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Characterization of the minimal length of functional SecA in *Escherichia coli*

Bing Na, Zhipeng You, Hsiuchin Yang, Phang C. Tai *

Department of Biology, Center of Biotechnology and Drug Design, Georgia State University, Atlanta, GA 30303, United States

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ABSTRACT

Previous studies showed that certain regions of *E. coli* SecA can be deleted from its N- and/or C-termini to complement a SecA *amber ts* mutant. In this study, we determined and characterized the dispensability of both ends of SecA molecules. With N-terminal intact or 9-aa deleted, 826aa ($SecA_{1–826}$ and $SecA_{10–826}$, respectively) is the minimum for complementation activity, while with N-terminus deleted by 2–21aa, $SecA_{22–829}$ is the minimum. Further deletion at the C-terminus of $SecA_{1–826}$/$SecA_{10–826}$/$SecA_{22–829}$ abolished the complementation activity in the cells. A hydrophobic amino acid is required for the 826th residue in the minimal-length SecAs. Chemical crosslinking and gel filtration result showed that both purified $SecA_{22–828}$ and $SecA_{22–829}$ could form a dimer. Moreover, the *in vitro* ATPase and protein translocation activities of $SecA_{22–828}$ and $SecA_{22–829}$ were similar, though lower than wild-type SecA. The active mutants had more truncated SecA in soluble than membrane-bound form, but was more stably embedded in membranes. In contrast, the inactive mutants tended to have truncated SecA more membrane-bound than soluble form, and were more loosely bound and easily chased out. Thus, the loss of complementation appears to be related to their altered subcellular localization and stability in the membranes. This study defines the substantial regions of N- and C-termini of SecA that may be deleted without losing complementation activity.

1. Introduction

The majority of proteins destined for the periplasm or outer membrane in *Escherichia coli* are translocated by the general Sec secretion pathway [1–4]. The bacterial Sec translocase has been extensively studied in the Gram-negative bacterium *E. coli*. The core of the Sec translocon consists of SecA ATPase that acts as a molecular motor, and a heterotrimeric integral membrane protein complex composed of SecYEG [5–7]. SecB is a molecular chaperone that targets preproteins [2,8] to SecA. Other Sec components, SecD, SecF, YajC and YidC, facilitate protein translocation [9,10].

SecA is an essential component of bacterial Sec translocase that interacts with several translocation components through different regions: ATP [11], preprotein [12], SecYEG [13], SecB [14], lipids [15] and RNA [16]. SecA consists of two separable soluble functional domains: the N-terminal 68 kDa domain that possesses high ATPase activity, and the C-terminal 34 kDa domain that down-regulates the ATPase activity and is involved in dimerization and SecB/lipid binding [17,18]. The N-68 domain contains a nucleotide binding domain (NBD) [11] and an intramolecular regulator of ATP hydrolysis (IRA2) [19]. SecA recognizes different substrates through the ligands specificity in the N-68 domain [20]. Biochemical and biophysical studies have shown that native SecA forms stable dimer in solution [21–24]. While in solution, SecA undergoes monomer–dimer equilibrium reactions, and the oligomeric state of SecA depends on temperature, ionic strength and protein concentration [8]. The crystal structures of *E. coli* SecA show that SecA formed a dimer in solution. SecA dimerization has been attributed to the C-terminal domain as well as N68-domain [25].

It has been reported that SecA deleted of N-terminal 8-amino acids, though lacking interaction with SecG, is functional in cells [26], and deleted of certain both N-/C-termini retains complementation activity for a SecA *ts* mutant [24,27–29]. We previously identified two specific N-terminal domains that are crucial for initial SecA/membrane interactions [30]. The first small helix, the linker and part of the second helix (Δ2–22) were found to be dispensable for SecA activity in complementing the growth of the SecA *ts* mutant. However, deletions of N-terminal aminoacyl residues #23–25 resulted in severe progressive retardation of growth. Moreover, a decrease of SecA activity caused by N-terminal dele-

* Corresponding author at: 161 Jesse Hill Jr. Drive, 592 Petit Science Center, Department of Biology, Georgia State University, Atlanta, GA 30303, United States. Fax: +1 404 413 5301.
E-mail address: biopct@gsu.edu (P.C. Tai).

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tions correlated to the loss of SecA membrane binding, formation of lipid-specific domains and channel activity [30]. Here we further investigated the roles of the C-terminal residues of SecA with or without N-terminal deletions. We identified the regions of N- and/or C-termini that can be deleted without losing complementation activity in *E. coli*.

2. Materials and methods

2.1. Media, bacterial strains and plasmids

Culture medium TAG containing 10 g/L of Tryptone, 5 g/L of NaCl, 40 mM potassium phosphate buffer (pH 7.0), 7.6 mM ammonium sulfate, 1.6 mM sodium citrate with 0.5% glucose was used as liquid and solid (with 1.5% agar) medium. Where appropriated, 100 μg/ml of ampicillin was added.

The bacterial strains and plasmids used in this work are listed in STable 1. *E. coli* strain DH5α was used as a host for plasmid construction and SecA *ts*/*amber* mutant BL21.19 (*secA13*(*Am*) *supF*(*Ts*) *trp*(*An*) *zch*::*Tn10 recA*::*CAT claA*::*KAN*) for complementation without over-expression and induced for protein over-expression [31].

Plating efficiency of colony forming units (CFU) at 30 °C and 42 °C was as described [24,28,30].

2.2. Overexpression and purification of mutant SecAs

BL21.19 cultures containing plasmids were grown at 30 °C in TAG medium until OD_{600} 0.8 and then were induced by 0.5 mM IPTG at 20 °C overnight. The cell culture was collected by centrifugation. SecA was purified according to procedures previously described [32]. The purification of $SecA_{22-828}$ and $SecA_{22-829}$ was similar to that of SecA except that they were eluted on SP-Sepharose column by 100 mM NaCl at pH 6.1 which separated well from wild-type SecA which was eluted at higher NaCl concentration.

2.3. Separation of cytosol and membrane SecA

BL21.19/pET5a–$SecA_{22-828}$ and BL21.19/pET5a–$SecA_{22-829}$ were grown in TAG/Amp medium at 42 °C to deplete wild-type SecA until BL21.19/pET5a–$SecA_{22-828}$ stopped growing. One ml of cell culture with OD_{600} 1.0 was harvested, and the pellet was re-suspended in 0.5 ml of 30 mM Tris–HCl, pH 8.0 containing 1 mM dithiothreitol (DTT), and 50 μg lysozyme in 10 mM EDTA (pH 8.0). The samples were treated by freezing–thawing 3 times to lyse the cells. $MgCl_2$ (10 mM) and 6 μg DNase I were added. The mixtures were sonicated in ice-water bath, and centrifuged to remove unbroken cells, followed by centrifugation to pellet membranes. The membranes were dissolved in the same volume as cytosol. SecAs were detected by immunoblotting and quantified by Bio-Rad GS710 densitometer.

2.4. In vitro chase of [^{35}S]-SecA in membrane

[^{35}S]-Met labeled $SecA_{22-828}$ and $SecA_{22-829}$ membranes were prepared from strains grown in TAG medium with Amp at 30 °C. 0.5 mM of IPTG was added to the cells when OD_{600} reached 0.8. After 90 min, the cells were labeled with 5 mCi [^{35}S]-Met for 10 min, harvested, washed and resuspended in 30 mM Tris–HCl, pH 8.0 containing 1 mM DDT. The cytosol and membrane fractions were separated as described [33]. [^{35}S]-labeled-membranes ($0.01A_{260}$) were incubated at 37 °C for 15 min with respective purified nonradioactive $SecA_{22-828}$ or $SecA_{22-829}$. The membranes were collected by centrifugation and the SecA was quantified as above.

2.5. Chemical crosslinking of SecA truncates and other in vitro activity assays

Purified SecAs were cross-linked as described [25]. Reaction mixtures (20 μl) contained: 20 μg purified SecAs, 1 mM 1-ethyl-3-(3-dimethylaminopropyl carbodiimide (EDC) in 0.1 M 2-Morpholinoethanesulfonic acid, monohydrate (MES) pH 6.4. Samples were separated by 5% SDS–PAGE and detected immunologically.

Three levels activities of ATPase (intrinsic, membrane and translocation) were assayed as described [34]. *In vitro* translocation of [^{35}S]-Met-labeled proOmpA was as described [32].

2.6. Reagents and chemicals

All reagents and are reagent grades and obtained from Sigma unless otherwise indicated.

3. Results and discussion

3.1. Maximal N- and C-terminal deletions of SecA to retain complementation activity

Based on our previous work on the deletion of N-termini, we further define the minimal length of N-/C-termini of active SecA for complementation [30]. Wild-type SecA (901 residues) was deleted from its N- and/or C-termini and tested for complementing *ts* strain BL21.19 at 42 °C (Table 1).

With SecA N-terminal $SecA_{\Delta 2-9}$, 826th amino acid is the minimal length at the C-terminal for complementation activity, identical to intact SecA; the deletion of first 9 SecA-aminoacyl-residues have no effect on complementation activity. With $SecA_{\Delta 2-21}$, C-terminal 829th residue is the minimum (Table 1). $SecA_{1-825/10-825}$ and $SecA_{22-828}$ completely lost their complementation activity, as reflected in the difference of the colony forming units (Fig. 1A). Therefore, the minimal length at the SecA C-terminus to complement can vary, depending on the length of the N-terminal region. It is interesting to note that the first 9 aminoacyl residues ($SecA_{\Delta 2-9}$) have no effect on SecA function, and that the additional deletion of N-terminal 12 residues $SecA_{\Delta 2-21}$ can be compensated

Table 1
Summary of complementation activity of N[¶]-/C-terminal truncated SecA mutants.

Δ(2–9) N-terminal deletions	42 °C	Δ(2–21) N-terminal deletions	42 °C
$SecA_{10-829}$–$SecA_{10-901}$	+++	$SecA_{22-829}$–$SecA_{22-901}$	+++
$SecA_{10-828}$	+++	$SecA_{22-828}$	–
$SecA_{10-826}$	+++	$SecA_{22-828}$ K828D, E, I, F, M, V, L	–
$SecA_{10-826}$ L826G, S, E, R, K	–	$SecA_{22-829}$ V829G, D, E, R, K, I, F, M, L	+++
$SecA_{10-826}$ L826I, F, M, V	+++	$SecA_{22-829}$ L826E, R	–
$SecA_{10-825}$ T825M, V, L, E	–	$SecA_{22-829}$ L826M, V	+++
$SecA_{10-821}$	–	$SecA_{22-829}$ K828R, H, F, V, L	+++

The Δ(2–9) N-terminal deletions were identical in complementation efficiency as intact SecA in all tests including mutations (for simplification, data not shown).
[¶] The numbers indicate the beginning and ending amino acid of SecA variants.

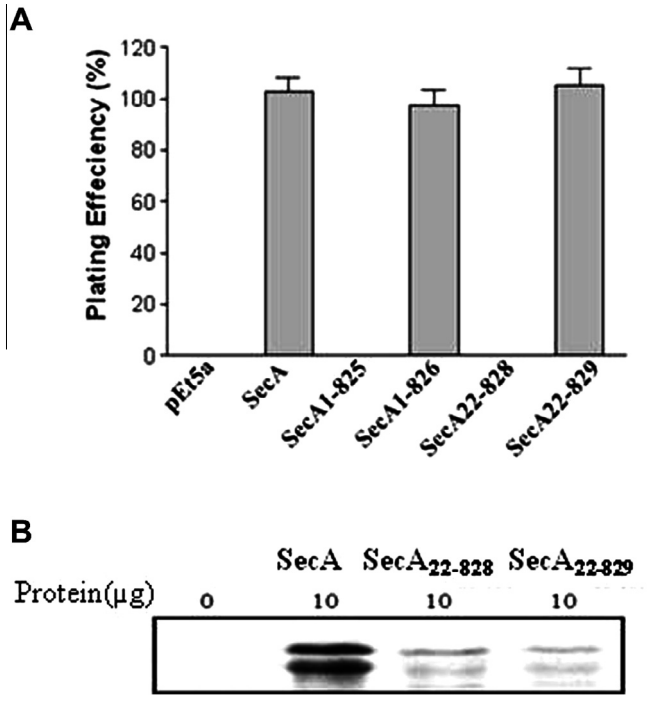

Fig. 1. Activities of truncated SecA mutants. (A) Plating efficiency of SecA and its truncated mutants as percent at 42 °C/30 °C. (B) *In vitro* protein translocation. Newly synthesized proOmpA was incubated with the same amount of SecA, $SecA_{22-829}$ or $SecA_{22-829}$ with SecA-depleted membranes.

by C-terminal three residues (from 826 to 829). Previously, N-/C-truncated SecAs have been shown to be active, or inactive, depending on the constructs, and the level of protein expression in the cells [24,28,29,35].

3.2. The importance of 826th aminoacyl residue in the hydrophobic side of α-helix

Since the active and inactive truncated SecA in our constructs differ only in one amino acid ($SecA_{1-825/1-826}$, $SecA_{10-825/10-826}$ and $SecA_{22-828/22-829}$), amino acid type might be important for SecA function. To test this possibility, the last amino acid was mutagenized and tested for complementation (Table 1).

For $SecA_{1-826}$–$SecA_{10-826}$, mutating Leu^{826} to hydrophobic Ile, Phe, Met and Val retained complementation while mutations to hydrophilic Ser, Glu, Gln, Arg, or Lys lost complementation (Table 1). Mutations (L826G/L826P) breaking α-helix could not complement either. $SecA_{1-825}$–$SecA_{10-825}$ could not complement regardless whether Thr^{825} was mutagenized into hydrophobic or hydrophilic residues (Table 1), indicating that $SecA_{10-826}$ is the minimal length of SecA, and the first 9 amino acids were dispensable regardless whether C-terminus is intact.

For $SecA_{22-829}$, Val^{829} could be replaced without losing complementation activity (Table 1). $SecA_{22-828}$ or mutating Arg^{828} could not complement (Table 1), indicating that 22–829aa is the minimal length for complementation. Leu^{826} of $SecA_{22-829}$ can be replaced with hydrophobic residues ($SecA_{22-829}$ L826M and $SecA_{22-829}$ L826V), but not hydrophilic residues ($SecA_{22-829}$ L826E and $SecA_{22-829}$ L826R) for complementation (Table 1). Hence, hydrophobic 826th residue is required for the minimal length SecAs to complement BL21.19.

The reported crystal structure of *E. coli* SecA lacks several residues [36]. Modeling supplemented by the crystal structures of *Bacillus subtilis* [37] and *Mycobacterium tuberculosis* SecA [38–40] using SWISS-MODEL server [41–43] shows overall structure of *E. coli* SecA (SFig. 1A), similar to *B. subtilis* and *M. tuberculosis* SecA. The hydrophobic Leu^{826} was located in a long α-$helix_{810-828aa}$ (SFig. 1B). In the α-helix, Phe^{811}, Leu^{815}, Leu^{818}, Val^{822} and Leu^{826} appear every 3–4 amino acids forming one turn of the α-helix, and compose a hydrophobic side (SFig. 1B). It is conceivable that a hydrophobic residue is required at the 826th position to maintain the hydrophobicity and the α-helix integrity. Lys^{828} is not located in the hydrophobic side, so it could be changed to hydrophobic or hydrophilic amino acids without impairing complementation activity as long as Val^{829} is intact. On the other hand, Val^{829} is located outside of the α-$helix_{810-828aa}$. Therefore, the change of Val^{829} will not affect the structure of the α-$helix_{810-828aa}$ and the hydrophobic side, thus having no effect on the complementation activity.

The α-$helix_{810-828aa}$ forms a Helix-Turn-Helix structure with α-$helix_{762-782aa}$ [44] (SFig. 1C), which was proposed to function as an intramolecular regulator of ATP hydrolysis (IRA1) [45]. Like α-$helix_{810-828aa}$, α-$helix_{762-782aa}$ also forms a hydrophobic side (SFig. 1B), which interacts with the hydrophobic side of α-$helix_{799-828aa}$ (SFig. 1B and C). These two α-helices further interact with the hydrophobic C-terminal portion (651–667aa) of the α-$helix_{621-667aa}$ (Fig. 1C). This long α-$helix_{621-667aa}$ stretches throughout SecA (SFig. 1A) with one end interacting with NBD and the other end with IRA1 regulatory domain, enabling the communication between the functional and regulatory domains [19].

3.3. ATPase and translocation activity do not account for complementation

To better understand the mechanism underlying the complementation ability of the truncated SecA, the complementation active and non-active SecA proteins were overexpressed and purified. Since $SecA_{1-825/826}$ failed to be overexpressed in our hands, we purified $SecA_{22-828}$ and $SecA_{22-829}$ and used them as representatives of the complementation non-active and active minimal length SecA proteins, respectively. The intrinsic-, membrane- and translocation-ATPase levels of the two complementation-active and non-active truncated SecAs were similar, though greatly reduced comparing to wild-type (data not shown), indicating that high ATPase activity is not obligatory for cell viability, as shown previously [30].

We next compared the protein translocation activity of $SecA_{22-828}$ and $SecA_{22-829}$ (Fig. 1B). The two mutant proteins, although one supported cell viability and the other did not (Fig. 1A), showed similar impaired protein translocation activity, much lower than wild-type SecA (Fig. 1B). Thus, high protein translocation activity seems not essential for cell viability either [30].

Taken together, the data suggest that *in vitro* ATPase and protein translocation activities, although important, are not directly correlated to cell viability, at least in the complementation ability under the conditions used. As suggested earlier [30], SecA needs not run at the maximal capacity *in vivo* to support the cell viability. The results suggest that the *in vitro* ATPase and protein translocation activities, albeit routinely used as criteria in evaluating SecA activity, do not necessarily represent the reflect SecA functions of the protein *in vivo*.

3.4. Dimer formation of truncated SecAs

SecA was found in equilibrium of monomer and dimer in solution [8] and exists as a dimer [25]. The C-terminal domain of SecA is involved in dimerization [22,25]. It has been reported that $SecA_{11-831}$ functions as a monomer [29] but has been disputed [24,28,46]. Deletion of the SecA C-terminus may affect its dimerization ability, thus impairing its complementation ability. We therefore examined the oligomeric state of the truncated SecAs

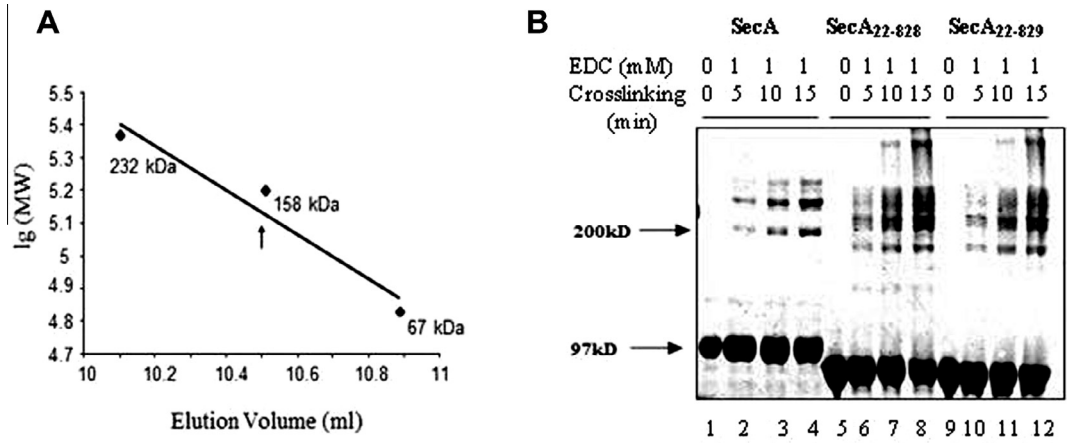

Fig. 2. (A) Size chromatography. Proteins (1 mg/ml) were chromatographed on a Superose 12–10/30 column. The arrow indicates the elution peak $SecA_{22-828}$ and $SecA_{22-829}$. (B) EDC crosslinking of SecA, $SecA_{22-828}$ and $SecA_{22-829}$. Molecular weight markers were as indicated.

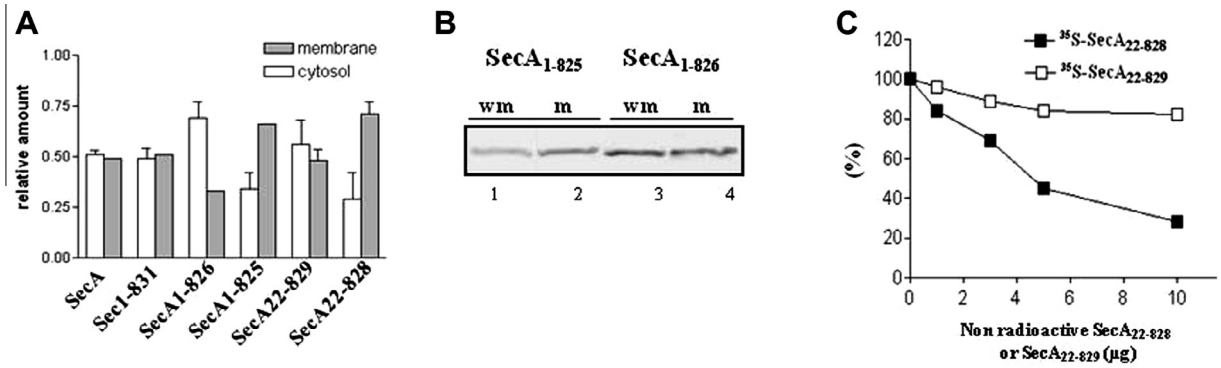

Fig. 3. The subcellular distribution and stability of SecA truncates. (A). SecA distributions in cytosol and membrane were detected by immunoblotting. (B). $SecA_{1-825}$ was loosely bound to membrane. Membranes were washed by 6 M urea (wm) or 30 mM Tris–HCl pH 7.6 (m), and SecA were analyzed. (C). Retention of [^{35}S]-SecA on membranes. [^{35}S-Met]-membranes of $SecA_{22-828}$ and $SecA_{22-829}$ were incubated at 37 °C for 15 min with 0–5 μg of cold purified $SecA_{22-828}$ or $SecA_{22-829}$ respectively. The membranes were collected and [^{35}S]-labeled-SecA were analyzed. [^{35}S]-labeled-SecA without cold protein was set as 100%.

by gel filtration and by chemical crosslinking. Both $SecA_{22-828}$ and $SecA_{22-829}$ were eluted in the size exclusion chromatography at around 160 kDa which is the size of dimers (Fig. 2A). Similar to previous data [46], when wild-type SecA was treated with EDC [22], several high molecular weight bands were observed at around 200 kDa and above (Fig. 2B, lanes 2–4), representing different crosslinking species of SecA. The band intensity increased with longer incubation time with EDC. A similar pattern of the high molecular weight bands were observed when $SecA_{22-828}$ and $SecA_{22-829}$ were treated with EDC (Fig. 2B, lanes 6–8 and 10–12), suggesting different crosslinking sites in the molecules. The crosslinking products of $SecA_{22-828}$ and $SecA_{22-829}$ were observed even when their concentration was low (data not shown). The gel filtration and crosslinking results indicate that substantial N-/C-terminal deletions did not affect SecA dimerization, and dimerization alone did not affect complementation activity of truncated SecA.

Previous study showed that the region of SecA from 675–831aa was involved in dimer formation [25], and that the four cysteine residues in SecA were not involved in dimerization [47]. SecA may form dimer via non-covalent bonds, like salt bridge, hydrophobic interaction and van der Waal's interaction, but probably not by one disulfide bond. One amino acid loss is less likely to result in significant change in these non-covalent interactions. Since both $SecA_{22-828}$ and $SecA_{22-829}$ form dimers, the loss of complementation activity is unlikely due to loss of dimerization.

3.5. The difference of subcellular distribution and stability of SecA truncates in the membranes

Since SecA is involved in lipid binding, and functions in the membrane [15], we examined their subcellular localization, Wild-type SecA distributes equally between cytosol and membrane fractions, while SecA51(Ts) mutant protein localizes in favor of the membrane-bound form [31]. When SecA was deleted from N- and/or C-termini, the complementation active derivatives of SecA, $SecA_{22-901}$, $SecA_{22-831}$ and $SecA_{22-829}$, had increased ratio of cytosol form, which was 60–70% of their total proteins (Fig. 3A, data no shown). On the contrary, the complementation inactive $SecA_{22-828}$, had significantly reduced cytosol form, which is about 30% of its total proteins. Similar observation was found in other active and inactive C-terminal truncated SecA mutants, $SecA_{1-828}$, $SecA_{1-826}$ and $SecA_{1-825}$ (Fig. 3A, data not shown). Since SecA functions in the membrane, and inactive SecA ($SecA_{1-825}$ and $SecA_{22-828}$) had more membrane-bound form, we determined whether these forms have normal conformations that are embedded in the membrane and resistant to urea treatment [32]. The membranes containing $SecA_{1-825}$ or $SecA_{1-826}$ were treated with 6 M urea: Urea-washed $SecA_{1-825}$ membrane contained less than 40% of total $SecA_{1-825}$ (Fig. 3B lanes 1 and 2), while more than 90% of $SecA_{1-826}$ remained in the membrane (lanes 3 and 4) under same conditions. These results indicated that most $SecA_{1-826}$ integrated

properly into the membrane [32]. In contrast, $SecA_{1-825}$ only loosely bound to membranes. To substantiate the result, we determined the stability of $SecA_{22-828}$ and $SecA_{22-829}$ on membrane. The membranes of BL21.19/pET5a–$SecA_{22-828}$ and BL21.19/pET5a–$SecA_{22-829}$ labeled with [^{35}S]-Met were chased by increasing amount of respective purified unlabeled $SecA_{22-828}$ and $SecA_{22-829}$. The amount of labeled $SecA_{22-828}$ and $SecA_{22-829}$ on the membranes after chase was quantified (Fig. 3C). Only about 10% of labeled $SecA_{22-829}$ was chased off by 10 μg of unlabeled $SecA_{22-829}$, indicating $SecA_{22-829}$ was stably embedded in the membrane. In contrast, about 70% of $SecA_{22-828}$ was chased out by 10 μg of unlabeled $SecA_{22-828}$, indicating its instability in membranes. These data suggested that the nature of the binding of inactive $SecA_{22-828}$ to the membranes differ significantly to that of its active counterpart $SecA_{22-829}$.

The localization and integration of SecA in the membrane is critical for SecA functions. Membrane is the site where SecA functions. On the other hand, membrane is the site of the limiting biochemical defect in SecA51(Ts) mutant protein [31]. The inactive mutant ($SecA_{22-828}$) with changed conformation is enriched in membranes in cells. The loss of the C-terminal lipid binding site may weaken its interaction with membranes. On the contrary, $SecA_{22-829}$ is active *in vivo* even with lowered ATPase and translocation activity *in vitro*. $SecA_{22-829}$ has slightly reduced membrane-bound form (Fig. 3B) but stably integrated into the membrane (Fig. 3C).

In summary, we define the dispensable regions of N- and/or C-termini SecA that could be deleted without losing complementation activity in the cells. The minimum lengths of functional *E. coli* SecA are 10–826aa and 22–829aa for active complementation. The conformation and stability of SecA in the membrane appear to contribute to the *in vivo* activity.

Acknowledgments

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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.2014.11.061.

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