minutes (**A**) but the ratio of ~30-kDa/~28-kDa intermediates continues to increase (**B**) indicating that the ~2-kDa translocation step occurs after the translocons have been jammed.

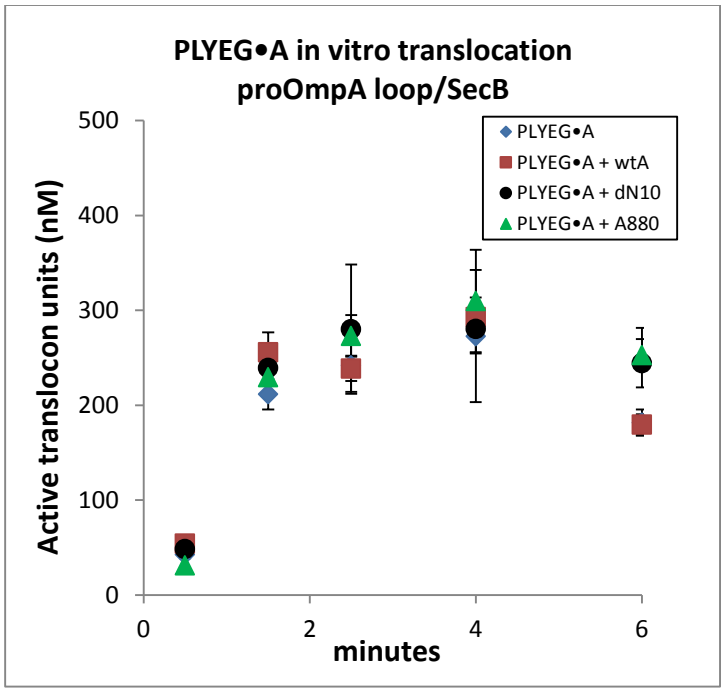


Figure 24. *In vitro* translocation of proOmpA L59(ox) with PLYEG•A. All translocons are jammed after 2.5 minutes. No difference is observed if wild type SecA, SecAdN10 or SecA880 is added.

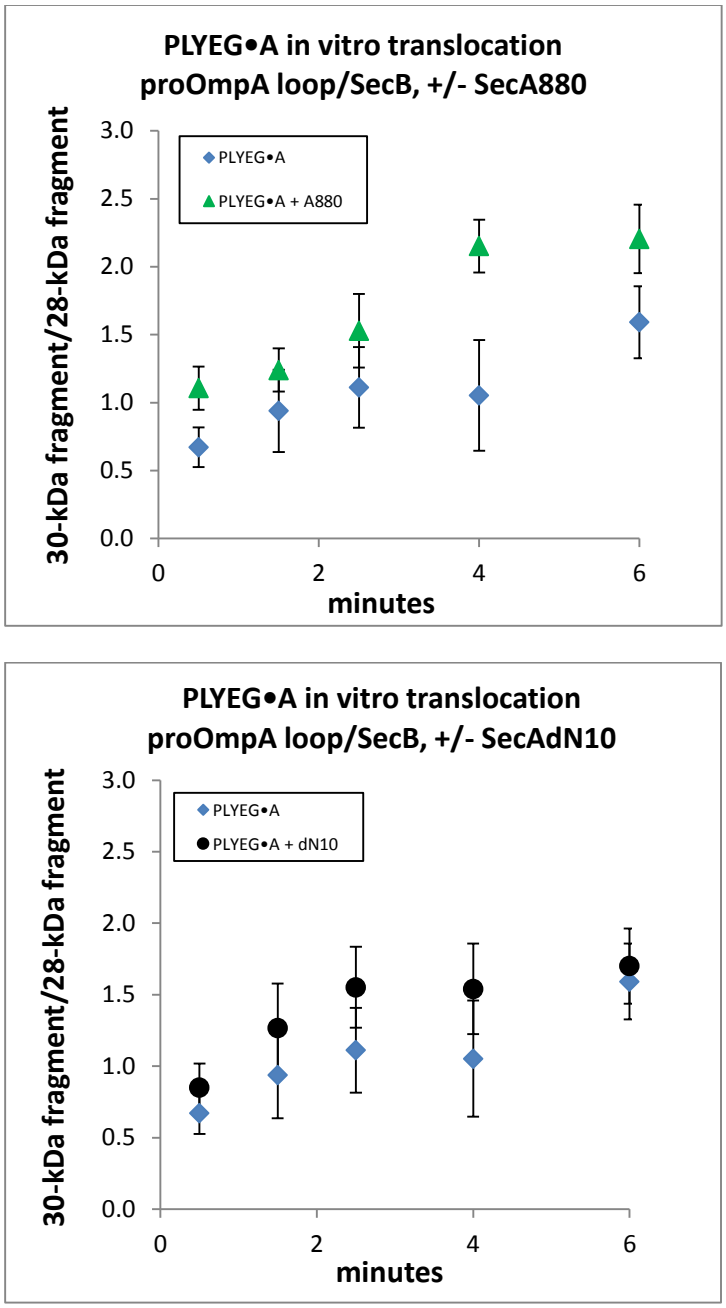
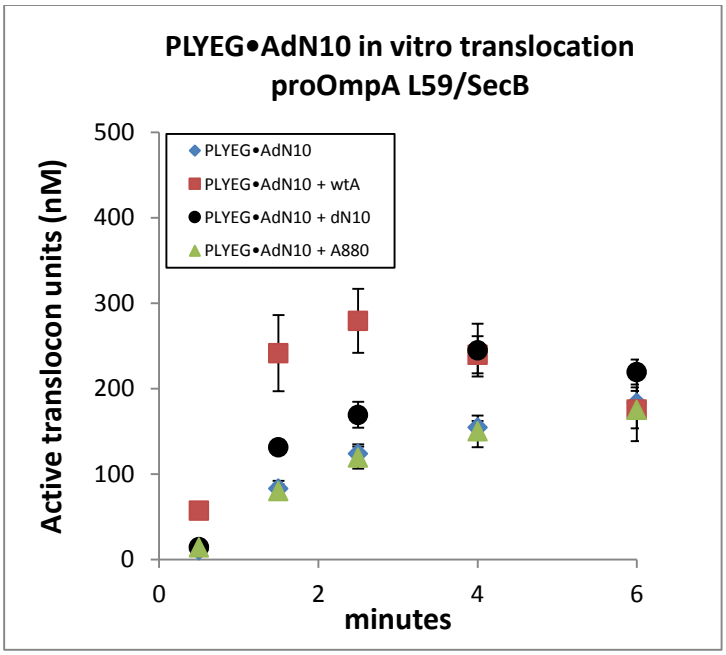
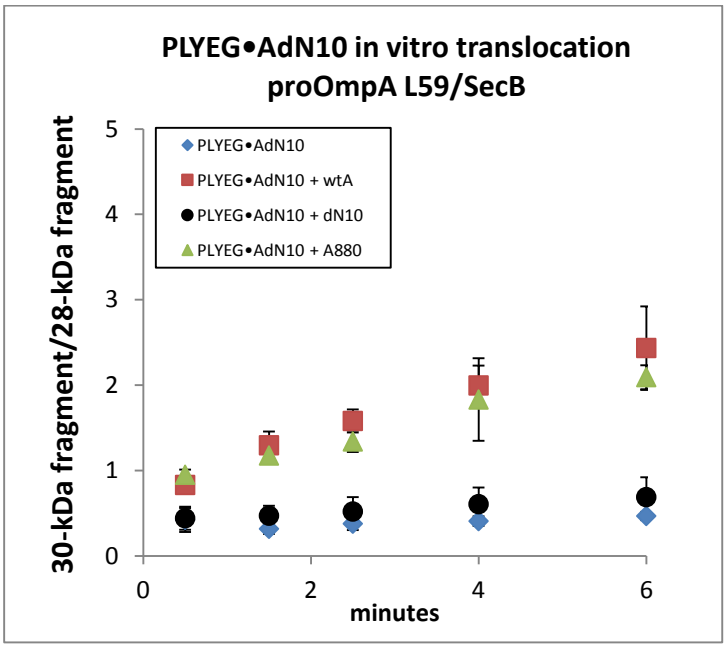


Figure 25. *In vitro* translocation of proOmpA L59(ox) with PLYEG•A. The ratio of 30-kDa/28-kDa fragments increases after 2.5 minutes if SecA880 is added (**A**) but not if SecAdN10 is added (**B**). This indicates that the N-terminal ten residues of SecA stimulate the ~2-kDa step of translocation. For translocation data and number of active translocons see Figure 24.

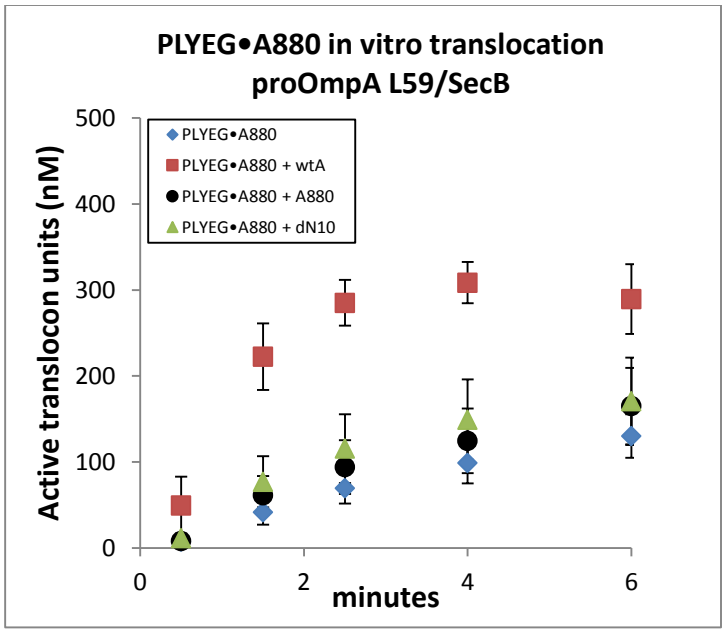


A

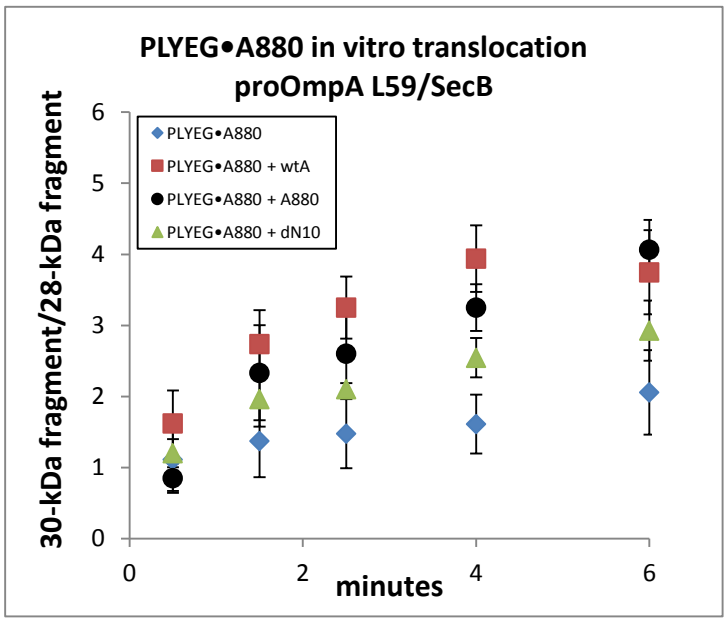


B

Figure 26. *In vitro* translocation of proOmpA L59(ox) in PLYEG•SecAdN10. Addition of wild type SecA or SecAdN10 increases the rate of translocation (**A**) but only wild type SecA and SecA880 stimulate the ~2-kDa translocation step (**B**).



A



B

Figure 27. *In vitro* translocation of proOmpA L59(ox) in PLYEG•SecA880. Addition of wild type SecA increases the rate of translocation (**A**) and addition of wild type SecA, SecAdN10 or SecA880 stimulate the ~2-kDa translocation step (**B**).

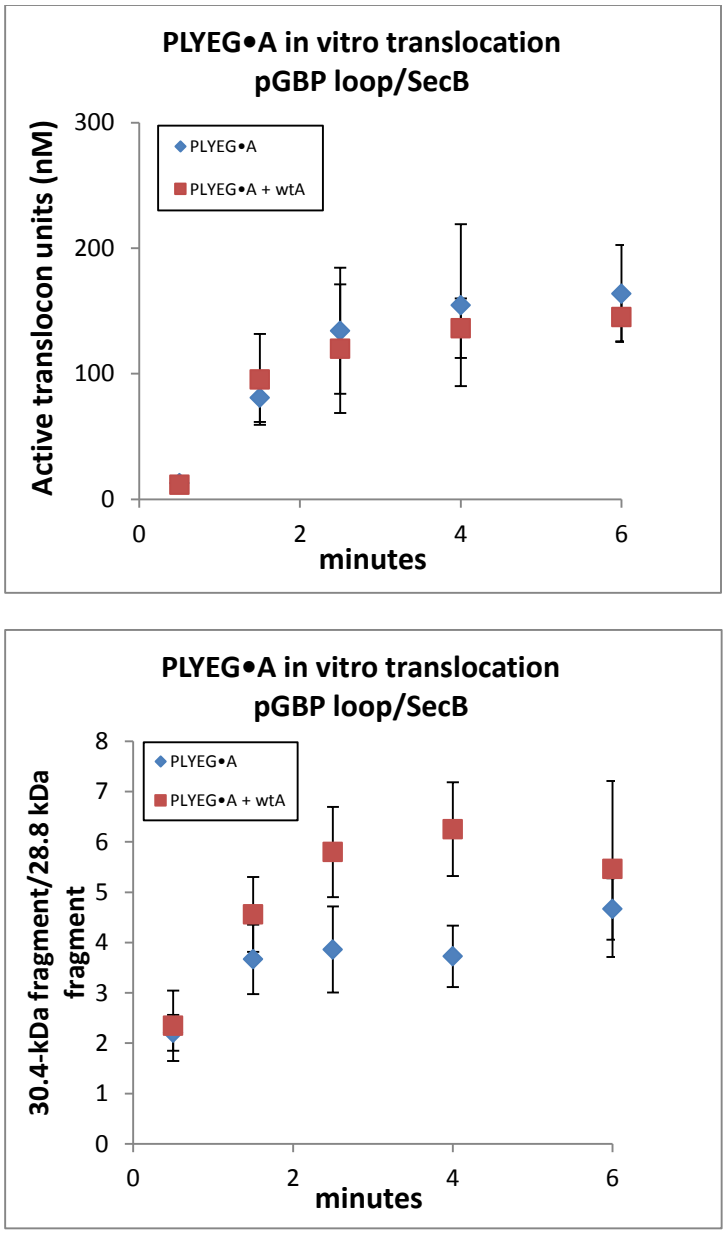
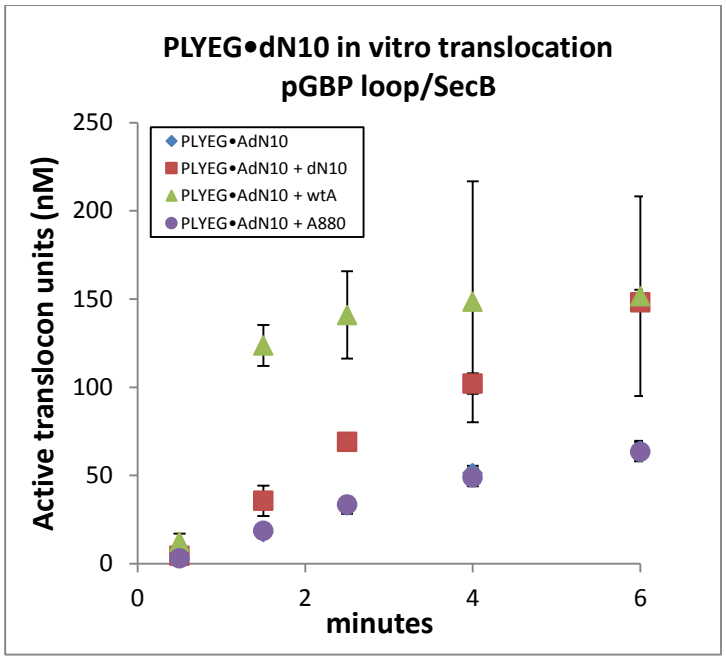
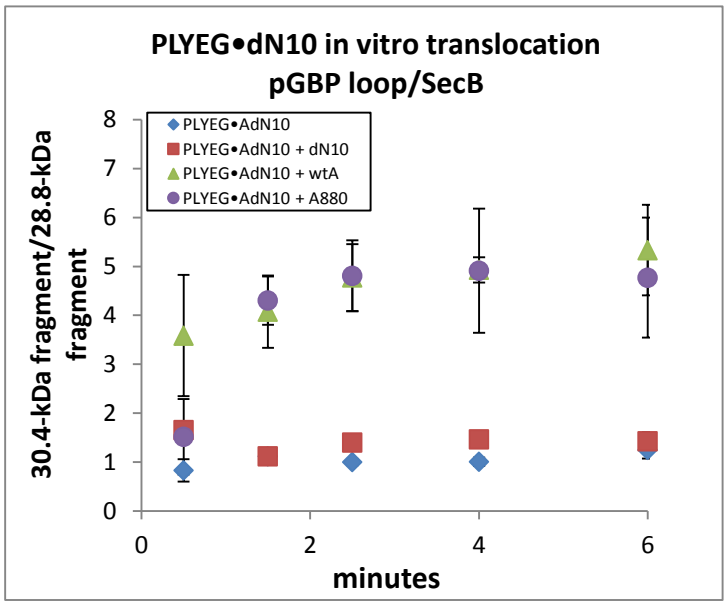


Figure 28. *In vitro* translocation assay of the pGBP loop in PLYEG•A. Additional wild type SecA does not change the number of active translocons or the rate of translocation (**A**) but does stimulate the ~2-kDa translocation step (**B**).



A



B

Figure 29. *In vitro* translocation of the pGBP loop in PLYEG•SecAdN10. Addition of wild type SecA or SecAdN10 increases the rate of translocation (**A**) but SecAdN10 does not stimulate the ~2-kDa step of translocation as do wild-type SecA and SecA880 (**B**).

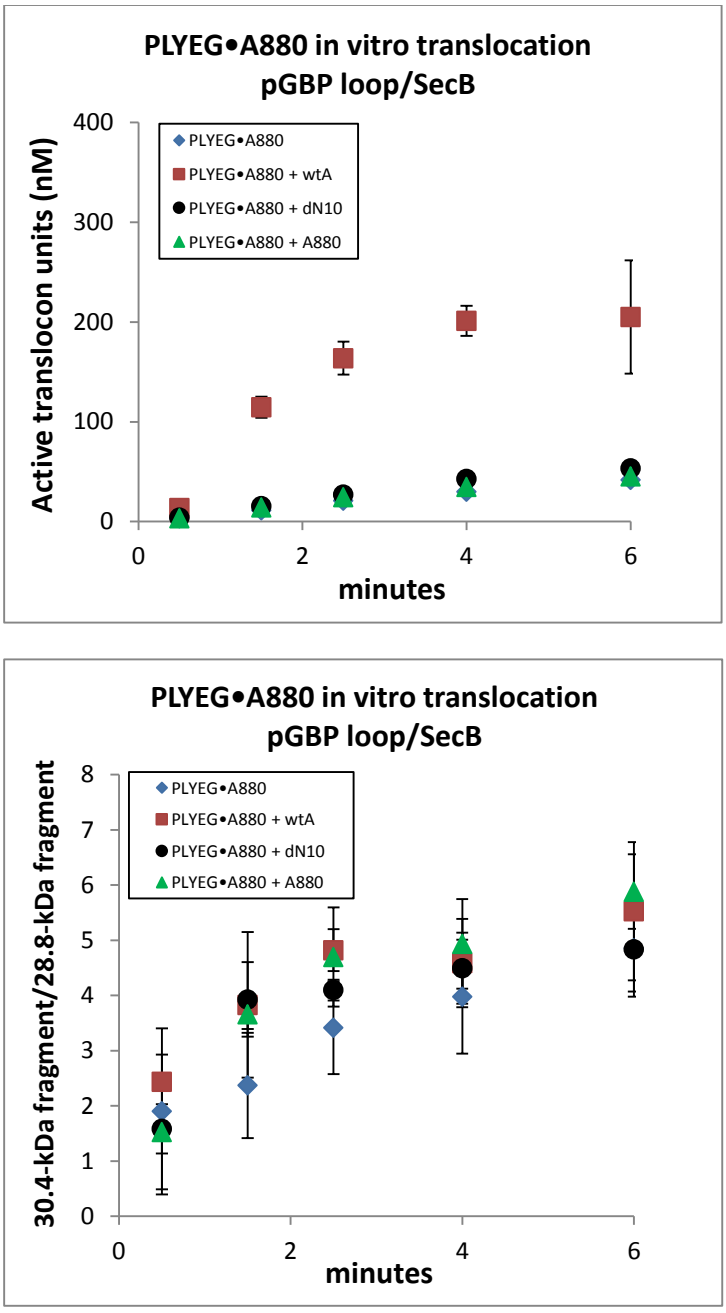


Figure 30. *In vitro* translocation of the pGBP loop in PLYEG•A. Addition of wild type SecA increases the rate of translocation (**A**). No stimulation of the ~2-kDa step of translocation observed when other SecA species are added (**B**) indicating that SecA880 is not deficient in this step of translocation.

The argument that the N-terminal ten residues are important for the ~2-kDa translocation step but not the initial translocation of the precursor up to ~28-kDa is summarized as follows. When the number of active translocons has plateaued in PLYEG•A (i.e., all the translocons have been occupied), the ratio of the larger molecular weight translocation intermediates to lower smaller molecular weight intermediates increases and this increase can be stimulated by either wild type SecA or SecA880 but not by SecAdN10. PLYEG•SecAdN10 is deficient in the ~2-kDa translocation step as the ratio of translocation intermediates does not change during the assay, but adding wild type SecA or SecA880 complements SecAdN10 and the ~2-kDa translocation step occurs. Addition of SecAdN10 does not allow elongation but does increase the rate of translocation. Addition of SecA880 to PLYEG•SecAdN10 does not increase the rate of translocation but does allow the ~2-kDa translocation step. This and the fact that SecAdN10 translocates precursor faster than does SecA880 suggests that the C-terminal residues are crucial for the initial translocation of the precursor up to ~28-kDa and the N-terminal residues are not.

These novel data show details of the mechanism of the general secretory system that have previously been unknown to researchers. The data presented here show that different parts of SecA participate in discrete steps of the translocation process and they may also address the question of how many SecA protomers are involved in translocation. These data show a step of translocation that is stimulated by additional SecA molecules, consistent with evidence that multiple copies of SecA cycle on and off the membrane during translocation (Morita et al. 2012). Furthermore the data implicate the N-terminal and C-terminal residues making different contributions to the process of

translocation. These results further demonstrate that our *in vitro* translocation assay is capable of offering new and valuable insights into the general secretory system that have been unknown previously.

Chapter 5

Discussion

This work contains novel data concerning the stoichiometry of SecYEG in translocation and the relevance of studies done with SecYEG and SecA in proteoliposomes. For the first time an *in vitro* translocation assay using reconstituted proteoliposomes has been described that replicates the activity of the translocase in its native environment. This work also contains data that are consistent with the body of work that indicate an important role for the N-terminal ten residues of SecA in associating with the bacterial inner membrane. The translocation, AFM, and proteolysis data from our studies suggest that biologically relevant SecA-SecYEG binding modes are prevalent in PLYEG•A but not in PLYEG + A. Thus conclusions based on previous studies in reconstituted proteoliposomes containing SecYEG and extraneously added SecA should be reconsidered in light of the possibility that the systems contained biologically irrelevant binding modes.

Co-assembly of SecYEG and SecA in proteoliposomes (PLYEG•A) caused a greater total amount of precursor translocated than PLYEG + A for both proOmpA and pGBP. Jamming the translocon with the proOmpA or pGBP loops showed that PLYEG•A contains a higher number of active translocons than does PLYEG + A. For proOmpA, there are 300 nM active translocons in PLYEG•A and 50 nM active translocons in PLYEG + A. For pGBP there are 180 nM active translocons in PLYEG•A and 35 nM active translocons in PLYEG + A. Thus co-assembly increases the number of active translocons by up to six-fold. Proteoliposomes made with only SecYEG were incubated with SecA at room temperature for 2.5 hours to simulate the incubation time of fabrication for PLYEG•A but no increase in activity was observed. When the number of active translocons was determined in inverted membrane vesicles (IMVs) using the

oxidized loop precursors, they showed an equivalent number of active translocons (corrected for sidedness of SecYEG) to PLYEG•A. Thus we conclude that the addition of SecA during reconstitution of SecYEG in proteoliposomes preserves the active state of SecYEG *in vivo* that is lost during purification and reconstitution. Furthermore we observed that when proOmpA is the substrate for our system there are twice the number of active translocons as when pGBP is the substrate. We conclude that the number of active translocons in our assay varies upon the precursor species used. This may depend on whether the substrate is a membrane protein or a soluble protein and future work will explore this. In future work this will be studied using other Sec system substrates such as pMBP (precursor maltose binding protein), LamB (precursor maltoporin) and mutant proteins of proOmpA that have only the membrane-integral or the cytoplasmic portions.

Translocation activities of PLYEG•A proteoliposomes made by co-assembly of variant SecA species with SecYEG indicate that there is a specific interaction between SecA and SecYEG that causes the increase in the number of active translocons. Of the SecA species used, those containing the N-terminal ten residues (wild type, SecA880, SecAC4, SecAN664) had similar numbers of active translocons (250-300 nM). PLYEG•SecAdN10 had an intermediate number of active translocons (150 nM) and PLYEG•SecAC619 and PLYEG•SecAC662 had no increase in active translocons over PLYEG + A. More work must be done to determine what part of SecA causes the increase in active translocons but it is clearly not within the C-terminal portion of SecA after residue 664. Future studies will use smaller N-terminal fragments of SecA in co-assembled proteoliposomes to probe the region of SecA that activates SecYEG. The intermediate level of active translocons in PLYEG•SecAdN10 relative to

PLYEG•SecAwt indicates that when SecAdN10 is bound to SecYEG it does activate SecYEG. The N-terminal residues of SecA most likely stabilize the binding interaction. Thus in their absence fewer SecAdN10 are bound to SecYEG. This interaction could be stabilized by a membrane-integral conformation of SecA or by a binding interaction between SecA and SecYEG that is stabilized when the N-terminal residues bind the lipid bilayer.

The N-terminal ten residues of SecA are important for SecA to associate with the membrane (Das et al. 2008) and have been demonstrated to be situated in the lipid bilayer (Bahar Tuba Findik, unpublished observation) in a helical structure. The data from the exchange experiments confirm that these ten residues are important in membrane association as SecAdN10 has very little affinity for the membrane and it also has impaired ability to displace other SecA species that contain the ten N-terminal residues. The 21 C-terminal residues of SecA have also been demonstrated to interact with the lipid bilayer (Breukink et al. 1993) and are also important in the association of SecA with SecYEG as revealed in the exchange experiments.

The exchange experiments also address the possibility of two different membrane-associated states of SecA that have been postulated previously (Chen et al. 1998). One would expect built-in wild type SecA in PLYEG•A to be completely displaced in the presence of a twenty-fold excess of wild type SecA added as a competitor; however, we observed only 60 percent of the wild type SecA displaced. This result is similar to what was observed with radio-labeled wild type SecA in inverted membrane vesicles (Chen et al. 1996). This suggests that there are two membrane-associated forms of SecA: one that is readily exchanged by externally added SecA and

one that is highly resistant to exchange by externally added SecA. Further evidence for two membrane-associated forms of SecA include the fact that the 44-kDa and 68-kDa proteolytic fragments observed in PLYEG•A cannot be breakdown products from the same SecA conformation and when the concentration of SecA increases in the translocation assay the 68-kDa fragment increases but the 44-kDa fragment does not.

The translocation intermediate data of the proOmpA and pGBP loop precursors are also consistent with two different roles of SecA in translocation. For both precursor species the rate of accumulation of these intermediates is higher for PLYEG•SecAdN10 than it is for PLYEG•SecA880. This suggests that part of the initial step of the translocation process requires the C-terminal tail of SecA and not the N-terminal ten residues while the elongation step of translocation requires the N-terminal ten residues of SecA independently of the C-terminal tail. These data are consistent with a proposed model of protein translocation (Morita et al. 2012) where one membrane-associated SecA molecule translocates precursor in 8-kDa steps in a manner coupled to ATP hydrolysis, holds the partially translocated protein in SecYEG and a second, cytosolic, ATP-bound SecA molecule further translocates the substrate by ~2 kDa. If we fit our data with this model the membrane-bound SecA molecule would translocate precursor by ATP hydrolysis in a manner dependent on the C-terminus of SecA and a cytosolic ATP-bound SecA molecule would translocate the precursor by ~2 kDa in a manner dependent on the N-terminal ten residues of SecA. It is possible; however, that one SecA molecule translocates the precursor alone. In that case SecAdN10 and SecA880 would have to alternate during translocation of the ~2-3 kDa portion of the substrate.

The results presented here show the importance of preserving the active state of membrane proteins when reconstituting them into lipid bilayers. Furthermore these findings establish the stoichiometry of SecYEG in translocating different precursor species and the role of the N-terminus in binding of SecA to the membrane and in translocating precursors. These results will be the foundation for further investigation of how SecA binds the bacterial inner membrane and the mechanism of translocation.

Chapter 6

Materials and Methods

Purification of Proteins

The translocon, SecYEG, was purified from a strain C43(DE3) suitable for overexpression of membrane protein (Miroux and Walker 1996) harboring a plasmid encoding secE with a Histag at the N terminus, secYC329S, C385S, and secG (Cannon et al. 2005). Cells were broken by passage through a French pressure cell (8,000psi), and the membranes were isolated by centrifugation and solubilized in dodecyl-β-maltoside (DBM). SecYEG was purified by chromatography, using a HisTrap column (GE Healthcare),and stored at −80 °C in 20 mM Tris-Cl at pH 8, 0.3 M NaCl, 10%(wt/vol) glycerol, 0.6 mM DBM, and 2 mM DTT. SecA ATPase and SecA variants were purified as described (Randall et al. 2005), with the following modifications: Intact washed cells were incubated on ice for 30 min with 8 mM EDTA to chelate Mg^{2+} in the cell envelope. The cells were pelleted and washed twice to remove the EDTA before being lysed by three cycles of freezing and thawing in the presence of lysozyme. The removal of EDTA before lysis is crucial to prevent the extraction of zinc from SecA. After centrifugation, SecA species were purified from the relevant supernatants by chromatography, using QAE (TosoHaas) and/or HiTrap Blue affinity columns (GE Healthcare). The purified proteins were dialyzed into 10 mM Hepes at pH 7.6, 0.3 M potassium acetate (KOAc), 2 mM DTT, and stored at −80 °C. Cultures for the chaperone SecB purification were grown as described (Randall et al. 1998), the cells were disrupted with a French press at 8,000 psi, and the protein was purified from high-speed supernatants, using a QAE column (TosoHaas). The purified SecB was stored in the same solution as that used for SecA. Concentrations of the proteins were determined spectrophotometrically at 280nm, using coefficients of extinction as follows: SecB tetramer, 47,600 $M^{-1} \cdot cm^{-1}$; SecA wild-

type and SecAdN10 monomers, 78,900 $M^{-1}\cdot cm^{-1}$; SecA880, 77,200 $M^{-1}\cdot cm^{-1}$; and SecYEG,45,590 $M^{-1}\cdot cm^{-1}$. SecAdN10 has aminoacyl residues 2 though11 deleted and SecA880 is truncated to remove the C-terminal 21 aminoacyl residues that coordinate zinc.

Preparation of Radiolabeled Proteins

The precursors of outer membrane protein A (OmpA) and periplasmic galactose-binding protein (GBP) were produced from plasmids (pAL612 andpAL663, respectively) carrying the ompA gene or the mglB gene altered to generate polypeptides with only two cysteine residues.

In OmpA, C290 was substituted by serine and G244 by cysteine, and the native C302 was retained. For GBP, two cysteines were introduced, L267C and D310C. The proteins were radiolabeled by the addition of [^{35}S]methionine or a mixture of ^{14}C-L-amino acids(Perkin-Elmer) to cells growing in M9 minimal media, as described(Mao, Hardy et al. 2009). Precursor OmpA (proOmpA) was purified as described(Mao, Hardy et al. 2009). Precursor GBP (pGBP) was purified as follows: cells were disrupted with a French press, inclusion bodies containing pGBP were collected by centrifugation, solubilized with urea, and loaded onto a HiTrap QAE column (GE Healthcare).

Precursors were stored at −80 °C in 10 mM Hepes at pH 7.6 with 1 mM Tris(2-carboxyethyl)phosphine (TCEP) to maintain the sulfhydryls in reduced form; for proOmpA, 0.1 M KOAc and 4 M urea; and for pGBP, 0.3 M KOAc, 1 N GnHCl, and 1 mM EGTA. Disulfide bond-stabilized loops were generated after removal of TCEP by the addition of 0.1 mM copper phenanthroline to the solution and incubation for 5 min on ice. Copper phenanthroline was removed using a NAP10 column (GE Healthcare). The

^{3}H SecA variants were produced and purified as described for SecA in Purification of Proteins above, except that growth of the cultures was in the M9 minimal media with addition of ^{3}H-Leu and ^{3}H-Met (Perkin-Elmer) and the other amino acids, nonradiolabeled at 0.5 mM, with the exception of Ile and Val.

Proteoliposomes

Unilamellar liposomes were prepared by extrusion of *Escherichia coli* (E. coli) polar lipids (Avanti) suspended in 10 mM Hepes at pH 7.6, 30 mM KOAc, 1 mM $Mg(OAc)_2$ through membranes with a 100-nm pore diameter, using a Liposofast (Avestin). To form proteoliposomes, the liposomes were swelled but not disrupted, using a ratio of detergent to lipids of 4.65 mM DBM to 5 mM lipids (Lambert, Levy et al. 1998). After swelling for 3 h at room temperature, the proteins to be incorporated were added: SecYEG at 5.2 μM, and for coassembly of SecA, SecA at 5 μM dimer unless otherwise specified. Incubation was continued for 1 h at room temperature, followed by addition of BioBeads SM-2 (BioRad) to remove the detergent. The proteoliposomes were isolated by centrifugation at 436,000 × g, 20 min at 4 °C, in a TL100.1 rotor (Beckman). The pellet was suspended in the same buffer and centrifuged again as earlier. The final pellet was suspended to give a concentration of ~10 mM lipid and ~10 μM SecY. The suspension was stored at -80 °C.

Translocation Assay

Translocation of 1 μM radioactive proOmpA or pGBP, labeled with a ^{14}C-L-amino acid mixture or [^{35}S]methionine, into proteoliposomes or inverted membrane vesicles was carried out at 30 °C under conditions of limiting SecY (1 μM) with SecA (1.2 μM dimer), SecB (1 μM tetramer), and EGTA (1 mM) to prevent pGBP from

folding, as described (Mao et al. 2009), with the following modifications: An ATP-regenerating system consisting of 7.5 mM phosphocreatine and 37 mg/mL of creatine phosphokinase was present in the reaction. The precursors are stored in denaturant (4 M urea for proOmpA or 1 N GnHCl for pGBP) and diluted into the translocation assay, which thus contains either 44 mM urea or 11 mM GnHCl. In experiments using precursors containing internal disulfide bonded loops, DTT was omitted from the reaction mix. Proteinase K (5 units/mL, 15 min on ice) was used for degradation of untranslocated proteins, and digestion terminated by trichloroacetic acid precipitation. The washed precipitate was dissolved in gel sample buffer containing DTT (10 mM) for analysis by electrophoresis.

Analyses of Translocation Data

The radioactivity in the protein bands in the gels of the translocation assays was measured using a Fujifilm FLA 3000 phosphorimager in the linear range of its response. For the experiments in which the number of active translocon units was determined, the radioactive precursors used contained internal disulfide bonded loops. When oxidized precursors were used as substrates, the protected protein intermediates observed spanned molecular weight ranges of 23.8–30.5kDa (full length, 37.2 kDa) for proOmpA and 28.8–31.7 kDa (full length, 35.7 kDa) for pGBP. Only species that could be converted to full-length precursors after reduction of the disulfide bond in the loop were included in the estimate of the molarity of the intermediates. We assumed that all protected bands were N-terminal fragments of the precursor, estimated their lengths from a standard curve derived from the molecular weight markers on the gel, and corrected for the missing ^{14}C-labeledsequences or missing [^{35}S]methionines to calculate the molarity. To determine

molarity of the full-length precursors and the intermediate-length species protected from degradation by Proteinase K, samples of the reaction mixture were taken at t = 0 min and processed without addition of protease. The t = 0 sample was taken in duplicate, as this value was the basis for calculation of molarity for each point in the time course.

Exchange of built-in SecA in PLYEG•A with competing SecA species

Proteoliposomes were made with SecYEG in the presence of radiolabeled (^{3}H-Leu and ^{3}H-Met) variant SecA species. The ratio during coassembly for SecAdN10 was 5 μM SecA dimer to 5.2 μM SecY, and for SecA880, 2.5 μM dimer to 5.2 μM SecY. Exchange of wild-type SecA for the incorporated variant species was achieved by incubation of the proteoliposomes at 30 °C for 5 min in10 mM Hepes at pH 7.6, 250 mM KOAc, and 5 mM $Mg(OAc)_2$with wild-type SecA at either an equal concentration to that of SecA in the proteoliposomes for SecAdN10 or an eightfold molar excess for SecA880. The proteoliposomes underwent 3cycles of centrifugation (Beckman TL100.1 rotor at 436,000 × g,4 °C, 15 min) and suspension to remove soluble SecA before they were assayed for the number of active translocons at 1 μM SecY and 1 μM oxidized proOmpA. The amount of variant ^{3}H-SecA displaced was determined by liquid scintillation counting of samples of the supernatant fractions after each centrifugation and of the final pellet. Because the radiolabel on SecA was ^{3}H, which is not detected by the phosphorimager, it did not interfere with the assay of activity using ^{14}C- and ^{35}S-labeled precursors.

To study the affinity of the various SecA species for the membrane proteoliposomes were made with SecYEG in the presence of radiolabeled (^{3}H-Leu and ^{3}H-Met or ^{14}C-L-amino acid mixture) variant (SecAdN10, SecA880, SecAC4) or wild-

type SecA species. The ratio during coassembly for the different SecA species was 5 μM SecA dimer to 5.2 μM SecY. Exchange of externally added SecA for the incorporated species was achieved by incubation of the proteoliposomes at 30 °C for 5 min in10 mM Hepes at pH 7.6, 250 mM KOAc, and 5 mM $Mg(OAc)_2$ with SecY at 0.5 μM and either no competitor, or the competing SecA species at either an equal concentration to that of SecA in the proteoliposomes or an excess of competitor. Ten μL of each mixture were sampled in duplicate to 5-mL vials containing 3 mL 30% Scintisafe and counted on a scintillation counter for input. Three 20-μL aliquots of the proteoliposome mixtures underwent centrifugation (Beckman TL100.1 rotor at 436,000 × g,4 °C, 5 min) and the supernatants were removed and the pellets were suspended in SDS sample buffer. The amount of input radio-labeled SecA displaced was determined by liquid scintillation counting of 10 μL of the samples of the supernatant fractions after centrifugation and of an equivalent amount of the suspended pellet.

Concentration of Protein and Lipid

The concentrations of the radiolabeled precursors were determined using SDS polyacrylamide electrophoresis and comparing the intensity of the protein bands with those of standards run on the same gel. The concentration of proteins used as standards was determined by quantitative amino acid composition of proteins having purities of greater than 95%. The concentration of purified SecY, as for the other proteins, was determined by SDS polyacrylamide electrophoresis and a standard run on the same gel. In IMVs the concentration of SecY was determined by Western blot. The intensity of bands was quantified using TotalLab Software. The concentration of lipids was

determined as described on the AvantiPolar Lipids Web site (www.avantilipids.com), using an average molar mass for E. coli lipids of 741 Da.

Proteolysis of SecA in PLYEG + A and PLYEG•A

Samples of PLYEG + A and PLYEG•A were prepared for proteolysis with Proteinase K. Final concentrations were 1.4 µM SecY and 1.1 µM SecA dimer for PLYEG + A and 1 µM SecY and 1.1 µM SecA dimer for PLYEG•A. Proteinase K was added from 19 U/ml to 5.4 U/ml and incubated 15 minutes on ice in buffer conditions of 9 mM HEPES pH 7.6, 230 mM KOAc, 3.6 mM $Mg(OAc)_2$. PMSF was then added to a concentration of 1 mM to stop proteolysis and an aliquot was sampled for the 'total' fraction. The remainder of the solution was centrifuged in a Beckman TL100.1 rotor at 436,000 × g, 4 °C, for 5 min. The supernatant was removed for the 'supernatant' fraction and the pellet was suspended in 9 mM HEPES pH 7.6, 180 mM KOAc, 3.6 mM $Mg(OAc)_2$, 0.8 mM PMSF for the 'pellet' fraction. All samples were subjected to trichloroacetic acid precipitation to ensure that no further proteolysis occurred when the proteins were denatured in sample buffer. The samples were analyzed by Western blotting using region-specific anti-SecA antisera at dilutions of 1:500 for αA1 and 1:1000 for antisera αA2-αA6.

Washing SecA off the membrane of PLYEG + A and PLYEG•A

Solutions of proteoliposomes PLYEG and PLYEG•A were made at 0.5 µM SecY (SecA was added to a concentration of 0.6 µM A_2 in PLYEG + A) in 10 mM HEPES pH 7.6, 250 mM KOAc, 5 mM $MgOAc_2$. The solutions were sampled to generate a standard curve of SecA to load on SDS PAGE gels as input. Twenty µl aliquots were added to TL100.1 tubes and centrifuged (Beckman TL100.1 rotor at 436,000 × g, 4 °C, 5 min) to

remove excess SecA. The samples were suspended in various solutions to 20 µl to remove membrane-associated SecA: 1 M NaCl 10 mM HEPES pH 7.6, 1 M KOAc 10 mM HEPES pH 7.6, 5 M Urea 10 mM HEPES pH 7.6, 10 mg/ml heparin 10 mM HEPES pH 7.6, 0.9 M hydroxylamine pH 8, 0.2 M Na_2CO_3 pH 11, 10 mM HEPES pH 7.6 250 mM KOAc 5 mM $MgOAc_2$ (control). The solutions were incubated for 30 min on ice (37°C for hydroxylamine) and centrifuged (Beckman TL100.1 rotor at 436,000 × g, 4 °C, 6 min) to separate the extracted SecA from the proteoliposomes. The supernatants were subjected to trichloroacetic acid precipitation and the washed precipitates were dissolved in gel sample buffer containing DTT (10 mM) for analysis by electrophoresis.

Iodination of TIDBE

The precursor compound 4'-(3-Trifluoromethyl-3H-diazirin-3-yl)-2'-tributylstannylbenzyl benzoate was purchased from Toronto Research Chemicals. Fifty nmol of the precursor compound was dissolved in anhydrous chloroform and taken to dryness in a Wheaton glass vial under argon and then dissolved in 35 µL of acetic acid. 0.5 mCi (in 1 µL) of $Na^{125}I$ (Perkin Elmer) was added followed by 10 µL of 39% peracetic acid (in acetic acid) solution. The vial was vortexed every 10 seconds and then continuously after 30 seconds for a total of two minutes. Next 2 µL of 25 mM aqueous sodium iodide was added and the vial was vortexed an additional two minutes. Next 10 µL 100 mM aqueous NaI and 200 µL 10% w/w aqueous $NaHSO_3$ were added to quench the reaction. Ethyl acetate (200 µL) was added and the vial was vortexed to extract the product—3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl) diazirine benzoic acid ester (^{125}I TIDBE)—into the organic phase. The organic phase was removed to an amber Reactivial

and the solvent was evaporated under argon. Anhydrous ethanol (100 µL) was added to the Reactivial to dissolve the product and the sealed vial was stored at -30 °C.

Labeling of membrane-integral proteins with ^{125}I TIDBE

Solutions of proteoliposomes were made in 10 mM HEPES pH 7.6 250 mM KOAc 1 mM $Mg(OAc)_2$. SecA species were added to a concentration of 1 µM monomer for 2 µM SecY in the case of PLYEG + A. Solutions of PLYEG•A were made at 1 µM SecY. Solutions of liposomes were made at 1 mM lipid and SecA was added to a concentration of 2 µM monomer in 10 mM HEPES pH 7.6 250 mM KOAc 1 mM $Mg(OAc)_2$. For every 100 µL of sample 1 µL of ^{125}I TIDBE was added and incubated for 30 minutes on ice before the photolysis reaction. Samples were handled under low light conditions from the time ^{125}I TIDBE was added until photolysis occurred. For the photolysis reaction the samples were irradiated with a Quartz-Mercury Arc lamp for 30 seconds with the spot plate 4.5 cm from the lamp.

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