



Reconstitution of functionally efficient SecA-dependent protein-conducting channels: Transformation of low-affinity SecA-liposome channels to high-affinity SecA-SecYEG-SecDF-YajC channels

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ABSTRACT

Previous work showed that SecA alone can promote protein translocation and ion-channel activity in liposomes, and that SecYEG increases efficiency as well as signal peptide specificity. We now report that SecDF-YajC further increases translocation and ion-channel activity. These activities of reconstituted SecA-SecYEG-SecDF-YajC-liposome are almost the same as those of native membranes, indicating the transformation of reconstituted functional high-affinity protein-conducting channels from the low-affinity SecA-channels.

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1. Introduction

Protein secretion is an important physiological process in all living cells. Sec translocon, the major protein translocation system across membranes in bacteria, has been studied for decades [1]. It has been proposed that during protein translocation, SecYEG function as a core of protein-conducting channel embedded in the membrane [2–4] while SecA acts as an ATPase to push the precursor through the SecYEG core in the cytoplasmic membrane to its final destination. Other accessory membrane proteins such as SecDF-YajC or YidC, serves as stabilizers that keep the translocation machinery intact and enhance translocation [3,4]. SecY contains the lateral gate and the plug domain which regulate the opening of the protein-conducting channels. The plug domain has been considered to be a proofreading gate that discriminates among precursors [5–7]. Partially or fully deletion on the SecY plug domain, like PrlA suppressor, lose the precursor selectivity but the translocation still takes place [6,8].

Although SecYEG has been proposed to be the core of the Sec channel, its essential role in Sec-translocation has long been ques-

tioned [9]. Several studies have shown that SecA-dependent protein translocation can occur without SecYEG [10–13]. Indeed, we recently showed that SecA alone is sufficient to promote ion-channel activity and translocation in liposomes [14]. 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 *Escherichia coli* [3,4,15,16]. SecA-liposomes, like PrlA suppressor and SecY plug-domain mutants [6,8,17,18] lack the signal peptide proofreading function. Moreover, the SecA-liposomes are not very efficient, requiring more SecA and ATP for functional activity [14]. Addition of SecYEG confers specificity, and improves efficiency, though not to the same extent as in membranes, especially at low SecA concentrations. 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 [14,19]. These findings suggest that additional membrane proteins other than SecYEG are required to form a fully functional protein-conducting channel.

SecDF-YajC and YidC have been shown to facilitate the protein translocation. The SecDF-YajC complex is encoded by *secD* operon [20,21]. Previous studies indicates that SecDF-YajC enhances the protein translocation by regulating the SecA ATPase cycling and membrane cycling, and works with SecYEG core to improve the translocation process [22–25]. In addition, it has been reported that SecDF is involved in maintaining proton motive force in late translocation process [26,27]. In this study, we use reconstituted SecA-liposomes to examine the function of SecDF-YajC and YidC

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in electrophysiological ion-channel activity and *in vitro* protein translocation activity in the presence of saturated ATP, thus the proton motive force does not play an important role [26,28,29]. We show that the functional efficiency of reconstituted SecA-liposomes with SecYEG and SecDF-YajC is equivalent to that of native membranes under the condition used. Thus, a fully functional protein-conducting channel can be reconstituted from the low-affinity SecA-liposomes channels.

2. Materials and methods

2.1. Liposome and membrane preparation

The preparation of liposomes is as described previously [14]. Briefly, *E. coli* total lipids (Avanti) were dried, re-suspended in 150 mM KCl, and sonicated in an ice water bath until the solution became clear (usually for 3–5 min), resulting in an average size of liposomes about 130 nm. Samples of these liposomes were stored at -80°C and thawed only once before use. BA13 SecA-depleted membranes were from *E. coli* BA13 cells grown at 42°C to deplete the SecA [19]. *E. coli* 773 is an ompA-deleted strain from our lab stock; the OmpA-depleted 773 membranes were used for assaying proOmpA translocation. Inverted membrane vesicles were prepared using sucrose gradients as described [11,13].

2.2. Protein purification

SecA was purified from BL21(λ DE3)/pT7-SecA as described [30]. ProOmpA was prepared as described [31,32]. SecDF-YajC was purified from BL21(λ DE3) cells containing plasmid pET543 *secDF-yajC* (from Dr. A. Driessen) as described [33]. SecYEG was prepared from *E. coli* BL21(λ DE3) strain harboring pBAD/*secE_{his}*YG (from Dr. F. Duong) and purified as described [34]. Briefly, cell-free lysates were passed through a Ni-NTA affinity column (Qiagen), the SecYEG fractions were further purified by a SP-Sepharose cation exchange chromatography (GE Healthcare). SecYEG complex was eluted at 300–400 mM NaCl in Tris-HCl pH 7.9 buffer containing 1% Triton X-100, 10% glycerol and 1 mM DTT; samples were stored at -80°C in the same buffer until use. The SecYEG and SecDF-YajC complexes were more than 90% pure as revealed by Coomassie Blue Stain or Silver Stain after SDS gel electrophoresis.

2.3. *In vitro* protein translocation

The translocation of pOmpA into liposomes is as described previously [14]. SecYEG and SecDFC were diluted in H_2O at $10\text{--}20\times$ before reconstituting into sonication-prepared liposomes. Reconstituted liposomes, SecA and pOmpA were added to the translocation buffer [14] separately. Liposomes (10 μg) or OmpA-deleted 773 membranes (3.5 μg) with 10 μg or indicated amounts of SecA were used in 0.1 ml translocation mixtures [14]. The reactions were at 37°C for 45 min, and the mixtures were processed for immunoblots as previously described [14].

2.4. *Xenopus* oocyte preparation and injection

Oocytes were obtained from live frog *Xenopus laevis* (Xenopus Express, Inc) and injected with sample mixtures as described previously [35]. Briefly, the 50 nl sample mixtures were injected into dark pole site of oocytes using Nanoject II injector (Drummond Scientific Co., Broomall, PA). The effective concentration of each component was based on the average volume of 500 nl oocytes. The ion current was recorded after three hours of incubation at 23°C . Unless otherwise noted, the amount for each component is 120 ng liposomes, 120 ng

SecA, 14 ng proOmpA, 2 mM ATP, and 1 mM Mg^{++} and where indicated, 0.47 ng of SecYEG and 0.53 ng of SecDF-YajC.

2.5. Voltage clamp measurement

The voltage clamp adapted from an electrophysiological method was used to measure the opening of protein conducting channels as described previously [14,19,35]. The current was recorded after 3 h of incubation after injection at 23°C . Inward currents and outward currents were recorded to measure the net currents for channel opening.

2.6. Protein quantification

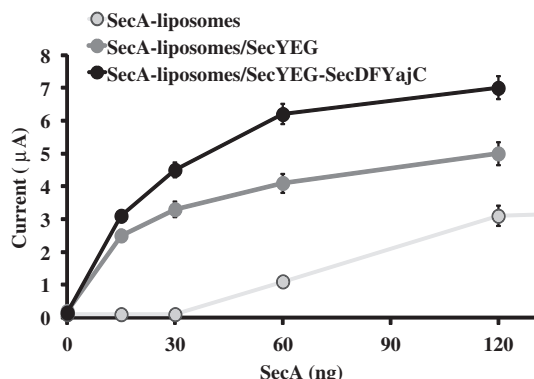
Protein amounts were estimated from A_{280}/A_{260} ratios, and confirmed by Bradford assay (Bio-Rad) using BSA as standards or by SDS-PAGE with sliver staining using BSA as standards. The relative amounts of purified SecYEG and SecDF-YajC compared to the membrane were determined by immunoblots with SecY and SecF antibodies. The amounts were quantitated by Bio-Rad Quantity One software and were fitted by linear regression (SI Figs. 1 and 2).

3. Results and discussion

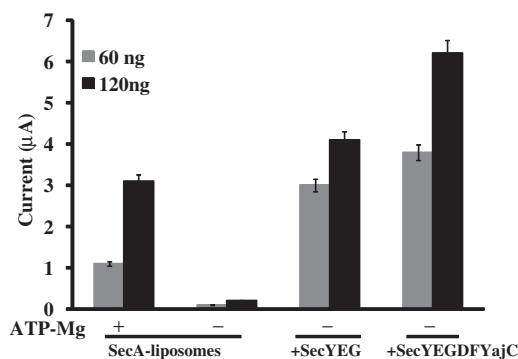
3.1. Reconstitution of efficient ion-channels

Recently, we have shown that SecA-liposomes alone are capable of promoting protein translocation *in vitro* and for eliciting ion-channel opening activity in the oocytes [14]. However, the SecA-liposomes require increased amounts of SecA, and additional ATP-Mg for channel activity; and even then the activity is only about 50% of the native membranes [14]. Since SecYEG plays a role in the channel efficiency, the SecYEG dose titration assay was performed to determine its relation with channel activity in the SecA-liposomes (SI Fig. 1). With 120 ng SecA-liposomes, 0.47 ng of SecYEG increased the ion-channel activity from 3 μA to 5 μA . Additional SecYEG failed to facilitate the channel activity further. However, the ion-channel activity of SecA-SecYEG liposomes is only about 70% of native membrane vesicles containing intrinsic SecYEG [14,35], suggesting that other membrane proteins or factors may be required for a fully functional ion-channel activity [10]. We further tested other Sec accessory proteins which may play a role for efficiency, such as YidC or SecDF-YajC [14]. Purified YidC had no additional activity (data not shown). SecDF-YajC have been shown to interact with SecYEG and to regulate the SecA membrane cycling and to maintain proton motive force during the later steps of protein precursors translocation in the cells [22–27]. SecDF-YajC like SecYEG by themselves were not active for channel activity without SecA (Fig. 1A, and data not shown). Addition of SecDF-YajC further enhanced the channel activity of SecA-SecYEG (Fig. 1A). The increase of activity of SecA-liposomes was more pronounced at lower concentrations of SecA (Fig. 1A). Conversely, in the presence of SecYEG and/or SecDF-YajC, less SecA was required to achieve the same level of channel activities (Fig. 1A). Thus, in SecYEG reconstituted liposomes, 15 ng SecA was sufficient to activate the SecA-liposomes channel activity which otherwise requires 120 ng SecA [14]. Moreover, previous studies showed that the SecA-liposomes need additional ATP-Mg to elicit channel activity in the oocytes [14] even though there is sufficient ATP in the oocytes to support the channel activity with native membranes containing SecYEG [17]. Here we found that additional ATP-Mg was no longer required in the presence of 0.47 ng of SecYEG and/or 0.52 ng of SecDF-YajC for SecA-liposomes to function as ion-channels in the oocytes (Fig. 1B). These data show that SecA-SecYEG liposomes not only regain the signal

A. Reduced SecA Requirements



B. Reduced ATP Requirement



C. Efficiency of SecYEG-liposomes

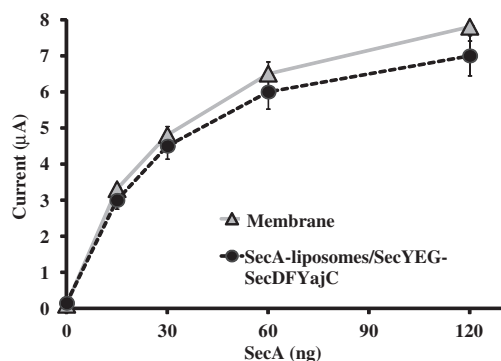


Fig. 1. SecYEG and SecDF:YajC increase efficiency of SecA-liposomes ion-channel activity. (A) SecA-liposomes were injected into oocytes with ATP-Mg, proOmpA in the presence of 0.47 ng of SecYEG or together with 0.53 ng SecDF:YajC and various amounts of SecA. (B) Reduced ATP-Mg requirement of SecA-liposomes channel activities as in (A). The channel activities were measured with or without additional 2 mM ATP + 1 mM MgCl₂. (C) Addition of SecDF:YajC fully restores efficiency of SecA/SecYEG-liposomes channel activity to the same extent as with native BA13 membranes (SecY needed was about 16% as in the membranes). *n* = 40.

peptide recognition function [14,19] but also increase the ion-channel efficiency. It is further noted that for the liposomes to function, SecA was in excess over SecYEG, consistent with the findings that there are more SecA than SecYEG in the cell [36,37].

Indeed, the channel activity of SecA-SecYEG complex together with SecDF:YajC were comparable to those of native membranes containing SecYEG (Fig. 1C). These data indicated that the reconstituted proteo-liposomes with SecYEG-SecDF:YajC were almost fully functional as native membranes for ion-conducting channels. To assess the amount of SecYEG used for reconstituted proteo-liposomes, we quantified the amount of SecY (Fig. 1C) by Western

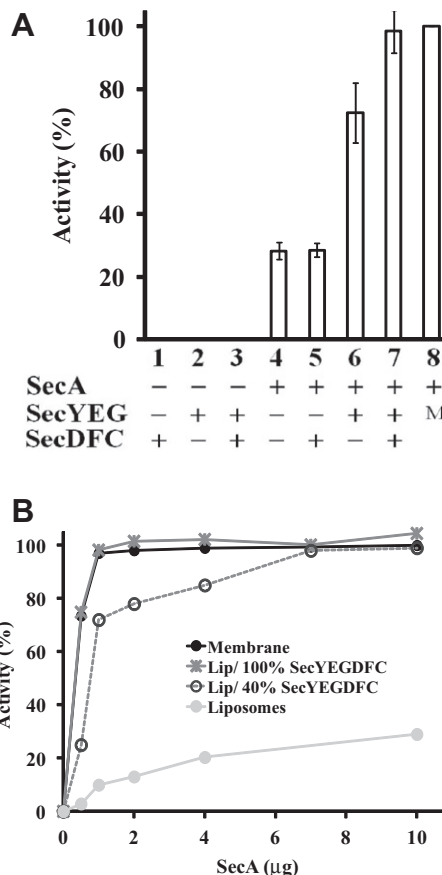


Fig. 2. Stimulation of pOmpA translocation by SecDF:YajC of SecA-liposomes in the presence of SecYEG. (A) The translocation activity of proOmpA with membranes was set as 100%. SecDF:YajC increased the precursor translocation efficiency. Lane 1–7, 10 μg liposome and 10 μg SecA were mixed with translocation buffer and energy source, and 66 ng of SecYEG and 55 ng SecDF:YajC were reconstituted into SecA-liposomes mixture. Lane 8, translocation activity with 3.5 μg of native 773 membrane vesicles (M) containing 165 ng of SecYEG (See SI Fig. 2, Upper panel) and 1 μg SecA. (B) Reduced requirements of SecA for SecA-liposomes with increased SecYEG/SecDF:YajC as in 3.5 μg of 773 membrane vesicles. The amounts of purified SecYEG used were 40% (66 ng) or 100% (165 ng) of the amount of SecY in membranes. *n* = 4.

immune blots as described in the Methods (SI Fig. 2A, Upper panel). Based on the linear equation generated from native membrane vesicles, the amount of purified SecY needed to achieve > 90% activity (Fig. 1C and SI Figs. 1 and 2A, Lower panel) was only 1/6 of that membrane used in the assays. Increasing SecYEG and SecDF:YajC to equivalent amounts found in native membranes marginally improved the channel activity (data not shown). Thus SecA-liposomes, when reconstituted with SecYEG and SecDF:YajC maintain ion-channel activity that is almost equivalent to native membranes containing these same components, under the condition that proton motive force is not a factor.

3.2. Reconstitution of efficient protein translocation channels

Previous work [14] has showed that SecA-liposomes can promote protein translocation. However, such SecA-liposomes lack signal peptide specificity because unfolded OmpA, without signal peptide, can also be translocated [14]. Moreover, such SecA-liposomes are far less efficient, requiring considerably more SecA to achieve only 50% of the translocation activity when both SecA and SecYEG are present. As shown previously, addition of SecYEG to the SecA-liposomes enhanced translocation activity (Fig. 2A,

Lane 6). More importantly, the translocation activity of the variably reconstituted liposomes was almost able to reach the same level as that of native membranes: from ~30% translocation of SecA-liposomes (Lane 4) to 75% with SecA-SecYEG (Lane 6), when compared to native membranes (Lane 8). In addition, as with ion-channel activity, the efficiency of proOmpA translocation is also increased to more than 97% when SecDF-YajC is reconstituted with SecA-SecYEG-liposomes (Fig 2A, Lane 7). It is also worthy of note that SecYEG (Fig. 2A, Lane 2) and SecDF-YajC (Lanes 2–3), absent SecA, is not active, and that SecDF-YajC (in the absence of SecYEG) exhibits no additional activity with SecA-liposomes (Lanes 4, 5).

We have examined the efficiency of the reconstituted liposomes for the requirements of SecA in the translocation of proOmpA. Much less SecA was needed to more efficiently translocate proOmpA by SecYEG-SecDF-YajC-liposomes (Fig. 2B, asterisk) than with SecA-only liposomes (Fig. 2B, open circle). These effects are much more pronounced at low SecA concentrations, and are also dependent on the amount of SecYEG in the reconstituted liposomes. The SecYEG complex used in the translocation contained only about 40% of SecY in the membranes (Fig. 2A, Lane 8); there was an appreciable difference in the efficiency of proOmpA translocation at the lower concentration of SecA (Fig. 2B). However, when similar amounts of purified SecYEG were used in the liposomes as were present in the membranes (Fig. 2B, closed circle), decreasing the concentration of SecA had virtually no effect on the efficiency of proOmpA translocation between the reconstituted SecYEG-SecDF-YajC and membrane vesicles (Fig. 2B).

Thus, the reconstituted SecA-liposomes with both SecYEG and SecDF-YajC function almost as efficiently in ion-channel activity (Fig. 1C) and protein translocation (Fig. 2B) as do in the native membrane protein-conducting channels. It is noted that the protein-conducting channel of the reconstituted proteo-liposomes does not contain signal peptidase, thus the signal peptide is not cleaved from the translocated proOmpA. Nevertheless the translocation of precursors by reconstituted channels is almost fully functional as native membranes containing SecYEG-SecDF-YajC.

Tsukazaki et al. [27] has recently reported the fine structures of SecDF from *Thermus thermophilus*, and identified an ATP-independent step in protein translocation that requires both SecDF and proton motive force in a pH-dependent manner. They proposed that SecDF serve as membrane integrated chaperones, driven by proton motive force to achieve ATP-independent protein translocation. While it has previously been shown that SecDF regulates the proton motive force during the translocation process, which is precursor specific, the proton motive force by SecDF-YajC has little effect on proOmpA translocation when ATP is saturated in assays [26]. In our studies here, SecDF-YajC were shown to enhance translocation of proOmpA and the ion-channel activity of SecA-liposomes in the presence of SecYEG with sufficient ATP and constant pH, under which proton motive force does not play an important role [28,29]; indeed, SecDF-YajC itself has no channel activity without SecYEG. As a consequence we would suggest that, under the conditions used here, SecDF-YajC are able to enhance translocation of proOmpA through a mechanism other than proton motive force.

3.3. Molecular evolution?

The evolution of the Sec components and pathways has been reviewed [19,35]. Previously, some of the evolutionary implications of having two SecA-dependent protein-conducting channels in membranes [14,38,39]: the low-affinity SecA-only channels and high-affinity SecA-SecYEG- channels [40–44], which now should include SecDF-YajC. In demonstrating the conversion of low-affinity channels to high-affinity channels we have, in effect, been able to mimic a form of *in vitro* molecular evolution. It should be emphasized again that SecYEG liposomes even with SecDF-YajC

are not active in translocation, while SecA-liposomes are active, albeit with lower efficiency and specificity, much like Prl suppressors [14,18,45]. It is reasonable, therefore, to consider again the question of whether the two SecA-dependent protein-conducting channels have evolved independently or that SecA started as the basic primitive core channels, and evolved (with the recruitment of SecYEG-SecDF-YajC) to form the more efficient and more specific channels that are found today: much like the development of a number of amino acid transport systems in bacteria, as well as the recruitment of various sigma factors by RNA polymerase [14]. Though this evolutionary argument is philosophical and difficult to prove experimentally, it is tempting to speculate that SecA-alone channel was the most primitive prototype channel. The SecA-alone channel promotes ion-channel activity translocation of proteins even without signal peptides and no other membrane protein in a process that requires only ATP but was inefficient and with low specificity [14], like PrlA suppressors [18,45]. Such channel would have then gained increased efficiency and specificity with the addition of SecYEG-SecDF-YajC. Presently, both types of channels are functional in bacteria (with SecA analogs still present in chloroplasts), which provide an energy-rich environment that enables both types of channels to function under rapid growth conditions. The high ATP requirement for SecA to function as both a motor and a core channel protein may have ultimately led to its evolutionary extinction in higher organisms, which primarily employ SecYEG analogs with the Sec61 complex. In these more highly evolved complexes the co-translational secretion derives the necessary energy from protein synthesis and thus does not require SecA-mediated ATP hydrolysis to cross the membranes.

In summary, this work shows that SecA-liposomes reconstituted with SecYEG-SecDF-YajC are as fully functional as native membranes in protein-conducting channels for protein translocation and ion-channel activity. Moreover, this study also establishes that the low-affinity SecA-channels can be transformed into higher efficient high-affinity SecA-SecYEG-SecDF-YajC channels with higher specificity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2013.01.042>.

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