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Electrophysiological Characterization of SecA-dependent Protein-conducting Channel

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Electrophysiological Characterization of SecA-dependent Protein-conducting Channel

by

Ying-Hsin Hsieh

Under the Direction of Phang-Cheng Tai

ABSTRACT

Sec translocon is the major machinery for protein translocation in *E.coli* including SecYEG, SecA and other Sec proteins. It is generally assumed that during translocation process, SecYEG serves as a protein-conducting channel and transports the protein across membranes by using SecA ATPase as driving force. However, previous work suggested that protein translocation can occur without SecYEG. In order to understand the role of SecA in this SecYEG-independent process, we use voltage clamp recording as a tool to study the ionic activity of SecA-dependent protein-conducting channel. In a major deviation from the conventional view, we found that SecA alone is sufficient to promote the channel activity with liposomes made of *E.coli* phospholipids in both whole cell recording in the oocytes and in the single channel recording with patch clamp. The activity is strictly dependent on the presence of functional SecA, including those from different species of bacteria. However, this SecA-alone dependent channel

activity is less efficient compared to the membranes containing SecYEG. Furthermore, the channel activity loses the signal peptide specificity. Addition of purified SecYEG restores the signal peptide specificity as well as the efficiency. This channel activity is more sensitive to SecA-specific inhibitors compared with membranes containing wild-type SecYEG but is less sensitive to membranes containing suppressor proteins. This is the first time it has been shown that SecA binds to lipid low-affinity site and functions as a protein-conducting channel.

To further characterize the structural roles of SecA as the core of the channel, we use several SecA variants to reconstitute with liposomes to determine the domains involved in forming functional channels. Using deletion truncated domains of 901 residues SecA and liposomes in the oocytes recordings, we identify two critical SecA domains for the formation of pore channel activity: with phospholipids alone, and for interacting with SecYEG to gain higher activity. These data provide fundamental understanding for the SecA-dependent protein – conducting channels. Our findings also suggest the possible evolution process on the protein translocation pathways from prokaryotes through eukaryotes.

INDEX WORDS: SecA, SecYEG, Liposomes, Channel activity

Electrophysiological Characterization of SecA-dependent Protein-conducting Channel

by

Ying-Hsin Hsieh

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

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in the College of Arts and Sciences

Georgia State University

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Electrophysiological Characterization of SecA-dependent Protein-conducting Channel

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December 2011

DEDICATION

To my lovely husband, Hsin, and my parents who has the faith in me

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It is a pleasure to thank those people who made this dissertation possible. A greatest hearty thankful to my advisor, Dr. Phang C. Tai, whose encouragement, supervision, and support from preliminary to the concluding levels enables me to pass through the difficulties of scientific research and help me make this dissertation into reality. I also want to thank Dr. Chun Jiang and Dr. Ningren Cui for all the instructions and technical assistances of electrophysiological experiments. Special thanks to Dr. Chung-Dar Lu, Dr. Parjit Kaur, and Dr. Zehava Eichenbaum for being my committee and all the precious advices for my research. In addition, Dr. Hsiu-Chin Yang for great consultation; Dr. Jin-shan Jin and Dr. Ying-Ju Huang for providing me the materials for my research. A special thanks for Mrs. Ying-Mei Tai for supporting me all those years in Atlanta.

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TABLE OF CONTENTS

DEDICATION	v
ACKNOWLEDGEMENT.....	vi
LIST OF TABLES.....	viii
LIST OF FIGURES.....	ix
BACKGROUND INTRODUCTION	1
MATERIAL AND METHODS.....	8
RESULTS PART I	16
RESULTS PART II	38
RESULTS PART III	54
RESULTS PART IV	74
CONCLUSION.....	92
REFERENCES.....	95
APPENDICES	115
APPENDIX I	115
APPENDIX II	148

LIST OF TABLES

Table 2.1 Rose Bengal IC50 on various membrane vesicles	73
Table 2.2 Rose Bengal IC50 on SecA homologs channel activity	73
Table 2.3 Comparison of SecA inhibitors.....	73

LIST OF FIGURES

Figure 1 Oocyte injection and whole cell recording.....	15
Figure 1.1 SecA and liposomes alone are capable for promoting ion-channel activity in the oocytes.....	28
Figure 1.2 Optimization of SecA ion-channel activity in the oocytes	29
Figure 1.3 Patch clamp measurements of ionic channel activity in SecA-liposomes	30
Figure 1.4 Channel openings of SecA-liposome by proOmpA stimulation	31
Figure 1.5 Channel openings of SecA-liposome by LamB signal peptide stimulation	32
Figure 1.6 Loss of signal peptide specificity of SecA-liposomes	33
Figure 1.7 SecY suppressor mutant membranes lose proofreading function	34
Figure 1.8 Proofreading function of channel activity on SecY-plug domain deletion	35
Figure 1.9 SecYEG restores the proofreading function of SecA-liposomes.....	36
Figure 1.10 Requirements of SecYEG or SecA on ion-channel activities in oocytes.....	37
Figure 2.1 Dependence of SecA for channel activity in BA13 membranes.....	47
Figure 2.2 Dependence of SecA for channel activity in RE-BA13 membranes	48
Figure 2.3 Channel activity of BA13 or reconstituted BA13 membranes with various SecAs addition.....	49
Figure 2.4 Channel activity of reconstituted BA13 by proPhoA stimulation	50
Figure 2.5 Loss of proofreading function in SecYEG minus and reconstituted BA13 membranes	51
Figure 2.6 Dependence of SecA for channel activity in BA13 membranes.....	53
Figure 3.1 Inhibition by Sodium Azide on MC4100 and RE-MC4100 membrane vesicles.....	64
Figure 3.2 Inhibition of Sodium Azide of BA13 and RE-BA13 membrane vesicles	65
Figure 3.3 Inhibition of Rose Bengal on MC4100 or RE-MC4100 membranes	66
Figure 3.4 Inhibition of Rose Bengal on BA13 and RE-BA13 membranes.....	67
Figure 3.5 Channel activity on SecA liposome in the presence of Rose Bengal	68
Figure 3.6 Inhibition of Rose Bengal on channel activity of SecYEG minus membrane	69

Figure 3.7 Channel activity of SecY supressor mutant PrIA4 membrane	70
Figure 3.8 Channel activity of PrIA665 and RE-PrIA665 with Rose Bengal treatment	71
Figure 3.9 Channel activity on SecY mutant membranes in the presence of Rose Bengal	72
Figure 4.1 SecA C-terminal deletion mutants	81
Figure 4.2 Channel activity of SecA C-terminal deletion mutants in the oocytes	82
Figure 4.3 SecA C-terminal deletion mutants with liposomes in the oocytes	83
Figure 4.4 BA13 membrane channel activity with SecA-deletion mutants in the oocytes	84
Figure 4.5 SecA liposomes channel activity on SecA1-828 to SecA1-831 in the oocytes	85
Figure 4.6 Channel activity of C-terminal deletion mutants coupled with C34 in BA13 membranes in the oocytes	86
Figure 4.7 Channel activity of C-terminal deletion mutants coupled with C34 in liposomes in the oocytes	87
Figure 4.8 SecA-N-terminal mutants	88
Figure 4.9 Channel activity of SecA N-terminal deletion mutants in BA13 membranes in the oocytes	89
Figure 4.10 SecA N-terminal deletion mutants' channel activity in liposomes in the oocytes	90
Figure 4.11 Channel activity of SecA C95.43-828 and C95.43-829 in the oocytes	91

Background Introduction

In all living organisms, the movement of proteins across cytoplasmic membrane is termed protein translocation, and the exported proteins are named secretory proteins. The mechanism underlying the protein translocation was originally postulated by Blobel and Dobberstein with signal peptide hypothesis (Blobel and Dobberstein 1975). The N-terminal sequence of secretory proteins serves as a recognition tag to initiate translocation and proteolytically removed during the process (Gilmore, Blobel et al. 1982; Gilmore, Walter et al. 1982).

In bacteria, proteins are transported to defined destination. After post-translation in cytoplasm, the nascent protein travel across the lipid bilayers to periplasm or outer membrane. In recent years, genetic and biochemical studies of bacteria translocation have significantly advanced the understanding of the mechanism and components involved in protein export. So far, there are at least six different types of protein secretion pathway (type I-type VI) (Economou, Christie et al. 2006). Type I secretion is independent of Sec system; this type secretion also known as ABC transporter (ABC binding cassette). Type II GSP, general secretory pathway, is Sec translocation dependent. Most of proteins in bacteria transport to their destination (most of time is periplasm in Gram-negative bacteria) through this type of system, which involved the signal sequence recognition and cleavage. Type III is another Sec-independent translocation system similar as type I. This type of secretion usually involved in pathogen protein injection into host eukaryotic cells. Type IV is widespread secretion system responsible for conjugation system; and the most proteins secreted through this system are designated to outer membrane (Lengeler, Drews et al. 1999). Type V is the autotransporter system originally from

type IV. Last, there are couple novel transpoter systems discovered from Gram-negative bacteria is named type VI (Economou, Christie et al. 2006).

Sec translocation system

The Sec translocation machinery is composed of many gene products. In *E.coli*, translocase itself comprises 7 proteins, including a chaperone protein (SecB), an ATPase (SecA), an integral membrane complex (SecY, SecE and SecG), and three additional membrane proteins that promote the release of the mature peptide into the periplasm (SecD, SecF and YajC) (Akimaru, Matsuyama et al. 1991). The chaperone protein SecB is a highly acidic homotetrameric protein that exists as a "dimer of dimers" in the bacterial cytoplasm. SecB maintains pre-proteins in an unfolded state after translation, and targets these to the peripheral membrane protein ATPase SecA for secretion (Economou 2002; Driessen and Nouwen 2008).

The proteins destined to secret are synthesized as a nascent protein chain with N-terminal signal sequence. In post-translational translocation, the pre-protein chain associates with the chaperone SecB, and the complex moves toward to the inner membrane where the Sec translocon locates. Once the complex reaches the translocation machinery, the pre-protein is released from SecB and re-associated with the motor protein, SecA. During the translocation, SecA hydrolyses ATP as energy pushing the pre-protein across the channel composed by SecYEG complex. During or after translocation, the N-terminal signal peptide of pre-protein is cleaved to become mature folded protein (Economou 2002; Driessen and Nouwen 2008).

In Sec system, SecYEG complex has been proposed to serve in the channel formation for pre-protein secretion. SecY is the largest subunit of the translocation channel with 443 amino acid residues. SecY is composed of 10 transmembrane helices (TM1–10) and separated to N-terminal and C-terminal domains. There is a loop between TM5 and TM6 to connect both domains into a clamshell conformation with a central funnel-like pore (Akiyama et al., 1987). The primary sequence of SecY is highly conserved. All conditional lethal mutants identified so far map in the conserved regions. Dominant loss of function mutations in SecY have only been identified in the cytoplasmic loop 5, which has been implicated in SecA binding (Mori et al., 2001).

SecE is a small protein containing three TMs (Schatz et al., 1989). Its cytoplasmic domain 2 that connects TM2-TM3 and the third TM including the C-terminal appear to be essential for protein translocation (Schatz et al., 1991). SecG is the smallest subunit of the protein conducting channel with a molecular mass of 12 kDa. By Co-immunoprecipitation, SecG interacts only with the SecY subunit in SecYEG complex (Homma et al., 1997). Although SecG is not essential for protein translocation, it stimulates protein translocation *in vitro*, in particular at lower temperatures and when the proton motive force (PMF) is low or absent (Hanada et al., 1996).

SecA

SecA protein is the only component that is essential for protein secretion *in vitro*. *E. coli* SecA is originally identified as a cytoplasmic protein of 901 amino acid residues (Oliver and Beckwith 1982). SecA is a 102kDa protein contains N-terminal 68 kDa (N68) and C-terminal 34 kDa (C34) domains and could be separated as two functional peptides. The N-terminal peptides

contains the nucleotide binding domain I (NBD1) with high affinity and the IRA2 domain for with low affinity nucleotide binding (Pugsley 1993). The NBD1 is composed by the Walker A and the Walker B motifs which are conserved in most of ATPase. The IRA 2 domain does not bind to nucleotide, however, it serves as an intramolecular regulator of ATP hydrolysis by controlling ADP release and optimal ATP catalysis at NBD1. The N68 fragment of SecA is also the homologous to helicase superfamily II DEAD and DExH helicase (Oliver and Beckwith 1982). The C-terminal fragment has been proposed to involve in the dimerization, and it contains IRA1 domain and the regions for SecY or SecB interaction (Li, Schulman et al. 2007) .

In *E.coli*, SecA exists in soluble and membrane-bound state in balance (Akiyama and Ito 1987; Rusch and Kendall 2007). SecA binds to the acidic phospholipids of cytoplasmic membrane with low-affinity; however, membrane-bound SecA binds to the SecYEG with high-affinity (Hartl, Lecker et al. 1990). Once bound to the SecYEG, the SecA-SecYEG has high-affinity interaction with SecB/preprotein to initiate translocation. SecYEG-bound SecA also recognizes the signal sequence and the mature part of preprotein through substrate specificity domain (SSD) of N68 fragment (Mori and Ito 2001). This process stimulates SecA ATPase to hydrolyze ATP as energy source.

Soluble SecA forms monomer-dimer equilibrium with a disassociation constant determined to be 0.25 μ M-0.5 μ M (Eichler, Brunner et al. 1997). The monomeric and dimeric SecA population is affected by temperature, salt concentration, and by the presence of translocation ligands (Cabelli, Dolan et al. 1991). In crystal structure data from *Bacillus subtilis* and *Mycobacterium tuberculosis*, SecA has been proposed to be an antiparallel homodimer in cytoplasm by C-terminus (Nishiyama, Suzuki et al. 1996; Yang, Lian et al. 1997; Wang, Chen et al. 2003). How-

ever, a C-terminal truncated SecA mutant remains dimeric. Recently, the size chromatography and crystal structure reveal that there may be a dimerization domain in N-terminus (Nishiyama, Suzuki et al. 1996; Jilaveanu and Oliver 2006).

There are evidences of the SecA-lipid bound form exists as a ring-like structure (Wang, Chen et al. 2003). Under the Transmission Electron microscopic and Atomic force microscopic analysis, the liposome-bound SecA could form a channel like structure in the lipids. Furthermore, SecA has been proposed to function as a dimer in the translocation process (Jilaveanu and Oliver 2006; Wang, Na et al. 2008); however, the mechanism by which SecA exerts its function in protein export is still not clear. SecA is the center motor component in the Sec translocon (Pugsley 1993; Rusch and Kendall 2007).

Previous data suggests that SecA ATPase activity is inhibited by Sodium azide and Rose Bengal. In oocyte electrophysiological study, the channel activity is decreased when MC4100 membrane and proOmpA are injected with azide. In addition, Rose Bengal abolishes the channel activity of MC4100 membrane in 10 μ M. The IC₅₀ for Azide is about 4 mM, and the IC₅₀ for Rose Bengal is 5 μ M (Bor-ruei Lin, Electrophysiological studies on *Escherichia coli* protein-conducting channel).

Although SecYEG has been proposed as the protein-conducting channel, there are several studies showed that the translocation process is active without SecYEG. Blobel and colleagues have presented strong evidence that reconstituted *E. coli* membranes lacking detectable SecY efficiently translocate proLamB. They suggested that SecY is not the only receptor for SecA and that it may not be essential for protein translocation (Watanabe, Nicchitta et al. 1990). Our previous work confirmed these observations with other precursors, and showed fur-

ther that either SecE-deficient or SecY-deficient membranes derived from genetically manipulated cells also efficiently translocate certain precursors *in vitro* (Yang, Lian et al. 1997; Yang, Yu et al. 1997). Moreover, a set of proteins, mostly inner and outer membrane proteins, were minimally affected in *E. coli* cells upon depletion of SecE (Baars, Wagner et al. 2008), indicating a differential requirement of SecYEG. Thus, apparently neither SecE nor SecY is essential for the translocation of all proteins. Moreover, SecA, which is often referred as being peripheral subunit of preprotein translocase with ATPase activity (Wickner and Leonard 1996; Driessen and Nouwen 2008), has been found to integrate into membranes (Kim, Rajapandi et al. 1994; Chen, Xu et al. 1996). It does not cycle on and off the membrane during protein translocation (Chen, Xu et al. 1996), and it is an integral part of the protein-conducting channel (Chen, Xu et al. 1996; Chen, Brown et al. 1998). Recently, we found that upon binding to anionic phospholipids, SecA undergoes conformational changes to form ring-like structures, and that alone it may form the core of protein-conducting channels (Wang, Chen et al. 2003; Chen, Tai et al. 2007; Wang, Na et al. 2008). These observations suggest that there may be a separate Sec pathway in which SecA plays a channel-forming role.

By using the electrophysiological approach (Lin, Gierasch et al. 2006), we monitor the channel opening by injecting the *E. coli* membrane or liposomes coupled with other Sec components into oocytes. This method has been used for channel studies in eukaryotes for years and has been a sensitive and more accurate in bacteria protein translocation study. In this study, we show that SecA alone with liposomes are able to maintain the translocation activity and channel activity in the absence of SecYEG. However, the channel activity loses the signal peptide specificity, efficiency and is more sensitive to SecA inhibitor. This study helps us to determine the

role of SecA, and also the fundamental machinery for the protein translocation in the absence of SecYEG

Material and Methods

Strains and plasmids

MC4100 is derived from *Escherichia coli* K12 strain (F- lacU169 araD136 relA- rpsL150 frbB5301 deoC7 ptsF25 thi-) obtained from J. Beckwith (Silhavy and Beckwith 1983); *E. coli* strains BA13 (MC4100 *supF*^{ts} *trp*^{am} *secA13*^{am} *zch::Tn10*) (Cabelli, Chen et al. 1988) was obtained from D. Oliver. The SecY suppression mutants PrIA4 and PrIA665 are from T. Silhavy (Emr and Silhavy 1983); SecYEG- membrane was a SecE depleted membrane prepared from *E. coli* strain PS289 (MC1000, leu+, ara+, phoAΔPvull, pcnB80, zadL:: Tn10 (Tcs Strr), SecEΔ19-111, recA:: cat/pBAD22 secE+, a gift from C. Murphy and J. Beckwith, Harvard medical School, Boston, MA. (Wang, Na et al. 2008). SecY half plug domain deletion and plug domain deletion mutants are gifts from T. Rapoport (Li, Schulman et al. 2007). The Half plug domain deletion is SecY deleted from amino acid residue 65-70; the plug domain deletion is SecY deleted from amino acid residue 60-74. These strains are expressed in a *E. coli* strain harboring a temperature sensitive SecY alleles (Shiba, Ito et al. 1984). Plasmids containing *secAL43P* and *secAR509K* were from H. Takamatsu (Takamatsu, Nakane et al. 1994) and D. Oliver (Mitchell and Oliver 1993), respectively. Plasmid encoding SecY_{his}G was from F. Duong (Collinson, Breyton et al. 2001; Maillard, Lalani et al. 2007).

Inner membrane vesicle (IMV) preparation

IMV was prepared from *E. coli* strains. After an overnight culture, the cells were diluted to A₆₀₀ O.D. ~0.1 and inoculated at desired temperature until growth eased (about A₆₀₀ O.D 2.0). For MC4100, the growth temperature is 37 °C; and the BA13 is at 30°C and shifted to 42 °C (in

order to deplete the SecA amount). The cells were then harvested and washed once by cold 0.2 M Tris-HCl and re-suspended in ST/Cm (0.5 M Sucrose, 20 mM Tris-HCl and 5 % Chloramphenicol) for spheroplast formation. Add 1.5 ml of 50 mM EDTA (150 μ l of 0.5 M EDTA) drop by drop, and then add 1.5 ml of 0.4 % lysozyme (fresh prepared in H₂O) drop by drop with slowly swirling. After about 10 min, 150 ml of dd- H₂O was added slowly in room temperature with rapid swirling. Follow spheroplast formation under microscope (about 5-10 min, not to exceed 20 min. The *E. coli* cells are rod-like shape, and the spheroplast is rounded shape). After 80~90 % spheroplasts are formed, 1ml of 1 M Mg(OAc)₂ was added for 5 min on ice to stabilize spheroplasts.

After 8000 \times g centrifugation, the pellet was re-suspended in Lysis buffer (1 mM DTT and 20 mM EDTA) and lysed by passage through French pressure twice or until the solution is clear. The lysed cells were spin down at 8000 \times g for 10 mins and the supernatant was loaded on to a two-step 5 ml sucrose gradient (2.5 ml of 1.4 M and 2 ml of 0.8 M sucrose) for ultracentrifugation (37000 rpm, 3 hour, 4 $^{\circ}$ C). The dense brown band was collected and diluted with DE buffer (1 mM DTT and 5 mM EDTA) and reloaded to second sucrose gradient (2 ml of 1.4 M, 3 ml of 1.2 M, 2 ml of 1 M, and 2 ml of 0.8 M) and centrifuged in a Beckman SW41 rotor for 37000 rpm, 18 hour, 4 $^{\circ}$ C. The clear brown-yellow fraction I and II were collected and centrifuged to obtain the pellet. The pellet was re-suspended in DTK (1 mM DTT, 10 mM Tris-HCl pH7.6, 50 mM KCl) buffer and store at -80 $^{\circ}$ C for further experiments.

For reconstituted *E.coli* inner membrane vesicles (IMV) preparation, the IMV was prepared as described above. Then the solution was treated with 1 % sodium cholate on ice for 1 hour (Blobel and Dobberstein 1975; Yang, Lian et al. 1997; Lin, Gierasch et al. 2006). The cells

were centrifuged at 90K for 30 mins to spin down the SecY complex. The supernatant was dialyzed in Dialysis buffer (0.25 M Sucrose, 0.4 M KOAC, 20 mM Triethanolamine hydrochloride pH7.5, 1.5 mM MgOAC and 1 mM EDTA) at 4 °C for 15 hours. The dialyzed solution was centrifuged at 90K again to collect the reconstituted membrane vesicles. The pellet was suspended with DTK byffer (1 mM DTT, 10 mM Tris-HCl pH 7.6, 50 mM KCl) and stored at -80 °C freezers before use.

SecYEG-His-tagged membranes were prepared from a C43 strain carrying a pBAD/*secE_{his}YG* plasmid and SecYEG was purified by affinity to SecE-His as described (Collinson, Breyton et al. 2001; Maillard, Lalani et al. 2007). The preparation is a lab stock.

Liposome preparation

E. coli total lipids extracts (Avanti Polar Lipid, Inc) were dried in the Thermo Savant vacuum and resuspended in 150 mM KCl or TAK buffer containing Tris-HCl 50 mM pH 7.6, 20 mM NH₄Cl and 25 mM KCl. The suspension was subjected to sonication (Fisher Scientific Sonic Dismembrator Model 500) at the amplitude of 70% for 4 to 5 minutes with one minute pause in a 0 °C ice-water bath. The particle sizes of opalescent liposomes were measured by a Beckman Coulter N5 submicron particle size analyzer and showed a normal distribution with a peak around 130 nm. The liposomes were aliquoted and stored at -80 °C until use.

For *E.coli* membrane fused liposomes for Patch Clamp, the *E.coli* inner membrane vesicles and the *E.coli* total lipids liposomes were prepared as described above. The fusion of *E.coli* membrane and liposomes were prepared as described (Delcour, Martinac et al. 1989). The membrane vesicles and liposomes (1:5 ratios) were mixed and centrifuged at 4°C 95,000 ×g

for 1 hour. The pellet was then resuspended in 20-40 μ l of 10 mM 3-[N-morpholino]propanesulfonic acid (MOPS), pH7.2 containing 5% ethylene glycerol. The solution was then dropped on a clear glass slide and rehydrated at 4°C in large desiccators for ~4 hours. After rehydration, a desired amount of rehydration buffer (150 mM KCl, 0.1 mM EDTA, 10^{-5} M CaCl_2 , 5 mM HEPES, pH7.2) was dropped on the surface of dried lipid film to make a 90 mg/ml solution. The glass containing dried lipid film and rehydration buffer was placed in a Petri dish containing a wet filter paper pad and rehydrated in 4°C for overnight. The membrane-fused liposomes were used after rehydration and only used on the day they are prepared.

Protein purification

E. coli SecA, derivatives and its homologs were purified similarly from BL21(λ DE3)/pT7-SecA as described (Cabelli, Chen et al. 1988). Purified proOmpA, proPhoA, PhoA, LamB wild type signal peptide (WT) and LamB deletion mutant (DM) were prepared as described (Chen et al., 1987; Chen et al., 1996). SecAL43P and SecAR509K were over-expressed and purified similarly with wild-type SecA. SecAL43P, SecAR509K and the SecA deletion fragments were laboratory stocks purified by L. Yu and Y. Huang respectively. SecA homologs from other bacteria were purified and obtained from J. Jin. Protein concentration was determined by A280/260 ratio or Bradford assay.

Xenopus oocyte preparation and injection (Fig 1)

Oocytes were obtained from *Xenopus laevis*. The frogs were anesthetized in 0.3 % 3-aminobenzoic acid thyl ester for 30-40 mins (Lin, Gierasch et al. 2006). A small abdominal incision was made and ovaries were taken out as needed. The incision was closed and the frog was

placed in recover tank until wake up. The oocytes clusters were digested with 2 mg/ml of Type IA collagenase in the OR2 solution (82 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 5 mM HEPES, pH7.4) for 25-30 mins or until 70 % of tissue digestion was done. After three times of wash with OR2 solution, the second digestion was done for less than 5 mins to make sure 90 % of digestion was completed. The oocytes then washed with excess OR2 solution to remove the collagenase and then incubated with 2 % Fetal Bovine Serum (FBS) for 5 mins to remove the tissue residues. After wash, the oocytes were maintained in 16 °C for 2-4 days in order to keep stable and constant results.

E.coli inner membranes vesicles and SecA with or without proOmpA were mixed in the TK buffer (10 mM Tris-HCl, pH7.6 and 50 mM KCl) and incubated at 30 °C for 4 mins before injection. The 50 nl sample mixture was then injected into dark pole site of oocytes by using Nanoject II injector (Drummond Scientific Co., Broomall, PA). The injected oocytes were stored at 23 °C in ND-96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, 40 mg/l sodium pyruvate, and 100 mg/l geneticin, 100 mg/l tetracycline, pH 7.4) for 3 hours. The working concentration for each component is 60 ng membranes, 60 ng SecA, 0.28 pmole proOmpA. For SecA-liposome experiments, the working condition is 120 ng liposomes, 120 ng SecA, 2 mM ATP, 1 mM MgCl₂, 14 ng proOmpA, or 9 ug Signal peptides. The effective concentration of chemical in the oocytes was estimated based on the average volume of oocytes at 500 nl with 50 nl injection.

Voltage Clamp Measurement

Voltage Clamp was performed to measure the opening of protein conducting channels. When the channel on the cell membrane was opened, ions pass through the membrane and generate the ionic current. Thus, the recording of ionic current could also mean the opening of the protein conducting channel. Currents through the plasma membranes of oocytes were measured after the oocytes were injected with tested materials. The cells were placed in a recording chamber (BSC-HT, Medical System, Greenvale, NY) on a supporting nylon mesh, so that the perfusion solution bathed both the top and the bottom surface of the oocytes. Two electrode voltage clamping was performed using an amplifier (Grunzeclamp 500, Axon Instruments Inc., Foster City, CA) at room temperature (23-25 °C). Cells were impaled using electrodes filled with 3 M KCl. One of the electrodes (1.0-2.0 M Ω) used for voltage recording was connected to the HS-2 \times 1L headstage (input resistance, 10^{11} Ω), and the other electrode (0.3-0.6 M Ω) was used for current recording to the HS-2 \times 10 MG headstage (maximum current, 130 μ A). The electrode was connected through a silver wire that was chloridized freshly for each experiment. Oocytes were used for further experiments only if their leak currents measured as the difference before and after a leak subtraction were less than 10% of the peak currents. The leak subtraction was not applied for data acquisition and analysis. Current records were low-pass filtered (Bessel, 4-pole filter, 3 db at 5 kHz), digitized at 5 kHz (12 bit resolution), and analyzed using pClamp6 (Axon Instruments). The highest and lowest records were eliminated, and then the data are presented as mean \pm S.E. (standard error; n, number of oocytes). The expression rates for each injection sample were also collected as a record to determine the channel activity efficiency.

Patch clamp measurement

Currents flowing through ionic channels formed on liposomes were monitored by inside-out patch clamp technique. Liposomes prepared from total *E. coli* lipids extracts were frozen and thawed once before dehydrating on the poly-lysine coated glasses at room temperature. After dehydration, the dried liposomes were rehydrated in the solution containing 150 mM KCl, pH 7.4 at 4 °C for 48 hours. The glass micropipette, filled with 150 mM KCl, pH 7.4, was pressed against the re-hydrated liposomes and suction was applied to assist patching sealing. Once the seal was formed, the pipette was withdrawn to obtain inside-out patch and the intracellular surface of liposome was exposed to the bath solutions in 0.1 ml containing where indicated 2.5 mM ATP, 1 mM MgCl, 1.8 µg LamB signal peptides or 2.8 µg proOmpA and 24 µg SecA (Activation bath). After incubation in the Activation bath, the active patch was moved into Inhibition bath with 10 mM EDTA, pH 8.0. The recordings were initiated after 15-20 mins incubation in each bath solution. In the ramp recording protocol, a command of -100 mV to +100 mV was given, whereas a steady potential of -80 mV was applied for the continuous protocol. The total recording time for the continuous protocol was 1 min.

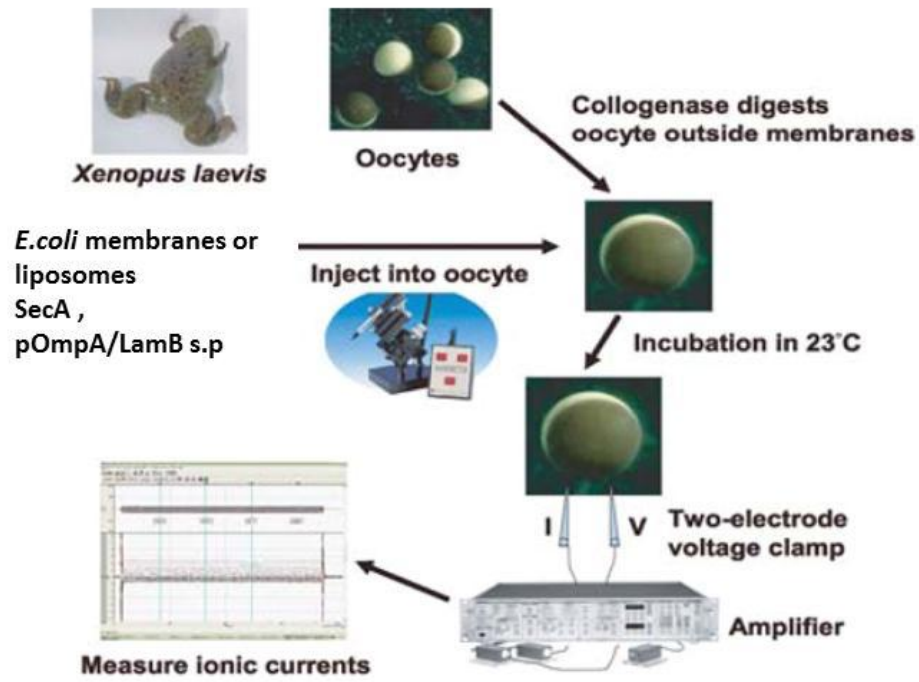


Figure 1. The oocyte injection and whole cell recording (modified from Bor-ruei Lin).

Results Part I

SecA Alone Can Promote Protein Ion-Channel Activity:

SecYEG Increases Efficiency and Signal Peptide Specificity

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Most proteins destined to traverse cytoplasmic membranes through the Sec-dependent pathway carries a transient N-terminal hydrophobic signal peptide that is cleaved during, or shortly after, translocation. In bacteria both biochemical and genetic approaches have provided important and complementary insight into the mechanism of this Sec secretion pathway. The current and prevailing model for Sec-dependent translocation depicts the bacterial SecYEG complex as providing the essential, protein-conducting channel, with SecA acting as a peripheral component of the translocase hydrolyzing ATP (Chen and Tai 1985; Chen and Tai 1987), as it cycles on and off the cytoplasmic membrane during protein translocation (Wickner and Leonard 1996; Driessen and Nouwen 2008). This model of SecYEG as being the essential protein-conducting channel arises in part from the homology of SecY to Sec61p, which has been proposed to be a component of the translocation channel in yeast and mammals (Hanein, Matlack et al. 1996; Beckmann, Bubeck et al. 1997; Hamman, Chen et al. 1997; Pohlschroder, Hartmann et al. 2005). The Sec61 complex is assumed to form 2 nm pores, which further assemble into 4-6 nm pores, through which nascent peptides in the ribosomes pass through the endoplasmic reticulum (Hanein, Matlack et al. 1996; Beckmann, Bubeck et al. 1997; Hamman, Chen et al. 1997). The X-ray structure of an archaea SecYEG complex shows similar pore size. The *E. coli* SecYEG translocation complex (Breyton, Haase et al. 2002) is significantly smaller than both the yeast Sec61 complex and the more complete SecA/SecYEG_{EC}, as defined by more recent X-ray structural analysis (Zimmer, Nam et al. 2008).

However, Blobel and colleagues have presented strong evidence that reconstituted *E. coli* membranes, lacking detectable SecY, efficiently translocate proLamB. They suggested that SecY is not the only receptor for SecA and that it may not be essential for protein translocation

(Watanabe, Nicchitta et al. 1990). Our previous work has confirmed these observations with other precursors, and showed further that both SecE-deficient or SecY-deficient membranes, derived from genetically manipulated cells, are also capable of efficiently to translocating certain precursors *in vitro* (Yang, Lian et al. 1997; Yang, Yu et al. 1997). Indeed, a set of proteins (mostly inner and outer membrane proteins) were only minimally affected in *E. coli* cells upon depletion of SecE (Baars, Wagner et al. 2008), indicating a differential requirement of SecYEG. Thus, neither SecE nor SecY is apparently essential for the translocation of all proteins. “Moreover, SecA, often referred as a peripheral subunit of pre-protein translocase with ATPase activity (Wickner and Leonard 1996; Driessen and Nouwen 2008), has been found to integrate into membranes (Kim, Rajapandi et al. 1994; Chen, Xu et al. 1996). It does not cycle on and off the membrane during protein translocation (Chen, Xu et al. 1996), and it is an integral part of the protein-conducting channel (Chen, Xu et al. 1996; Chen, Brown et al. 1998). Indeed, it has been found that SecA can bind to membranes at SecYEG high-affinity sites and at phospholipids low-affinity sites (Kim, Rajapandi et al. 1994; Chen, Xu et al. 1996). We have also found that, upon binding to anionic phospholipids, SecA undergoes conformational changes to form ring-like structures, and that these may form the core of the protein-conducting channels (Wang, Chen et al. 2003; Chen, Tai et al. 2007; Wang, Na et al. 2008). These observations suggest that there may be a Sec pathway in which SecA plays a channel-forming structural role. In this study, we show that SecA alone can indeed promote protein translocation and elicit ion-channel activity in liposomes, and that SecYEG restores the lost efficiency and specificity.

SecA and liposomes are necessary and sufficient for eliciting ion-channel activity in oocytes.

We previously developed a semi-physiological assay in *Xenopus* oocytes to measure bacterial SecA-dependent ion-channel activities in an effort to complement the *in vitro* protein translocation assay (Lin, Gierasch et al. 2006). Owing to findings that SecA-liposomes can promote protein translocation (Hao Zhang), we expected similar results for the ion-channel activity in the oocyte system. However, no significant activity was observed, unless ATP-Mg²⁺ was added together with SecA-liposomes (Fig.1.1A), even though the oocytes contain sufficient ATP to promote ion-channel activity with regular BA13 membranes (Fig.1.1A, bar 2; (Lin, Gierasch et al. 2006). Optimization of the system revealed that more ATP-Mg²⁺ and SecA are required by SecA-liposomes (Fig.1.1A and Fig.1.2) than by BA13 membranes to elicit the ion-channel activity, but only at a level of about 50 % (Fig.1.1B). The amount of SecA needed to reach the maximal activity is about 1 - 2 μ M, which is similar to the amounts needed for *in vitro* protein translocation system (Hao Zhang), and in the bacterial cells as noted above. The localized concentration of SecA that forms the channels in the membrane might be even higher (Das, Stivison et al. 2008). These data indicate that SecA can elicit ion-channel activity, albeit with lower efficiency.

We next examined whether other bacterial SecA homologs can promote ion-channel activity in oocytes. We found that purified SecA's of *Bacillus subtilis* and *Pseudomonas aeruginosa*, as well as the genetically constructed *E. coli* tandem SecA-SecA (Wang, Na et al. 2008), were all active in eliciting ion-channel activities to about the same extent (Fig.1.1C). Neither of the inactive SecA L43P (Oliver and Beckwith 1981; Yu, Yang et al. 2006), or SecA R509K (Mitchell and Oliver 1993), however, demonstrated any ion-channel activity of note (Fig.1.1C).

These data provide further evidence that functional SecAs alone can elicit ion-channel activities in liposomes.

SecA alone promotes single channel activity with liposome patch-clamp recording.

Recordings of the ionic current in the oocytes reflect the activities of a population of several hundred thousand channels (Lin, Gierasch et al. 2006). To explore and characterize the requirements of SecA-liposome channel activity and to determine the specificity of precursor or signal peptides for opening the channels, we developed a liposome patch-clamp recording system. We found that SecA-induced channel activities were activated by ATP-Mg⁺⁺ in the presence of LamB signal peptides (Fig.1.3A and Fig.1.5). These channel activities (Fig.1.3A.b and Fig.1.5) were strictly dependent on the presence of both SecA (Fig.1.3A.c and Fig.1.5) and signal peptides (Fig.1.3A.d and Fig.1.5), whereas they were inhibited by EDTA (Fig.1.4A.e and Fig.1.5). Similar results were obtained with proOmpA (Fig.1.3B and Fig.1.4). The SecA-dependent channels mediated by the signal peptide often opened and closed rapidly (Fig.3A.b and Fig.1.4), while those mediated by proOmpA appeared to have extensively “open states” (Fig.3B.a, b and Fig.1.4). The sizes of the single channels varied from 40 to 120 pS, a range similar to those reported for membrane-liposome fusions (Simon and Blobel 1992). However, the majority of channels with SecA-liposomes appeared to be around 50 pS (Fig.1.3C). These results further provide strong evidence that in the presence of signal peptides or precursors SecA alone can elicit ionic channel activities in the liposomes.

SecA-liposomes lose signal peptide specificity in protein translocation and in the opening of ion channel.

We further characterized the features of SecA-liposomes activity in the oocytes which allow easier measurement of ionic activities with the sum of hundred-thousand channels (Lin, Gierasch et al. 2006). To determine the signal peptide specificity, we used puromycin to release the oocyte endogenous signal peptides such that the channel activity is strictly dependent on the addition of exogenous signal peptides or precursors. As shown in Fig. 1.6A, SecA-liposomes were active in generating ion-channel activity with unfolded mature OmpA without signal peptides (Fig.1.6A), while BA13 membranes were not active as expected (Lin, Gierasch et al. 2006). By comparison, proOmpA was able to elicit ion-channel activities in either BA13 membranes or SecA-liposomes (Fig.1.6A). Similar results were obtained with precursor protein proPhoA or mature unfolded PhoA (by urea and DTT treatment); though the mature PhoA without unfolding was not active (Fig.1.6B), indicating some limitation for activities.

Similarly, SecA-liposomes were less efficient but also lost the specificity for proofreading recognition of signal peptides (Lin, Gierasch et al. 2006). Thus, while BA13 membranes differentiated the wild-type from defective LamB signal peptides for channel activity (Fig.1.6A,(Lin, Gierasch et al. 2006), SecA-liposomes generated similar channel activity with both LamB WT and LamB DM signal peptides, indicating that the SecA-liposomes channels were also opened by defective signal peptides (Fig.1.6A).

The loss of proofreading of signal peptides recognition in SecA-liposomes resembles those of PrIA/SecY mutants (Simon and Blobel 1992; Duong and Wickner 1999; Li, Schulman et al. 2007; Tang, Pan et al. 2010). We therefore tested the SecY suppressor mutant, PrIA4, which

enables translocation of pre-proteins with defective or missing signal peptides (Li, Schulman et al. 2007; Tang, Pan et al. 2010), for the proofreading function of channel activity in the oocytes. The PrIA4 membranes showed current activities with OmpA or LamB DM (Fig.1.7A), and PhoA (Fig.1.7B), while wild-type MC4100 membranes did not. As expected, proOmpA (Fig.1.6A), and proPhoA (Fig.1.6B) stimulated similar ionic currents with wild-type as well as PrIA4 suppressor membranes.

Similar results were obtained with membranes isolated from SecYEG plug-mutants that also lose proofreading specificity (Li, Schulman et al. 2007). These membranes elicit similar ion-channel activities with unfolded OmpA or proOmpA, and LamB wild-type or defective signal peptides (Fig.1.8A). Similar results were obtained with PhoA or proPhoA protein (Fig.1.8B). These data indicate that SecA-liposomes lose the signal peptide recognition specificity, mimicking the SecY/PrIA mutants in eliciting ionic channel activities in the oocytes.

SecYEG restores signal peptide specificity, and enhances ion-channel activities

Since the SecA-liposomes have the same ion-channel activity characteristics as PrIA4 and SecY-plug mutants, we further tested whether purified SecYEG reconstituted into liposome could restore the proofreading. The purified SecYEG liposomes had no ion-channel activity without SecA-liposomes in the oocytes (Fig.1.9). The ion-channel activities elicited by SecA-liposomes with unfolded OmpA and LamB DM were abolished in the presence of SecYEG-liposomes (Fig.1.9A, bars 6, 8) while the proOmpA or LamB WT were able to stimulate the ion-channel activity in SecA-liposomes and SecA/SecYEG liposomes (Fig.1.9A, bar 5, 7). In the presence of SecYEG the activities were about twice as high as in its absence (Fig.1.9A and Fig.1.10A),

and reduced the requirements of increased SecA and ATP in the oocytes (Fig.1.10B). Similar results are obtained with *in vitro* protein translocation system (Hao Zhang). These results indicate that SecYEG not only restores the signal peptide proofreading capabilities to discriminate among different functional signal sequences, but also enhances the ion-channel activity of SecA-liposomes.

Thus, the SecA-liposomes are fundamentally functional to promote ion-channel and protein translocation activities, but lose efficiency and signal peptide specificity, both of which can be restored by SecYEG.

Discussion

SecA-mediated ion channel activity at lipid low-affinity binding sites.

In this study, we demonstrate that SecA alone can promote protein translocation and ion-channel activities in the liposomes, indicating that SecA could function as a protein-conducting channel. Similar results are obtained with protein translocation assays in our lab. The activity and signal peptide specificity of SecA-liposomes can be enhanced by the addition of SecYEG. It is known that bacterial cells contain more molecules of SecA than SecYEG (Yang, Yu et al. 1997), and that the excess SecA could integrate into the anionic phospholipids at “low-affinity” sites. Similar phenomena were observed earlier that Ben de Kruijff and his colleagues showed SecA insertion into lipid bilayers (Breukink, Demel et al. 1992; Keller, Snel et al. 1995) in which two distinct pools of membrane-bound SecA were identified: one is lipid-bound and other is SecYEG-bound (van Voorst, van der Does et al. 1998). Thus, the two membrane-bound SecAs that differ in the degree of lipid association enlighten our observation that lipid-bound SecA, in the absence of SecYEG, plays a structural role to penetrate into lipids forming protein-conducting channels (Wang, Chen et al. 2003). We hypothesize, therefore, that there are potentially two kinds of SecA-dependent protein-conducting channels: One that forms when SecA binds with high-affinity to the SecYEG complexes and another that forms when SecA binds, with low-affinity, to anionic phospholipids (Driessen and Nouwen 2008). There are many examples of pairs of high and low-affinity transport systems for particular amino acids, which presumably offer a selective advantage. Thus, the existence of two SecA-dependent protein-conducting channels in bacteria would also offer a similar selective advantage for protein translocation. The current prevailing view is that bacterial SecYEG is the essential protein-conducting channel,

while SecA is the peripheral ATPase that pushes precursor peptides through these high-affinity channels. Our findings indicate an alternative understanding, that SecA alone can integrate into the membrane to form a low-affinity site protein-conducting channel. In support of this view, we have previously observed that SecA forms a ring-structure of 8.4 nm with a potential pore size of 2 - 3 nm (Wang, Chen et al. 2003; Tang, Pan et al. 2010) upon interaction with anionic phospholipids, while (together with SecYEG) SecA forms a 10.9 nm-diameter structure with a 3.0 - 4.8 nm cavity (Tang, Pan et al. 2010). The latter parameters are remarkably similar to those of the Sec61 complex. Thus, our work supports the idea that SecA, in the absence of SecYEG, forms channels in phospholipids that are capable of mediating protein translocation and eliciting ion-channel activity.

In our lab, we have used both electrophysiological conductance and protein translocation assays to analyze activities of SecA-dependent protein conducting-channels in liposomes. The two assays measure somewhat different activities. Even so, it is likely that conductance measures only opening of ion channels, not necessarily complete translocation process. Thus, LamB signal peptides and proPhoA can open the channel, but still cannot be secreted (Yang, Lian et al. 1997; Yang, Yu et al. 1997). For proOmpA, both translocation and channel activities are mediated by SecA alone in liposomes. We have also measured ion-channel activities within SecA-liposomes by both oocyte whole-cell and single-channel recordings with similar conclusion that provide confidence for using oocytes as a tool for measuring SecA channel activities. While single-channel recordings provide specific information about the channel characteristics, the oocyte whole cell recordings are easier to manipulate with large numbers of test samples (typically 20-30 oocytes can be measured for each experimental condition), and yield the sum of

more than a hundred-thousand channel activities (Lin, Gierasch et al. 2006). With the oocyte recordings, we further show that inactive EcSecA has no channel activity, but that other SecA homologs yield comparable channel activities. Moreover, these same experiments show that SecYEG-liposomes alone without SecA have no activity in either ion-channel or protein translocation activities.

Earlier studies have shown that protein translocation can take place in the absence of SecYEG (Watanabe, Nicchitta et al. 1990; Yang, Lian et al. 1997; Yang, Yu et al. 1997; Baars, Wagner et al. 2008); see also Introduction). Our findings that SecA-liposomes alone can promote protein translocation and ion-channel activities provide a plausible explanation and basis to reconcile with the then inconceivable observations.

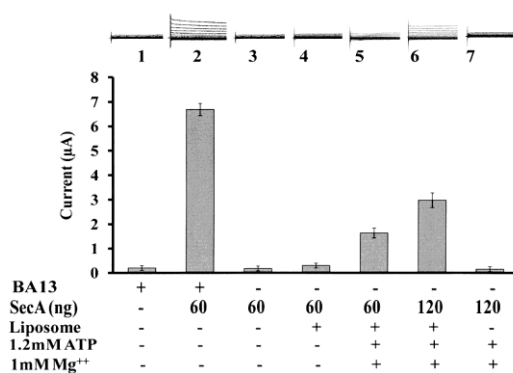
Reduction of efficiency and loss of signal peptide specificity of SecA-liposomes and the restoration by SecYEG.

The SecA-liposomes are active in promoting both protein translocation and ion-channel activities, but they are less efficient and lose the specificity for signal peptides. Thus, more SecA and ATP (though still in the concentration ranges found in the bacterial cells) are required for SecA-liposomes to reach maximal activities of about 50% activities obtained from SecYEG-containing membranes, and the defective, or no signal peptides can still promote activities (Fig.4). The loss in the ability of SecA-liposomes to differentiate specificity of signal peptides resembles that of PrIA suppressors and the “no-plug” SecY mutant. In SecYEG complex, SecY serves as a “gating” mechanism to select the proper precursors with typical signal peptides for translocation (Li, Schulman et al. 2007). Removal of SecYEG plug causes the similar translocat-

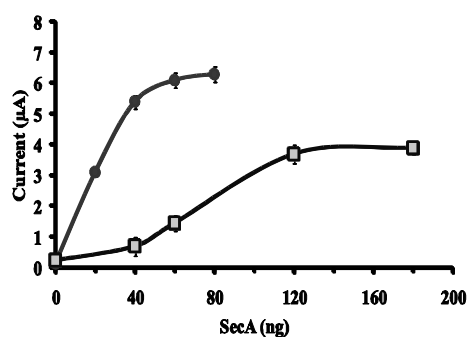
tion defects as PrlA suppressor mutants which allow the translocation of proteins lacking of signal peptide such as OmpA and PhoA in the cells (Derman, Puziss et al. 1993; Duong and Wickner 1999), as have been observed in this study with SecA-liposomes. Taken together, though SecA-liposomes lose the proofreading function and is less efficient, they may represent the basic essential core for the Sec-dependent protein-conducting channels.

In support of this idea, we show that the reduction of efficiency and loss of signal peptide specificity in SecA-liposomes can be restored by SecYEG. Thus, the addition of purified SecYEG to the SecA-liposomes not only enhances both protein translocation and ion-channel activities, but also regains the ability to differentiate the specificity of signal peptides. Moreover, the presence of SecYEG reduces the requirements of more SecA and ATP in the oocytes. We propose that binding of SecA at low-affinity sites in the membrane constitutes the basic protein-conducting channel, while the binding at SecYEG high-affinity sites confers additional efficiency and signal peptide-specificity. Though SecA-liposomes resemble PrlA/SecY mutants in the loss of signal peptide specificity, whether such low-affinity SecA sites are functional in the cells remains to be determined. It is worth noting that certain precursors may have different requirements: proPhoA cannot be translocated in our *in vitro* system in the absence of SecYEG (Yang, Lian et al. 1997; Yang, Yu et al. 1997) and certain proteins are differentially affected during SecYEG depletion in the cells (Baars, Wagner et al. 2008).

A. SecA-dependent activity



B. Reduced efficiency



C. SecA homologs functional

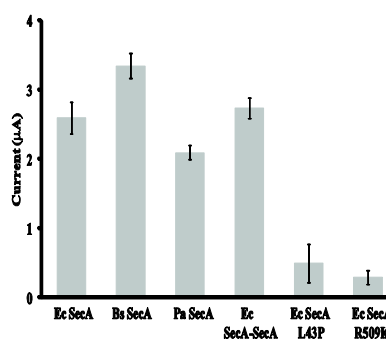


Figure 1.1. SecA and liposomes alone are capable for promoting ion-channel activity in the oocytes.

(A). Additional SecA and ATP-Mg²⁺ were required for achieving optimal channel activity with liposomes than BA13 membranes containing SecYEG. (B). Additional SecA is needed for channel activity with SecA-liposomes (■) as compared to BA13 with SecYEG (●). (C). Homologous SecAs but not non-functional *E. coli* SecAs can promote the ion-channel activity in the oocytes. Various SecAs, 120 ng each, liposomes mixtures were injected together with proOmpA, ATP and Mg²⁺ into oocytes. Ec: *E. coli*, EcSecA-SecA, EcSecAL43P, EcSecAR509K; Pa: *Pseudomonas aeruginosa* PAO1; Bs: *Bacillus subtilis* 168.

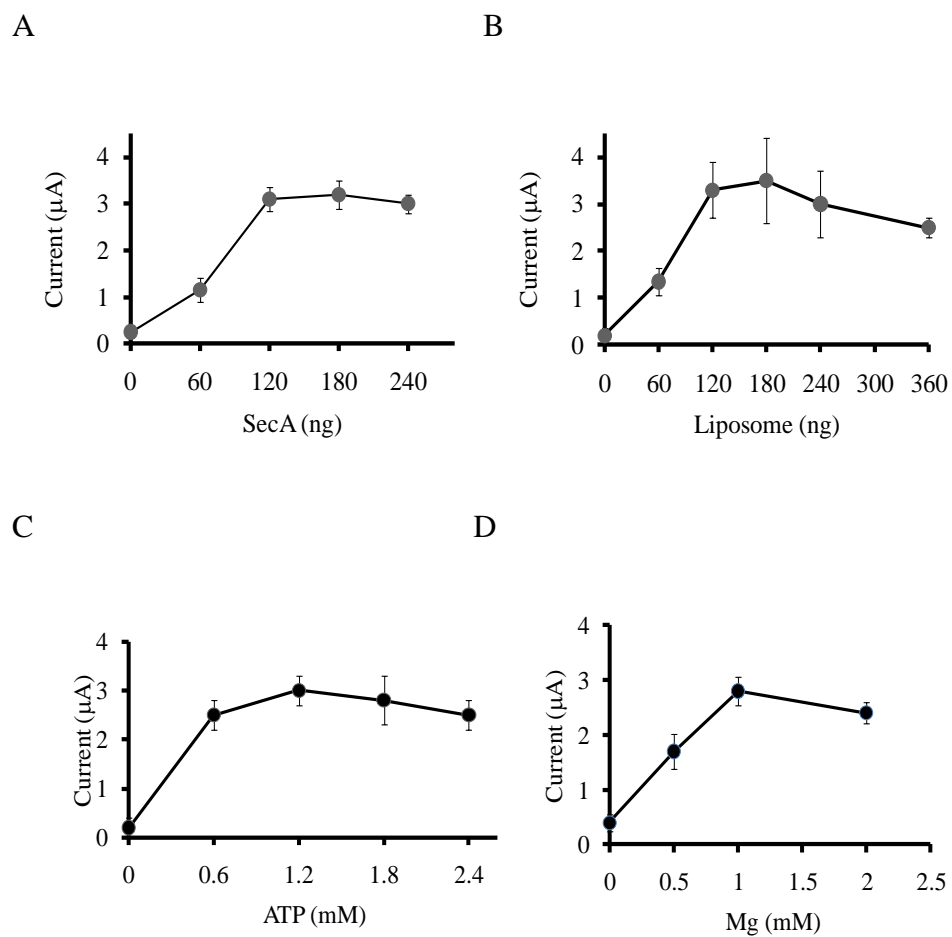
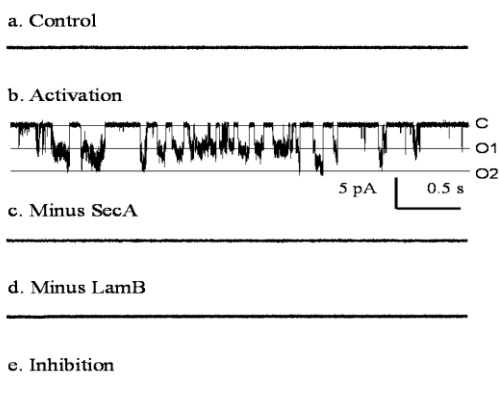


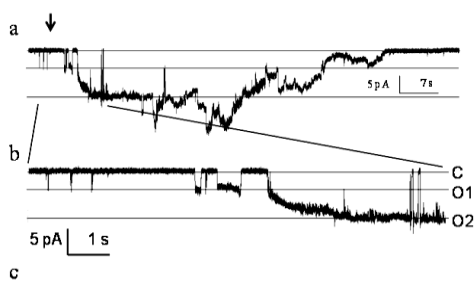
Figure 1.2. Optimization of SecA ion-channel activity in the oocytes.

Titration of (A) SecA, (B) Liposomes, (C) ATP, and (D) Mg^{2+} . Injection mixtures in 50 nl contained: 120 ng liposomes, 1.2 mM ATP, 1 mM Mg^{2+} , 14 ng proOmpA and 120 ng SecA, excepted the tested component. The recording was 3 hours after injection.

A. LamB Signal Peptide



B. pOmpA



C. Unitary conductance by pOmpA

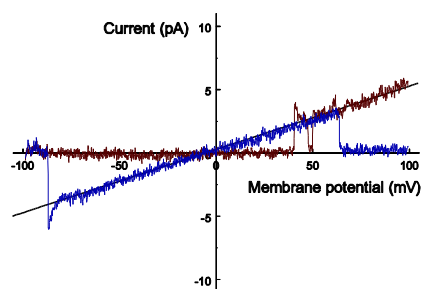


Figure 1.3. Patch clamp measurements of ionic channel activity in SecA-liposomes.

The intracellular side of the inside-out patch was exposed to various solutions (0.1 ml). [C, closed baseline, O1, single opening, O2, double channels opening.] (A). Channel activity was observed only in the presence of LamB, SecA, ATP and Mg^{2+} . The channels opened and closed rapidly, and two channels were active, both with the same conductance of about 50 pS. The time showed is about 4s. (B). Activation of SecA channels by proOmpA. Some channels opened by the Activation solution showed long opening with a large conductance (~ 120 pS), while no channel activity was seen in Minus SecA solution. The arrow zoom-in area in B.c. is 10s. (C). Single channel conductance by proOmpA stimulation. Single channel conductance was measured in activation treatment from Fig.3B. The ramp voltage was from -100 mV to +100 mV. An active channel was observed, and the slope indicates the single channel conductance is 50 pS.

A. Control**B. Activation****C. SecA minus****D. proOmpA minus****E. Inhibition**

3pA
5s

Figure 1.4. Channel openings of SecA-liposome by proOmpA stimulation.

Inside-out patch clamp was performed on BA13 membrane vesicles. The intracellular side of the patch was exposed to various solutions including: control solution, activation solution, minus SecA solution, and inhibition solution. The component of solutions was described in the material and methods. The total time for each solution recording was 1 min.

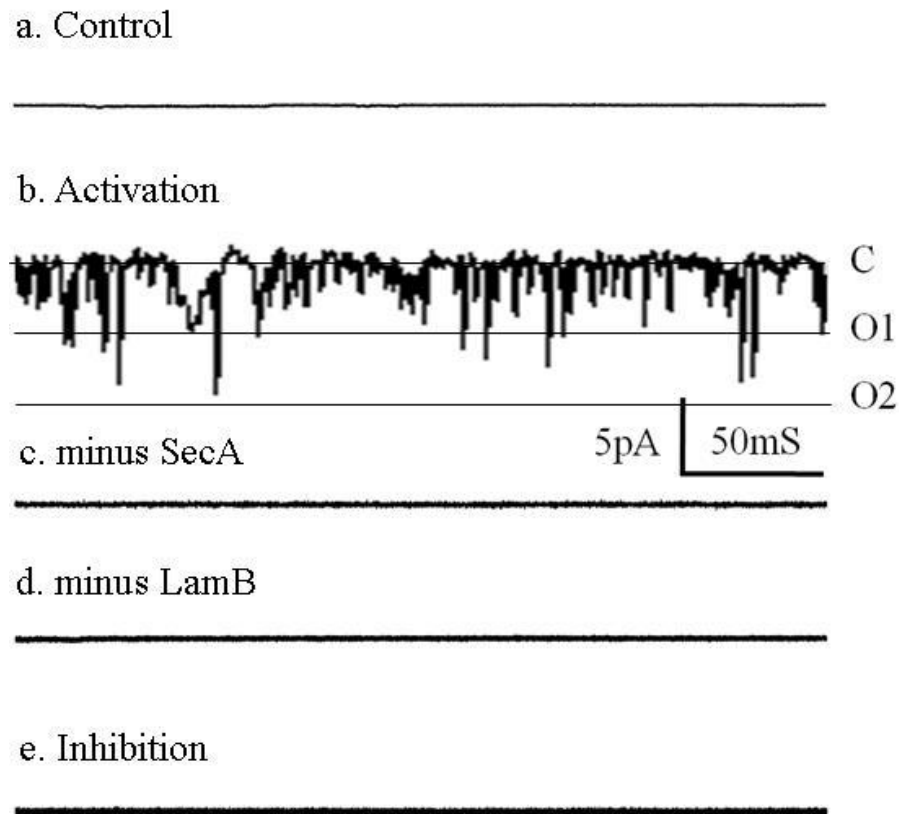
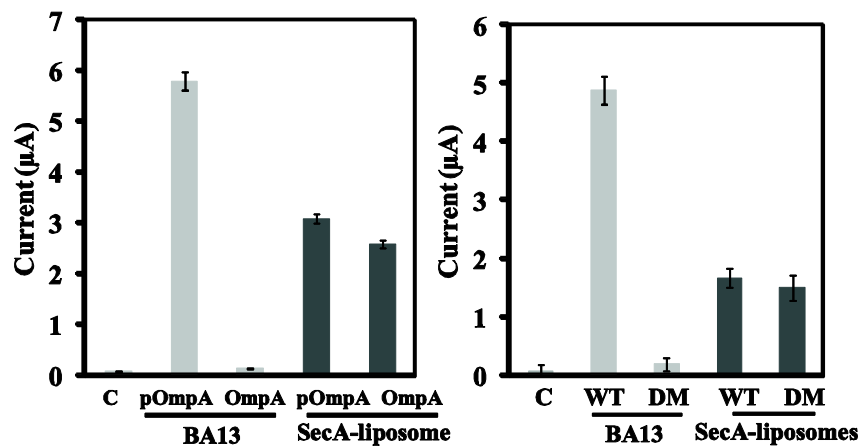


Figure 1.5. Channel openings of SecA-liposome by LamB signal peptide stimulation.

Inside-out patch clamp was performed on E.coli liposome. The intracellular side of the patch was exposed to various solutions including: control solution, activation solution, minus SecA solution, minus LamB solution, and inhibition solution. The component of solutions was described in the material and methods. The total time for each solution recording was 1 min. A. The recording of channel opening by LamB signal peptide stimulation. The time showed here is 6s.

A.



B.

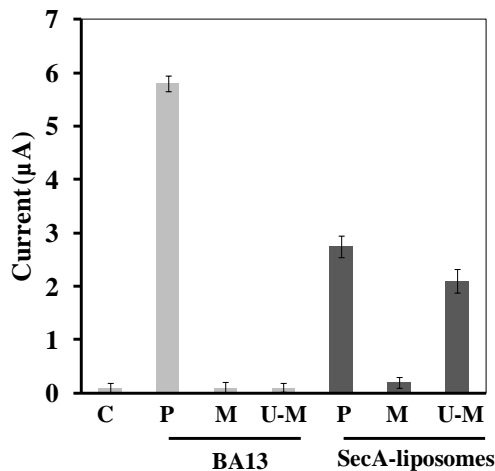
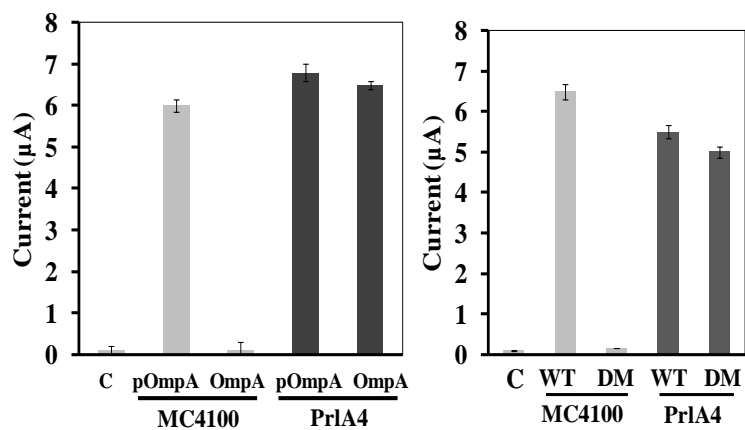


Figure 1.6. Loss of signal peptide specificity of SecA-liposomes

Ion-channel activities in oocytes in the presence of 4 mM puromycin. Unfolded OmpA, proOmpA, LamB wild-type (WT), deletion mutant (DM) signal peptides, proPhoA (P), Mature PhoA (M), or Unfolded mature PhoA (U-M) were injected with 120 ng SecA-liposomes.

A.



B.

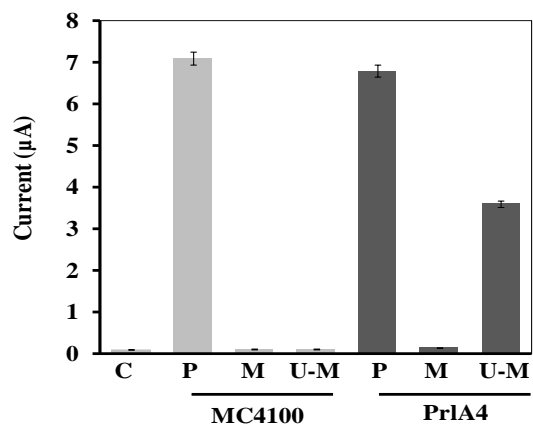
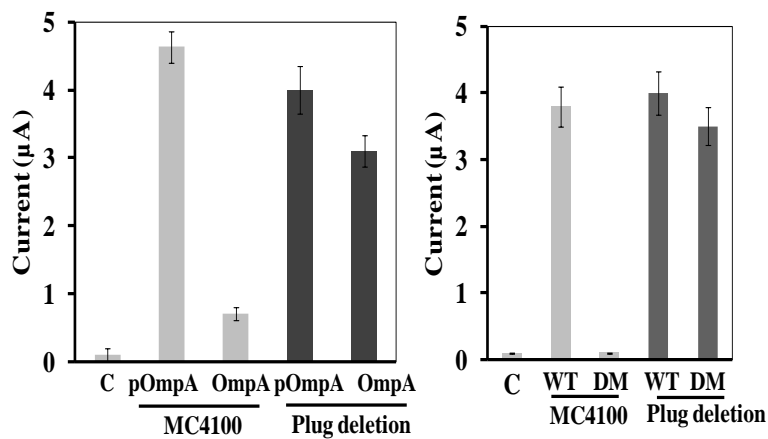


Figure 1.7. SecY suppressor mutant membranes lose proofreading function.

The wild type MC4100 or SecY suppressor mutant PrlA4 membranes were injected with proOmp or OmpA (A) or ProPhoA, mature PhoA, Unfolded PhoA (B). The channel activity was recorded after 3h injection. C. control, the injection without precursor. P. proPhoA. M. mature PhoA. U-M. Unfolded mature PhoA. n =20

A.



B.

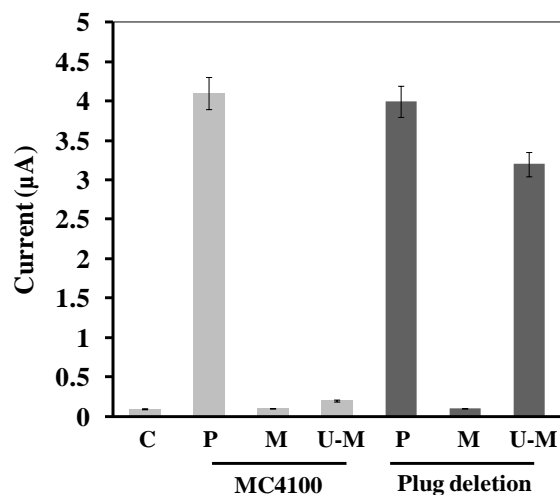
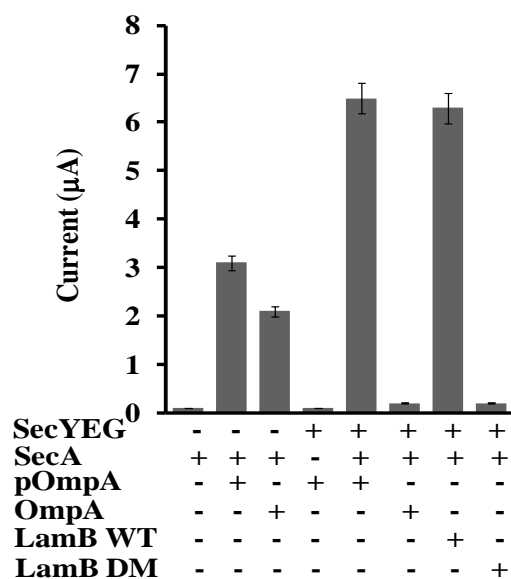


Figure 1.8. proofreading function of channel activity on SecY-plug domain deletion.

The wild type MC4100 or SecY plug deletion mutant membranes were injected with proOmpA or OmpA (A) or ProPhoA, mature PhoA, Unfolded PhoA (B). The channel activity was recorded after 3h injection. C, control, the injection without precursor. P, proPhoA. M, mature PhoA. U-M, Unfolded mature PhoA. n =20

A.



B.

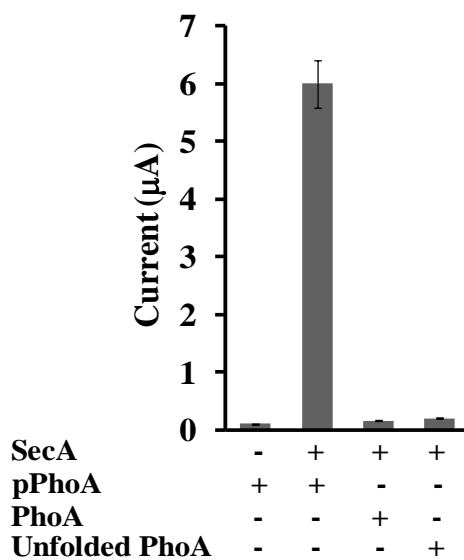
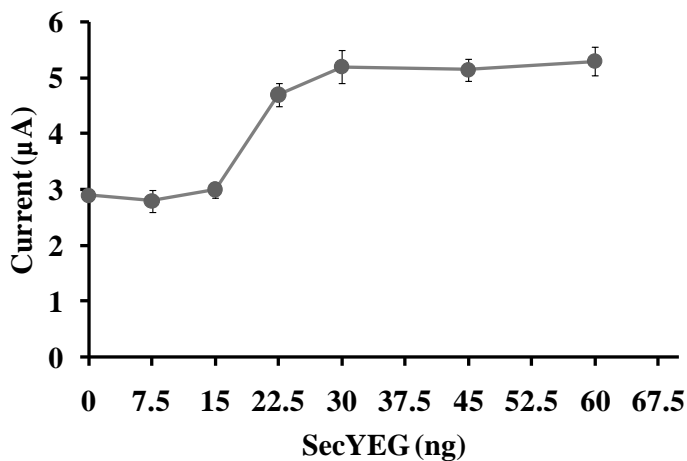


Figure 1.9. SecYEG restores the proofreading function of SecA-liposomes.

120 ng SecA-liposome, 120 ng SecYEG, 2 mM ATP, 1 mM Mg, and 4 mM puromycin was injected into oocytes with various precursors or signal peptides. The recording was performed after 3h injection. Each experiment set was repeated twice; and each time, the n=20

A.



B.

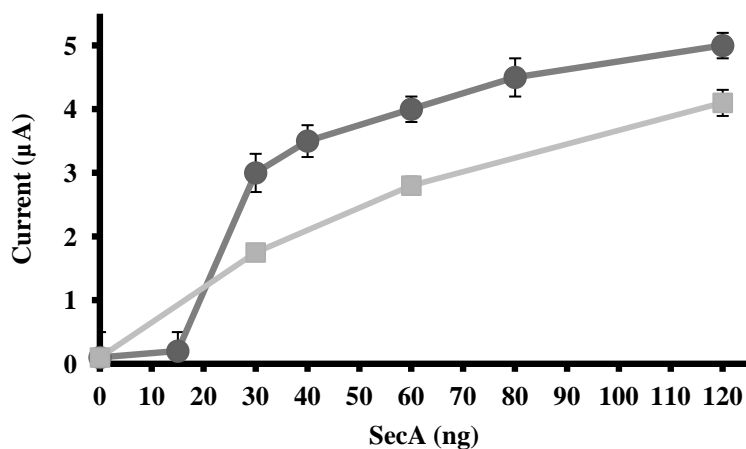


Figure 1.10. Requirements of SecYEG or SecA on ion-channel activities in oocytes.

(A). Ion-channel activity of SecA-liposomes by increasing SecYEG addition. 120 ng SecA-liposomes was injected with various amounts of SecYEG into oocytes. The channel activity reaches the maximum at 30 ng SecYEG addition. (B). SecA requirement of ion-channel activity with SecYEG. The injection mixtures in oocytes included 30 ng SecYEG, 120 ng liposomes, proOmpA and SecA with (open circles) or without (closed circles) additional ATP-Mg. n=10

Results Part II

SecA-Dependent Protein-Conducting Channel:

Loss of Proofreading and Specificity in the Absence of SecYEG

Part of this chapter has been published in Hsieh et. al. 2011 Journal of Biological Chemistry

The characteristics of SecA and SecYEG complex involved in *E. coli* protein translocation have been studied extensively over the last few decades. SecA has been widely viewed as a peripheral protein that is able to cycle on and off during the protein translocation. By hydrolyzing ATP as the energy source, it is thought that SecA is able to insert part of its domain into the SecYEG protein-conducting channel, and thereby drive preproteins across the translocase complex (Manting and Driessen 2000; Mori and Ito 2001; Veenendaal, van der Does et al. 2004). However, the centrality of the SecYEG complex has also been brought into question. It has been found that certain precursor proteins can be translocated *in vitro* with membranes depleted of either SecY or SecE (Watanabe, Nicchitta et al. 1990; Yang, Lian et al. 1997; Yang, Yu et al. 1997), and that, from crystallographic analysis, that the SecYEG complex does not form a pore large enough for protein translocation (Van den Berg, Clemons et al. 2004). Consequently, the role of SecA in protein-conducting channels is still in flux. We have shown that SecA is permanently embedded in *E. coli* cytoplasmic membranes (Chen, Xu et al. 1996), and that is capable of forming ring-like pore structures in the presence of anionic phospholipids (Wang, Chen et al. 2003). From *in vitro* studies, dimeric, cross-linked SecA has been shown to be active for protein translocation (Jilaveanu and Oliver 2006). Through the use of *in vivo* of sulphhydryl domain specific labeling, the membrane-embedded SecA has been shown to possess multiple domains that face and are exposed to the periplasmic side of the membrane (Jilaveanu and Oliver 2007), and also that increased concentrations of SecA compensate for several defects in protein translocation (Fandl and Tai 1987; Cabelli, Chen et al. 1988; Fandl, Cabelli et al. 1988; Kusters, Huijbregts et al. 1992).

We have recently devised a sensitive method to measure the opening of protein-conducting channels in *E. coli* membranes by injecting inverted bacterial membrane vesicles into *Xenopus* oocytes (Lin, Gierasch et al. 2006). We found that ionic currents through such membranes were SecA-dependent and could be blocked by such SecA inhibitors as sodium azide or adenylyl 5'-(β , γ -methylene)-diphosphonate (AMP-PCP). However, the major component in the openable protein-conducting channel has not been identified. Thus, using this electrophysiological method, we are able to examine the central components of protein-conducting channel in bacterial membranes, and determine the function of Sec proteins at various steps in protein translocation.

In this study, we observed ionic currents through *Xenopus* oocytes following injection of inverted membrane vesicles removed SecYEG (reconstituted membranes), thus providing direct evidence for the opening of protein-conducting channel in the absence of SecYEG complex. The protein-conducting channel in these reconstituted membranes can be opened by oocyte-endogenous precursor proteins bearing signal sequences, which can be inhibited by puromycin. The current can then be restored by addition of LamB signal peptides, proOmpA, or proPhoA as the case with wild-type membranes. However, in contrast to results with membranes containing wild-type SecYEG, current through those lacking SecYEG can be stimulated by added mature precursors or defective signal peptides, suggesting a bypass of the proofreading function of SecY for signal sequences. These electrophysiological studies combined with previous biochemical and physical evidence (Chen, Xu et al. 1996; Yang, Lian et al. 1997; Yang, Yu et al. 1997; Wang, Chen et al. 2003; Chen, Tai et al. 2007) suggest that although it is important for channel

efficiency and proofreading, SecYEG is not essential for opening the SecA-dependent protein-conducting channel, and that SecA may be the central core component of that channel.

Reconstituted membranes in the absence of SecYEG with added SecA stimulate ionic currents.

In order to determine the role of SecA and SecYEG in ionic currents, we use the BA13 membranes and reconstituted BA13 membranes as tools for channel activity. BA13 strain is a SecA-depleted strain containing physiological amount of SecYEG. The channel activity of BA13 was dependent of SecA addition (Fig.2.1). Without SecA, there was not ion current detected in BA13 with SecYEG. This data indicated that SecYEG is not essential for the channel activity which is compatible with result I's finding. The channel activity could be replaced by SecA homologs from other bacteria (Fig.2.1B). Similar result was observed by using the reconstituted BA13 membranes removed SecYEG (Fig.2.2). The reconstituted BA13 membrane obtained the channel activity with SecA addition without SecYEG (Fig.2.2A). However, the activity from reconstituted BA13 required more SecA compared to the activity from BA13. In addition, the reconstituted BA13 also generated ionic current with SecA homologs (Fig.2.2B). We further replaces the *E.coli* SecA with other SecA homologs from either Gram-positive or Gram-negative bacteria, and all of those SecA homologs could generated channel activity with BA13 or reconstituted BA13 (Fig.2.3). These data suggested that SecA is essential for the channel activity but not SecYEG.

proPhoA can elicit current activities with reconstituted membranes without SecYEG

It has been shown that the translocation of proPhoA through SecE-depleted membranes was severely diminished as compared with translocation of proLamB, proOmpA, or proLpp *in vitro* (Yang, Yu et al. 1997). Here, we demonstrate that either proPhoA or proOmpA can still cause ionic currents with reconstituted BA13 membranes without SecYEG in the presence of puromycin, but only when excess SecA is added (Fig.2.4). In contrast to the biochemical results with protein translocation into membranes vesicles (Yang, Yu et al. 1997), both of the precursor proteins can stimulate ionic currents in either SecYEG⁻ or RE-BA13 (Fig.2.4) membranes. Thus, these current activities reflect a discrete step in the opening of the protein-conducting channel in the oocyte system not necessarily capacity for protein translocation.

The restoration of proofreading function by SecYEG

Since ionic currents can be recorded through SecYEG⁻ minus condition, we investigated the possible role of SecYEG in the protein-conducting channel for protein translocation. As SecY is suggested to provide the proofreading function for the signal peptides (Osborne and Silhavy 1993), we investigated whether these SecYEG⁻ minus or reconstituted BA13 membranes lose their proofreading capacity for signal peptides in the oocyte system. As shown in the previous studies (Emr, Hanley-Way et al. 1981; Sako and Iino 1988; Bieker and Silhavy 1989), the mature precursors or defective signal peptides are not active in stimulating current activity of wild-type membranes containing SecYEG (Lin, Gierasch et al. 2006), however, they were almost as active as wild-type precursors or signal peptides in eliciting current activities with the injection of SecYEG depleted membranes in the presence of puromycin (Fig.2.5). The ionic current was ob-

served when the proOmpA, proPhoA and LamB wild type signal peptides were injected. Furthermore, the ionic currents were also detectable with mature precursors (OmpA or Unfolded PhoA) or a deletion mutant of LamB signal peptide (Fig.2.5A, B), indicating that the proofreading function for SecYEG⁻ or reconstituted BA13 membranes was lost.

Since the SecYEG is responsible for the proofreading function, we expect the restoration of signal peptide recognition when the purified SecYEG was added into the SecYEG depleted membranes. In Figure 2.6, the 30 ng purified SecYEG proteoliposomes was injected with either SecYEG⁻ minus or reconstituted BA13 membranes. The SecYEG proteoliposome alone did not generate any ion current (Fig.2.6A, bar 1, 2, 3 and 5) unless the SecA and the regular precursors were added. The ionic current was disappeared when the OmpA or LamB DM was injected in the SecYEG⁻ minus membranes (Fig 2.6A, bar 4 and 6). The same result was observed in the reconstituted BA13 membranes (Fig 2.6B). The proofreading function was restored in RE-BA13 with purified SecYEG in the presence of OmpA or LamB DM (Fig.2.6B, 4 and 6). These data suggested that the SecYEG indeed is important for the proofreading function.

Discussion

We have shown that ionic currents can be observed through membranes in the absence of detectable SecYEG using the electrophysiological studies with *Xenopus* oocytes. With the injection of *E. coli* wild-type membranes, as little as 5 ng of SecA can stimulate ionic currents with or without co-injected proOmpA, and 20 ng of SecA can recover the ionic currents with proOmpA or LamB signal peptides in the presence of puromycin, which was added release endogenous oocyte signal peptides (Lin, Gierasch et al. 2006). However, higher amounts of SecA (60 ng to 120 ng of current saturation) were needed to stimulate the ionic currents when reconstituted BA13 membranes were injected into oocytes. LamB signal peptides or precursor proteins, proOmpA, and proPhoA, can open the channel in reconstituted BA13 membranes in the presence of puromycin, with SecA addition. Thus, the excess SecA needed to open protein-conducting channel may compensate for the function lost in the absence of SecYEG, causing the channel to adopt a conformation that favors the opening by precursors. These data indicate that SecY is not essential for protein translocation, as determined in both biochemical and electrophysiological studies.

It has been reported that SecY contains a high-affinity binding site for SecA (Matsumoto, Yoshihisa et al. 1997; Mori and Ito 2006) and a “plug domain” to gate the channel (Tam, Maillard et al. 2005; Li, Schulman et al. 2007). Consequently, SecA conformation and function in the membranes might be affected by the absence of SecYEG. Consistent with this idea, we noted that excess SecA was required to recover the ionic currents when SecYEG⁻ minus membranes were injected with LamB signal peptides and puromycin, even though SecA was over-expressed in those SecYEG⁻ minus membranes (Bor-Reui Lin). Exogenous SecA was also needed

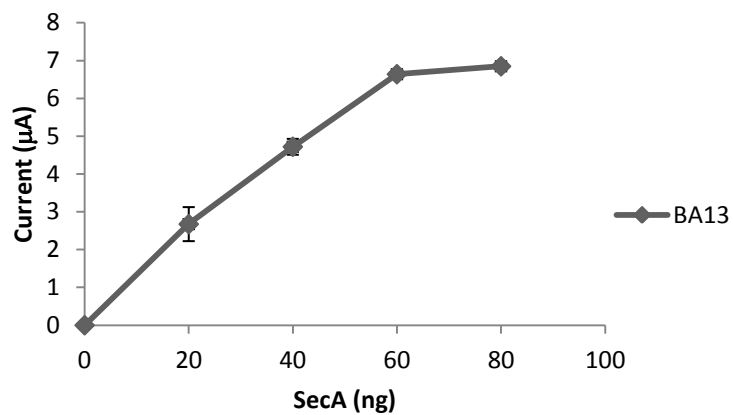
for unfolded PhoA and to stimulate ionic currents with SecYEG⁻ membranes. Therefore, in the oocyte system, we suggest that exogenous SecA interacts with precursor proteins (or signal peptides), and the SecA embedded in the membranes combines to conduct the successful initial steps of protein translocation. Furthermore, in the reconstituted membranes, additional SecA was required to elicit the current activities. It also provides the evidence that the SecA instead of SecYEG is essential for the ion current.

Since SecYEG complex is not essential for the opening of the SecA-dependent channel according to our electrophysiological results, these proteins may play other important roles in maintaining the structural integrity of the channels, and enhancing the efficiency of protein translocation. Proofreading of signal sequences is one of the functions of SecY fulfills in the early stage of protein translocation. Wild-type membranes having functional SecYEG rejected the mature precursors or defective LamB signal peptides ability to open of the protein-conducting channel. Defective LamB signal peptides, OmpA or unfolded PhoA can bypass such recognition and were able to induce the opening of protein-conducting channel in either SecYEG⁻ minus membranes or reconstituted BA13 membranes. However, unfolded BSA did not cause ionic currents, which indicates that the proofreading function of the protein-conducting channel cannot be bypassed by a non-specific unfolded protein (Bor-Reui Lin), and that these channels retain some degree of specificity for secretory proteins or signal peptides, albeit to a lesser degree. Moreover, the crystal structure of SecY from *Methanococcus jannaschii* (Bostina, Mohsin et al. 2005), suggested that SecY possesses a plug domain, which is not essential for cell viability in either *E. coli* (Li, Schulman et al. 2007; Maillard, Lalani et al. 2007) or yeast (Junne, Schwede et al. 2006). This SecY plug might be involved in the channel gating and for the specificity of signal

peptides (Li, Schulman et al. 2007; Maillard, Lalani et al. 2007). Indeed, studies have shown that removal of half or the complete the plug domain suppresses the effects of a defective signal sequence of alkaline phosphatase. In our data, the channel activity loses its signal peptide recognition function without the SecY-plug domain. The Unfolded Phoa A and LamB DM could promote the ionic current in the presence of puromycin. These results suggest that SecY does represent a check point for signal sequences or precursor proteins, and also selects the entrance of protein-conducting channel proficiently before protein translocation is initiated.

In this report, we provide evidence for active SecA-dependent channels in the absence of SecYEG, which may be supported by interacting with other membrane proteins such as SecD-FYajC, but lose the channel specificity and proofreading function for signal peptides. Thus, the minimum requirement for a functional protein-conducting channel still needs to be elucidated. SecD-SecF-YajC or other membrane proteins may be involved in this apparatus, supporting the basic SecA channel structure for active protein translocation. The use of electrophysiological voltage clamping and patch clamping techniques on reconstituted proteoliposomal complexes may provide another perspective of this dynamic system.

A.



B.

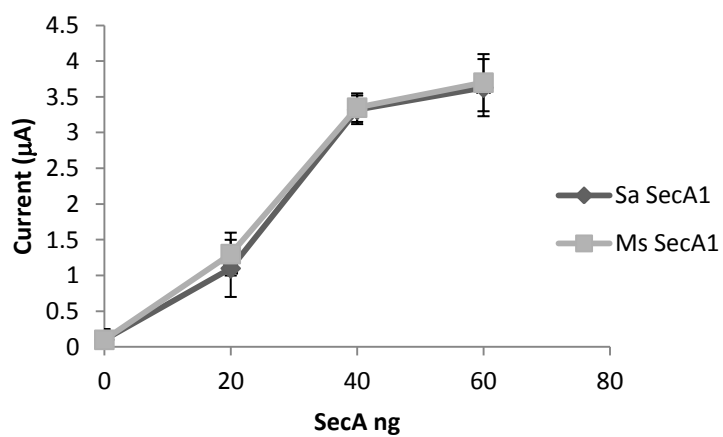
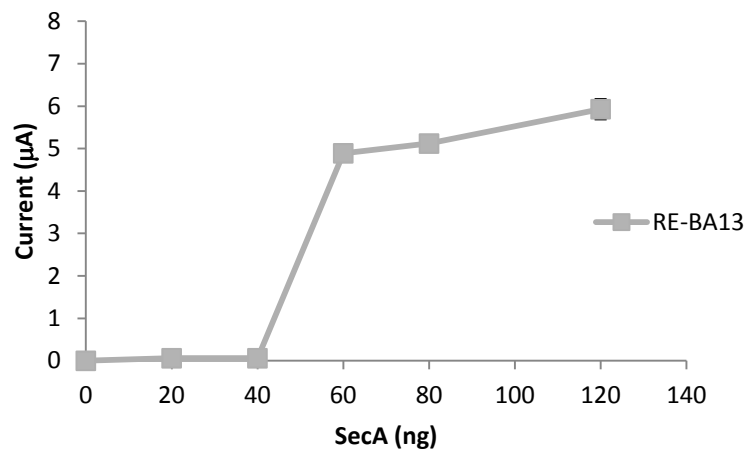


Figure 2.1. Dependence of SecA for channel activity in BA13 membranes.

60 ng BA13 membrane vesicles was injected into oocytes with various amount of *E.coli* SecA (A) or SecA homologs (B). All the recordings were done after 3h injection. Each experiment set was repeated three times; and each time, the n=10

A.



B.

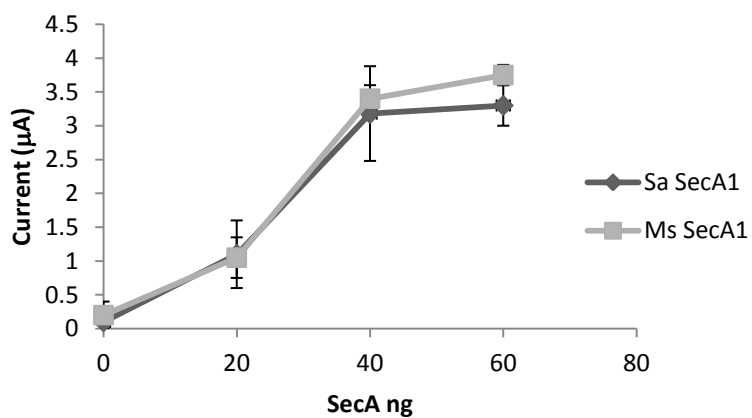
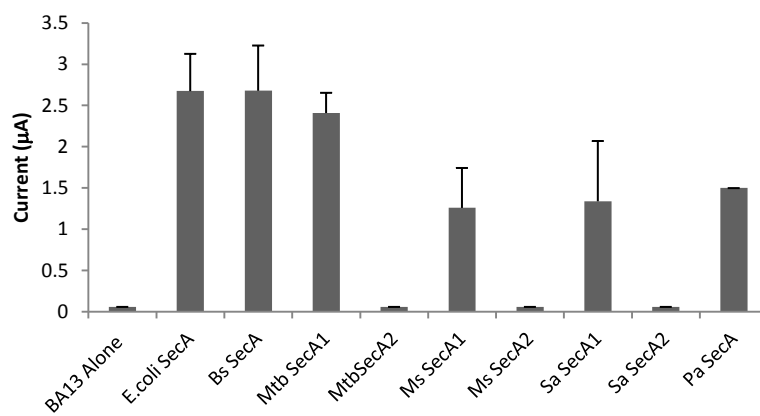


Figure 2.2. Dependence of SecA for channel activity in RE-BA13 membranes.

60 ng RE-BA13 membrane vesicles was injected into oocytes with various amount of *E.coli* SecA (A) or SecA homologs (B). All the recordings were done after 3h injection. Each experiment set was repeated three times; and each time, the n=10

A.



B.

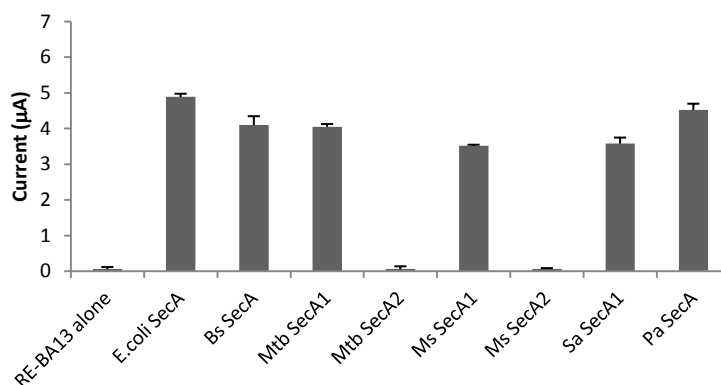


Figure 2.3. Channel activity of BA13 or reconstituted BA13 membranes with various SecAs addition.

60ng of BA13 or reconstituted BA13 membrane vesicles and various species of SecA were jointly injected into oocytes in the presence of proOmpA. Ionic current was recorded after 3h injection. Each experiment set was repeated three times; and each time, the n=10

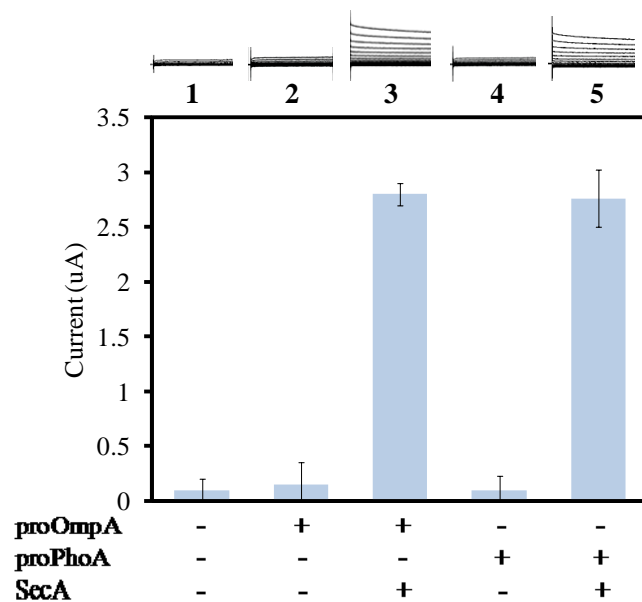
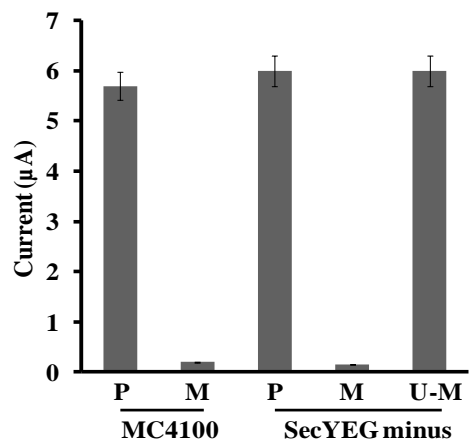
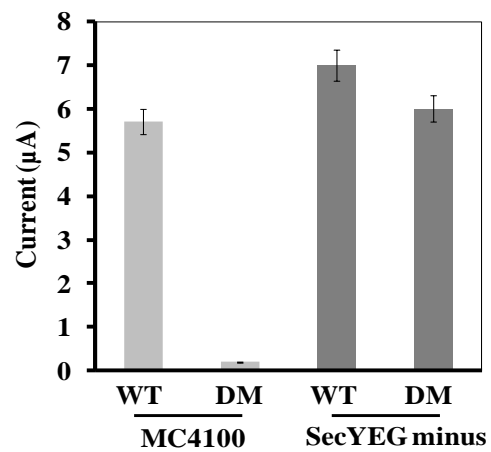
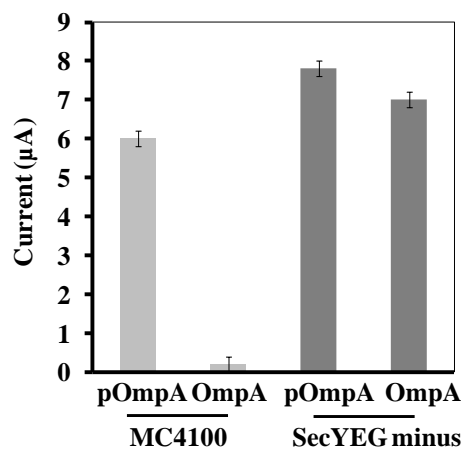


Figure 2.4. Channel activity of reconstituted BA13 by proPhoA stimulation

Reconstituted BA13 (RE-BA13, 60 ng) were injected with proPhoA (0.15 pmol) or proOmpA (0.15 pmol) with or without exogenous SecA (60 ng).

A. SecYEG minus



B. Reconstituted BA13

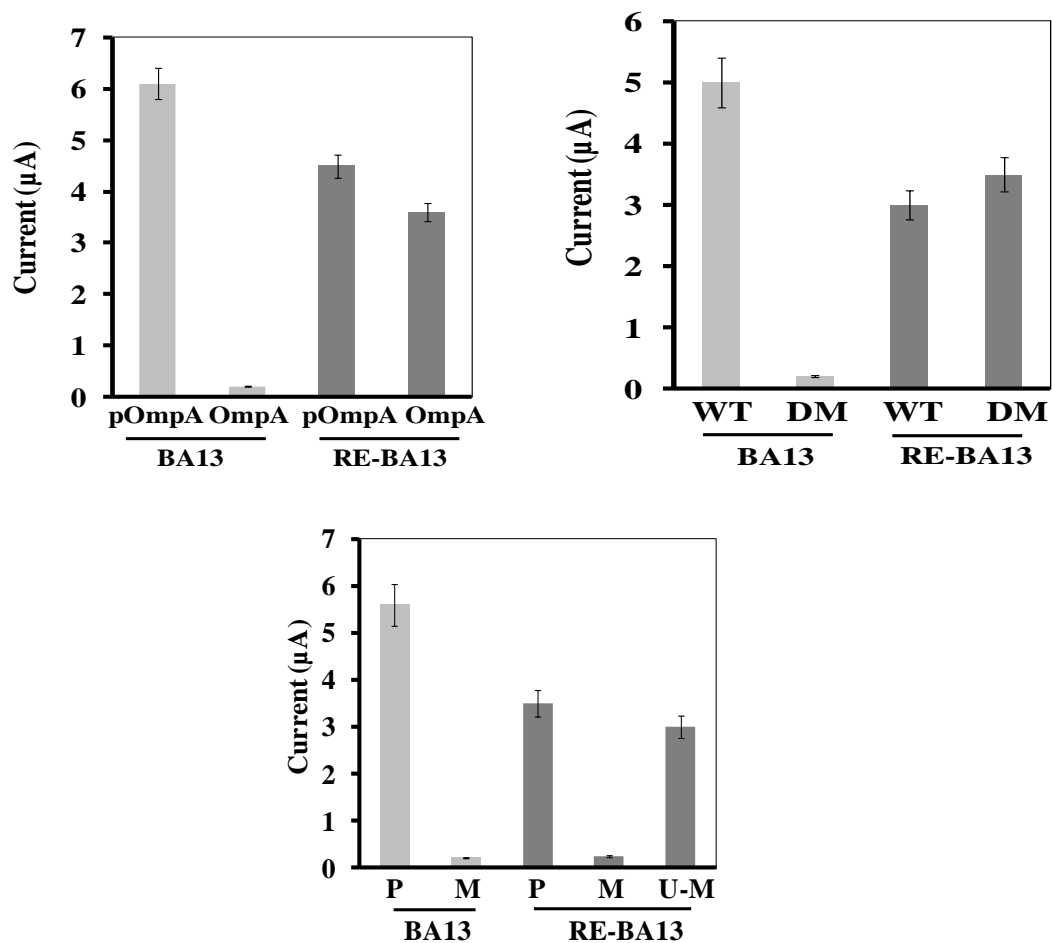
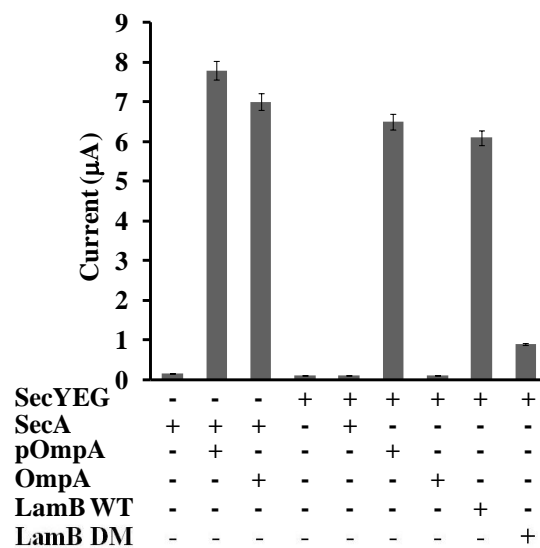


Figure 2.5. Loss of proofreading function in SecYEG minus and reconstituted BA13 membranes

SecYEG⁻ (A) and RE-BA13 (B) membranes were injected with precursors (proOmpA, pro-PhoA) or mature precursors (OmpA, PhoA) or LamB wild type (WT) signal peptides, or LamB defective mutant (DM) in the presence of 4 mM puromycin. P, proPhoA; M, mature PhoA; U-M, Unfolded mature PhoA.

A. SecYEG minus



B. Reconstituted BA13

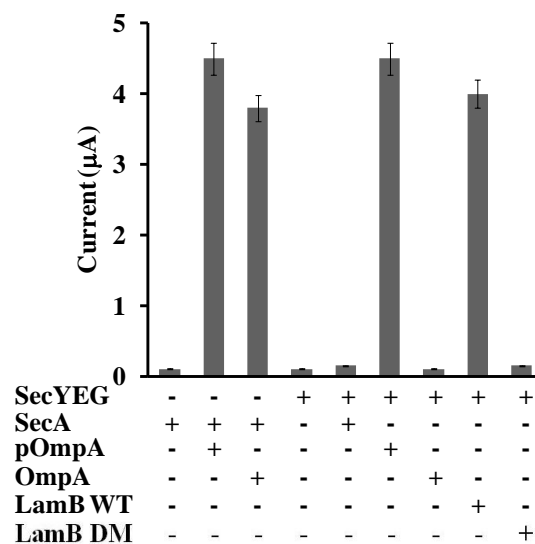


Figure 2.6. Re-gain proofreading function on membranes without SecYEG.

SecYEG proteoliposomes (30 ng) was injected with (A) SecYEG⁻ or (B) RE-BA13 membranes (60 ng) in the presence of 4 mM puromycin.

Results Part III

Inhibitions of SecA with SecYEG affect the drug-sensitivity

Previous studies identified that Sodium Azide, Rose Bengal (RB), and Erythrosine B are SecA sufficient inhibitors. These inhibitors abolish SecA ATPase activities depending on their specificity effect. On the other hand, the SecA-dependent channel activity could be inhibited by injecting inhibitors with wild type MC4100 *E.coli* membrane in the presence or absence of exogenous precursors. Although these three inhibitors are good inhibitors, Erythrosine B in the oocytes system is less sensitive to the channel activity. In order to understand the SecA-inhibitor sensitivity on channel activity under SecYEG+ or SecYEG minus condition, we used the membrane containing SecYEG, the reconstituted membrane, and SecA-liposomes to test the effect of Sodium azide and Rose Bengal. Furthermore, we will focus more on the Rose Bengal inhibition due to its sensitivity.

Azide inhibition

Sodium azide is a well-known SecA inhibitor during protein translocation in bacteria. We have observed that azide can inhibit SecA-dependent ionic currents when *E.coli* wild type MC4100 membrane was injected with proOmpA. However, the inhibitory effect is less efficient without the *E.coli* specific precursor (Fig. 3.1A). With proOmpA injection, the IC₅₀ for MC4100 membranes is 2.5 mM. For reconstituted MC4100 membrane, the channel activity was slightly affected by azide without proOmpA; which is similar like regular MC4100 membranes. The IC₅₀ for RE-MC4100 is 3 mM with proOmpA (Fig.3.1B). These data are compatible with previous findings (Lin, Gierasch et al. 2006), thus, we further use SecA-dependent membranes to determine the effect of azide. Compared to MC4100, the azide inhibition for BA13 and RE-BA13 is similar (Fig.3.2). Without the *E.coli* proOmpA, the BA13 and RE-BA13 could still obtain 30%

channel activity under 4 mM sodium azide treatment. And the IC₅₀ for BA13 and RE-BA13 is about 2.5 mM in the presence of proOmpA. These data indicated that although the channel activity can be active without exogenous *E.coli* precursor, the azide inhibition was relied on the *E.coli* precursor. In addition, this inhibition is not interfered by the presence or absence of SecYEG which suggested that azide binding site is not affected by the SecYEG interaction.

Rose Bengal inhibition

Rose Bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein) is a commonly used food stain and also a SecA specific ATPase inhibitor. Previous data (Y. Huang) indicated that Rose Bengal (RB) is a strongest inhibitor compared to the Sodium azide and Erythrosine B. In the presence or absence proOmpA, MC4100 membrane was totally inhibited by 10 μM RB treatment with an IC₅₀ about 5.3 μM (Fig.3.3A). In the absence of SecYEG, the reconstituted MC4100 membranes were more sensitive to the RB; the IC₅₀ is about 0.6 μM with or without proOmpA injection (Fig.3.3B). For the SecA-dependent membranes, the IC₅₀ for BA13 is 4.7 μM and the IC₅₀ for RE-BA13 is 0.4 μM (Fig.3.4). Compared to the wild type MC4100, BA13 (SecA-depleted membrane) is slightly more sensitive to the RB. Furthermore, both reconstituted membranes without the SecYEG had huge decreasing on channel activity with RB treatment. This data indicated that the RB is inhibitorier without SecYEG.

To determine the role of SecYEG in RB inhibition, we further tested the SecA-liposomes channel activity (Fig.3.5). Indeed, the channel activity was abolished at 1 μM RB treatment; the IC₅₀ of RB is about 0.4 μM. The channel activity generated by SecA homologs-liposomes has similar IC₅₀ of RB (table 3.2). The comparison table of RB inhibitory effect in the presence or

absence of SecYEG is summarized in Table 3.2. The channel activity is more resistant to RB with membranes containing SecYEG, for example BA13. Without SecYEG, SecA and its homologs are more sensitive to the RB. Interaction with SecYEG may alter the SecA conformation by RB inhibition.

Rose Bengal inhibition on SecY mutants

Due to the observation above, we hypothesized that SecA may have different conformation changes under different situation; we choose couple SecY mutant membrane (PrIA4, PrIA665) and SecYEG- minus membrane to see if there are more SecA conformation changes. SecYEG minus membrane is a membrane deleted the chromosome SecE. In the figure 3.6, SecYEG minus membranes was less sensitive to the RB treatment in the presence or absence of proOmpA. Under 100 μM RB treatment (20X times higher than IC₅₀ on BA13), there was still 20% channel activity observed. PrIA4 and PrIA665 are SecY suppressor mutants which enable translocation of pre-protein with a defective or missing signal sequence (Lin, Gierasch et al. 2006; Or and Rapoport 2007). For PrIA4, the IC₅₀ of RB is about 93 μM which is also about 20X higher than the one on BA13 or 100X higher than reconstituted BA13 or SecA-liposomes (Fig.3.7). Similar results were observed with PrIA665 (Fig.3.8A). The PrIA665 membranes is also resistant to RB; the IC₅₀ is about 100 μM . However, after removing SecYEG by Sodium cholate, the reconstituted PrIA665 lost its resistance to RB and the channel activity was inhibited by 1 μM RB. The IC₅₀ for RE-PrIA665 is about 0.7 μM .

Rose Bengal inhibition on SecY plug domain mutants

The three SecY plug domain mutants were obtained from Dr. Tom Rapoport. Previous study suggests that the plug domain function as a “gate” to recognize the signal peptide (Li, Schulman et al. 2007). Removing partial or full plug domain has no effect on the translocation process and allows no-specific precursor translocation (Li, Schulman et al. 2007). To test if the plug domain plays a role on the RB inhibition effect, we first inject the membrane to test the activity and also the activity after RB treatment (Fig.3.9). In the oocytes system, the channel activity could be detected in all the SecY mutants in the presence or absence of proOmpA (Fig. 3.9A). With the increasing amount of RB, the SecY WT membrane activity was inhibited; however, the half plug deletion and whole plug deletion mutants were less sensitive on RB treatment (Fig. 3.9A). On the other hand, after removing the SecYEG, all the channel activities on SecY wild type or plug domain mutants were disappeared when 1 μ M RB was added (Fig.3.9B).

The IC₅₀ of RB inhibition on various membrane vesicles is summarized on the Table 3.1. In the presence of SecYEG, the channel activity is more resistant to the RB and the reconstituted membrane without SecYEG seems to be more sensitive. Furthermore, even with intact SecYEG, the RB resistance is still varies based on the nature of SecYEG. This indicates that the SecA may have different conformation for SecYEG interaction related to RB inhibition.

The data on RB inhibition with various SecA homologs is summarized in Table 3.2. All the SecAs from other species of bacteria tested in here enable to generate the channel activity in either BA13, or reconstituted BA13, or SecA-liposomes experiments. And all these SecAs channel activities could be inhibited by Rose Bengal. The IC₅₀ of RB is similar in different mem-

branes, and the data without SecYEG is more sensitive which consistent with our other observations is.

Discussion

Sodium azide is identified as an effective Sec translocation inhibitor by screening the *azi* mutants (Oliver, Cabelli et al. 1990). The effect on the Sodium azide is focus on the SecA protein. This compound inhibits 50% of SecA ATPase activity at 5 mM, and 27% of SecA ATPase activity at 10 mM. In SecA-dependent translocation activity, 3 mM sodium azide decreased 50% of translocation activity (Tang, Pan et al. 2010). In the oocytes whole cell recording, the result is slight different compared to the translocation activity. In oocyte system, with or without the proOmpA, the exogenous *E.coli* precursors interfere the inhibition effect on the azide. Since the oocytes contain endogenous signal peptide which could also be recognize by the *E.coli* translocation machinery, the inhibition effect may depends on the ratio between oocytes signal peptides and *E.coli* signal peptides. This also indicates that the SecA may have conformation changes during the translocation based on the type of precursors. For reconstituted membranes without SecYEG, the IC₅₀ of azide on RE-MC4100 and RE-BA13 membrane is about 2 mM in the presence of proOmpA. Compared to the membrane containing the SecYEG, the inhibition effect is similar. These data indicates that the azide binding site on SecA in not affected by the SecYEG. In the presence of absence of SecYEG, the SecA's conformation may or may not change, and the channel activity is less sensitive to azide without *E.coli* precursors.

Rose Bengal is commonly used as a histological staining dye in general. It has also proposed as a treatment for cancers and skin condition (<http://www.wtvq.com/health/8992-rose-bengal-for-melanoma->). Previous studies in our lab (Y. Huang) shown that it can use as an inhibitor of SecA protein at μM level compared to the Sodium azide. By examing the channel activity on the membranes containing SecYEG, for example MC4100 or BA13, the IC₅₀ is 5 μM . inte-

restingly, the channel activity is more sensitive to the Rose Bengal treatment when SecYEG is removed. Reconstituted membranes lost its channel activity at 0.6 μM . This result is not due to the weakness of membrane vesicle itself. Although the reconstituted BA13 membrane is a SecA-depleted membrane and may contain some stress factor during the growth to weaken the membrane, the channel activity of reconstituted BA13 seems has similar sensitivity for Rose Bengal like reconstituted MC4100 membranes under normal condition. This result indicates that the Rose Bengal binding site of SecA may be close to the SecYEG binding. By removing the SecYEG, the conformation of SecA changes for interaction with Rose Bengal to result in loss of activity. The SecA may change its conformation based on the availability or functionality of SecYEG.

This hypothesis is further supported by data using SecY mutant membranes, such as PrIA4, PrIA665, and SecY-plug domain deletion. The PrIA mutants are originally isolated by their ability to restore the translocation activity of protein with defective signal sequence (Silhavy and Beckwith 1983; Shiba, Ito et al. 1984). These mutations loosen the SecYEG interaction, allow the non-typical preprotein across the inner membrane (Maillard, Lalani et al. 2007); and increase the interaction affinity with SecA (van der Wolk, Fekkes et al. 1998). Previous studies also reveal that those mutations are located on the periplasmic loop 1 and TM7/TM10 on SecY which is closed to the plug domain (Duong and Wickner 1999; Lin, Gierasch et al. 2006). On the Rose Bengal treatment, the channel activity of PrIA4 and PrIA665 membranes are more resistant. The Rose Bengal IC₅₀ on PrIA mutants is 93.5-100 μM which is almost 15X higher than wild type MC4100 membrane, and is almost 100X higher than reconstituted membrane without SecYEG. After removing the SecYEG on PrIA mutants, the reconstituted PrIA membranes are

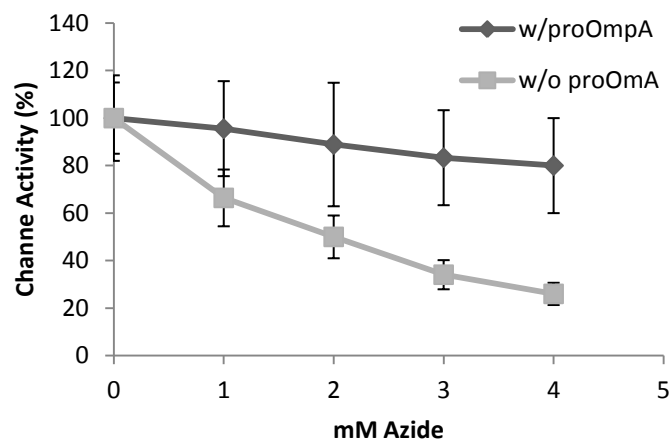
sensitive to the Rose Bengal. The IC₅₀ is about 0.7 μ M. These data support our hypothesis that the Rose Bengal binding site on SecA is related to the site of SecYEG interaction; and the status of SecYEG affects SecA conformations that affect the Rose Bengal resistance. The same result was observed when the SecY-plug domain is tested. The SecY-plug domain is located on the SecY TM2a. The plug domain has been determined to serve as a “gate” to select the preprotein with correct signal peptide acrossing the membrane (Li, Schulman et al. 2007). Deletion of partial or full plug domain could still maintain the translocation activity; however, it lost the selectivity on the preprotein. The plug domain mutation loosens the structure of SecYEG from the “seal” state to the more “open” state (Emr and Silhavy 1983). This result is also confirmed by using the oocyte whole cell recording. Furthermore, the SecY plug domain mutations are resistant to the Rose Bengal. Additionally, removing the SecYEG from the plug domain mutant membranes could increase the inhibition effect of Rose Bengal treatment.

The only exception is the SecYEG minus membrane prepared from cells depleted of SecYEG. The IC₅₀ for SecYEG minus membrane is around 50 μ M which is 10 X higher than that of the reconstituted membrane. One possibility is that due to the deletion of SecYEG, the cells produce more SecA up to 10-20 folds (Yang, Yu et al. 1997; Baars, Wagner et al. 2008). With excess SecA in cells, the resistance of SecYEG membrane to Rose Bengal may be higher than the membrane chemically removed SecYEG.

The IC₅₀ of Rose Bengal on different membranes is summarized in Table 3.1. These data indicate that the SecA may change its conformation in the absence of SecYEG. This conformation change affects the sensitivity of the inhibitors depending on the state of SecYEG. For Sodium azide, the inhibition effect is the same at both conditions. On the other hand, the sensitiv-

ity of SecA on Rose Bengal is affected by SecYEG interaction. In the absence of SecYEG, the translocation is functional, and the Rose Bengal has greater effect on SecA inhibition. This suggests that the Rose Bengal binding site of SecA is affected by the SecYEG interaction site. Taken together, these results could provide more details on the relationship of SecA, SecYEG and the inhibitors in protein translocation.

A.



B.

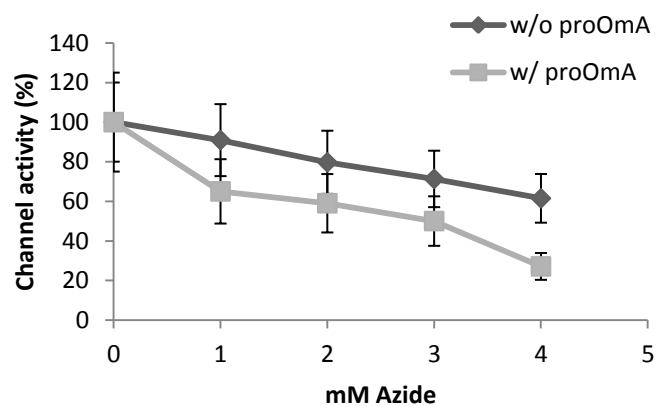
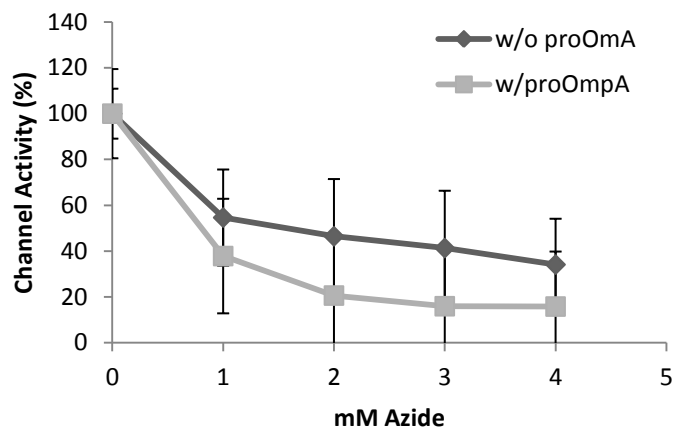


Figure 3.1. Inhibition by Sodium Azide on MC4100 and RE-MC4100 membrane vesicles.

60 ng *E.coli* membrane and different amount of Azide was mixed and injected into oocytes in the presence or absence of proOmpA. The whole cell recording was done after 3h injection. The channel activity without Azide treatment is set of 100% channel activity. Each experiment set was repeated three times; and each time, the n=10

A.



B.

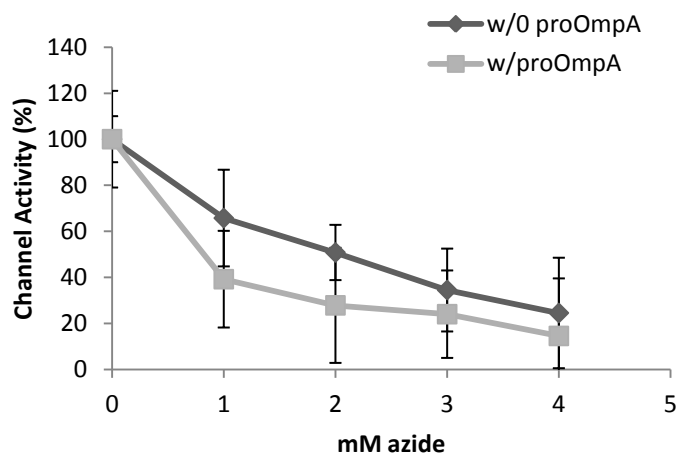
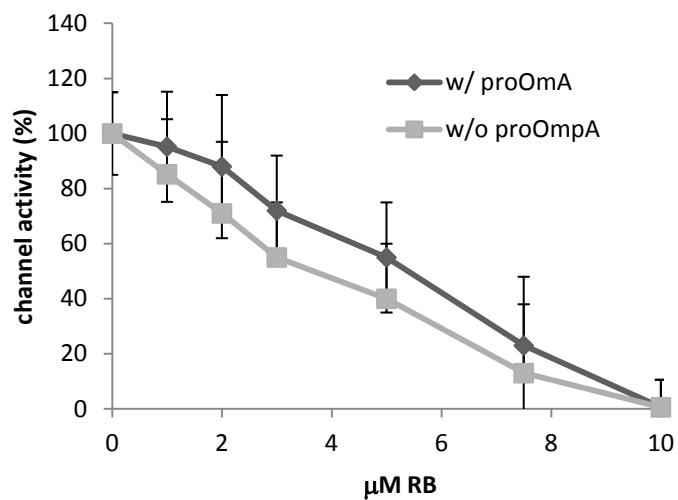


Figure 3.2. Inhibition of Sodium Azide of BA13 and RE-BA13 membrane vesicles.

60 ng *E.coli* membrane and different amount of Azide was mixed and injected into oocytes in the presence or absence of proOmpA. The whole cell recording was done after 3h injection. The channel activity without Azide treatment is set of 100% channel activity. Each experiment set was repeated three times; and each time, the n=10

A.



B.

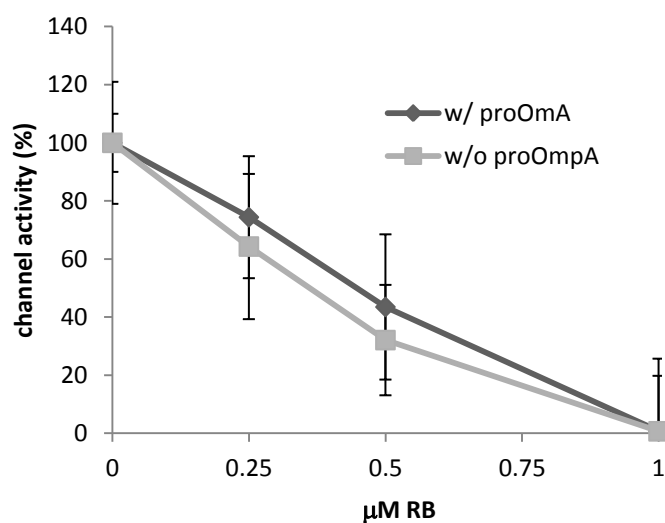
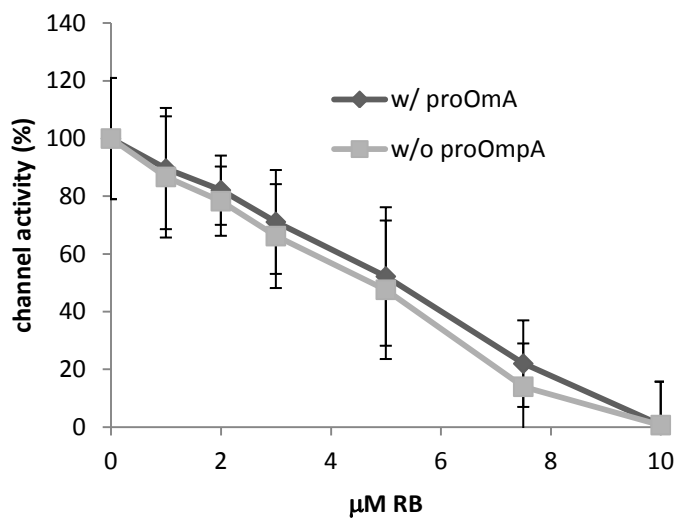


Figure 3.3. Inhibition of Rose Bengal on MC4100 or RE-MC4100 membranes.

60 ng *E.coli* membrane with 60 ng SecA was injected into oocytes in the presence of absence of proOmpA. After 3h injection, the oocytes were ready for whole cell recording to determine the channel activity. The channel activity without the Rose bengal treatment is set of 100% channel activity. Each experiment set was repeated three times; and each time, the n=10

A.



B.

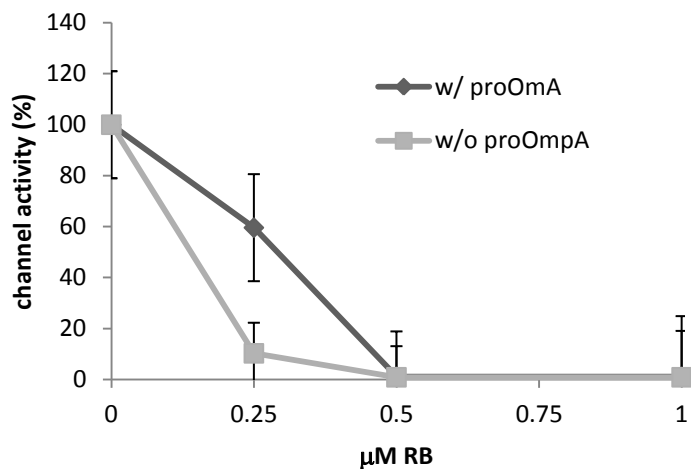


Figure 3.4. Inhibition of Rose Bengal on BA13 and RE-BA13 membranes.

60 ng *E.coli* membrane with 60 ng SecA was injected into oocytes in the presence of absence of proOmpA. After 3h injection, the oocytes were ready for whole cell recording to determine the channel activity. The channel activity without the Rose bengal treatment is set of 100% channel activity. Each experiment set was repeated three times; and each time, the n=10

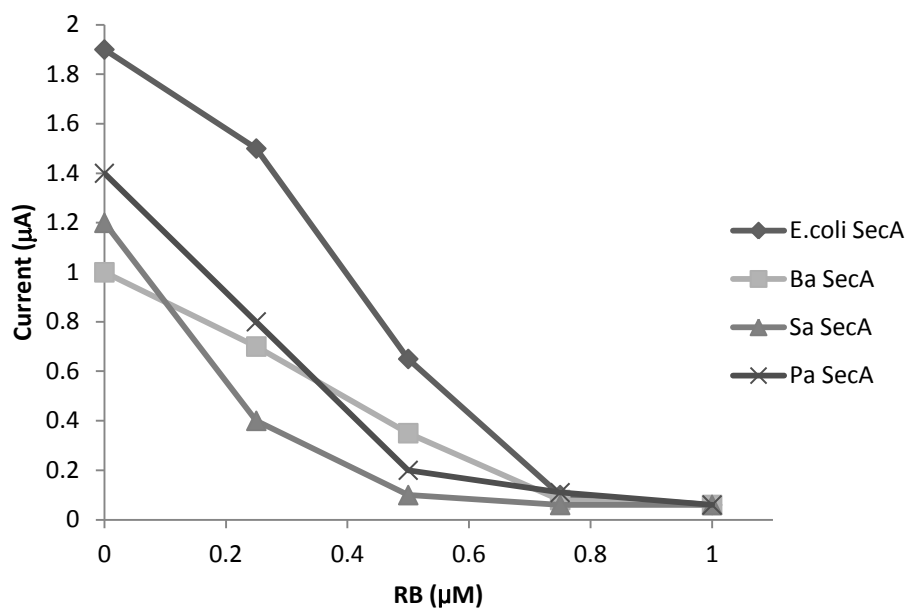


Figure 3.5. Channel activity on SecA liposome in the presence of Rose Bengal.

120 ng liposomes was injected with 120 ng SecAs in the absence of proOmpA with 2 mM ATP and 1 mM MgCl₂. The mixture was injected into oocytes for 3h. The channel activity was determined by whole cell recording. n=10

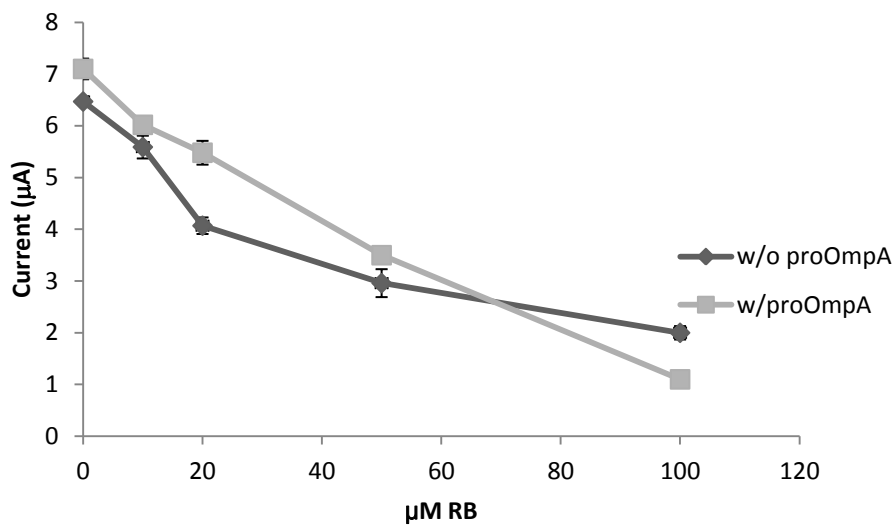
SecYEG minus membrane

Figure 3.6. Inhibition of Rose Bengal on channel activity of SecYEG minus membrane.

60 ng membrane with 60 ng SecA was co-injected into oocytes with or without proOmpA. Channel activity was determined after 3h injection. Each experiment set was repeated twice; and each time, the n=10

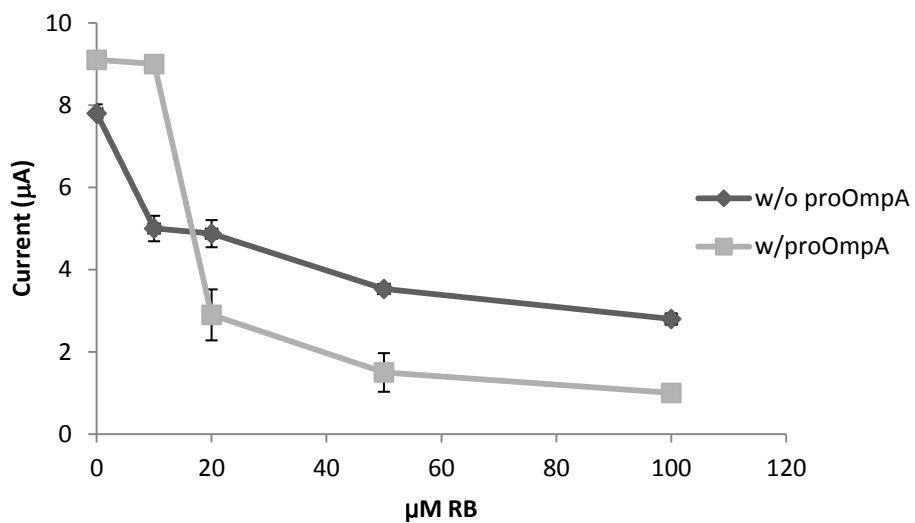
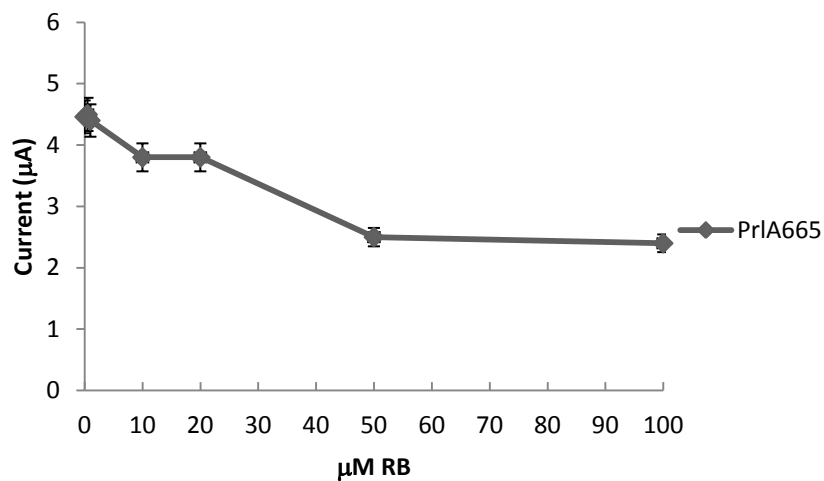
PrlA4

Figure 3.7. Channel activity of SecY supressor mutant PrlA4 membrane.

60 ng membrane with 60 ng SecA was co-injected into oocytes with or without proOmpA. Channel activity was determined after 3h injection. Each experiment set was repeated twice; and each time, the n=10

A.



B.

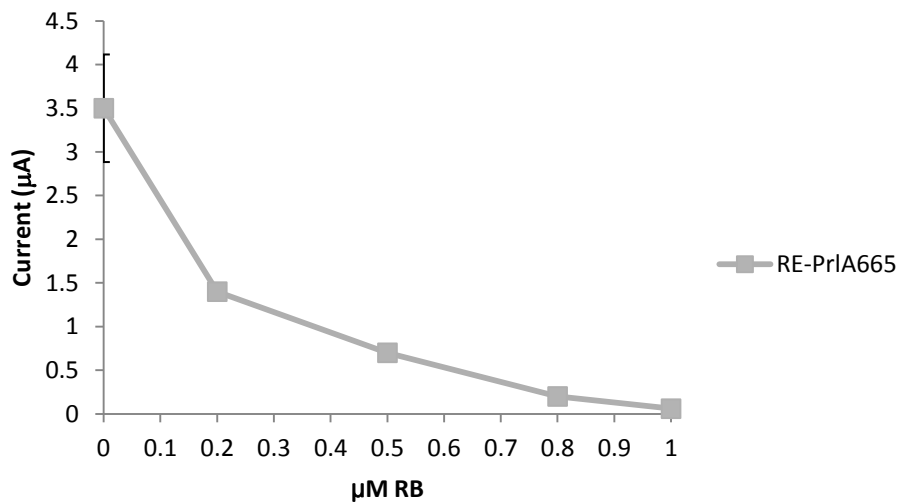
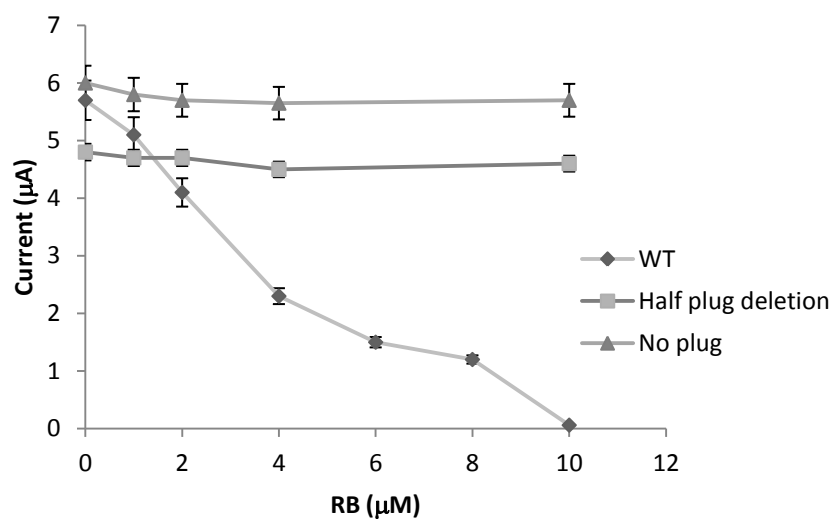


Figure 3.8. Channel activity of PrIA665 and RE-PrIA665 with Rose Bengal treatment.

The 60 ng PrIA665 and RE-PrIA665 was mixed with 60 ng SecA in the absence of proOmpA for injection. After 3h, the channel activity was determined by whole cell recording. A. PrIA665, B. RE-PrIA665 Each experiment set was repeated three times; and each time, the n=10

A.



B.

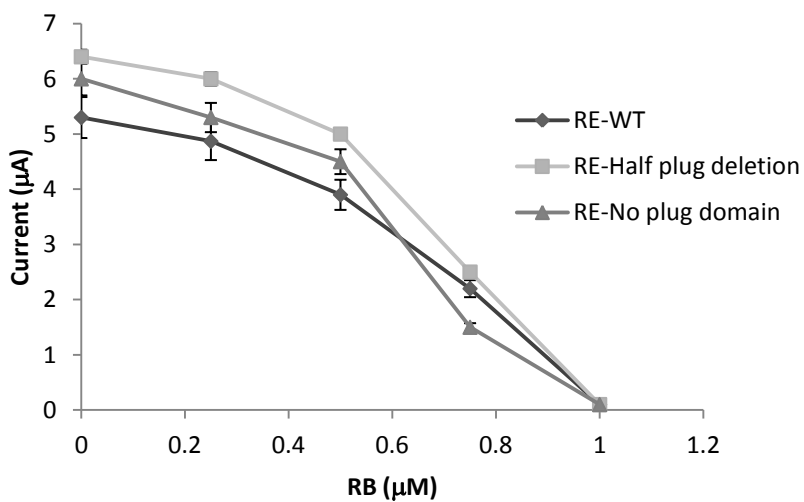


Figure 3.9. Channel activity on SecY mutant membranes in the presence of Rose Bengal.

60 ng membrane was mixed with 60 ng SecA in the absence of proOmpA. The mixture was injected into oocytes for 3h. The channel activity was determined by whole cell recording. A. regular membrane, B. reconstituted membrane. Each experiment set was repeated twice; and each time, the n=10

Table 2.1. Rose Bengal IC50 on various membrane vesicles.

	MC4100	RE-MC4100	BA13	RE-BA13	SecYEG minus	PrIA4	PrIA665	RE-PrIA665	SecY-plugWT	SecY-plug mutant	SecA-liposome (120 ng)
RB IC50 (μ M)	5.3 \pm 0.1	0.6 \pm 0.1	4.7 \pm 0.2	0.4 \pm 0.1	67 \pm 0.1	93.5 \pm 0.1	100 \pm 0.1	0.7 \pm 0.3	No inhibition at 10	0.7 \pm 0.1	0.4 \pm 0.2

For inner membrane vesicles, the amount for each injection was 60 ng membrane and 60 ng SecA . For SecA-liposome, the amount for each injection is 120 ng SecA-liposome, 1 mM ATP, 1 mM Mg, and proOmpA.

Table 2.2. Rose Bengal IC50 on SecA homologs channel activity.

	<i>E.coli</i> SecA	<i>Bs</i> SecA	<i>Ba</i> SecA1	<i>Mtb</i> SecA1	<i>Ms</i> SecA1	<i>Sa</i> SecA1	<i>Pa</i> SecA1
IC50 (μ M) in BA13	4.7 \pm 0.1	5.8 \pm 0.3	6.1 \pm 0.3	NA	NA	6.1 \pm 0.3	5.1 \pm 0.1
IC50 (μ M) in RE-BA13	0.4 \pm 0.1	0.5 \pm 0.5	0.5 \pm 0.1	0.5 \pm 0.2	0.4 \pm 0.5	0.5 \pm 0.3	0.3 \pm 0.5
IC50 (μ M) in SecA-liposome	0.4 \pm 0.5	0.4 \pm 0.2	0.3 \pm 0.2	NA	NA	0.4 \pm 0.1	0.3 \pm 0.2

For inner membrane vesicles, the amount for each injection was 60 ng membrane and 60 ng SecA . For SecA-liposome, the amount for each injection is 120 ng SecA-liposome, 1 mM ATP, 1 mM Mg, and proOmpA

Table 2.3. Comparison of SecA inhibitors

IC50 (μ M)	RB	SCA-15	SCA-21	SCA-41	SCA-107
<i>Ec</i> SecA	0.4	4.2	2.4	3.4	1.6
<i>Ba</i> SecA1	0.4	2.3	1.5	3.8	NA
<i>Sa</i> SecA1	0.4	2.8	1.6	3.4	0.6

120 ng SecA-liposomes were injected with various inhibitors in the presence of 2 mM ATP, 1 mM Mg, and proOmpA.

Results Part IV

SecA domain structure and function in channel activity

We have identified a SecA-dependent protein conducting channel activity and determine its characteristics and the differences with the channel containing SecYEG. We have proposed that SecA contains two binding sites for protein translocation: One with low-affinity phospholipids and one with high-affinity SecYEG. We further define the critical domains on SecA for these two binding sites channel activity.

The SecA contains 901 amino acids, which is about 102 kDa. Our laboratory had constructed serial C-terminal deletion mutants of SecA for us to test the channel activity. The SecA deletion mutants are listed as Figure 4.1. There was no channel activity in N39, N68, and N69 in the presence of 60 ng membranes containing SecYEG. The N70D, N71.5D and N71.6 had little channel activity with less expression efficiency (Fig.4.2A). However, higher amounts of N68-N71.6 increase the channel activity efficiency (Fig.4.2B). The channel activity was observed when N72K, N73, or N75 was injected with BA13 membranes (Fig. 4.2A) at 60 ng protein which indicated that the high affinity SecYEG interaction site may be located on SecA 640-649 amino acids. We further test the activity of those fragments with liposomes system, and none of them had positive channel activity (Fig. 4.3A). These activity could be restored when 30 ng purified SecYEG was added with N70D, N71.5D, N71.6, N72K, N73 or N75 (Fig. 4.3B). Although the channel activity was restored by SecYEG addition, the ionic current and the expression efficiency in the oocytes were less effective. Compared to the BA13 membranes containing SecYEG, the data indicates that there may be some other components in the BA13/SecYEG channel system which is important for the channel activity. In addition, the length of SecA 1-668 fragments are not enough to maintain the SecA-liposomes channel activity.

We further extended the C-terminus to amino acid 828, 829 or 831 to determine the ion currents with BA13 and liposomes. Surprisingly, there was no activity on C828 or C829 at 60 ng with BA13 membranes (Fig.4.4A). Additions of 80-120 ng of these two fragments were able to maintain the currents in BA13 indicating that this loss of activity in 60 ng protein may be due to the poor enzyme activity of SecA 1-828 and SecA 1-829 (Fig.4.4B). The fragment 1-831 was able to express the BA13 channel activity at 60 ng in contrast to SecA 1-829, indicating the intriguing function of residues 829-831. On the other hand, this 829 amino acids fragment was enough to maintain the SecA-liposomes channel activity (Fig4.5A). The SecYEG-liposomes activity was not affected either (Fig. 4.5B). Thus, the amino acids 668-828 are important for liposomes binding.

In order to identify the requirement of the SecA C-terminus for the SecA-liposomes channel activity, we used a short C34-SecA fragment, from 610-901, to redeem these N-terminal deletion mutant SecAs-liposomes channel activity. We first tested these combinations with the BA13 membranes, and N73 and C75 were active with C34 addition at 60 ng proteins (Fig. 4.6A). However, the N68+C34 and N70+C34 and N71.6D+C34 were less efficient at 60 ng protein. Increasing the proteins amount to 120 ng enhanced the ionic currents and the expression efficiency on N68, N70D, and N71.6 in the presence of C34 (Fig 4.6B). Furthermore, the channel activity was observed in SecA-liposomes in N73+C34, and N75+C34 with high channel activity but N68+C34, N70+C34 or N71.6+C34 combinations produced low channel activity (Fig. 4.7A). These N-terminal deletion fragments complement the C-terminus domain to restrain the channel activities; however, those activities in liposomes were not efficient, only 40% expression rates in the oocytes as compared to more than 70% with wild type SecA.

We also added the purified SecYEG into these combination experiments, and the data in figure 3.7B showed that those activities were active except the N68+C34. Compared to the data in BA13 (Fig. 4.6), the liposomes activities with SecYEG were 2X less indicating that there are still more accessory proteins required to the channel activity in SecYEG system (Fig. 4.7B).

So far, for the SecYEG high-affinity site, amino acids 640-649 of SecA are required. Moreover, there are other accessory proteins which may involve in the SecYEG/SecA function for the channel activity efficiency. For the low-affinity phospholipids site, the 668-828 amino acids of SecA are required. Next, to see the importance of SecA N-terminus in channel activity, we used combination of N-terminal deletion mutants of SecA to test its ion channel activity. The list of the N-terminal deletion fragments are in Figure 4.8. All those fragments are tested with BA13, liposomes, and the SecYEG addition similar as previous experiments. In figure 4.9, C34 (610-901) had no activity with BA13 but C95.54 (9-901), C95.43 (18-901) and C95 (61-901) produced the channel activities. This result suggested that the N-terminal amino acids 1-61 is not important for the SecYEG binding channel activity. Surprisingly, deletion of first 10 amino acids abolishes the SecA-liposomes channel activity (Fig. 4.10A) which suggested that the N-terminal end of SecA is required for the liposomes binding. SecYEG addition restores the channel activity on SecA-liposomes with 9-901, 18-901, and 61-901 (Fig. 4.10B). Thus, the amino acids 1-9 are another critical site for the liposomes activity which can be compensated by the addition of SecYEG.

Previous lab data (Bing Na) showed that C95.43-828 (20-828) and C95.43-829 (20-829) are able to complement the bacteria growth in SecA-depleted strain. We further used them to test the channel activities, and the data was shown in the Figure 4.11A. These two fragments

were able to maintain the channel activity in the BA13 but not the SecA-liposomes. Purified SecYEG restore the activity for SecA-liposomes (Fig. 4.11B).

Discussion

E. coli SecA contains 901 amino acids and exists in the cytoplasm as membrane- or soluble form. During the translocation, SecA provides the mechanical energy by hydrolyzing pushing the precursor into channel (Lengeler, Drews et al. 1999). Since SecA is a ATPase, It belongs to the super family 2 helicase (SF2) and contains the DEAD motor. This DEAD motor is composed of two sub-domains: Nucleotide binding domain I (NBD), and the Intra-molecular regulator of ATPase (IRA2) (Lengeler, Drews et al. 1999). Besides the DEAD motor, SecA also contains several domains including the preprotein binding domain (PBXD), the helical scaffold domain (HSD), the helical wing domain (HWD) and the C-terminal linker (CTL) (Driessen, Manting et al. 2001; Driessen and Nouwen 2008). There are conserved Walker A and Walker B motifs among the NBDs. Previous report identifies that the NBD is located in a.a. 1-220 and 378-420 and the IRA2 is located in a.a 421-610 (Lengeler, Drews et al. 1999). Between NBD and the IRA2, the a.a 221-377 is the preprotein binding domain and also a connecting bridge for the DEAD motor (Lengeler, Drews et al. 1999). The C-terminal domain is proposed as a lipid binding site and SecB binding domain (Hendrick and Wickner 1991; van Voorst, van der Does et al. 1998; Keller 2011).

In Chapter I, we proposed that there are two binding sites for SecA: one with high-affinity SecYEG, and the other one with low-affinity liposomes. Both sites are SecA –essential and the channel activity was varying depending on the energy efficiency. By using the deletion mutation of SecA, we are able to map the critical domains responsible for the channel activity on SecA/SecYEG or SecA-liposomes. The amino acid residues 640-649 are critical for the SecYEG interaction and the fragment of SecA less than 71-72 kDa lost the affinity with SecYEG. Similar result was observed in the study from Das and Oliver (Breukink, Demel et al. 1992). By site-

directed *in vivo* photocrosslinking, they identify the amino acids 59, 226, 340, 350, 600, 640, and 661 of SecA is essential for SecYEG interaction. This data is also similar with the finding from Copper et al (Cooper, Smith et al. 2008). Our data support their observations that the area on amino acid 640 are responsible for the SecYEG interaction, and the N-terminal end from 1-60 is not involved in the SecA/SecYEG channel activity function. However, the reconstitution of SecYEG into SecA-liposomes doesn't generate the high ionic current as observed with BA13 membranes containing SecYEG. This may suggest that the translocation machinery components in the BA13 membrane have more than just SecA, SecYEG, and the energy. Other accessories proteins also play an important role for the translocation channel activity.

In additions, our data also reveals two sites for the liposomes binding: one is located on the amino acids 1-9 and the other one is located on 668-828. It has previously proposed that SecA contains two lipids binding-sites located at both N-terminal (1-100) and C-terminal (600-900) ends (Hendrick and Wickner 1991; van Voorst, van der Does et al. 1998; Keller 2011). The data we collected was compatible with previous findings. Moreover, we are able to narrow down the regions to amino acid 1-9 and 668-828 for the lipids binding critical for forming channels. The certain domains of the SecA is required to maintain the ring structure formation.

Theses SecA domain mapping data allow us to understand how the SecA interacts with other components to maintain the channel activity. By studying the structures of SecA domains, it would help to design the potential antibiotics for particular bacteria pathogens using SecA as a major protein transportation system.

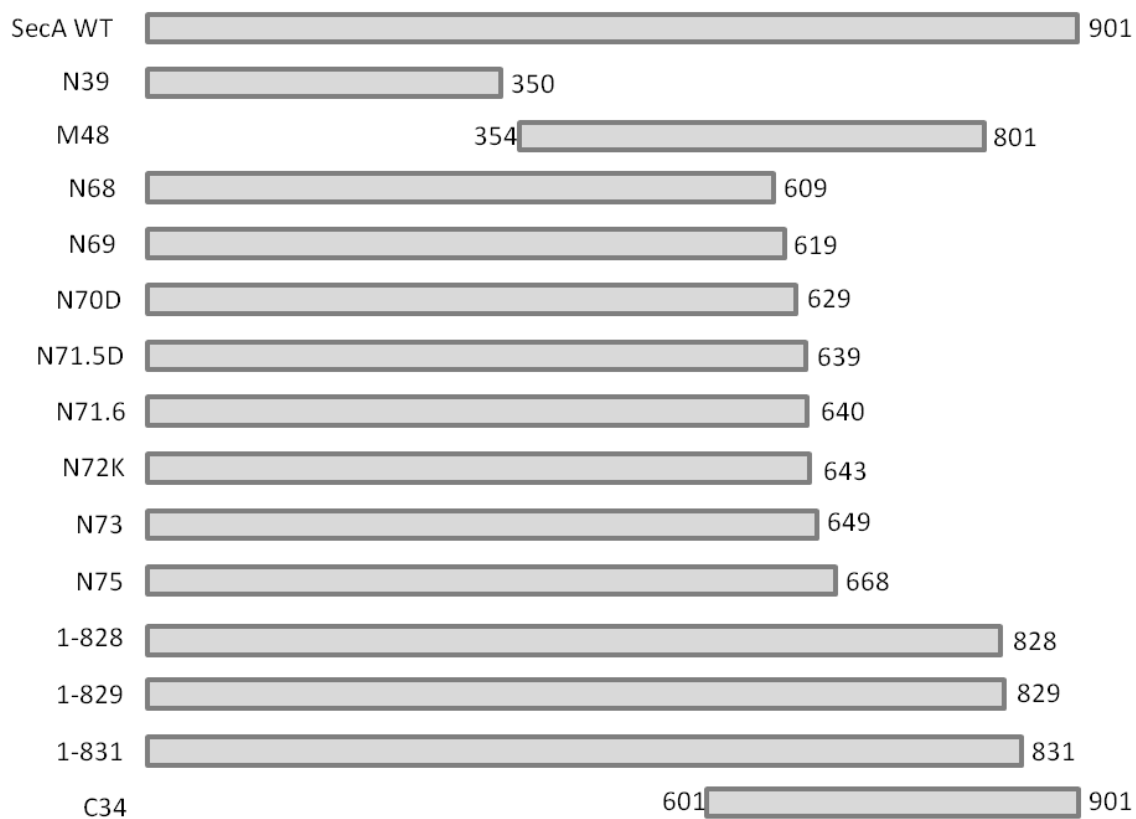
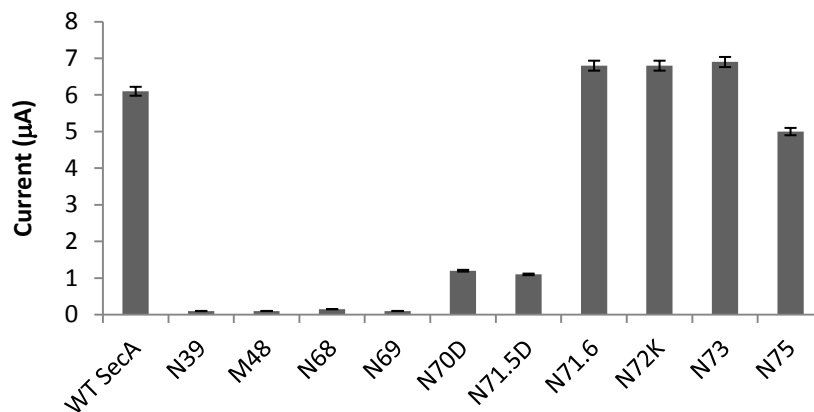


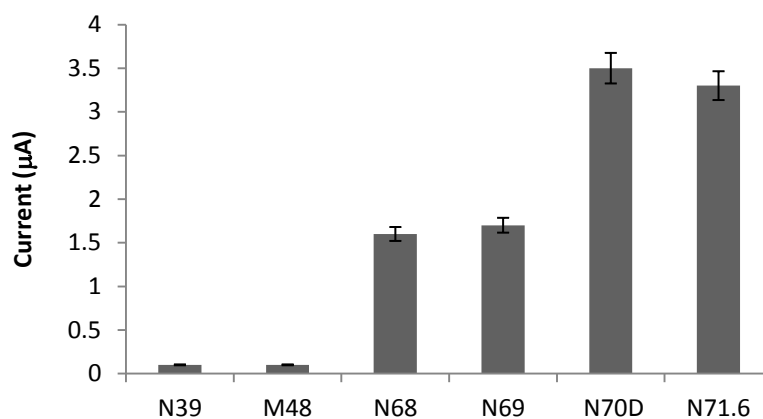
Figure 4.1. SecA C-terminal deletion mutants.

A.



	N70D	N71.5D
BA13	4/20	4/20

B.

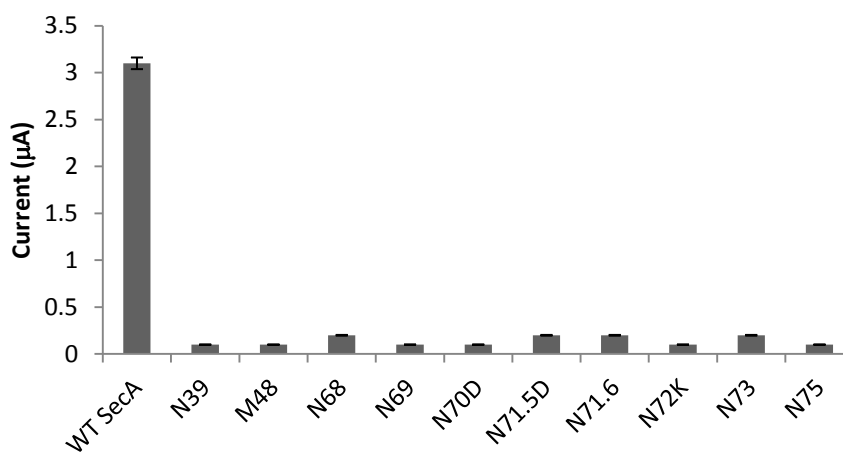


	N68	N69	N70D	N71.6
BA13	2/20	3/20	15/20	15/20

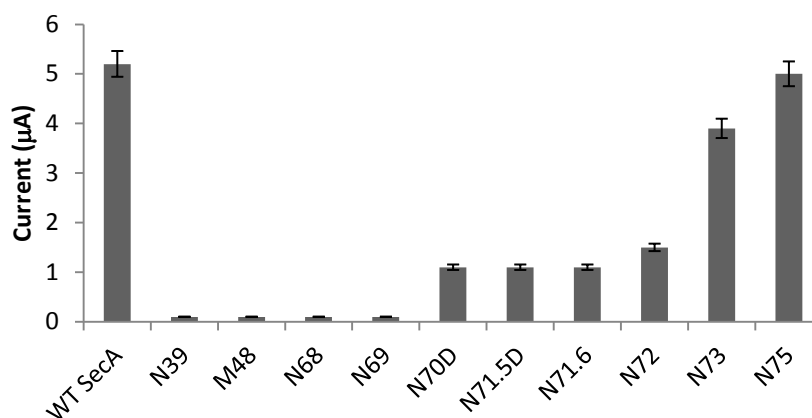
Figure 4.2. Channel activity of SecA C-terminal deletion mutants in the oocytes.

(A) 60 ng of BA13 membranes and 60 ng SecA were injected into oocytes in the presence of proOmpA. (B) 120 ng SecA protein was used for the experiment as (A). The recording was done after 3h injection. n=20

A



B.

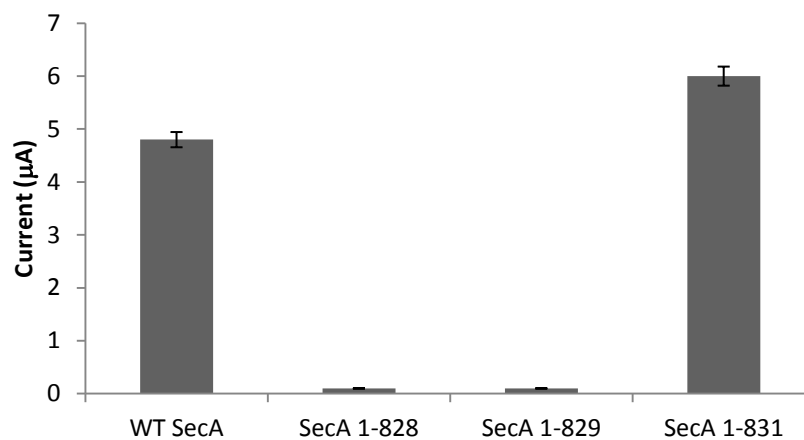


	N70D	N71.5D	N71.6	N72K	N73	N75
SecA-liposomes with SecYEG	4/20	4/20	6/20	10/20	15/20	15/20

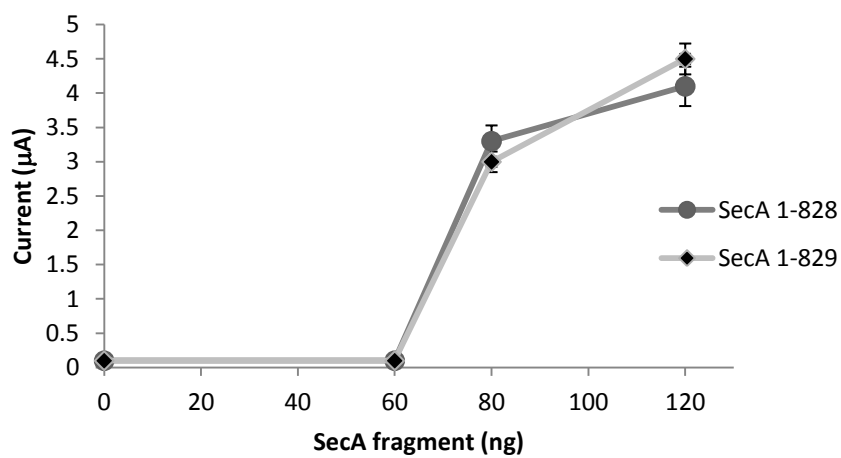
Figure 4.3. SecA C-terminal deletion mutants with liposomes in the oocytes.

120 ng SecA-liposomes, 2 mM ATP, 1mM Mg, and proOmpA were injected in the absence (A) or presence (B) of 30 ng SecYEG. After 3h, the whole cell recording was performed to collect the ion currents. n=20

A



B.



	60 ng	80 ng	120 ng
SecA 1-828	0/20	10/20	18/20
SecA 1-829	0/20	9/20	15/20

Figure 4.4. BA13 membrane channel activity with SecA-deletion mutants in the oocytes.

60 ng of SecA (A) were injected with BA13 membranes into oocytes in the presence of proOmpA. The SecA fragments protein titration in 60 ng BA13 membranes. n=20

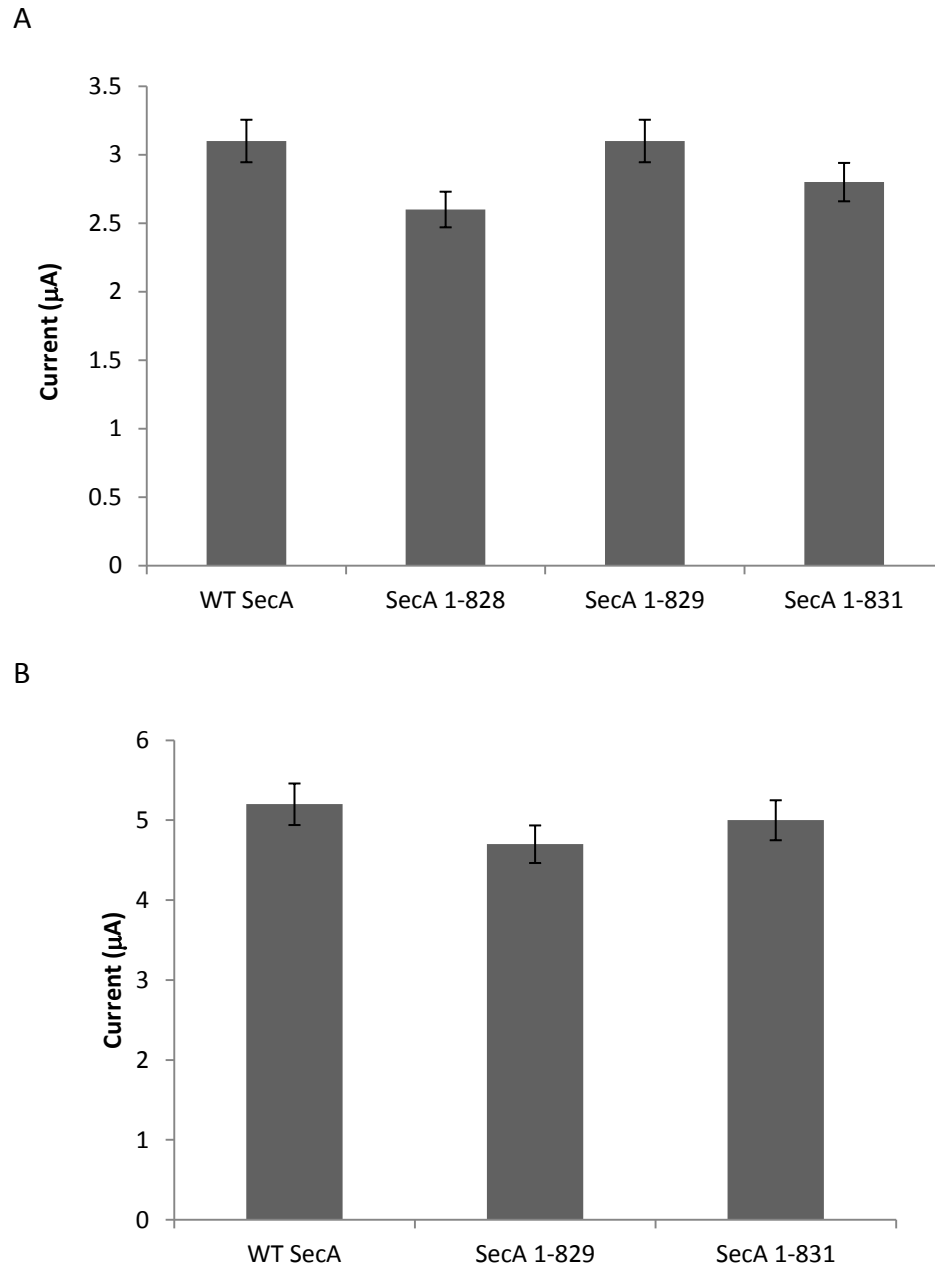
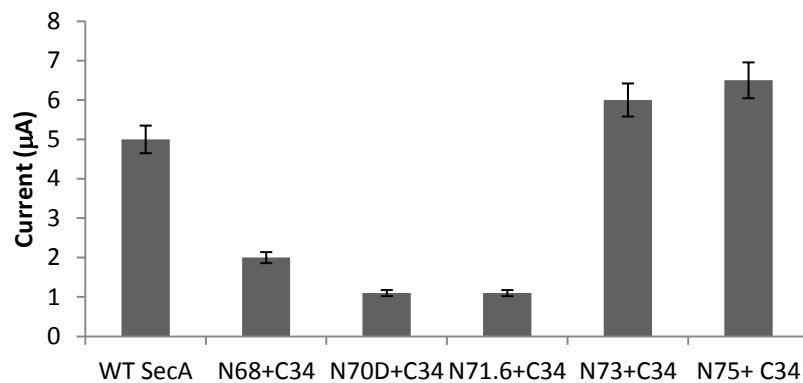


Figure 4.5. SecA liposomes channel activity on SecA1-828 to SecA1-831 in the oocytes.

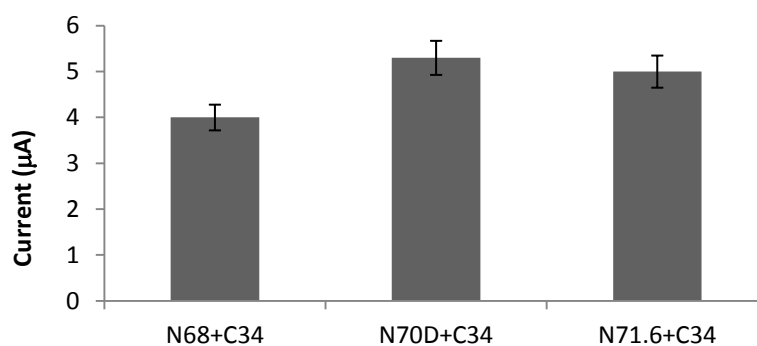
120 ng SecA-liposomes, 2 mM ATP, 1 mM Mg and proOmpA were injected without (A) or with (B) 30 ng SecYEG. n=10

A.



	N68+C34	N70D+C34	N71.6+C34
60 ng SecA	6/20	2/20	3/20

B.

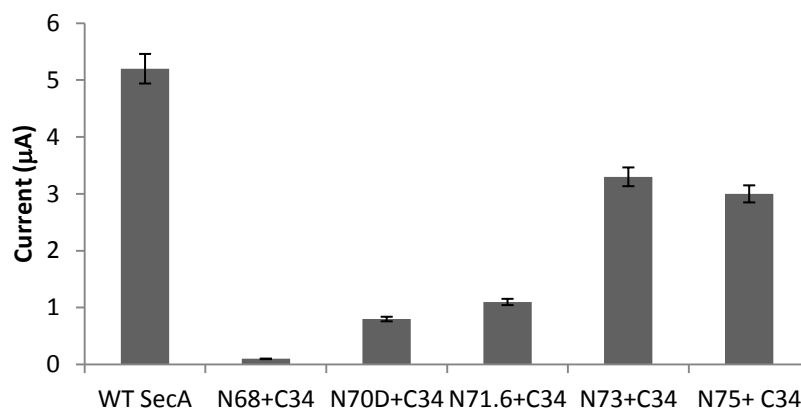


	N68+C34	N70D+C34	N71.6+C34
120 ng SecA	15/20	17/20	17/20

Figure 4.6. Channel activity of C-terminal deletion mutants coupled with C34 in BA13 membranes in the oocytes.

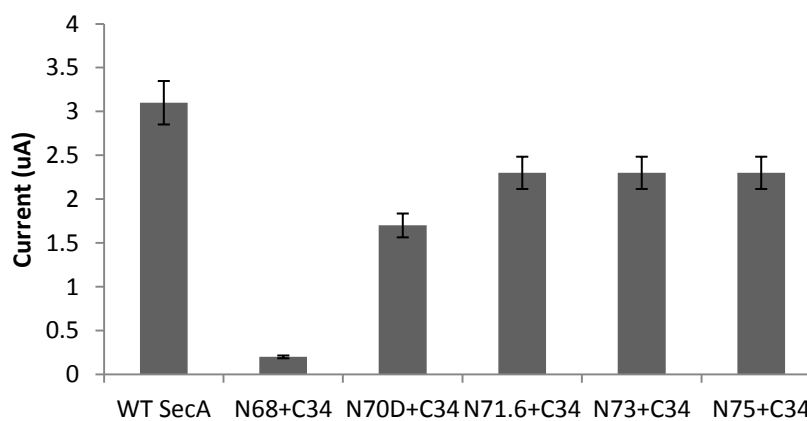
(A) 60 ng SecA, 60 ng BA13, and proOmpA was injected with C34-SecA. The recording was done after 3h injection. (B) 120 ng of SecA was used for the experiments. n=20

A



	N68+C34	N70D+C34	N71.6+C34	N73+C34	N75+C34
60ng	1/20	5/20	5/20	15/20	15/20

B.



N68+C34	N70D+C34	N71.6+C34	N73+C34	N75+C34
8/20	8/20	8/20	10/20	10/20

Figure 4.7. Channel activity of C-terminal deletion mutants coupled with C34 in liposomes in the oocytes.

120 ng SecA-liposomes, 2 mM ATP, 1 mM Mg, and proOmpA were injected with C34-SecA in the absence (A) or presence (B) of 30 ng SecYEG. n=10

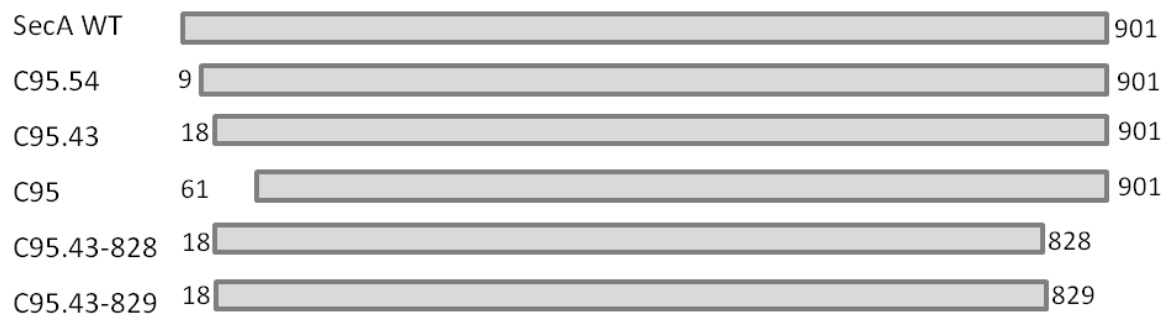


Figure 4.8. SecA-N-terminal mutants.

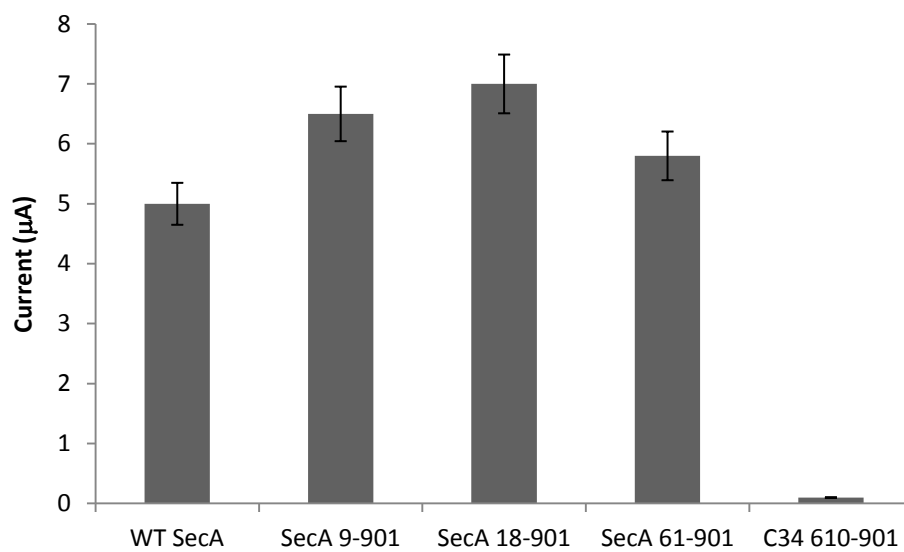


Figure 4.9. Channel activity of SecA N-terminal deletion mutants in BA13 membranes in the oocytes.

60 ng SecA was injected with 60 ng BA13, and proOmpA into oocytes. n=10

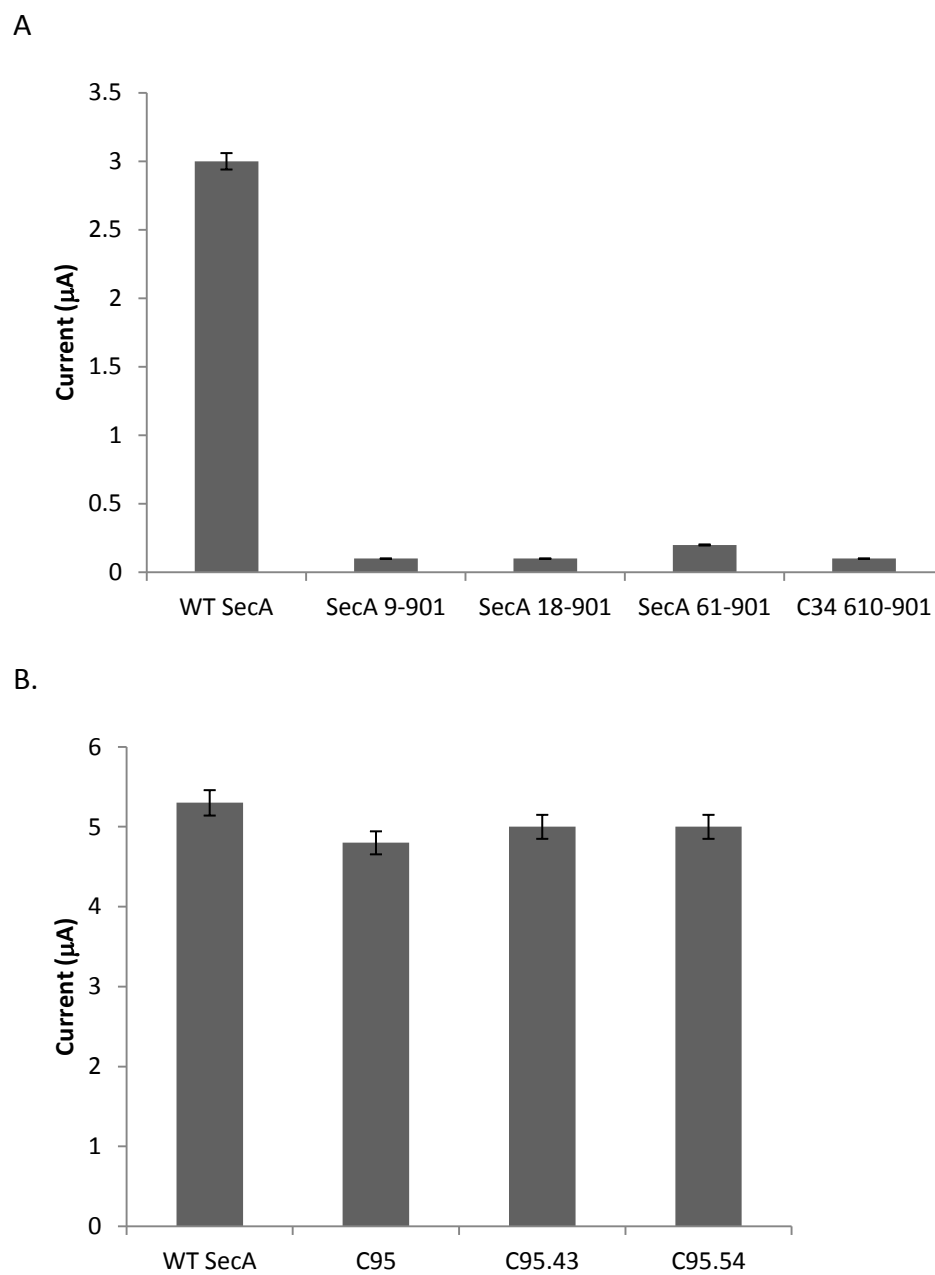
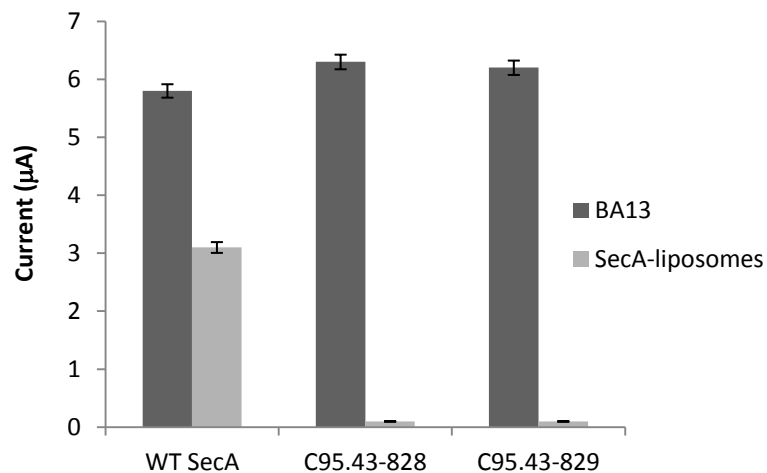


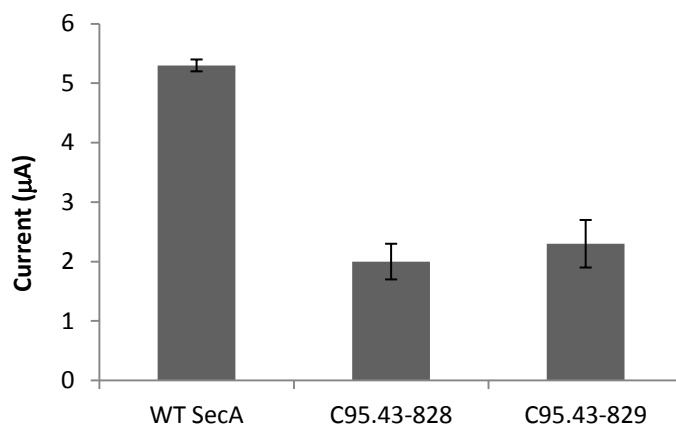
Figure 4.10. SecA N-terminal deletion mutants' channel activity in liposomes in the oocytes.

120 ng SecA-liposomes, 2 mM ATP, 1 mM Mg, and proOmpA were injected with (A) or without (B) 30 ng SecYEG. C95: 61-901, C95.43 18-901, C95.54: 9-901. n=10

A.



B.



	C95.43-828	C95.43-829
With SecYEG	4/20	4/20

Figure 4.11. Channel activity of SecA C95.43-828 and C95.43-829 in the oocytes.

(A) 60 ng SecA and 60 ng BA13 were injected into oocytes in the presence of proOmpA. 120 ng

SecA-liposomes were injected with 2 mM ATP, 1 mM Mg, and proOmpA. (B) 30 ng SecYEG was

added into the SecA-liposomes for channel activity. n=20

Conclusion

SecA serves as a motor protein in the Sec translocon in bacterial protein translocation. During the translocation, the SecA hydrolyzed ATP and transfer the preprotein into the SecYEG channel. However, several studies had revealed that without the SecYEG, the translocation is still active. Furthermore, the SecA, which exists as soluble and membrane forms, is shown to form a ring-like structure as a donut hole shape in the presence of lipids (Wang, Chen et al. 2003), and also is essential to maintain the translocation activity in the absence of SecYEG. In this study, we use the oocytes electrophysiological system to study the channel activity. This method has been used for channel research in decades, and is sensitive and accurate for the channel opening. By injecting the SecA-liposome with addition ATP-Mg⁺⁺, the channel is active; however, this activity loses its precursor selectivity. In addition, the channel activities with SecYEG restore the efficiency and reduce the requirement of SecA. In our study, we fully characterize the SecA dependent channel activity and also identify the difference between the channel activity with or without SecYEG.

Based on the data obtained, we propose a possible explanation to address this finding in cells. There are two different SecA-dependent protein-conducting channels: One with SecYEG as high affinity site and one with inner membrane as low affinity site (Driessen and Nouwen 2008). Bacterial cells contain more molecules of SecA than SecYEG (Yang, Yu et al. 1997). The excess SecA might integrate into the anionic phospholipids at the low affinity sites. There are many examples of pairs of high- and low- affinity transport systems for particular amino acids, which presumably offer a selective advantage. Thus, the existence of two SecA-dependent protein-conducting channels may also offer a selective advantage in bacteria. Besides, the electron

microscopy data revealed that SecA-liposome forms a ring-structure of 8.4 nm with a potential pore size of 2-3 nm (Wang, Chen et al. 2003; Ting, Pan et al. 2010), smaller than the SecYEG channel with a 10.9 nm-diameter structure and a 3.0-4.8 nm cavity (Ting, Pan et al. 2010). These parameter also supported that SecA binds to lipids with low-affinity and requires more SecA and energy to maintain the channel activity.

Possible evolution of a Sec protein conducting channel

This hypothesis also provides a theme for the protein translocation evolution. The general Sec secretion pathway is evolutionally conserved from prokaryotes to eukaryotes. The SecY homolog, Sec61, is been found widely--from mammals to bacteria-- and the signal recognition particles (SRP) from elephants to *E. coli* (Wolin 1994). While SRP is an obligatory component of cotranslational translocation in the endoplasmic reticulum, the bacterial SRP analog is but one of the several targeting components required for only some membrane protein integration but not for others. Yet each component of FtsY/Ffh/4.5S RNA of the bacterial SRP is essential for cell growth. Similarly, the *secY* or *secE* gene is essential for cell growth, but not for the translocation of all secreted proteins. Thus, there is no compelling reason why SecYEG should be the only core translocase in bacteria, despite the extensive literature that is based mainly on *in vitro* reconstitution of proteoliposomes with SecYEG. The system has undergone significant evolutionary modification. SRP is essential for eukaryotic co-translational secretion, but in bacteria it is only one of the possible components in membrane protein biogenesis system; many proteins can be secreted without it and most can be translocated post-translationally. Evolutionary conservation of a molecule is not proof that the molecule contin-

ues to play the same role. SecA is the only essential component of all Sec secretion pathways in bacteria, yet it does not survive evolution of eukaryotes.

Thus, while it is possible that two SecA-dependent pathways have evolved independently (one with and the other without SecYEG) that reflects the diverse pathways in bacterial secretion; we suggest a possible simpler evolutionary route: there is only one SecA-dependent secretion pathway. In it, SecA provides the protein-conducting channel. SecYEG confers higher affinity and specificity -- much the same as RNA polymerase being the core enzyme in bacteria and sigma factors conferring specificity. SecA function is markedly energy profligate, expending one ATP for each 25-aa length of precursors that passes through the membrane (Wickner and Leonard 1996), either co- or post-translationally (Chen and Tai 1985; Chen and Tai 1987). Although such costs can be tolerated by the relatively energy-rich bacteria, selective pressure -- exerted by the sub-compartmentalization of cells into organelles in eukaryotes-- perhaps intensified the needs for energy conservation, leading to the extinction of SecA. Under such selective pressure, Sec61p might have evolved to becoming a channel, mediating mainly a co-translational process that utilizes energy from protein synthesis to push the nascent chain across the channels.

Our data do not differentiate these evolutionary routes: whether two independent SecA-dependent secretion pathways (one at the high affinity binding site with SecYEG, and another at the low affinity binding site with phospholipids, albeit with lower efficiency and specificity), or a single SecA-dependent pathway, which has diverged into two. Whatever the mechanism, it is clear that SecA is necessary and sufficient to promote bacterial protein translocation and ion-channel activity, even in the absence of SecYEG.

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Appendix I

Mutational analysis on catalytic triad of CvaB transporter in the secretion of Colicin V

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Running title: Mutational analysis on catalytic triad of CvaB transporter

Abstract

CvaB is the central membrane transporter of Colicin V secretion system that belongs to an ATP-binding cassette superfamily. Previous data showed that N-terminal and C-terminal domains of CvaB are essential for the function of CvaB. N-terminal domain of CvaB possesses Ca²⁺-dependent cysteine proteolytic activity, and two critical residues – Cys32 and His105 - have been identified. In this study, Asp121 is identified as the third residue of the putative catalytic triad of the active site. The mutations of Asp121 lose their Colicin V secretion activity and N-terminal proteolytic activity. The adjacent residue Pro122 also plays a critical role for the Colicin V secretion. However, the reversal of the two residues D121P-P122D results in loss of activity. Based on protein sequence alignment, several residues adjacent to the critical residue Cys32 and His105 were also examined and characterized. Site-directed mutagenesis of Trp101, Asp102, Val108, Leu76, Gly77, and Gln26 indicate that the neighboring residues around the catalytic triad affect Colicin V secretion. Most mutated CvaB proteins with defective secretion tested are structurally stable. These results indicate that the residues surrounding the identified catalytic triad are functionally involved in the secretion of biologically active Colicin V.

Introduction

CvaB is the central membrane transporter of the bacteriocin Colicin V secretion system in *Escherichia coli*. It has been shown that CvaB, together with CvaA and TolC proteins, directs the secretion of the Colicin V with the cleavage of a double-glycine leader peptide (Gilson, Mahanty et al. 1990; James, Lazdunski et al. 1992; Fath, Zhang et al. 1994). Sequence alignments indicate that CvaB protein belongs to the ABC superfamily (Gilson, Mahanty et al. 1990). It is predicted to span the membrane six times and contains an ATP-binding site in its C-terminal cytoplasmic domain (Gilson, Mahanty et al. 1990; Zhong and Tai 1998). The amino acid sequence of this ATP-binding domain is highly conserved among many proteins involved in export processes, including the mammalian MDR family of drug

exporters (Endicott and Ling 1989; Borst and Elferink 2002) and CFTR, the chloride channel defective in patients with cystic fibrosis (Riordan, Rommens et al. 1989; Aleksandrov, Aleksandrov et al. 2002). For the export of bacteriocin precursors containing a short leader peptides of the double-glycine type, such as Colicin V, it has been proposed that the substrate-binding site in the proteolytic domain binds a bacteriocin precursor, and hydrolysis of ATP molecule(s) induces a conformational change in the ABC transporter that results in the simultaneous cleavage and translocation of the peptide (Havarstein, Diep et al. 1995). Point mutations in the CvaB N-terminal putative protease domain and C-terminal nucleotide-binding motif severely affect Colicin V secretion, suggesting that these two domains are critical for CvaB function (Zhong and Tai 1998; Wu and Tai 2004).

Previous work (Wu and Tai 2004) shows that the N-terminal domain of CvaB (BntD) possesses Ca^{2+} -dependent cysteine proteolytic activity. Mutations on critical residues Cys32 and His105 totally abolish its proteolytic activity and Colicin V secretion, indicating that the BntD indeed is involved in ColV precursor processing. By protein sequence blast, the BntD belongs to the C39 peptidase superfamily which contains the Cys-His-Asp as catalytic triad. Furthermore, Dessens *et al.* (Dessens and Lomonossoff 1991) reported that Asn forms H-bond with His to help orient the imidazole ring of His to be in a favorable position for Cys. In this work, sequence alignment and site-directed mutagenesis indicate that Asp121 forms the catalytic triad and is important to Colicin V secretion. Molecular modeling and sequence alignment have been used to predict 3-D protein structure and locate potentially important residues in the vicinity of active sites of the cysteine protease. Pro122, Gln26, Trp101, Asp102, Val108, Leu76, and Gly77 in the neighborhood of critical Cys32 and His105 and Asp121 are shown to be functionally important in the secretion of biological active Colicin V.

Results

Identification of Asp121 as the third residue for CvaB proteolytic catalytic triad. Previous studies (Wu and Tai 2004) showed that Cys32 and His105 are critical residues for cysteine proteolytic activity of N-terminal CvaB and for Colicin V secretion activity. We searched the third residue of the catalytic triad. It has been suggested that Asn or Asp residue forms catalytic triad with Cys-His for cysteine proteolytic activity (Dessens and Lomonossoff 1991) and Asn or Asp is involved in hydrogen bonding with His to stabilize the thiolate-imidazolium ion pair (Vernet, Tessier et al. 1995; Bromme, Bonneau et al. 1996). In the protein sequence alignment with C39 family, the Asp121 is the conserved amino acid (Fig 1.). In addition, the molecular modeling of BntD showed that Asn70 and Asn115 are the only two Asn residues close to His105 (data not shown), though neither is aligned within the conserved region comparable to the C39 protease family. Nevertheless, site-directed mutagenesis on these two residues with Gln and Lys were carried out; the mutations did not affect Colicin V secretion (Table 1), indicating that neither Asn70 nor Asn115 is involved in the catalytic triad of proteolytic activity or secretion. On the other hand, according to the catalytic triad sequence alignment (Fig. 1), Asp121 could be the third conserved residue of CvaB involved in catalytic triad formation. Indeed, we found replacement of Asp121 with Ala, Ser, and Asn lost all of Colicin V secretion activity (Table 1). All the mutants were tested at 30°C, 37°C, and 42°C, and none of them showed secretion activity indicating that Asp121 is essential for Colicin V secretion activity. Surprisingly, mutant D121E showed partial secretion activity (about 25%) at 30°C and 37°C, but not 42°C, indicating that the carboxyl group of Asp might be important for Colicin V secretion. We also cloned and purified the N-terminal CvaB mutant, BntD-D121E, to determine its proteolytic activity. Using LA-pNA as a substrate, the protease activity was found to decrease ~30-40% comparing with wild-type. Pro122 is a conserved residue in the N-terminal CvaB around Asp121. The mutations of Pro122 including P122A and P122S resulted in the defective Colicin V secretion at 30°C, 37°C, or 42°C (Table 1). Based on the 3D model prediction, Pro122 residue located at alpha-helix turn which might be important for helix

structure. We further mutated the Pro122 into Gly, Phe, and Val, however, there was no Colicin V activity found (Table 1). The reversal of the two residues, a double mutation of D122P/P122D, also results in no secretion (Table 1), showing the critical positions of these two amino acids. These results indicate the importance of D121/P122 for Colicin V secretion activity.

Important residues surrounding His105. Based on sequence alignment, there are several possible residues around Asp121 which may form H-bond interactions with His105 imidazole ring including Trp101, Asp102, Val108, Leu76 and Gly77. The mutations of these residues were characterized for their involvement in Colicin V secretion activity.

Trp101. It has been reported that Trp is proximal to His and Cys in the active site of *Conidiobolus* alkaline protease (Tanksale, Vernekar et al. 2000). Trp101 of CvaB is 4.15 Å to His105 residue based on the molecular modeling prediction. Replacement of Trp101 with Phe and Tyr showed retention of 75% of wild-type ColV secretion activity while 50% ColV secretion was observed when replaced with Ala or Ser. Asp replacement showed only 25% secretion activity. However, replacement with His, Pro, and Lys showed virtually no secretion activity (Table 2). The results suggest that Trp101 is an important residue for activity, and that phenyl group can efficiently substitute for the indole ring of Trp101 to potentially stabilize His105 residue during catalytic reaction.

Asp102. It has been reported that Asp residue is involved in hydrogen bonding network to His residue of cysteine protease in order to stabilize the active-site thiolate-imidazolium ion pair and catalytic activity (Menard, Khouri et al. 1990; Menard, Carriere et al. 1991). The oxygen atom of Asp102 in CvaB is 3.2 Å to nitrogen atom of His105 imidazole ring based on the molecular modeling prediction. To test whether Asp is important in this function, Colicin activity assays in selected mutants were performed. Replacement of Asp102 to Lys, Tyr, Gly, Thr, Gln, Asn, and His showed the same secretion activity comparable to wild-type. Ser, Cys, and Glu replacements showed 75% of wild-type secretion activity, but Ala, Phe, and Pro replacements completely abolished Colicin V secretion activity (Table 2). Double mu-

tations of W101D & D102W totally abolished the secretion activity, indicating that exchange of residues at position 101 and 102 cannot complement the amino acid property and the indole ring is important at the position of residue 101. Based on these mutagenesis results, hydrophilic residues appear to be required at the position 102.

In addition to Trp101, Phe103 is another residue carrying phenyl ring in the close distance to His105 residue, although not next to each other in 3-D structure prediction. Replacement of Phe103 to Tyr or Trp showed 100% secretion activity comparable to wild-type; moreover, replacements to hydrophobic Ala, hydrophilic Ser, and charged His, did not change ColV secretion activity, indicating that Phe103 is not involved in secretion (Data not shown).

Val108. Val108 is 3.4 Å away from His105 residue based on CvaB molecular modeling. Replacement of Val108 to Leu reduced secretion activity slightly to 75%; replacement with Ser reduced activity to 50%; replacement to Ala reduced activity to 25%. Mutations to charged residues Lys and Asp totally abolished secretion activity (Table 2). Interestingly, when V108A mutation combined with any other second mutation, the ColV secretion activity was totally abolished (data not shown). These results indicate that Val108 is involved in Colicin V secretion activity.

Trp101, Asp102, and Val108 mutants are structurally stable. To determine whether the mutations that abolish secretion activity are due to functional or structural defects, we determine the stability of CvaB, and its interacting partner, CvaA. Flag-epitope was inserted into the C-terminal of CvaB wild-type, Trp101, and Asp102 mutants (SI Table 1) to detect CvaB; the insertions of Flag-epitope have no effect on the secretion of active Colicin V. To determine whether the lost activity of several Trp101 mutants is due to functional defect or structural stability, membrane protein preparations from these mutants were run on SDS-PAGE and immunoblottings were performed. The results showed that the mutated CvaBs are structurally stable and can be detected immunologically in inactive W101Y, W101K, W101P, and W101H mutants as detected by Flag antibodies (Fig. 2A). Similar results were obtained with Asp102 mutants

D102F, D102A, D102P, D102W&W101D, and D102K (Fig. 2B), suggesting that those Asp102 mutants lacking secretion activities are also due to functional defect.

Similarly, membrane proteins of V108A mutants were tested for CvaA and CvaB stability by immunoblottings. The results showed that CvaA (Fig. 2C) and CvaB proteins (Fig. 2D) of V108L, V108S, V108A, V108K, and V108D mutants could all be detected immunologically, indicating that secreting defect of these mutants are not due to structural instability. We conclude that Trp101, Asp102 and Val108 are functionally involved in Colicin V secretion, probably interacting with critical residue His105.

Important residues around Trp101 and Asp102. Molecular modeling showed that Leu76, Gly77, and Thr80 residues are around important residues Trp101 and Asp102 (Fig. S1A). Leu76 is 2.9Å to Asp102 and 3.1Å to Thr80. Gly77 is 3.7Å to Trp101, 4.3Å to Asp102, and 4.3Å to His105. Thr80 is 3.2Å to both Trp101 and Asp102. To determine whether these three residues might be involved in Colicin V secretion activity, site-directed mutagenesis was performed. Replacement of Gly77 to Ala or Ser reduced Colicin V secretion activity to 50%; replacement of Gly77 to Pro totally abolished Colicin V secretion activity; replacements of Leu76 to Ala, Ser, and Val reduced Colicin V secretion activity to 50% at 37 °C. Replacement of Thr80 to Ser had no effect. Interestingly, replacements of Leu76 to Ala, Ser, or Val retained most activity at 30°C, but not at 42°C (Table 3 and data not shown). These results indicated that Leu76 and Gly77 are temperature-sensitive mutations affecting Colicin V secretion activity.

Important residues surrounding Cys32: Gln26 is involved. Previous studies showed that Cys32 is also critical for Colicin V secretion activity and cysteine proteolytic activity of N-terminal CvaB (Wu and Tai 2004). Sequence alignment shows that Gln26, corresponding to Gln19 in papain, is conserved among cysteine proteases. It has been reported that Gln19 in papain forms an “oxyanion hole” with Cys25, which in turn stabilizes the Cys- and His- ion pair (Drenth, Kalk et al. 1976; Menard, Carriere et al. 1991; Menard and Storer 1992) structure of calcium-free human m-calpain shows oxygen atom of Gln99, conserved among cysteine protease, is at the distance of 7.73Å to its sulfur atom of critical residue Cys105

(PDB:1KFU) (Strobl, Fernandez-Catalan et al. 2000). 3-D modeling of BntD and sequence alignment predicts that oxygen atom of Gln26 is at the distance of 7.6Å to sulfur of Cys32, suggesting that Gln26 might be involved in cysteine proteolytic activity of BntD (Fig. 1 and Fig.S1B).

Site-directed mutagenesis was performed to determine if Gln26 is involved in Colicin V secretion activity. Replacement of Gln26 to Ala and Asn reduced minimally Colicin V secretion; replacements to charged residues Lys, Glu, and His, abolished Colicin V secretion at 37°C (Fig. 3). However, replacement of Gln26 to Glu only reduced Colicin V secretion to 50% at 30°C (data not shown). These results showed that Gln26 residue is involved in Colicin V secretion activity.

Molecular modeling also indicated that four residues, Asp49, Tyr52, Lys56, and Ala67, are in the vicinity of critical residue Cys32 at the distance of 3.83Å, 3.4 Å, 4.99Å, and 5.11Å, respectively (Fig. S1B).(Drenth, Kalk et al. 1976; Vernet, Tessier et al. 1995; Bromme, Bonneau et al. 1996; Ishii, Yano et al. 2006) Site-directed mutagenesis on Ala67, Lys56, and Tyr52 residues did not affect Colicin V secretion activity (data not shown). However, replacements of Asp49 to Glu and Ser reduced secretion activity to 75%; replacements to Lys and Ala reduced activity to 50% indicating that hydrophilic and acidic residues might also be involved in the secretion.

Discussion

Previous study identified Cys32 and His105 of BntD as catalytic residues for Colicin V secretion activity and cysteine proteolytic activity (Wu and Tai 2004). Most cysteine proteases contain Cys-His-Asn as a catalytic triad. However, it also has been reported that Asp, instead of Asn, serves as the third residue to form a critical hydrogen bond with the His imidazole ring to stabilize its interaction with Cys residue that contributes to the structural integrity (Drenth, Kalk et al. 1976; Vernet, Tessier et al. 1995; Bromme, Bonneau et al. 1996; Ishii, Yano et al. 2006). Molecular modeling of BntD shows that Asn70, in the distance of 7.68Å, and Asn115, in the distance of 14.9Å, are the only two Asn residues that are po-

tentially close enough to His105 to have any interaction. However, the distance is not close enough to form H-bonds. On the other hand, Asp121, a conserved residue in sequence alignment, was suggested as a third residue involved in catalytic triad formation (Ishii, Yano et al. 2006). The mutations, D121A and D121N, result in the complete defect on Colicin V secretion activity. Replacement of Asp121 with the common third catalytic residue Asn in cysteine protease does not restore the Colicin V secretion activity which suggests a role of the carboxyl side chain of Asp for Colicin V secretion. Although Asp cannot be considered as an essential catalytic residue in the cysteine protease (Asp, Bowra et al. 2004; Im, Na et al. 2004; Kim, Yoo et al. 2004), surprisingly, the mutant D121E protein loses most of its protease activity and is temperature-sensitive for secretion which indicated that Asp121 is involved for protease activity of CvaB. In the D121N mutation, the change of OH with NH₂ eliminates the negative charge in the van der Waals surface of the side chain atoms. The carboxyl group on the Asp has been suggested to be important for the catalytic activity in different pH (Neuvonen 1997). The protease activity of D121N would be more active in the high pH environment (Craik, Roczniak et al. 1987). Moreover, replacement of Asp with Glu partially retains the Colicin V secretion activity, suggesting that the carboxyl group is indeed important for the Colicin V secretion. It was reported that Trp- Phe- and Pro- of cysteine protease stack together to help forming and stabilizing the catalytic triad (Zhu, Shao et al. 2004). Based on the 3D modeling, the Pro 122 locates on the end of helix structure next to Asp121. All the mutations of Pro122 lose Colicin V secretion activity. Thus, these mutations may change the interaction with Trp and Phe to destabilize the catalytic triad. Interestingly, the reversal of the two residues, D121p/P122D, result in loss of activity.

For cysteine protease, it has been suggested that the hydrogen bond between the side chain of His and Asn is buried in a hydrophobic region composed mainly of the side chains of residues Phe and Trp (Loewenthal, Sancho et al. 1992). Moreover, a Trp indole ring interacts with a protonated His imidazole ring to increase the pK_a and stability of His (Loewenthal, Sancho et al. 1992; Tanksale, Vernekar et

al. 2000). The Trp-His interaction shields the hydrogen bond of His-Asn from external solvent to facilitate different steps in the catalytic mechanism of the enzyme (Bromme, Bonneau et al. 1996). Previous studies indicated that Trp is essential for the active site of an alkaline serine protease (Tanksale, Vernekar et al. 2000), and that the imidazole ring is essential for the proteolysis (Hata, Sorimachi et al. 2001). Since Asn is not involved in Colicin V secretion, Trp101 is the only Trp residue that could potentially interact with His105 in this way. Site-directed mutagenesis of Trp101 showed that Trp-to-Phe and Trp-to-Tyr substitutions can be tolerated for activity, but five-carbon ring His and Pro substitutions cannot. Thus, this His-Trp interaction in ColV system is probably important for the Cys-His ion pair stability and the catalytic activity through the effects of increasing the pK_a of His.

The molecular modeling predicts the charged Asp102 may interact with the nitrogen of the imidazole ring in His105 in the distance of 3.24 Å to form a hydrogen-bonding network. Replacements of Asp102 with hydrophobic residues, such as Ala, Trp, and Phe, totally abolish Colicin V secretion activity. On the other hand hydrophilic residue replacements retain 75 ~ 100% of wild-type activity, suggesting that hydrophilic residues are important in hydrogen-bonding network in this position. Menard *et al.*, (Menard, Khouri et al. 1990; Menard, Carriere et al. 1991) reported that Asp158 in papain is in proximity to active site His and is involved in stabilizing the thiolate-imidazolium ion pair by hydrogen-bonding interactions. However, the charge and the hydrogen bonds of Asp158 in papain both contribute to the activity of the enzyme. The molecular modeling of BntD showed that Leu76, Gly77, and Thr80 are in the close distance to Trp101 and Asp102. Replacement of Leu76 to Ala, Ser, and Val reduced Colicin V activity at 37°C, but totally abolished the activity at 42°C, indicating that Leu76 is involved in interaction result in temperature-sensitivity. Site-directed mutagenesis on Gly77 to Ala and Ser showed 50% Colicin V activity, but G77P lost all activity, suggesting that Gly77 residue is involved in secretion activity but is not involved in turning the structure.

Several residues surrounding critical residue Cys32 were tested and only Gln26 affected Colicin V secretion activity. Sequence alignment shows that Gln26 corresponding to Gln19 in papain is conserved among cysteine proteases. Gln19 is reported to form an “oxyanion hole” with Cys25 that stabilizes the Cys-His ion pair. In Colicin V system, Gln26 mutants show reduced ColV activity.

This work identifies Asp as the third residue of catalytic triad of His-Cys-Asp, and shows that additional surrounding residues contribute to the cysteine protease activity and Colicin V secretion.

Materials and Methods

Media and growth conditions. TB (10 g of tryptone and 8 g of NaCl per liter) was used as both liquid and solid (with 1.5% agar) growth media for transformation. TB plates with 0.7% agar as semi-solid growth media for lawn were used for Colicin V secretion assay (Gilson, Mahanty et al. 1990; Skvirsky, Gilson et al. 1991). Ampicillin and chloramphenicol were used at final concentrations of 100 µg/ml and 30 µg/ml, respectively.

Bacterial strains, plasmids and reagents. The strains and plasmids used in this study are listed in Supplement Table 1. *Escherichia coli* DH5α was used as the bacterial hosts. *E. coli* ColV sensitive strain 71-18 for Colicin V halo activity assay (Gilson, Mahanty et al. 1990; Skvirsky, Gilson et al. 1991), and colicin plasmids were obtained from Dr. R. Kolter (Harvard Medical School, Boston, MA). Plasmid pHK11-4 contains intact *cvaA* and *cvaC* but not *cvaB*. For Colicin V secretion assays, *cvaB* and its mutations were provided by a complementary plasmid pACYC184. Recombinant DNA manipulations were performed essentially as described by Sambrook *et al* (Maniatis, Fritsch et al. 1982). The expression vector pTrcHis2B with His₆-tag at C-terminus was from Invitrogen (Carlsbad, CA).

Site-directed mutagenesis. All mutagenesis were carried out by PCR using oligonucleotides containing the desired mutations. The standard PCR reaction in a total volume of 50 µl contained 20 ng of linearized plasmid template, 400 ng of each oligonucleotide primers, 200 µM of each deoxynucleotide tri-

phosphate, and 5 units of Ex *Taq* DNA polymerase (PanVera/TaKaRa, Madison, WI) in the buffer provided by the manufacturer.

To generate the desired site-directed mutagenesis constructs, a three-round asymmetric PCR strategy was used as previously described (Tzeng and Frey 2002; Wu and Tai 2004). All mutations were confirmed by sequencing using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and appropriate primers.

Protein sequence alignment and dynamic protein modeling The N-terminal CvaB (BntD, amino acids 1-171) has previously been determined as the protease domain (Wu and Tai 2004). The BntD protein sequence was aligned with other C39 peptidase family by using the ClustalW2 multiple sequence alignment (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) (Chenna, Sugawara et al. 2003).

Coordinates of the crystal structures of the *E. coli* translation inhibitor (PDB: 1JD1) and photosynthesis metal transporter (PDB: 1G8P) were obtained from protein database (www.rcsb.org/pdb) and used as templates for the molecular modeling. The BntD sequence was aligned with the templates using BLAST (www.ncbi.nlm.nih.gov/BLAST) and FASTA (www.ebi.ac.uk/fasta33). The corresponding residues of CvaB were graphically incorporated into the published crystallographic coordinates of templates using program AMMP (<http://www.cs.gsu.edu/~cscrw>), creating the structure for the dynamic modeling computations. The computations, including energy minimization and molecular dynamics, generate minimized structure, which is displayed using RasMol molecular display program (<http://www.bernstein-plus-sons.com/software/rasmol/>).

Colicin V activity assay. Direct colony halo assays were performed as described in Skvirsky *et al.* (Skvirsky, Gilson et al. 1991) with modifications (Wu and Tai 2004). DH5 α cells containing various plasmids grown overnight in TB broth were spotted on Colicin V sensitive 71-18-overlaid TB plates and incubated at 30°C, 37°C, or 42°C. All the results were shown as 37°C unless otherwise indicated. The halo area around colony was measured overnight or at different time as indicated during incubation. Amino

acids are shown in the one-letter code. The description of the mutations represented the amino acid change. Letters before the number indicates the original amino acid residues, and the letter after the number indicates the introduced amino acid residues. The symbol represented Colicin V secretion activity: Wild-type (++++) is used as 100% secretion activity. (++) is used as approximate 50% secretion activity compared to the wild type, + is used as 25%. Symbol “-“ indicates no secretion activity.

Protein Purification. N-terminal CvaB (BntD) was cloned, expressed, purified and used for proteolytic assay as previous described (Wu and Tai 2004).

Membrane preparations and Western blottings. DH5 α cells containing appropriate plasmids grown overnight in TB at the presence of appropriate antibiotics at 30°C were diluted to an OD₆₀₀ of ~0.1 and incubated for growth. When OD₆₀₀ reached ~0.5, the cells were spun down and washed once by TB, and the cells were re-suspended in TB, induced with 0.5 mM 2,2'-dipyridyl, and harvested 2 hr later. Cells were suspended in 50 mM Tris - HCl (pH 8.0) - 50 mM NaCl - 20% glycerol plus Complete Protease Inhibitor Cocktail (Roche, IN) and 1 mM *N*-ethylmaleimide (NEM), and lysed with a French Press as described (Wu and Tai 2004). Lysates were centrifuged for 8 min at 4,000 x *g* to remove cell debris, and the supernatants were centrifuged at 100,000 rpm for 20 min at 4°C in a Beckman TLA100.3 rotor. The pellet fractions containing total membranes were suspended in sample buffer containing 1 mM NEM, complete protease inhibitor cocktail, and 4% SDS. For detection of CvaB, membranes were incubated at 37°C for 30 min prior subjecting to SDS-PAGE. For Western immunoblottings, alkaline phosphate-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad Laboratories, Hercules, CA) was used as the secondary antibody for anti-CvaA (Hwang, Zhong et al. 1997) and anti-N-terminal CvaB antibody (Wu and Tai 2004); alkaline phosphate-conjugated goat anti-mouse immunoglobulin G (Promega, Madison, WI) was used as the secondary antibodies for anti-Flag antibodies which was obtained from Sigma (St. Louis, MO).

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Figure legends

FIG. 1. Alignment of N-terminal CvaB (BntD) with the C39 peptidase family. Amino acid multiple sequence alignments were performed using the ClustalW2 program. The sequences were taken from the Swiss Protein Database or the GenBank™/EBI Data Bank. Identical and similar residues are marked with *asterisks* and *dots*, respectively. The three conserved catalytic triad residues are marked as dark gray color. The *numbers* on the left and right refer to amino acid positions. The BntD is the N-terminal pro-

tease domain of CvaB (a.a. 1-171), ComA protein is a bacteriocin-processing peptidase from *Streptococcus pneumoniae*, Clyb protein is a mersacidin lantibiotic processing peptidase from *Streptococcus pneumoniae*, NukT is family C39 non-peptidase homologues from *Staphylococcus warneri*.

FIG. 2. Stability of CvaA and CvaB in the membranes of Trp101, Asp102, and Val108 mutants. Wild-type and mutants *cvaB* plasmids were co-transformed with pHK11-4 plasmid lacking *cvaB* and incubated at 37°C. (A) Trp101 mutants CvaBs were detected by Flag antibodies. Lane 1, wild-type; 2, W101Y; 3, W101K; 4, W101P; 5, W101H mutants. (B) Asp102 mutant CvaBs were detected by Flag antibodies. Lane 1, wild-type; lane 2, D102F; lane 3, D102A; lane 4, D102P; lane 5, D102W & W101D; lane 6, D102K mutants. Val108 mutant membranes were detected with CvaA antibodies (C) or by N-terminal domain of CvaB antibodies (D). Lane 1, wild-type; lane 2, V108L; lane 3, V108S; lane 4, V108A; lane 5, V108K; lane 6, V108D; lane 7, pHK11-4. The samples of membrane proteins were run on 10% SDS-PAGE and subjected to immunoblotting as described in Materials & Methods.

FIG.3. Direct colony assay for Gln26 site-directed mutants. Colicin V secretion assays were at 37°C. Wild-type and mutants were co-transformed with pHK11-4 plasmid and clear zones were measured at the time indicated. The zone diameters of wild-type at 24-hr point were used as Y-axis 100%. Error bars represent the S.D. of three repeats of experiment in triplicate.

Table 1. The Mutation of Conserved residues on Colicin V secretion

Residue	Mutation	Activity
Asn70	N70G, N70K	++++
Asn115	N115G, N115K	++++
Asp121	D121A, D121S, D121N	-
	D121E	+
Pro122	P122A, P122S, P122GP122F, P122V	-

Mutation and activity are defined as in Material and Methods

Table 2. Mutation of the residues around the His 105 on Colicin V secretion

Residue	Mutations	Activity
Trp101	W101Y, W101F	+++
	W101A, W101S	++
	W101D	+
	W101K, W101P, W101H	-
Asp102	D102K, D102G, D102Y, D102H, D102T, D102N, D102Q	++++
	D102S, D102E, D102C	+++
	D102W	+
	D102A, D102F, D102P,	-
	W101D -102W	-
Val108	V108L	+++
	V108S	++
	V108A	+
	V108K, V108D	-

Mutation and activity are defined as in Material and Methods

Table 3. Colicin V secretion activity on the conserved residue, Leu76, Gly77 and Tyr80.

Mutation ^a	37°C	42°C
CvaB WT	++++	++++
L76A	++	-
L76S	++	-
L76V	++	+
G77A, G77S	++	++
G77P	-	-
T80S	++++	++++

Mutation and activity are defined as in Material and Methods

Figure 1.

```

BntD      MTNRFNFRQIINLLDLRWQRVPVIHQETETAECGLACLAMICGHFGKNIDLIYLRKFNLS 60
ComA      -----MNMKFGKRHYRPQVDQMDCGVASLAMVFGYYSYFLAHLRELAKT 47
Clyb      -----MKIPMIYQMENSECGLACCAMILNYFKYEISLNLREIYPSS 42
NukT      -----LKIALQNSDQDCLLACYSMILSYFGKNVINSLSYKREMIP 40
          * . : * : * . : * : : : : : : : : : : * . . .

BntD      ARGATLAGINGIAEQIGMATRALSLELD-----ELRVLKTPCILHWDFS----HFVVLVS 111
ComA      MDGTTALGLVKVAEEIGFETRAIKADMTL---FDLPDLTFPFVAHVLEKGLLHYVVVTG 104
Clyb      RSGYSLLSISKVLGDFNISSHAFKASVR-----DLKPLSFPLICFWESS----HFIILEK 93
NukT      PDGLSISYKELNIKYELNMKVYRIKDKEKTFRVISKIKKPIIVHWDLN----HFVIVKN 96
          * : : : : : : : : : : : : : : : : : * : . . * : : :

BntD      VKRNRVVLHDPARGIRYISRE--EMSRYFTGVALEVWPGSEFQSETLQTRISLRSLNSI 169
ComA      QDKDSIHIAAPPDPGVKLTCLKLPRERFEEEWGVTLFMAPSPDYKPKHEQKNG-LLSFIPIL 163
Clyb      ISKNKFYILDPAKGRQRMSSIS--EFERHYSNIILTFKKLDSFMSRKNK---SPVLKYF 148
NukT      VKKNHIEIVNPEIG--KVKISKEMFLEHFSNVLLMFDPKSDFP--KDKEKIEFFDDLKSV 152
          . : : : : * * . . : . : : : * . . : . : : : : .

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Figure 2.

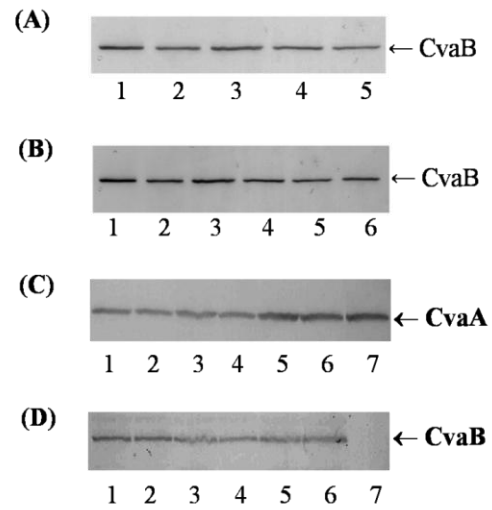
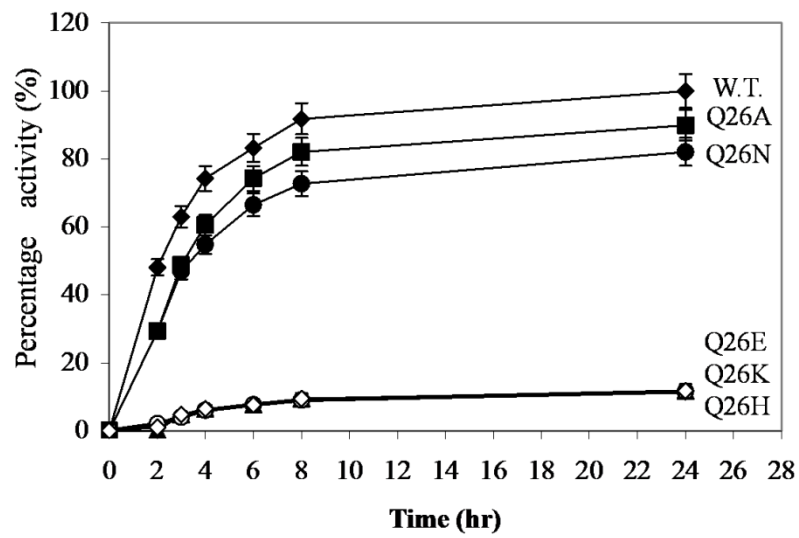


Figure 3



Supplement Information

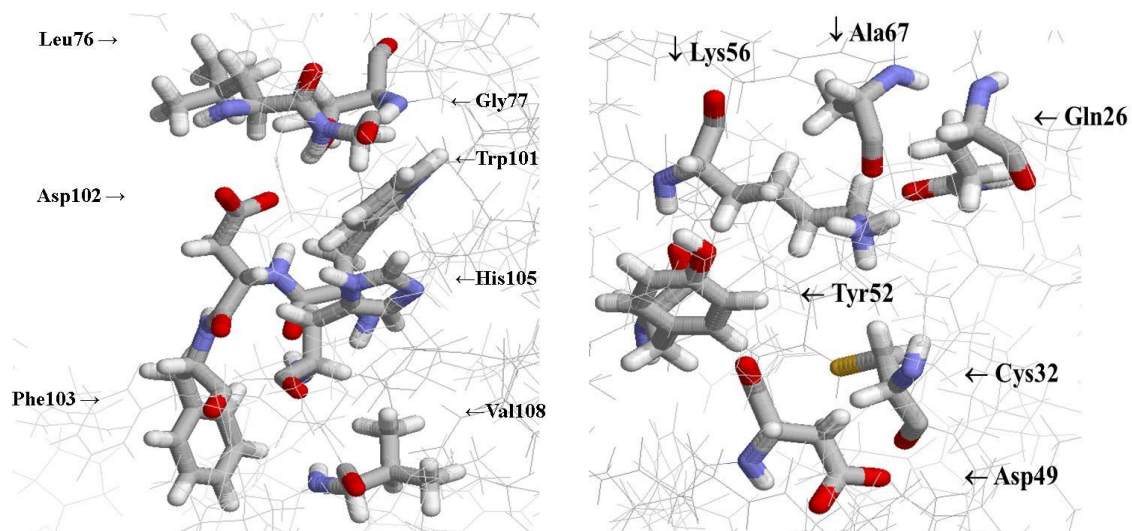
SI Table1. Bacterial strains and plasmids used in this study

Strain and Plasmids	Genotype or Description	Reference
Strain DH5 α	<i>supE44 ΔlacU169(ϕ80 lacZAM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahad 1983
Strain 71-18	<i>supE thi Δ(lac-proAB) F' [proAB+ lacIq lacZAM15]</i>	Yanisch <i>et al.</i> , 1985
Plasmids		
pHK11-4	pHK11 with <i>cv0B:: Tn5</i>	Gilson <i>et al.</i> , 1987
pKW67	pACYC184 with wild-type CvaB 698 amino acids	This Study
pKW182	Q26N from pKW67	
pKW183	Q26K from pKW67	
pKW184	Q26E from pKW67	
pKW364	Q26H from pKW67	
pKW177	N70K from pKW67	
pKW380	N70G from pKW67	
pKW185	N115G from pKW67	
pKW186	N115K from pKW67	
pKW187	W101A from pKW67	
pKW235	W101K from pKW67	
pKW239	W101S from pKW67	
pKW232	W101D from pKW67	
pKW233	W101F from pKW67	
pKW244	W101Y from pKW67	
pKW240	W101P from pKW67	
pKW250	W101H from pKW67	
pKW236	D102A from pKW67	
pKW245	D102E from pKW67	
pKW234	D102S from pKW67	
pKW241	D102K from pKW67	
pKW269	D102N from pKW67	
pKW270	D102Q from pKW67	
pKW138	D102C from pKW67	
pKW277	D102W from pKW67	
pKW280	D102F from pKW67	
pKW281	D102P from pKW67	
pKW287	D102G from pKW67	
pKW288	D102H from pKW67	
pKW289	D102Y from pKW67	
pKW279	W101D&D102W from pKW67	
pKW238	V108A from pKW67	
pKW237	V108S from pKW67	
pKW243	V108K from pKW67	
pKW242	V108D from pKW67	
pKW248	V108L from pKW67	
pKW212	Wild type CvaB with epitope flag at C-terminus	
pKW214	V108A CvaB with epitope flag at C-terminus	
pKW421	W101Y CvaB with epitope flag at C-terminus	
pKW422	W101K CvaB with epitope flag at C-terminus	
pKW423	W101P CvaB with epitope flag at C-terminus	
pKW424	W101H CvaB with epitope flag at C-terminus	
pKW306	D120K CvaB with epitope flag at C-terminus	
pKW301	D102F CvaB with epitopeflag at C-terminus	
pKW303	D102A CvaB with epitope flag at C-terminus	
pKW302	D102P CvaB with epitope flag at C-terminus	
pKW304	D102W&W101D CvaB with epitope flag at C-terminus	
pYH53	D121A from pKW67	
pYH59	D121S from pKW67	
pYH61	D121N from pKW67	
pYH55	P122A from pKW 67	
pYH63	P122S from pKW67	
pYH65	P122G from pKW67	
pYH68	D121E from pKW67	
pYH70	D122V from pKW67	
pYH72	D122F from pKW67	
pYH74	D122Y from pKW67	
pYH84	D121P from pKW67	
pYH86	P122D from pKW67	
pYH88	P122E from pKW67	
pYH90	P122N from pKW67	
pYH92	D121P&P122D from pKW67	

SI Figure 1.

S1A.

S1B.



Supplement Figure 1. Molecular modeling of CvaB N-terminal domain (BntD) in the vicinity of Cys32 and His105 residues. Dynamic protein modeling was performed as described in material and methods. (A) Trp101, Asp102, and Val108 are close to His105 to form potential H-bonds (4.15 Å for Trp101, 3.2 Å for Asp102 and 3.4 Å for Val108). Three residues, Leu76, Gly77, and Thr80, around Trp101 and Asp102 are also shown. (B) The critical residue Cys32 is shown with the surrounding residues, Gln26 (7.6 Å), Asp49 (3.83 Å), Tyr52 (3.4 Å), Lys56 (4.99 Å), and Ala67 (5.11 Å).

Appendix II

We previously identified a SecA alone-liposomes channel activity without SecYEG; however, this activity is less efficient and requires extra energy. After optimizing the amount of each essential component for this SecA-liposomes, the channel activity compared to the reconstituted membranes is still 2X less on ion current. Thus, we hypothesized that there are more accessories required to maintain the channel efficiency in SecA-liposomes system. In this chapter, we tried to determine the accessories proteins which could stabilize or help SecA with its channel activity.

SecDFYajC is the accessory complex in the Sec translocation. Previous data indicates that SecDF YajC stabilizes the structure of SecYEG during translocation. Since we identified a SecA-dependent channel activity with phospholipids with low affinity, we first check if SecDFYajC could function as an accessories proteins to enhance the channel activity of SecA-liposomes. SecDFYajC-His is a gift from Dr. Arnold Driessen; and the compatible expression host is SF100. We first check the SF100 strain for the oocytes channel activity and the result was shown in the figure A2.1. Compared to the MC4100, the channel activity in SF100 strain or the SF100/SecDFYajC were low, almost 2X less. The extra SecA addition had no increasing on the channel activity in SF100 and SF100/SecDFYajC. Based on the result we tested, we decide to overexpress the SecDFYajC in the MC4100.

Surprisingly, the reconstituted MC4100/SecDFYajC lost the channel activity in the presence of SecA addition (Figure A2.2 A). As expected, the MC4100 and RE-MC4100 maintain the channel activity with SecA. However, after removing the SecYEG from MC4100/SecDFYajC, there was no detectable ionic current even with 80 ng SecA addition (Fig. A2.2 B). In order to

confirm that the induction of SecDF was not toxic to affect the MC4100 or SF100 channel activity, we then overexpressed the SecDFYajC in regular BL21 or C41 strains (common strains used for protein expression) or D10-3. The BL21 and C41 are both protein expression strains. And the C41 is derived from BL21 which was specific for toxic protein expression. The result was shown in figure A2.3. The regular BL21 and C41 were okay for the protein overexpression, however, the channel activity were low or no activity (Fig. A2.3 A). In addition, the reconstituted BL21/SecDFYajC and reconstituted C41/SecDFYajC had no channel activity at all. The D10-3 also got the same results; after removing the SecYEG, the RE-D10-3/SecDFYajC lost its channel activity (Fig A2.3 B).

According to the data above, we started thinking there might be some toxins or inhibitors generating during the reconstituted membrane preparation. We checked the protein expression profile in the RE-MC4100 and the RE-MC4100/SecDFYajC. By western blot against SecD and SecF, the supernatant fraction after sodium cholate treatment in RE-MC4100 and RE-MC4100/SecDFYajC contains similar amount of SecDF (Fig. A2.4). This indicated that the loss of channel activity is not due to the loss of SecDF.

We further determine the protein expression profile under silver staining, and the result was shown in figure A2.5. We are able to identify at least 4 different protein expressed in the supernatant fraction of RE-MC4100 but not the RE-MC4100/SecDF. These four protein spots were further sequenced and one spot around 60 kDa was identified as GroEL.

From the study by Bing Na and RE-SecDFYajC protein expression profile, both indicate that GroEL may play a role during the protein translocation. The next thing would be focus on the GroEL and also finding the other accessories proteins.

GroEL is a chaperonin protein, and there are some references indicating that GroEL interact with SecA. Besides, previous lab data also identified that N39-SecA and M48-SecA could stabilize the structure and activity of SecA. N39-SecA is an N-terminal fragment of SecA, which previous identified to stabilize SecA. M48-SecA is a membrane domain of SecA, which seems having similar function of N39-SecA. Combine the data we had from SecDF experiments and the early lab research, we further determine the roles of GroEL, N39, and M48 in SecA-liposomes channel activity.

For the experimental design, the total *E.coli* lipid was sonicated 3mins and then stored at -80 before use. The liposome was freeze and thaw once for experiment. Wild type SecA, GroEL or N39-SecA or M48-SecA were mixed with 60 ng liposome, 1 mM ATP, and 1 mM Mg for the oocyte injection. After 3 hour injection, the oocytes were ready for whole cell recording.

1. GroEL

The channel activity could be detected when GroEL mixed with SecA (Fig. A2.6). However, the GroEL alone sometimes had the channel activity itself (Fig. A2.6B). It is not surprised because the GroEL is a chaperone; thus, it may have chance to form channel when mixed with liposome.

2. N39-SecA

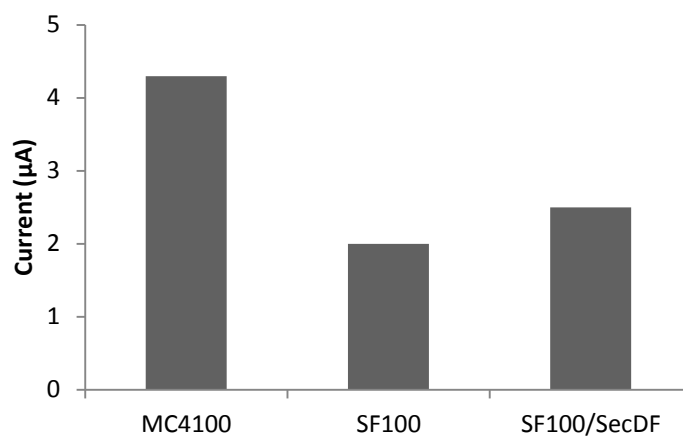
The N39-SecA alone didn't generate any channel activity unless the wild type SecA was added (Fig. 2.7A). When mixed with SecA, the channel activity could be induced almost 2X compared to the SecA alone (Fig. A2.7 B). However, when the N39-SecA was added to 180-240 ng, it seems compete with WT SecA for the channel formation. This result was shown on the expression rate. The higher amount of N39-SecA addition means the lower of expression rate.

3. M48-SecA

There were not ionic currents detected by M48-SecA alone with liposomes (Fig. 2.8 A). Compared to the N39-SecA, M48-SecA didn't induce the channel activity when mixed with WT SecA-liposomes (Fig. A2.8 B). Furthermore, M48-SecA may also compete with WT SecA for membrane insertion due to the expression rate was decreased when the M48-SecA amount was higher.

We also use Cytochrome C oxidase and CvaA as a negative control which have no functions with SecA to see if they could induce the channel activity. Indeed, these two proteins didn't stimulate the SecA-liposomes channel activity comparing to the SecA alone (Fig. A2.9). In summary, the GroEL seems act as a potential accessory protein which may stimulate the channel activity of SecA-liposomes. The further goal may need to adjudge the ratio between the SecA and GroEL coupled with the concentration of ATP-Mg to determine the maximum ionic currents.

A. Without SecA



B. SecA addition

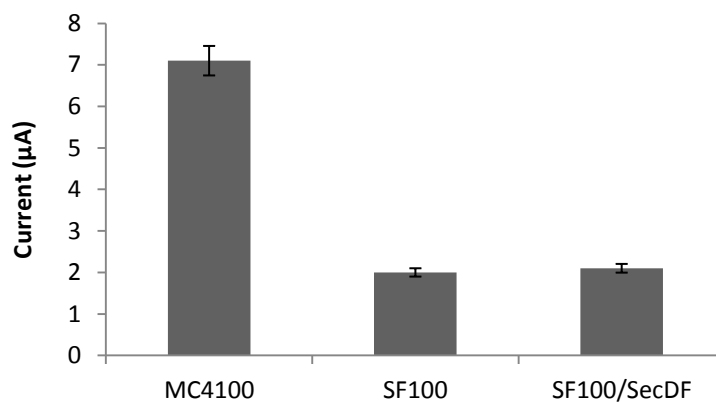
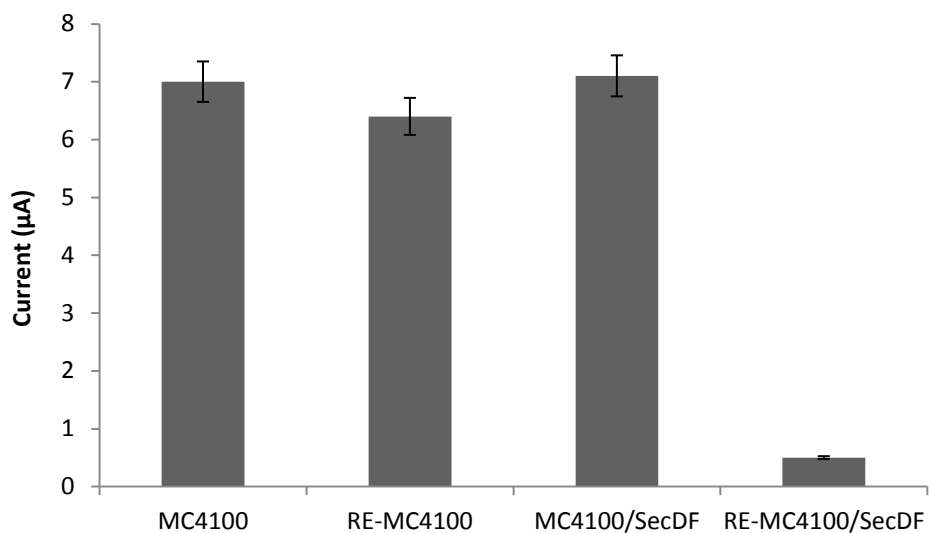


Figure A2.1. The channel activity in SF100 and reconstituted SF100 system.

(A) 40 ng MC4100, or SF100 or SF100-SecDF were injected into oocytes. The current was recorded after two hours injection. (B) The 40 ng SF100 or SF100/SecDF membrane and 80 ng SecA were injected into oocytes.

A.



B.

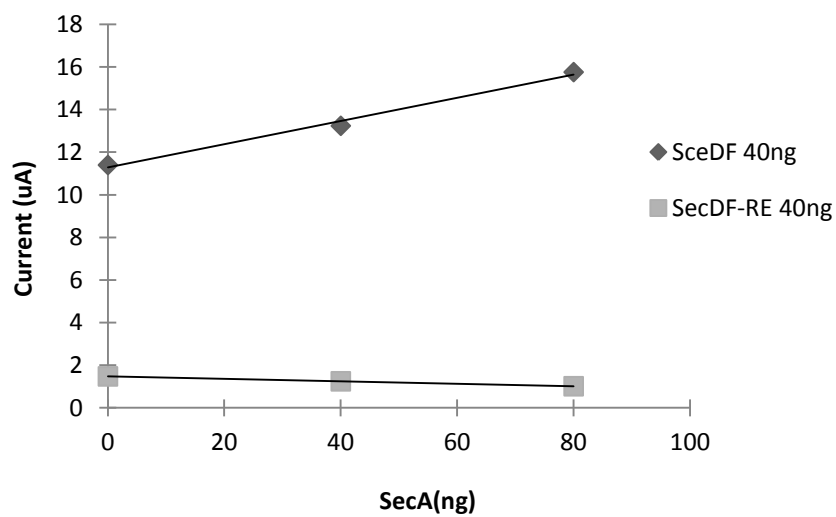
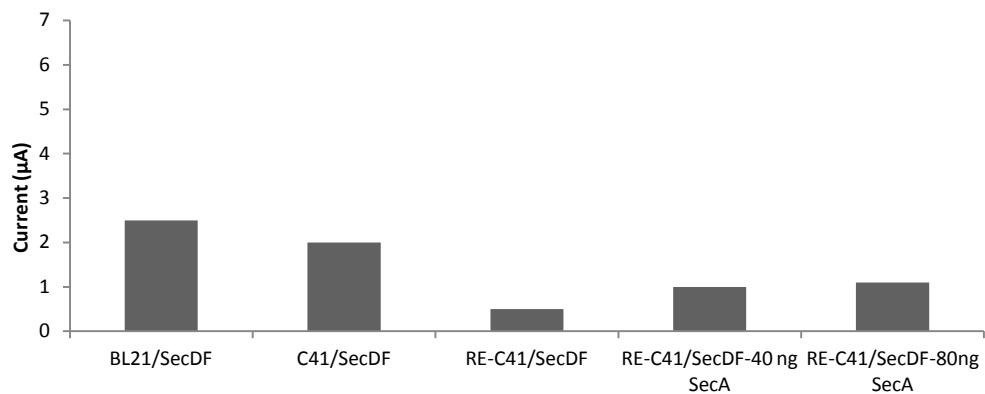


Figure A2.2. The channel activity of SecDF overexpressed in MC4100 or RE-constituted MC4100 (A) 40 ng membrane fraction with extra 60 ng of SecA. The current was recorded after two hours injection. RE-Mc4100+SecA: Reconstituted MC4100 membrane with 80 ng SecA, RE-SecDF: Reconstituted MC4100/SecDF membrane with 80 ng SecA.

A.



B.

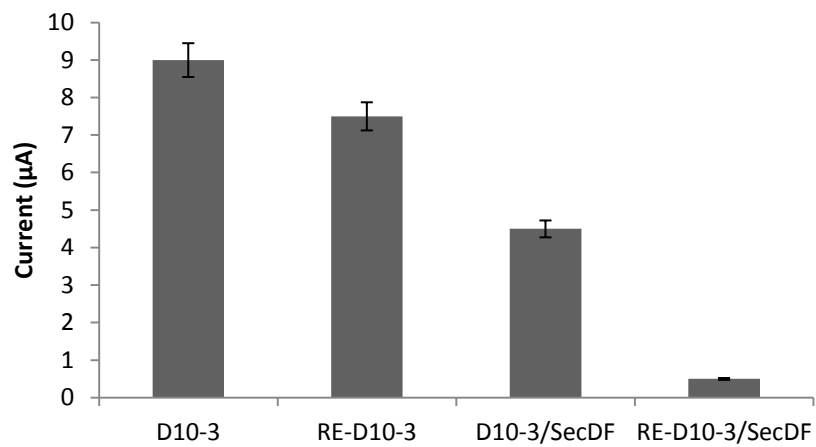


Figure A2.3. The channel activity in BL21 and C41 and D10-3 overexpressed SecDFYajC.

The BL21, C41, (A) and D10-3 (B) were overexpressed SecDFYajC plasmids. The IMV were prepared and injected into oocytes with SecA addition.

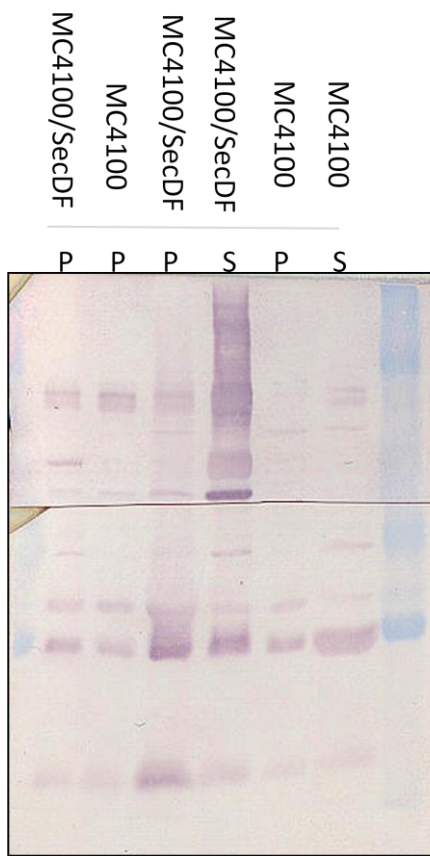


Figure A2.4. Western Blot of RE- MC4100 or RE-MC4100/SecDF membrane

The MC4100 or MC4100/SecDF membrane was treated with 1% sodium cholate on ice for 1 hour. The mixture than centrifuged at 90 K for 30 mins. The supernatant and pellet was collected and analyzed by 12% SDS-PAGE. Western Blot was against the Sec D and SecF.

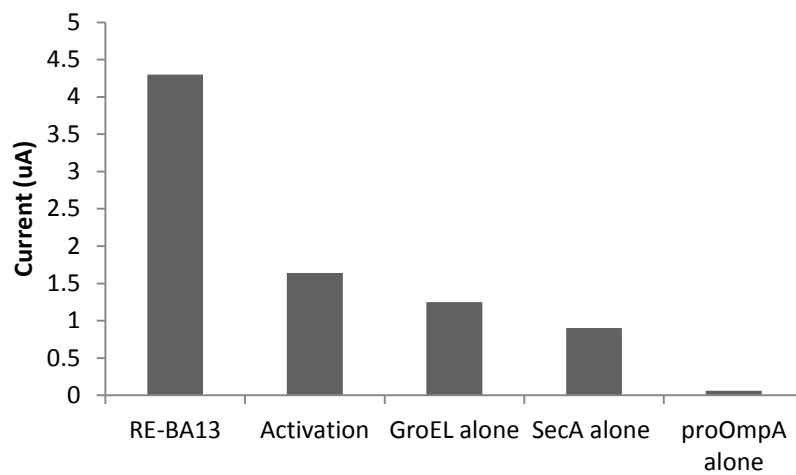


Figure A2.5. The protein profile of RE-MC4100 or RE-MC4100/SecDF.

All the membranes were treated with 1% SOD on ice for one hour. The sample then centrifuged at 90K for 30 mins. The sample was divided into supernatant and pellet fractions. The total 40 ug of each fraction was loaded to the 12% SDS-PAGE for commassive blue staining and Silver Staining. The star sign was the protein which is located in the RE-MC4100 but not the RE-MC4100/SecDF.

Five different spots from Silver stain were sent to the MAUDI-TOF analysis. Four of them had strong signal for the database blast.

A



B.

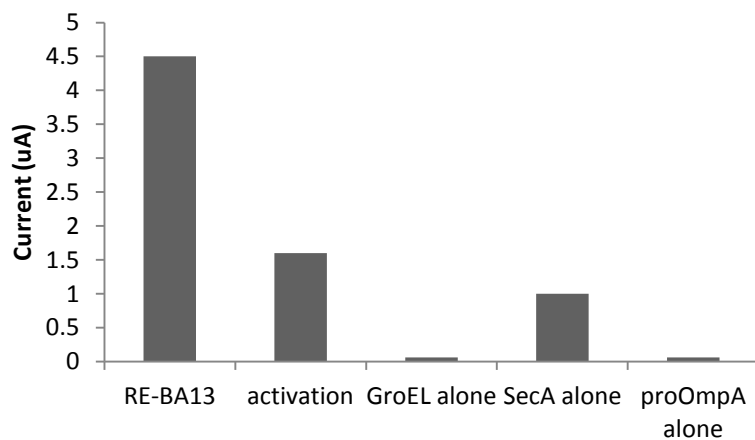


Figure A2.6. The channel activity of SecA-liposomes with GroEL

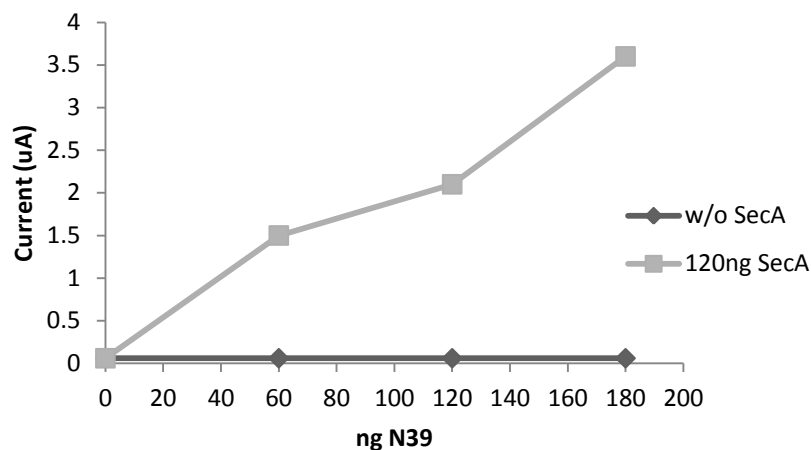
The oocytes were extract from the frogs and incubated at 16°C for 72hours. The Liposome, SecA, proOmpA, ATP, and Mg were mixed and injected into oocytes in the presence or absence of GroEL. n=30

Activation: 60 ng SecA-liposomes, 60 ng GroEL, 0.6 mM ATP, 0.5 mM Mg and proOmpA

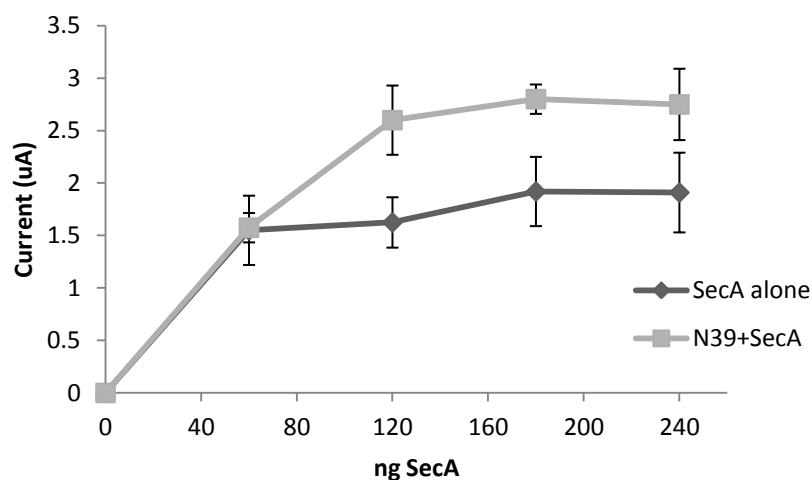
GroEL alone: 60 ng GroEL-liposomes, 0.6 mM ATP, 0.5 mM Mg and proOmpA

SecA alone: 60 ng SecA-liposomes, 0.6 mM ATP, 0.5 mM Mg, and proOmpA

A.



B.

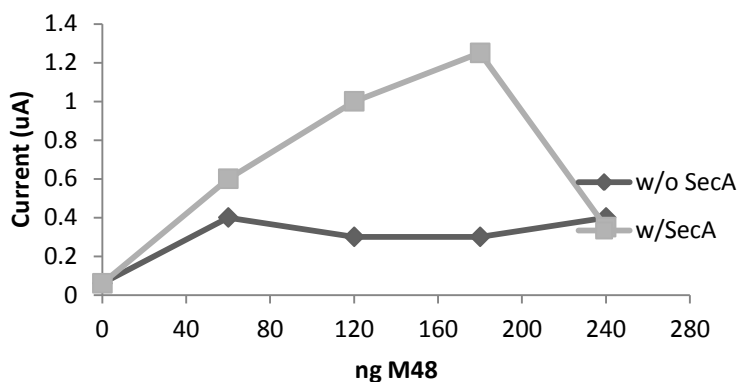


	60 ng SecA	120 ng SecA	80 ng SecA	240 ng SecA
w/o N39	5/20	11/20	16/20	15/20
w/N39	5/20	8/20	10/20	10/20

Figure A2.7. N39-SecA enhance the channel activity of SecA-liposomes.

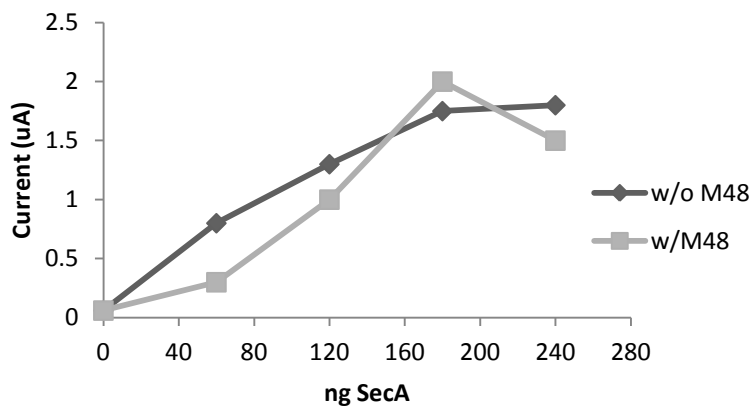
(A) 120 ng SecA, 60 ng liposome, proOmpA, ATP and Mg were mixed with different amount of N39. (B) Serial amount of SecA was injected with 60 ng liposomes, 0.6 mM ATP, 0.5 mM Mg and proOmpA in the presence or absence of 60 ng N39-SecA. The solution then injected into oocytes for three hours. After that, oocytes were ready for whole cell recording. n=30

A.



	60 ng M48	120 ng M48	180 ng M48	240 ng M48
w/o SecA	0/20	0/20	0/20	0/20
w/ SecA	2/20	4/20	1/20	0/20

B.

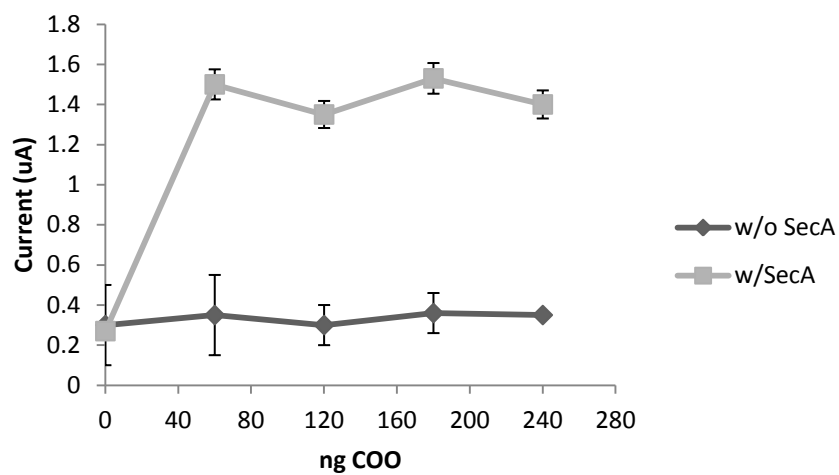


	60 ng SecA	120 ng SecA	180 ng SecA	240 ng SecA
w/o M48	3/20	8/20	10/20	12/20
w/ M48	5/20	5/20	2/20	3/20

Figure A2.8. Channel activity of SecA-liposomes with M48-SecA

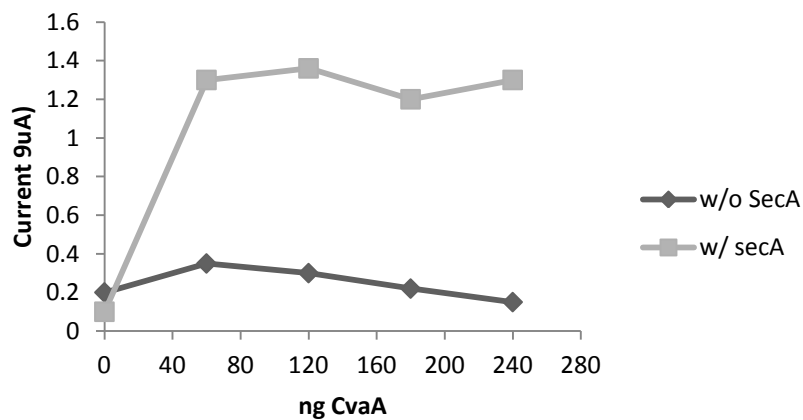
(A) 60 ng SecA-liposomes, 0.6 mM ATP, 0.5 mM Mg and proOmpA were injected with or without the M48-SecA. (B) 60 ng M48-SecA, 0.6 mM ATP, 0.5 mM mg, and 0.025 ug/ul proOmpA were injected with wild type SecA-liposomes, 60 ng.

A.



	60 ng COO	120 ng COO	180 ng COO	240 ng COO
w/o SecA	0/20	0/20	0/20	0/20
w/ SecA	6/20	7/20	7/20	9/20

B.



	60ng CvaA	120ng CvaA	180ng CvaA	240ng CvaA
w/o SecA	0/20	0/20	0/20	0/20
w/ SecA	7/20	7/20	7/20	9/20

Figure A2.9. Channel activity of SecA-liposomes stimulated by Cytochrome C oxidase or CvaA.

120 ng SecA, 60 ng liposome, 1 mM ATP, and 1 mM mg were mixed with various amount of Cytochrom C oxidase (A) or CvaA (B).