

*Biochem Biophys Res Commun.* Author manuscript; available in PMC 2014 July 26.

Published in final edited form as:

Biochem Biophys Res Commun. 2013 July 26; 437(2): 212–216. doi:10.1016/j.bbrc.2013.06.039.

# Specificity of SecYEG for PhoA precursors and SecA homologs on SecA protein-conducting channels

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#### **Abstract**

Previous studies showed that *E. coli* membranes depleted of SecYEG are capable of translocating certain precursor proteins, but not other precursors such as pPhoA, indicating a differential requirement for SecYEG. In this study, we examined the role of SecYEG in pPhoA translocation using a purified reconstituted SecA-liposomes system. We found that translocation of pPhoA, in contrast to that of pOmpA, requires the presence of purified SecYEG. A differential specificity of the SecYEG was also revealed in its interaction with SecA: EcSecYEG did not enhance SecA-mediated pOmpA translocation by purified SecA either from *Pseudomonas aeruginosa* or *Bacillus subtilis*. Neither was SecYEG required for eliciting ion channel activity, which could be opened by unfolded pPhoA or unfolded PhoA. Addition of the SecYEG complex did restore the specificity of signal peptide recognition in the ion-channel activity. We concluded that SecYEG confers specificity in interacting with protein precursors and SecAs.

#### Keywords

SecA-liposomes; SecYEG complex; pPhoA translocation; Protein-conducting channel; Channel activity

## Introduction

Protein secretion is a vital physiological process in all cells. In bacteria Sec-mediated translocon is the major system for moving proteins across membranes (9). It has been proposed that during such protein translocation, SecYEG function as a core of protein-conducting channel (12, 13, 30) while SecA acts as an ATPase to push the precursor protein through the SecYEG-SecDF•YajC core to its final destination (4, 5, 10, 28). However, the essential role SecYEG in Sec-translocation has long been questioned (37). Several studies have shown that SecA-dependent protein translocation can occur without SecYEG (1, 36,

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40, 41) Indeed, we recently showed that in liposomes SecA alone is sufficient to promote ion-channel activity and protein translocation. (15, 23). Such SecA-liposomes are likely to be located at low-affinity sites, associated with phospholipids that are different from those interacting with high-affinity binding sites that involve SecYEG in *E.coli* (6, 13, 19, 30). SecA-liposomes, like PrlA suppressor and SecY plug-domain mutants, lack a signal peptide proofreading function (15). Addition of SecYEG and SecDF•YajC confers specificity, and improves efficiency to the same extent as it does in membranes (16). Similarly, reconstituted membranes lacking SecYEG are less efficient and less specific than native membranes, but addition of SecYEG fully restores the efficiency of channel activity (15, 16). Thus, a fully functional, low-affinity protein-conducting channel can be constituted from SecA alone.

In contrast, it has been shown that precursors of alkaline phosphatase (pPhoA) cannot be translocated in membranes lacking SecYEG (40, 41), suggesting that translocation of pPhoA does require SecYEG (29, 41). In this study, we examined the precursor specificity of SecA channels in a liposomes reconstituted system. We found that the translocation, but not opening channel activity, of pPhoA strictly depends on SecYEG, and that SecYEG confers specificity for the precursors and for interacting SecAs.

#### **Materials and Methods**

#### Inner membrane vesicles preparation

Wild-type MC4100 membranes, OmpA-depleted 773 membranes, PrlA/SecY suppressor membranes were prepared as described (33). SecA-depleted BA-13 membranes were prepared from *secAts* BA13 mutant (from D. Oliver). Briefly, BA13 cells were grown at 30 °C until mid-log phase, then shifted to 42 °C until growth ceased due to SecA depletion. The cells were collected and the BA13 SecA-depleted membranes were prepare through the established preparation procedures (33).

### Liposomes preparation

Liposomes of *E. coli* total lipids (Avanti Lipids) were prepared as described previously (15). The lipids were dried by spin vacuum and resuspended in 150 mM KCl solution/water and sonicated 3–5 mins until the solution was clear. Preparations were stored at –80°C for later use; they were thawed only once.

#### Protein purification

EcSecA was purified from BL21(λDE3)/pT7-SecA as described (4, 15). PaSecA and BsSecA were purified as described (18, 42). Precursor PhoA (pPhoA) was prepared from BL21.19 containing plasmid pAE2.2 (41) as follow. The inclusion bodies of the overexpressed pPhoA were dissolved in 8 M urea/ 50 mM Tris-HCl pH 7.6 buffer containing 50 mM NaCl, then was diluted, centrifuged, and loaded onto a Q column. The pPhoA was eluted at 1M NaCl in 50 mM Tris-HCl pH 7.6 buffer and stored at -80°C. Purified mature alkaline phosphatase of E.coli (Sigma-Aldrich) was denatured with 6 M urea, 5 mM EGTA and 200 mM DTT to unfold PhoA where indicated. OmpA and pOmpA were prepared as previously described (6, 15). SecDF•YajC was expressed from BL21(λDE3) containing plasmid pET543 (obtained from A. Driessen) and purified as described (16). SecYEG was prepared from pBAD/secE<sub>his</sub>YG (obtained from F. Duong) in C43 strain as described (7). Briefly, cell-free lysates were passed through a Ni-NTA affinity column (Qiagen), followed by a Q-Sepharose cation exchange chromatography (GE Healthcare). SecYEG complex was eluted at 300 to 600 mM NaCl in Tris-HCl pH 7.9 buffer containing 1% Triton X-100, 10% glycerol and 2 mM DTT, aliquoted and stored at -80°C in the same buffer until use. Protein amounts were estimated from A<sub>280</sub>/A<sub>260</sub> ratios, and confirmed by Bradford assay (16).

In vitro protein translocation—The translocation of pOmpA into liposomes was conducted as described previously (15). To avoid precipitation, SecYEG and SecDF•YajC were diluted in  $H_2O$  at 10 to 20 folds and reconstituted into liposomes prepared by sonication. Unless otherwise indicated, the translocation mixtures in 0.1 ml contained 120  $\mu$ g of liposomes or 4.5  $\mu$ g of OmpA-depleted 773 membranes, 10  $\mu$ g SecA, 0.1  $\mu$ g SecB and 150 ng substrates (pOmpA and pPhoA) (15). The mixtures were incubated at 37 °C; 30 min for pOmpA/OmpA and 2 hours for pPhoA/PhoA. The translocation mixtures were treated with proteinase K at 400  $\mu$ g/ml in ice water for 30 min, and liposomes were collected by centrifugation. Translocated proteins were detected by immunoblots as described previously (15, 16).

#### Xenopus oocyte injection and whole cell recording

The oocytes were collected from *Xenopus laevis* and prepared as described (15, 21, 23). Live frog Xenopus laevis (Xenopus Express, Inc) oocytes were obtained and kept at 16°C for 48 hours before injection to obtain consistent expression and injected with sample mixtures as described previously (21). Briefly, the 50 nl sample mixture was injected into dark animal pole side of oocytes by using Nanoject II injector (Drummond Scientific Co., Broomall, PA). Unless otherwise noted, all the experiments were done in the presence of 4 mM puromycin to remove oocytes endogenous precursors; the amount for each component was 120 ng liposomes, 120 ng SecA, 14 ng protein (pPhoA or mature PhoA or unfolded PhoA or pOmpA), 2 mM ATP, and 1 mM Mg<sup>2+</sup>. The amount for SecYEG and SecDF•YajC was 30 ng for each complex (16). The effective concentration of the reagents in 50 nl injected mixtures was estimated based on the average volume of oocytes of 500 nl. The voltage clamp adapted from an electrophysiological method was used to measure the opening of protein-conducting channels as described previously (15, 21, 23). The current was recorded for 1 min after 3 hours of incubation at 23°C. The inward and outward currents were recorded with the two-electrode voltage clamp technique by using KCl as the bath solution to measure the net currents.

# Results

#### SecYEG is necessary for the translocation of pPhoA in reconstituted SecA-liposomes

Previous studies showed that SecYEG-deficient membrane were severely defective in translocation of pPhoA (41), suggesting a dependence on SecYEG. We re-examined this requirement by employing a newly developed reconstituted SecA-liposome translocation system (15, 16) in which SecA-liposomes alone in the absence of SecYEG can promote the translocation of unfolded pOmpA or OmpA. We found that the translocation of pPhoA was strictly dependent on the presence of SecA and SecYEG (Fig.1A), in contrast to that of pOmpA (15). Consistent with previous findings with SecYEG-deficient membranes, SecA-liposomes alone (Fig.1A, lane 6) or SecYEG alone (lane 7) were not capable of translocating unfolded pPhoA even with a typical signal peptide. Addition of SecYEG facilitates the translocation of pPhoA (Fig.1A, lane 8) but not unfolded PhoA (lane 4). Thus, translocation of pPhoA requires SecYEG, and in the presence of SecYEG, a signal peptide is also required. This is the first time that pPhoA has been shown to be translocated in the reconstituted liposomes system, and with specificity.

Next, we examined the requirements of SecYEG for translocation of pOmpA and pPhoA. Previous studies showed that translocation of pOmpA occurs in SecA-liposomes without SecYEG, but that the addition of SecYEG greatly increases the efficiency and reduces the amount of SecA required for activity (15, 16); see also Fig. 1C, lanes 8–9). As shown in Fig. 1B, relatively low amounts of SecYEG promoted maximal activity on pOmpA translocation. In comparison, translocation of pPhoA required much more SecYEG for maximal activity,

and the concentration-dependence over a wide range was much more pronounced. As expected, the addition of SecDF•YajC in the reconstituted system further increased translocation activity (Fig.1C, lanes 6, 7) as has been observed with pOmpA (Fig.1c, lanes 9, 10). However, at comparable amounts of SecYEG-SecDF•YajC (16), the translocation activity of pPhoA in the reconstituted system was only 50% of that of intact membranes at all SecA concentrations tested (Fig.1D), suggesting that there may be other additional factors involved in the pPhoA translocation.

#### SecYEG stimulates translocation by EcSecA, not PaSecA or BsSecA

Next, we examined the interaction between SecYEG and SecA. As expected from previous studies (16), purified *E. coli* SecA (EcSecA) alone promoted the translocation of pOmpA (Fig.1C, lane 8, and Fig. 2, lane11) and purified *E. coli* SecYEG-SecDF•YajC enhanced translocation (Fig.1C, lanes 9, 10; Fig. 2, lane 12, 13), indicating an interaction between SecA and SecYEG-SecDF•YajC. Purified SecA from *Bacillus subtilis* (BsSecA) and *Pseudomonas aeruginosa* (PaSecA) also promoted translocation of pOmpA (Fig. 2, lanes 5 and 8, respectively) to almost same extent as EcSecA, indicating a lack of substrate specificity of BsSecA and PaSecA for *E. coli* pOmpA. On the other hand, EcSecYEG had little effect on the translocation of pOmpA mediated by the BsSecA (Fig.2, lanes 6, 7) or PaSecA (Fig.2, lanes 9,10), but greatly enhanced the EcSecA-mediated translocation (Fig. 2, lanes 12, 13). These results indicate a preferential specificity of interaction among the pOmpA, SecA and SecYEG-SecDF•YajC.

## SecYEG is not required for pPhoA to elicit ion-channel activity

Electrophysiological voltage measurements to detect the opening of ion-channels in the semi-physiological oocyte system were used to complement our in vitro protein translocation studies (15, 21, 23). We next examined the specificity of pPhoA in eliciting the channel activity by co-injecting SecA-liposomes into the oocytes. In contrast to the lack of in vitro translocation in the SecA-liposomes (Fig.1A), pPhoA stimulated the ion-channel activity with SecA-liposomes in the absence of SecYEG (Fig. 3, lane 4), similarly to pOmpA (lane 2). This result is consistent with earlier findings of stimulation of ion-channel activity by pOmpA or wild-type LamB signal peptides (21). Moreover, unfolded mature PhoA without signal peptide (Fig.3, lane 6) but not folded mature PhoA (lane 5), also elicited the channel activity. These results indicated that SecA-liposomes without SecYEG also lost signal peptide proofreading specificity for PhoA protein to elicit the channel activity, even though SecYEG is required for translocation. The loss of signal peptide specificity for PhoA to elicit the channel activity resembles the SecY/PrlA mutant membranes in the oocyte system (SFig.1. The SecA-liposomes channel activity by the unfolded pPhoA or PhoA acted similarly as SecY suppressor mutants in losing the signal peptide specificity (SFig.1A,B).

#### SecYEG restores signal peptide specificity

Even though the unfolded mature PhoA can elicit channel activity in oocytes with SecA-liposomes, the addition of SecYEG to the SecA-liposomes restored the signal peptide specificity of pPhoA (Fig.3). The unfolded mature PhoA without signal peptide was no longer capable of eliciting the channel activity in the presence of SecYEG (Fig.3, lanes 10–12), similar to the lack of activity with unfolded mature OmpA (Fig.3, lane 9). This specificity has been observed with wild-type membranes (21). Surprisingly, the addition of SecYEG did not stimulate much of the channel activity of pPhoA (Fig.3, compare lanes 4 and 10), in contrast to pOmpA where SecYEG stimulated the channel activity (Fig.3, lanes 2 and 8). The signal peptide specificity by SecYEG (23) was also evident in the reconstituted SecYEG-deficient membranes for unfolded PhoA: the addition of SecYEG restored the specificity, so the unfolded mature PhoA was no longer active (SFig.1C lanes PhoA, U-

PhoA\*). These results indicated that though SecYEG is not required for eliciting for channel activity, the presence of SecYEG confers the signal peptide specificity in the reconstituted system, as well as in the wild-type membranes (21).

## **Discussion**

Both electrophysiological conductance and protein translocation assays were used to analyze SecA-dependent protein conducting-channels in reconstituted liposomes. The two assays yielded somewhat different results. Conductance measures only the opening of ion channels, not a complete translocation process. Thus, signal peptides and the unfolded pPhoA can open the channel, but pPhoA cannot be translocated without SecYEG in the reconstituted crude membranes (40) or in the reconstituted liposomes reported here. On the other hand, pOmpA can function for both channel activity and protein translocation. It is interesting to note that SecA-alone channels behave like PrlA/SecY suppresors (22, 26) which allow secretion of PhoA without signal peptides in the PrlA suppresor cells (11), and the presence of wild-type SecYEG confers the specificity of requiring proper signal peptides as reported here. Moreover, the SecYEG specificity extends to interaction with SecA as well: EcSecYEG does not stimulate the translcation activity of PaSecA and BsSecA.

The demonstration of the requirement of SecYEG for pPhoA translocation resolves a long standing puzzle. Watanabe et.al (37) have shown that not all proteins require SecY for translocation, which has been extended to SecYEG (1, 40, 41). However, reconstituted membranes depleted of SecYEG were not active in translocating pPhoA (40, 41). With the liposome reconstituted system in this study, we have shown that indeed translocation of pPhoA is strictly dependent on the presence of SecYEG, and its activity is concentration dependent. The presence of SecYEG-SecDF•YajC in the SecA-liposomes not only confers specificity, it also increases efficiency to the extent of native membranes for translocating precursors such as pOmpA (15, 16). However, this work showed that reconstituted SecA-SecYEG-SecDF•YajC liposomes are not as efficient as membranes for precursors such as pPhoA (Fig1D), indicating that additional factors in the membanes are also needed to achieve maximal translocation activity of reconstituted SecA-SecYEG-SecDF•YajC liposomes.

The requirement of SecYEG for the tanslocation of some proteins such as pPhoA expains why sec Y and sec E are essential for the bacterial viability, even though neither Sec Y or SecE is required for the translocation of other proteins such as pOmpA. This phenomenon resembles that exhibited by the bacterial signal recognition particle (SRP) (24, 25, 32, 34). While SRP is an obligatory component of co-translational translocation in the endoplasmic reticulum (35), the bacterial SRP analog is but one of several targeting components that is required for the integration of only some membrane proteins, and not for others (8). Yet each component of FtsY/Ffh/4.5S RNA of the bacterial SRP is essential for cell growth (24, 34). The secretory system has undergone significant evolutionary modification. Bacterial SecYEG has evolved into eukaryotic Sec61p to becoming the channel. In these more highly evolved complexes in eukaryotes, co-translational secretion derives the necessary energy to push the secretory proteins to cross the membranes from protein synthesis. Interestingly, SecA is the only essential component of all Sec secretion pathways in bacteria, yet it did not survive the evolutionary transition into eukaryotes. The high ATP requirement for SecA (27) to function as both a motor and a core channel protein (15, 16) may have led to its evolutionary extinction in higher organisms, which primarily employ SecYEG analogs with the Sec61 complex and SRP. Bacterial SecYEG confers efficiency and specificity, thus sparing some of SecA's expenditure of ATP, leading to its evolutionary survival.

We previously showed that bacteria may possess two SecA-dependent protein-conducting channels in their membranes: one functioning with SecYEG at high-affinity phospholipid sites, the other functioning without SecYEG at low-affinity phospholipid sites (15, 16). The former confers specificity and efficiency; the latter exhibits a looser recognition of signal peptides, reminiscent of PrlA/SecY suppressors (11, 23, 26). Interestingly, synthesis of Seccomponents varies with growth phase. We recently found that transcription and translation of SecY, SecD and SecF by both B. subtilis and E. coli decreased during the transition from exponential to stationary phase while expression of SecA increased, most pronouncedly in Bacillus (17). Protein secretion into the extracellular medium by Bacillus occurs mainly in the stationary phase (3, 17, 39). It has been reported that secretion of  $\alpha$ -amylase and leuvansucrase by Bacillus has different requirements for SecA (14, 20). Increasing SecA expression, or certain mutations in SecA, enhances protein secretion during the exponential phase (2, 3) and over-expressing SecYE allows high level secretion of  $\alpha$ -amylase (31). Perhaps fast growing Bacillus cells require the more efficient SecA-SecYE system in order to satisfy the needs for house-keeping proteins in the cell membrane and wall directed there via the cotranslational SRP-targeting to the Sec translocon. Bacillus may have evolved to regulate secretion of proteins that are important only during the stationary phase in order to produce degradative enzymes in search of nutients. Regardless of why major components of Sec-secretion machinery are expressed so differently during the growth phase, the late increase in expression of SecA and signal peptidases can explain why protein secretion occurs principally in stationary phase (17, 38).

# **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

# **Acknowledgments**

We thank A. Driessen, D. Oliver and F. Duong for plasmids, and C. Alexander for technical assistance, and J. Ingraham for comments. YHH and BRL were fellows of Molecular Basis of Disease Program at Georgia State University. The core facility is supported in part by Georgia Research Alliance. This study was supported in parts by NIH grant GM34766 (PCT) and by the Chinese National Basic Research program 2010CB833706 and 2011CB910500 (S.S.F.).

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# Highlight

1) Translocation of precursor PhoA only active with SecYEG.

- 2) PaSecA- and Bs-SecA mediated translocation not stimulated with EcSecYEG/DFC.
- 3) Channel activity elicited by pPhoA without SecYEG.
- 4) SecYEG confers signal peptide specificity.

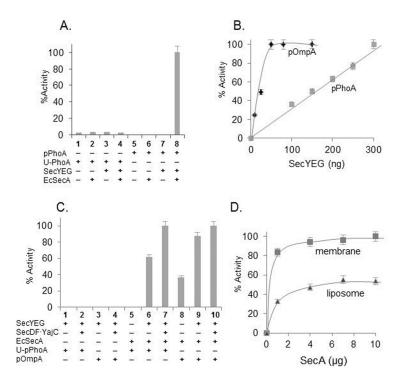
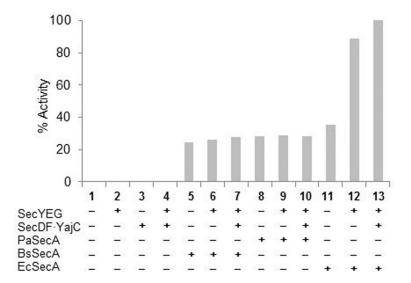


Figure 1. Requirement of SecYEG for pPhoA translocation (A) Dependence of SecYEG. The reactions were as described in Material and Methods. U-PhoA: unfolded PhoA. (B). Differential requirement of SecYEG for translocation of pPhoA and pOmpA with SecA (10  $\mu$ g). C. Enhancement of SecDF•YajC 55 (ng). (D). Differential translocation of reconstituted-liposomes with same amount of SecYEG (165 ng) in SecA-liposomes and 773 membrane.



**Figure 2.**Lack of stimulation of translocation by EcSecYEG for the translocation of pOmpA mediated by PaSecA and BsSecA.

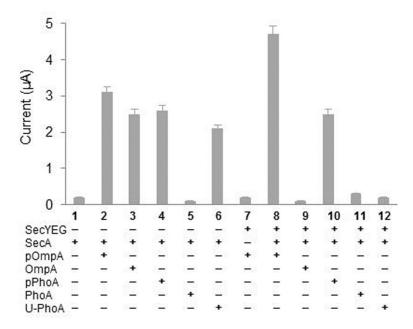


Figure 3. SecYEG confers signal peptide recognition in channel activities
SecA-liposomes were injected with varies precursors as described in M &M. PhoA: mature PhoA. U-PhoA: Unfolded mature PhoA.