Escherichia coli SecA truncated at its termini is functional and dimeric

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Abstract Terminal residues in SecA, the dimeric ATPase motor of bacterial preprotein translocase, were proposed to be required for function and dimerization. To test this, we generated truncation mutants of the 901aa long SecA of *Escherichia coli*. We now show that deletions of carboxy-terminal domain (CTD), the extreme CTD of 70 residues, or of the N-terminal nonapeptide or of both, do not compromise protein translocation or viability. Deletion of additional C-terminal residues upstream of CTD compromised function. Functional truncation mutants like SecA9-861 are dimeric, conformationally similar to SecA, fully competent for nucleotide and SecYEG binding and for ATP catalysis. Our data demonstrate that extreme terminal SecA residues are not essential for SecA catalysis and dimerization.

Keywords: SecA; ATPase; Dimerization; Translocase; Protein secretion

1. Introduction

SecA, the bacterial preprotein translocase motor, contains the ATPase core structure (hereafter ''Asp-Glu-Ala-Asp'' or ''DEAD'' motor) of Superfamily 2 helicases [1]. The DEAD motor is built of two sub-domains (nucleotide-binding domain; NBD and intra-molecular regulator of ATPase; IRA2) that form between them a mononucleotide cleft [2–5] (Fig. 1A). Two additional enzymatic specificity domains sprout out of the DEAD motor [3,6]: the Substrate Specificity Domain (SSD) and the C-domain (Fig. 1A). SecA binds to the inner membrane SecYEG channel [7] to form the essential protein translocase core.

Escherichia coli SecA, a homodimer in solution [8,9], folds via a dimeric intermediate [10] and was proposed to function as either a dimer [11] or a monomer [12]. Assignment of the dimerization interface has remained controversial: evidence supporting a role in dimerization for either the C-domain [6,9] or of the N-terminal N68 polypeptide (DEAD motor plus SSD) [13] (Fig. 1A) has been presented. In a *Bacillius subtilis* SecA structure, a dimeric species is formed through crystal contacts between N-terminal residues of one protomer and the C-terminal IRA1 sub-structure [6,14] of another protomer [4]. It was proposed that these contacts are essential for dimerization [12] and that they participate in forming the physiological SecA dimer in solution [15]. However, no such contacts occur in the *Mycobacterium* SecA structure where the dimerization interface is clearly different [5]. It was reported that dimerization of a mutant carrying six altered C-domain residues postulated to participate in dimerization in *B. subtilis* SecA is compromised [12]. However, dimerization is not affected by several other point mutations, deletions and insertions in the same region [6,14].

The extreme C-terminal domain (CTD; Fig. 1A) binds metal ions [16,17], the SecB chaperone and lipids [18] and appears important for the export of some preproteins [18] but not of others [8]. CTD is not present in all SecA proteins and is not required for C-domain binding to the DEAD motor [14].

To resolve the role of the SecA termini, we used truncation mutants to determine whether N-terminal and C-terminal residues of SecA are required for dimerization and function. Our data demonstrate unequivocally that neither the extreme N-terminus nor CTD is essential for SecA catalysis or dimerization during translocation.

2. Materials and methods

2.1. Bacterial strains and recombinant DNA experiments

Escherichia coli strains were grown and manipulated as described in [6]. Truncation mutations were constructed by PCR from pIMBB10, using the common forward primer X157 (5′GGCCCGTACATATGCTAATCAAATTGTTAAC3′) and reverse primers X179 (5′CGGCAGGGATCCTTAATCCTGATGGCTAAGC3′), X107 (5′CGCGGATCCTTAAGGCATACGTACCTGAACTTTG3′) and X111 (5′CGCGGATCCTTACTCAGCACCAACCACTTCTC3′), respectively. Gel-purified, *Nde*I/*Bam*HI-digested products were inserted in pET3a (Invitrogen) giving rise to pIMBB270 (N1–861), pIMBB342 (N1–831) and pIMBB494 (N1–757).

pIMBB269 (N9-901), pIMBB272 (N9-861) and pIMBB501 (N9-610) were constructed by PCR using pIMBB10 as template DNA, common forward primer X178 (5′GGCCCGTACATATGGTTTTCGGTAGTCGTAAC3′) and reverse primers X131(5′CGGCAGGGATCCTTATTGCAGGCGGCCATGGC3′), X179 (5′CGGCAGGGATCCTTAATCCTGATGGCTAAGC3′) and X143 (5′CGCGGATCCTTACAGTTTACGCATGCCGG3′), respectively. Gel-purified, *Nde*I/*Bam*HI-digested products were cloned in pET3a.

2.2. ATP hydrolysis measurements

All assays were in buffer B (50 mM Tris–Cl, pH 8, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mg/ml BSA and 1 mM ATP). SecA or N68 derivatives were added at 0.1 mg/ml. For membrane ATPase,

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Abbreviations: MANT, [2′-(or-3′)-*O*-(*N*-methylanthraniloyl)adenosine 5′-diphosphate]; IMV, inverted inner membrane vesicles

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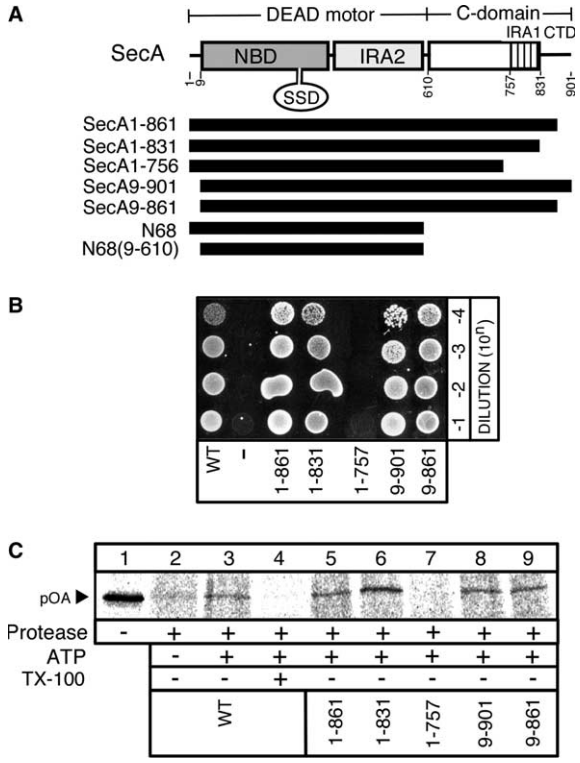

Fig. 1. (A) Map of SecA and derivatives. NBD, IRA1 and 2, SSD and CTD. (B) In vivo function. Genetic complementation of a *secAts* strain by SecA truncation mutants. BL21.19 cells carrying pET5 vector alone or the indicated cloned *secA* derivative were grown at 30 °C (12 h; LB). Cultures were adjusted to the same density (OD_{600} = 0.8). The indicated dilutions were spotted on LB/Ampicillin plates and incubated at 42 °C. (C) In vitro ATP-driven protein translocation of [^{35}S]proOmpA (60.000 cpm/μl) into SecYEG-proteoliposomes (0.2 mg protein/ml) catalyzed by SecA or derivatives. Lane 1: 100% of undigested input [^{35}S]-proOmpA; lane 4 = membranes were dissolved with Triton X-100 (1%) prior to addition of proteinase K (1 mg/ml). Proteins were analyzed by SDS–PAGE and visualized by phosphorimaging.

SecYEG-proteoliposomes (0.2 mg/ml) were also added. For translocation, ATPase proOmpA (20 μg/ml) was further added. Released phosphate was measured using malachite green detection [19].

2.3. Fluorescence measurements

Measurements were carried out in quartz cuvettes (1 ml; Hellma) with a Cary Eclipse fluorimeter supplemented with a Peltier temperature controller (Varian) as described [14]. Briefly, for determination of equilibrium dissociation constants SecA or SecA9-861 were added at increasing concentrations (0.025–5 μM) to MANT-ADP (0.1 M; Molecular Probes) in buffer B. Emission spectra were recorded (380–550 nm; excitation 356 nm; slits at 2.5 and 20 nm) at each step of the binding curve. K_D was determined by plotting the change of emission spectra upon SecA addition against SecA concentration using the equation $(F_1 - F_0)/F_0$ integral values (F_0 = no SecA added, F_1 = SecA added). To determine apparent Tm_{app}, changes in intrinsic tryptophan fluorescence emission of SecA or SecA9-861 (0.25 μM in buffer B) were monitored as a function of increasing temperature (4–82 °C; ramping rate 0.8 °C/min; exc. 297 nm/em. 345 nm; slits at 2.5 and 20 nm; data acquisition interval = 0.5 min), in the presence or absence of ADP (2 mM). Data were analyzed by non-linear regression.

2.4. Chemicals

Nucleotides were from Roche; DNA enzymes from Minotech; oligonucleotides from MWG; [^{35}S]Methionine (1000 Ci/mmol) and chromatography materials (except Ni^{2+} affinity; Qiagen) from Amersham; all other chemicals from Sigma.

2.5. Miscellaneous

Protein purification by metal affinity chromatography, analytical size exclusion chromatography, biochemical assays, preparation of inner membrane vesicles and proteoliposomes from SecYEG-overexpressing cells, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS- and native-PAGE) were as described [2,6,19]. Radioactivity was quantitated by phosphorimaging (Storm 840; Amersham). Ultracentrifugal sedimentation experiments were carried out in a bench-top ultracentrifuge (TLX120 Optima; TLA120.2 rotor; Beckman, USA) using polypropylene tubes (0.2 ml) as described in [20].

3. Results

3.1. In vivo and in vitro protein translocation of SecA truncations

To determine the role of SecA termini, we generated a series of truncation mutants. These remove different lengths of the C-terminus or the N-terminal nonapeptide thought to participate in dimer formation [12] or both (Fig. 1A). The ability of the mutant genes to rescue a *secAts* strain (BL21.19) [21], when carried on a plasmid, was examined at 42 °C. *secA1-861*, *secA1-831*, *secA9-901* and *secA9-861* (Fig. 1B) complemented the *ts* strain as efficiently as *secA*, while *secA1-757* that is missing additionally the complete IRA1 molecular switch [6,14] did not.

To examine their ability to support protein translocation, all mutant derivatives were purified to homogeneity and were added to an in vitro assay (Fig. 1C). With the exception of SecA1-757 that is completely defective (lane 7), all other mutant proteins (lanes 5, 6, 8 and 9) catalyzed efficient ATP-driven proOmpA translocation into SecYEG proteoliposomes similar to that of SecA (lane 3).

We concluded that the CTD domain and the N-terminal nonapeptide of SecA are dispensable for viability and protein translocation, while IRA1 is not.

3.2. SecAΔ9-861 binds SecYEG and hydrolyzes nucleotides

We next sought to compare the behavior of a functional mutant derivative with wild-type SecA in a series of representative biochemical sub-reactions. Since none of the singly or double truncated mutants has any measurable defect in vivo (Fig. 1B) or in vitro (Fig. 1C; data not shown; supplementary material), we chose to focus hereafter on SecA9-861. This is the only truncated SecA derivative that could be successfully crystallized and therefore its mechanism of dimerization can be studied in atomic detail (Papanikolau et al., in preparation).

We first examined the basal, membrane (i.e., SecYEG-stimulated) and translocation (i.e., SecYEG- and preprotein-stimulated) ATPase activity [19] of SecA9-861 (Fig. 2A). All three activities were similar to those of SecA (compare lanes 4–6 to lanes 1–3). To determine binding of the protein to SecYEG-membranes, we used a flotation gradient centrifugation assay (Panel B) [22]. Identical sedimentation behavior was seen between SecA and SecA9-861. Next, the affinity of the protein for nucleotide was determined using the fluorescent ADP analog MANT-ADP [14]. SecA9-861 binds MANT-ADP with high affinity (0.04 μM at 4 °C [Panel C]; 0.14 μM at 37 °C) that is slightly better than that of SecA (0.14 μM at 4 °C [Panel C]; 0.28 μM at 37 °C). Finally, ADP-driven conformational changes in SecA were studied by monitoring intrinsic fluorescence as a function of temperature (Panel D) [14,23]. Both SecA and SecA9-861 apoproteins melt with similar transitions (Tm_{app} of 42.5 and 43, respectively) and become

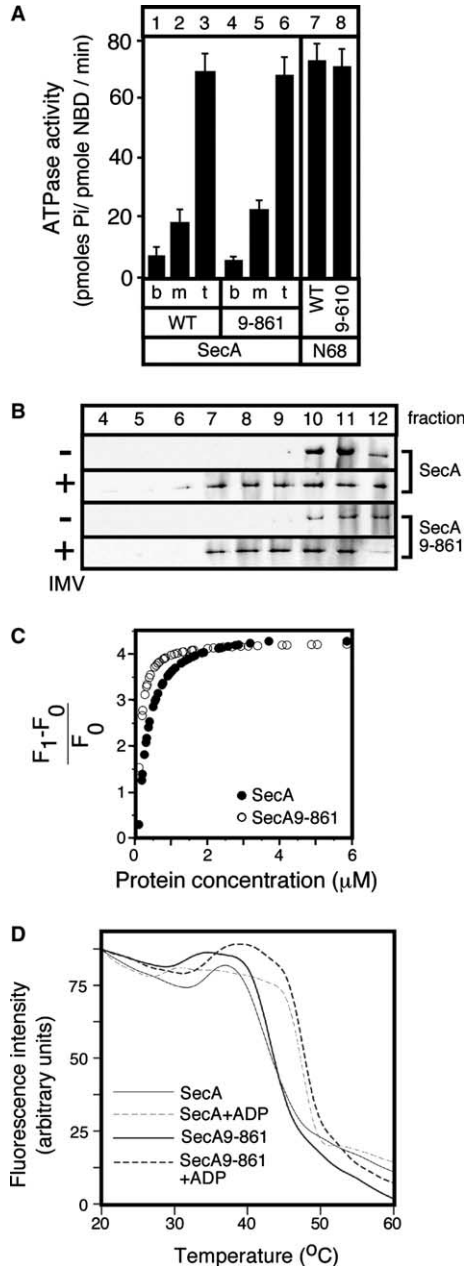

Fig. 2. (A) ATPase activities of SecA, SecA9-861, N68 and N68(9-610). Basal (b), membrane (m), translocation (t) were determined as described in [6,19]. (B) Binding of SecA and SecA9-861 to inverted inner membrane vesicles (IMVs) studied by flotation analysis using ultracentrifugal isopycnic sedimentation. SecA (11 μg) was mixed with IMVs (0.2 mg protein) in Buffer B or with the corresponding volume of buffer alone. Following incubation (4 °C; 5 min), reactions (50 μl) were adjusted to a final sucrose concentration of 1.6 M by addition of 100 μl of 2.4 M sucrose in Buffer B and deposited at the bottom of the centrifugation tube. Samples were overlaid with 3 consecutive layers (150 μl) of 1.4, 1.2 and 0.25 M Sucrose solutions. Following centrifugation (90 min; 436 000 × *g*), fractions (50 μl) were carefully removed, analyzed by SDS–PAGE and visualized by immunostaining with an α-SecA antibody as described in [6]. Only the bottom 9 fractions are shown. (C) Determination of equilibrium dissociation constants of SecA and SecA9-861 (0.025–5 μM in buffer B; 4 °C) for MANT-ADP performed as described [14]. 40–60 data points were used for each curve. The fit to the data, derived by non-linear regression analysis, is presented. (D) Tm_{app} of SecA and SecA9-861 (0.25 μM; buffer B), determined from fluorescence spectra acquired as a function of temperature in the absence or in the presence of ADP (2 mM) as described in [14].

stabilized to similar extents in the presence of ADP (Tm_{app} of 47.8 and 47.5, respectively). To ensure that the catalytic DEAD motor ATPase core is fully functional, N68(9-610) (Fig. 1A) was constructed and purified to homogeneity. The characteristically elevated basal ATPase of N68 (Fig. 2A; lane 7) [6] is as high as that of N68(9-610) (lane 8).

We concluded that in all biochemical sub-reactions tested, SecA9-861 is as active as SecA.

3.3. SecA9-861 and N68(9-610) can oligomerize

The N-terminal nonapeptide of *B. subtilis* SecA was seen in one structure to form contacts with an adjacent molecule in the crystal lattice [4] and was proposed to be an essential dimerization determinant [12]. To test this hypothesis directly, we compared the oligomerization state of SecA9-861 to that of SecA by size-exclusion chromatography (Fig. 3A). SecA chromatographs to a position consistent with an apparent MW of ~215 kDa, while SecA9-861 migrates as a ~190 kDa species. In native-PAGE experiments (Panel B) SecA migrates as a ~220 kDa (lane 1) and SecA9-861 as a ~180 kDa species (lane 2). The observed reduced size of SecA9-861 is attributable to

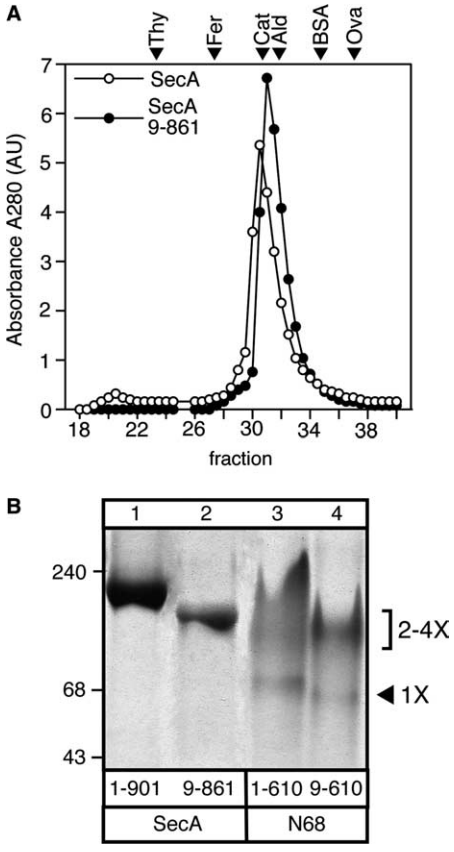

Fig. 3. (A) Size exclusion chromatography of SecA and SecA9-861. Proteins (0.25 mg/ml) were chromatographed on a Superdex 200 10/30 HR prepacked column and monitored online. Indicated MW markers were: thyroglobulin (667 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa) and ovalbumin (45 kDa). (B) Native-PAGE analysis of SecA, SecA9-861, N68 and N68(9-610). Proteins (10 μg) were analyzed by native-PAGE [6] and stained by Coomassie blue. N68 oligomeric species are indicated. MW markers were as in A.

the residues it is missing. Taken together the two hydrodynamic analysis experiments are consistent with a homodimeric organization for both SecA and SecA9-861.

We previously demonstrated that the N-terminal N68 polypeptide can form oligomers in solution at high concentrations [13]. Indeed, N68 analyzed by native-PAGE (Fig. 3B) migrates as a discreet ~68 kDa species (representing the monomer) and a major diffuse species that extent between ~150 and 240 kDa represents dimers and tetramers [13]. The N-terminal nonapeptide does not affect this property, since N68(9-610) (lane 4) migrates similarly to N68 (lane 3) and N68(9-610).

We concluded that removal of CTD and the N-terminal nonapeptide does not affect SecA oligomerization properties.

4. Discussion

We sought to determine a minimal functional core of SecA. Our analysis was achieved by removing sequences from either SecA terminus and characterizing the derived truncated derivatives in vivo and in vitro.

A functional SecA core must include the C-terminal IRA1 helix–turn–helix element (Fig. 1A) that acts as a global regulator [6,14]. SecA1-757 that is devoid of IRA1 is non-functional (Fig. 1B). This is in agreement with data showing that IRA1 is essential for catalysis [6,14]. IRA1 therefore defines the C-terminal essential boundary of the enzyme. CTD that is fused C-terminally to IRA1 and houses a SecB, metal ion and lipid binding site is dispensable (Fig. 1B). Derivatives that are devoid of CTD are fully functional in vivo and in vitro under our assay conditions and form dimeric proteins (Fig. 3A) [8,9,11,24]. This is in agreement with the notion that CTD is not conserved in all SecA proteins. However, we cannot rule out the possibility that under more stringent conditions CTD may be crucial for optimal catalysis [18].

At the N-terminal end, we only characterized the 1-9 aa deletion. Further deletions give rise to unstable proteins (data not shown) [25]. This region is surface exposed in the SecA structures [4,5,26] and was proposed to be important for an interaction with SecG [27]. In our experiments, this nonapeptide is clearly dispensable for function (Figs. 1B and 2). It was also proposed that this region may be essential to form the dimerization of *B. subtilis* SecA [12]. However, in *E. coli* SecA we failed to detect such a role, since in either size exclusion or native-PAGE experiments the absence of the nonapeptide did not affect SecA dimerization (Fig. 3A and B). We cannot exclude the possibility that there is variability between SecA from different bacteria. However, given the degree of genetic and biochemical conservation we think this possibility less likely. Rather, we opine that the dimerization core of SecA involves regions of the DEAD motor that do not include the extreme N-terminus. This is supported by our finding that N68 forms higher order assemblies [13] in the presence or absence of this region (Fig. 3B). In full agreement with this, SecA9-861 described here has been crystallized as a dimer (Papanikolau et al., in preparation).

Minimal enzyme cores define the bare essentials for catalysis. Constructs described here are invaluable tools for deciphering the structure of *E. coli* SecA, the essential features of the SecA nanomachine, its membrane topology and how it interacts with preproteins and the SecYEG "pore".

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version at doi:10.1016/j.febslet.2005.01.025.

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