THE BACTERIAL ATPASE SECA FUNCTIONS AS A MONOMER IN PROTEIN TRANSLOCATION

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The ATPase SecA drives the post-translational translocation of proteins through the SecY channel in the bacterial inner membrane. SecA is a dimer which can dissociate into monomers under certain conditions. To address the functional importance of the monomeric state, we generated an E. coli SecA mutant that is almost completely monomeric (>99%), consistent with predictions from the crystal structure of B. subtilis SecA. In vitro, the monomeric derivative retained significant activity in various assays and in vivo, it sustained 85% of the growth rate of wild type cells and reduced the accumulation of precursor proteins in the cytoplasm. Disulfide cross-linking in intact cells showed that mutant SecA is monomeric and that even its parental dimeric form is dissociated. Our results suggest that SecA functions as a monomer during protein translocation in vivo.

Many bacterial proteins are transported post-translationally across the inner membrane by the Sec machinery, which consists of two essential components (1-4). One is the SecY complex, which forms a conserved heterotrimeric protein-conducting channel in the inner membrane (5,6). The other is SecA, a cytoplasmic ATPase, which “pushes” substrate polypeptide chains through the SecY channel (7). SecA interacts not only with the SecY channel (8), but also with acidic phospholipids (9-11) and with both the signal sequence and the mature part of a substrate protein (12). It also binds the chaperone SecB, which ushers some precursor proteins to SecA (8,13,14). When associated with the SecY complex, SecA undergoes repeated cycles of ATP-dependent conformational changes, which are linked to the movement of successive segments of a polypeptide chain through the channel (15,16). However the mechanism employed by SecA to translocate substrates polypeptide chains through the SecY channel remains largely unknown.

An important issue concerning the function of SecA is its oligomeric state during translocation. SecA is a dimer in solution (17,18) and previous work argued that this is its functional state (19). An X-ray structure of B. subtilis SecA also indicates the existence of a dimer (7). However, recent evidence raises the possibility that SecA might actually function as a monomer: In solution, SecA dimers are in rapid equilibrium with monomers (20,21). Although the equilibrium favors dimers, it is shifted almost completely towards monomers in the presence of membranes containing acidic phospholipids or upon binding to the SecY complex (21). A synthetic signal peptide had a similar effect, although this result is controversial (22). A monomeric derivative of SecA containing six point mutations retained some in vitro translocation activity (21), but the low level of translocation precluded any firm conclusion. In addition, the previous results do not exclude models in which SecA cycles between monomeric and oligomeric states during the translocation of a polypeptide chain (22,23). Most importantly, the functional oligomeric state of SecA in vivo remains to be established.

In this report, we have tested whether SecA can function as a monomer in vivo. Guided by the X-ray structure of B. subtilis SecA (7), we generated a monomeric derivative of E. coli SecA that lacks the first eleven amino acids. The monomeric derivative retains significant activity in various in vitro assays and can substitute for
endogenous SecA in vivo. Cross-linking experiments in intact cells indicate that even the parental dimeric derivative is mostly present as a monomer. Our results therefore suggest that SecA functions as a monomer.

**MATERIALS AND METHODS**

_Bacterial strains and plasmids_ – Table 1 lists the bacterial strains and plasmids used in this work. The segment containing the arabinose promoter and the AraC gene on pBAD22 was amplified by PCR using the primers BclIBAD5pr (gtacgatgattctgacaataaagacac) and SecABAD3pr (gtgcgtgatcgttacgctcagaaactttagtttaacattt gatgattctgacaatttttgctgaaatgg). The kanamycin resistance gene was amplified from pBAD18 using BglIIKan3pr (gcttagatctgcctcgtgaagaaggtgttgctgac) and SecMKan5pr (gtgcgaacgctgttttcttaagcacttttcgcacaactttcattt gaagggttgaattaacatttt). The PCR-amplified fragments were digested with either BclI (araC-PBad) or BglII (kan) and ligated to each other. The ligation mixture was digested with BglII and BglIII and the product fragment was amplified by PCR using SecMKan5pr and SecABAD3pr. The amplified linear cassette [SecM(-50--1)-kan-araC-PBad-SecA(1-50)] was used to replace the chromosomal region between SecM(+1) to SecA(-1) by homologous recombination using strain DY378 as described (24). The resulting strain, EO527, requires arabinose for growth. Strain EO528 was generated by P1 transduction of the _prlA4_ allele into strain EO527. The presence of the _prlA4_ mutation was confirmed by sequencing. Strain EO529 was generated by P1 transduction of _kan-araC-PBad-SecA_ from EO527 into strain SMG96. Δ11/N95 was constructed from pT7N95-SecA (21) by PCR-based deletion of residues Leu2 to Gly11. N95 and Δ11/N95 were amplified by PCR and cloned between the Neol and BamHI sites of pDSW204 (25) generating pDSW204N95 and pDSW204Δ11/N95, respectively. Δ11/N95 was also cloned between the BamHI and NotI sites of pET21 (Novagene). The mutations S636C and Q801C were introduced into pT7N95-SecA(C98S) using PCR-based site directed mutagenesis (Invitrogen) and confirmed by sequencing. The double-cysteine construct, N95(CC), was used to generate Δ11/N95(CC) by PCR-based deletion. Both constructs were then amplified by PCR and cloned between the Neol and BamHI site of pDSW204 yielding plasmids pDSW204N95CC and pDSW204Δ11/N95CC, respectively.

**Over-expression and purification of proteins** - Expression and purification of SecYE _His6G_, SecY(prlA4) _EHis6G_, SecA and its derivatives and were done as described (21,26). Δ11/N95 was additionally purified by Superdex200 GF. ProOmpA was expressed and purified from inclusion bodies as described(27).

**Cross-linking and sucrose gradients** - SecA and derivatives in buffer (50 mM K-HEPES pH 7.5, 100 mM KCl, 4 mM MgCl2, 1mM DTT) were cross-linked with 20 mM EDC 1 or analyzed by sucrose gradient centrifugation as described (21).

**In vitro assays** - SecA and Δ11/N95 were labeled with 125Iodine using Iodogen (Pierce) as described (15). Liposomes containing reconstituted wild type SecY or SecY(prlA4) complexes were prepared as described (26). Proteoliposomes binding assays were done according to (8) as detailed by (21). ATPase and translocation assays were carried out at 30°C and 37°C, respectively, as described (21). To follow the kinetics of translocation 40 μl aliquots were withdrawn from a master mixture (380 μl) at different times, and mixed with 160 μl ice-cold 75 mM KCl, 50 mM K-HEPES pH 7.5. Samples were then processed as usual.

**Disulfide cross-linking of SecA derivatives** - Strains EO529 and EO527 expressing N95(CC) from pDSW204N95(CC) were grown in LB containing ampicillin (100 μg/ml) at 30°C for five hours. Where indicated, 150 μM IPTG was added after 3.5 hours. EO529 expressing Δ11/N95(CC) from pDSW204Δ11/N95(CC) were grown in LB containing ampicillin (100 μg/ml) at 30°C for five hours. Where indicated, 150 μM IPTG was added after 3.5 hours. EO529 expressing Δ11/N95(CC) from pDSW204Δ11/N95(CC) were grown in LB containing ampicillin (100 μg/ml) at 30°C for five hours. Where indicated, 150 μM IPTG was added after 3.5 hours. EO529 expressing Δ11/N95(CC) from pDSW204Δ11/N95(CC) were grown in LB containing ampicillin (100 μg/ml) at 30°C for five hours. Where indicated, 150 μM IPTG was added after 3.5 hours. EO529 expressing Δ11/N95(CC) from pDSW204Δ11/N95(CC) were grown in LB containing ampicillin (100 μg/ml) at 30°C for five hours. Where indicated, 150 μM IPTG was added after 3.5 hours. EO529 expressing Δ11/N95(CC) from pDSW204Δ11/N95(CC) were grown in LB containing ampicillin (100 μg/ml) at 30°C for five hours. Where indicated, 150 μM IPTG was added after 3.5 hours. EO529 expressing Δ11/N95(CC) from pDSW204Δ11/N95(CC) were grown in LB containing ampicillin (100 μg/ml) at 30°C for five hours. Where indicated, 150 μM IPTG was added after 3.5 hours.
centrifuged again. Pellets were air-dried and dissolved with 150 μl 20 mM iodoacetamide, 1% SDS, 0.1 M Tris-Cl pH 8.0. After one hour at 22°C 40 μl 6% SDS, 50% glycerol, 0.1% bromophenol-blue were added. In vitro cross-linking of SecA derivatives in buffer (50 mM K-HEPES pH 7.5, 50 mM NaCl, 4 mM MgCl2) was done with 40 μM diamide for 15 minutes at 22°C. The reaction was stopped with 30 mM iodoacetamide, 1% SDS, 50 mM Tris-Cl pH 8.0 and after one hour 12 μl 6% SDS, 50% glycerol, 0.1% bromophenol-blue were added.

In vivo pulse-chase labeling of proOmpA - Cells were grown at 30°C in minimal glycerol medium (M63 salts with 0.5% glycerol, vitamin B1 μg/ml, vitamin B5 μg/ml, 1 mM MgSO4, 18 amino acids, each at 50 μg/ml, and 0.005% yeast extract). Early log phase cells (7.5 ml) were pulsed with 0.1 mCi of [35S]methionine for 20 seconds and chase was initiated by adding 750 μl of 1% methionine, chloramphenicol 1 mg/ml. Samples (1 ml) were removed at the indicated times and precipitated with TCA as described (28). Acetone washed pellets were solubilized with 25 mM Tris-Cl, 1 mM EDTA, 1% SDS and OmpA was immunoprecipitated using OmpA antibodies and protein A.

RESULTS

Generation of a monomeric derivative of SecA. We previously generated a monomeric SecA derivative by mutagenizing into alanines six residues that we suspected to be important for dimerization (21). The X-ray structure of B. subtilis SecA (7) later showed that four of the six residues were indeed close to the interface between the monomers. However, the derivative exhibited low translocation activity in vitro, probably because the specific mutations had additional effects. The X-ray structure of B. subtilis SecA now offers a more rational design of a monomeric derivative: In the crystal, SecA is a dimer with the monomers arranged head-to-tail (7). Most of the intersubunit contacts are contributed by the first nine residues of each subunit (Met1-Phe9), which contact side chains in the C-terminal domain of the other subunit (Fig. 1). To test whether these residues are crucial for dimer stability, we deleted the corresponding sequence (L2IKLLTKVFG11) from N95, an E. coli SecA derivative that lacks the last 70 residues. We used N95 as a starting point, because it is shorter than SecA and yet is fully dimeric and functional (21,29,30). Also, the last 70 residues contribute slightly to dimer formation. After sucrose gradient centrifugation the parental N95 dimer (189kDa) migrated in fractions 15-17, close to full-length SecA (fraction 18, 204kDa; Fig. 2A). In contrast, the derivative Δ11/N95, lacking residues Leu2-Gly11, migrated in fractions 11-13 close to the position of BSA (fraction 9, 70kDa) and consistent with it being monomeric. Quantification of the experiment showed that at most 1% of Δ11/N95 exists as dimers. Cross-linking experiments using EDC (17,21) supported the conclusion that Δ11/N95 is monomeric (Fig. 3E).

The monomeric derivative Δ11/N95 is active in vitro. Next we compared the activity of Δ11/N95 with that of wild type, full-length SecA in several in vitro assays. We first measured their binding affinity to the SecY channel. Increasing amounts of SecA or Δ11/N95 were mixed with a constant amount of [125I]SecA and incubated with proteoliposomes containing the SecY complex (Fig. 3A). The amount of unlabeled competitor was increased, the level of [125I]SecA bound to reconstituted SecY decreased. A binding constant of 224±19 nM was calculated for wild type SecA (Fig.3A, circles), in close agreement with previous data (31). The monomeric derivative, Δ11/N95, had an only two-fold lower affinity of 447±32 nM (triangles). About 28% of the bound [125I]SecA could not be competed away by Δ11/N95, perhaps because the N-terminal residues of SecA have a secondary binding site at SecY.

Previous experiments had shown that the prlA4 signal sequence suppressor mutation in SecY (32) enhances the activity of a monomeric derivative of SecA approximately five-fold (21). We therefore tested proteoliposomes containing the mutant SecY complex for its ability to bind D11N95. The affinities for SecA and Δ11/N95 were a little higher than with wild type SecY (173±12 nM and 305±23 nM, respectively), and again, the monomeric SecA derivative,
Δ11/N95, had only a slightly reduced affinity for the SecY complex. We next compared the translocation ATPase activity of SecA and Δ11/N95 under conditions where the ATPase activity is proportional to the protein concentration (Fig. 3B). SecA and Δ11/N95 exhibited the same low basal ATPase activity (~6.5 mol ATP/mol protein/min; Fig. 3B, closed and open triangles, respectively). Addition of both SecY complex-containing proteoliposomes and translocation substrate (proOmpA), stimulated the ATPase activity of SecA and of Δ11/N95 by a factor of 16 and 10, respectively (closed and open circles, respectively). With SecY(prlA4) proteoliposomes the factor of stimulation was 13 and 12, respectively (closed and open squares). Thus, the monomeric derivative has almost the same level of translocation ATPase activity as wild type SecA.

To compare the translocation activities of SecA and Δ11/N95, they were incubated with SecY-complex containing proteoliposomes, ATP, and [35S-Met]-proOmpA. Translocated proOmpA was detected by its resistance to protease treatment (Fig. 3C). We found that Δ11/N95 translocated 16% of the amount of proOmpA compared to wild type SecA (Fig. 3C, ‘SecY’). With SecY(prlA4) complex-containing proteoliposomes, the translocation efficiency of Δ11/N95 increased to 74% (Fig. 3C, ‘prlA4’). The kinetics of translocation was almost the same for SecA and Δ11/N95 (Fig. 3D; see inset for raw data). As expected, no protease-protected proOmpA was seen in the absence of ATP, or when Triton X-100 was added during proteolysis (Fig. 3D, inset). Together, these results show that the Δ11/N95 monomer retains significant activity in several in vitro assays.

To exclude the possibility that Δ11/N95 dimerizes when bound to the SecY(prlA4) complex, we performed cross-linking experiments with the carbodiimide EDC. Wild type SecA on its own showed strong dimer crosslinks, and these were reduced upon addition of proteoliposomes containing SecY(prlA4) complex (Fig. 3E, lane 1), in agreement with previous experiments showing dissociation of wild type SecA under these conditions (Or et al., 2002). Δ11/N95 did not give rise to dimer crosslinks in the absence or presence of proteoliposomes (Fig. 3E, lanes 3,4), indicating that it remains monomeric when bound to SecY(prlA4).

The monomeric derivative Δ11/N95 supports cell growth. To test the activity of Δ11/N95 in vivo, we constructed E. coli strain EO527, in which the 5’ regulatory region of the chromosomal SecA gene (~300 bp) was replaced with the tightly regulated arabinose promoter. This promoter is active in the presence of arabinose, but is turned off completely in the presence of glucose. Indeed strain EO527 exhibited robust growth in arabinose (doubling time of 45 min), but upon a switch to glucose, the cells stopped growing after four hours (Fig. 4A). This was paralleled by the expression of SecA: while in the presence of arabinose SecA was expressed at similar levels throughout the experiment (Fig. 4B, ‘+Arabinose’), its expression dropped to negligible levels four hours after the switch to glucose (‘+Glucose’). These data are consistent with SecA being essential for viability of E. coli(33).

Next we tested whether Δ11/N95 can replace SecA and support growth of EO527 cells, when expression of chromosomal SecA is shut down. N95 and Δ11/N95 were cloned into the plasmid pDSW204 under the control of an attenuated IPTG-driven promoter. The two plasmids and, as a control, the empty vector were each introduced into EO527 cells. It should be noted that the deletion of the sequence coding for Leu2-Gly11 also removes a segment, which plays a key role in translation initiation (34), resulting in much lower expression levels of the Δ11/N95 construct. As expected, all strains grew on plates containing arabinose owing to the expression of chromosomal SecA (Fig. 4C, left plate, lower half). On glucose plates lacking IPTG, only the N95-expressing construct gave viable cells (middle plate, lower half). As expected, the incomplete repression of the promoter by the Lac repressor allowed N95 to be made at levels sufficient for growth. Immunoblotting showed that even in the absence of IPTG, N95 was still made in significant amounts (Supplementary Fig. S1). The Δ11/N95 construct did not support growth in the absence...
of IPTG, consistent with its negligible basal expression level (see Fig. 5D). In the presence of IPTG, both N95 and Δ11/N95 supported growth. Immunoblots showed that, in the presence of arabinose, the full-length SecA protein was synthesized from the chromosomal gene, while in the presence of IPTG and absence of arabinose, only the plasmid encoded Δ11/N95 protein was made (Fig. 4D, lane 2 versus 1). These data show that Δ11/N95 is responsible for cell growth under these conditions.

We performed similar experiments using strain EO528, which differs from EO527 by harboring the prlA4 mutation in its chromosomal SecY gene (Fig. 4C, upper halves of the plates). Again, Δ11/N95 supported cell growth only in the presence of IPTG (compare right and middle plates). Interestingly, N95 supported growth in the absence, but not in the presence of IPTG. This is explained by the massive overexpression of N95 in the presence of IPTG, which inhibits growth of strain EO528 (Supplementary Fig. S1).

To compare the efficiency of Δ11/N95 with wild type SecA in a quantitative manner, we determined the growth rates of cells expressing similar levels of either protein. In the presence of arabinose, EO527 cells had a growth rate that approached that of the parental strain DY378 (Fig. 5A). In the absence of arabinose and presence of IPTG, plasmid-born Δ11/N95 supported a growth rate that reached 85% of that of the parental strain DY378 (Fig. 5B). Immunoblotting for SecA showed that after induction, the levels of SecA and Δ11/N95 were about equal (Figs. 5C versus 5D). These data therefore indicate that the monomeric derivative is almost as efficient as wild type SecA in supporting cell growth. Similar results were obtained with strain EO528, which has a SecY(prlA4) background (Figs. 5E-H). Again, comparable growth rates corresponded to similar expression levels of SecA and Δ11/N95.

Finally, we tested whether Δ11/N95 can prevent the steady-state accumulation of precursor proteins with uncleaved signal sequences, which is observed under SecA deficiency. When EO527 cells were depleted of SecA by incubation in the absence of arabinose, precursors to DegP, MBP and OmpA were prominently present, as demonstrated by immunoblotting with specific antibodies (Fig. 6A, lane 1). In contrast, when wild type SecA was expressed in the presence of arabinose, only the mature forms were seen (lane 2). When Δ11/N95 was expressed, the accumulation of precursors was significantly diminished though not entirely eliminated (lanes 3 versus 1). With strain EO528, having a SecY(prlA4) background, precursor accumulation was further reduced (lanes 4-6): with MBP and OmpA the monomeric derivative was as effective as wild type SecA, and with DegP it was even more efficient (lanes 6 versus 5). Similar conclusions could be drawn from ‘pulse-chase’ experiments in which the processing of proOmpA into mature OmpA was followed (Fig. 6B). In EO527 cells expressing wild type SecA in the presence of arabinose, proOmpA was processed into mature OmpA within less than a minute (‘SecA’). In the absence of arabinose, even after 10 minutes only 50% of proOmpA was processed (‘none’). When Δ11/N95 was expressed, 50% of the labeled proOmpA was processed within one minute (‘Δ11/N95’). In conclusion, the monomeric SecA derivative clearly supports translocation in vivo, albeit less efficiently than wild type SecA.

Probing the oligomeric state in vivo by disulfide cross-linking. To test the oligomeric state of the SecA derivatives in vivo, we introduced cysteines at defined positions and tested their ability to form disulfide bridged SecA-dimers. Guided by the X-ray structure of the B. subtilis SecA dimer (7), we identified Gly587 and Arg750 as residues in close proximity across the subunit interface (Fig. 1). The equivalent residues of E. coli (SecA)N95, Ser636 and Gln801, were changed to cysteines generating the single cysteine mutants and the double mutant (N95(CC)). We first tested these mutants in vitro for their ability to form disulfide-bridged dimers. Guided by the X-ray structure of the B. subtilis SecA dimer (7), we identified Gly587 and Arg750 as residues in close proximity across the subunit interface (Fig. 1). The equivalent residues of E. coli (SecA)N95, Ser636 and Gln801, were changed to cysteines generating the single cysteine mutants and the double mutant (N95(CC)). We first tested these mutants in vitro for their ability to form disulfide-bridged dimers. The purified mutant proteins were treated with the oxidizing reagent diamide (35,36), and separated on a non-reducing SDS gel (Fig. 7A). The double mutant N95(CC) showed two main crosslinked products in the molecular weight region where dimers are expected (lane 2). In other studies crosslinked products containing the same components in
different linkages also had different mobilities in SDS gels (37,38). Several weaker bands were seen in the low molecular weight region, perhaps caused by crosslinking to contaminating proteolytic N95 fragments. The two dimer crosslinks were also seen when the single cysteine mutant proteins were mixed together before oxidation (lane 3). This is in agreement with previous experiments showing that SecA dimers are in equilibrium with monomers (Or et al., 2002). In addition, this experiment shows that both dimer crosslinks contain a single disulfide bridge. The single-cysteine mutant Ser636Cys did not give crosslinks (lane 4), as expected from the X-ray structure. Surprisingly, however, the single cysteine mutant Gln801Cys gave a strong crosslinked band (lane 5), even though in the X-ray structure residues 801 in the two subunits are quite distant (Fig. 1). This band corresponds to the lower crosslinked band seen with the double mutant (lane 5 versus lane 2). Thus, there seem to be two different conformations of the dimer, one corresponding to the X-ray structure, which gives rise to the upper crosslinked band, and one in which residues Gln801 in the two subunit come close, which gives rise to the lower band. All crosslinked bands disappeared when the samples were reduced (lanes 7, 8), indicating that they are indeed formed by disulfide bridges.

Next we performed crosslinking experiments in vivo. Since the cytoplasm of wild type cells is reducing and thus does not favor disulfide bond formation, we used strain EO529, which contains an oxidizing cytoplasm, owing to the absence of TrxB and Gor, two key enzymes in the thiol reducing pathway (36). N95(CC) was functional, and supported growth of EO529 cells in the absence of arabinose when expressed from a plasmid. To test whether N95(CC) can form dimers when highly over-expressed, cells were induced with IPTG and precipitated with TCA. The proteins were treated with iodoacetamide to block free cysteines, and separated on non-reducing SDS gels. Blotting with SecA antibodies revealed the monomer and, in addition, three high molecular weight bands in the 200kDa region (Fig. 7B, lane 3 ‘Dimers’). A similar pattern of three high molecular weight bands (21) was seen when purified N95 was treated with the cross-linking agent EDC (lane 12). Two of the bands were at the same position as the crosslinked products generated in vitro (Fig. 7A, lane 1 versus lane 2). The third upper band may be a dimer with two Cys636-Cys801 disulfide bridges. When IPTG-induced cells were pre-treated with diamide, the intensity of all three bands was enhanced (Fig. 7B, lane 4). As expected, the bands disappeared when the samples were reduced with DTT (lane 11). These data therefore suggest that over-expressed N95(CC) forms disulfide-linked dimers in vivo. The appearance of the cross-linked dimers was dependent on an oxidizing environment in the cytoplasm: they were not observed in EO527 cells with a reducing cytoplasm, unless diamide was added (lane 10 versus 9). The N95(CC) protein did not form dimers when expressed at lower levels in the absence of IPTG in either strain EO529 or EO527 (lanes 1 and 7), even though more material was loaded onto the gel to compensate for the lower expression level. Diamide treatment produced weak cross-links in strain EO527 (lane 8) but not in EO529 cells (lane 2). Thus, even the N95(CC) protein with an intact N-terminus is largely monomeric when expressed at basal levels in vivo. As expected, the derivative Δ11/N95(CC) did not give rise to disulfide-linked dimers in EO529 cells, even when diamide was added (Fig. 7B lanes 5 and 6). These data therefore support our assumption that this protein is also monomeric in vivo. It should be noted that deletion of the N-terminus of N95 abolished the crosslinks corresponding to both dimer conformations. In addition, whenever the dimer cross-links were diminished or absent (lanes 1, 2, 5 - 8), other prominent cross-links were seen (some are marked in lane 6 by asterisks); apparently, the monomeric form of SecA can interact with additional proteins in the cell.

DISCUSSION

Our results suggest that SecA functions as a monomer in protein translocation. Using sucrose gradient centrifugation and chemical cross-linking, we show that a SecA derivative, Δ11/N95, lacking the first 11 residues is fully monomeric. It remains monomeric in intact cells, as demonstrated by in vivo disulfide cross-
linking experiments. Δ11/N95 is functional in vitro, as demonstrated by its high affinity for the SecY complex, its translocation ATPase activity, and its ability to translocate proOmpA into reconstituted proteoliposomes. More importantly, we show that the monomeric derivative is active in vivo, supporting growth of the cells at ~85% the rate of wild type cells. Our in vivo results extend previous in vitro experiments that showed dissociation of SecA dimers in the presence of membranes (21,22) and provide strong evidence against the notion that SecA functions as a dimer (19). Interestingly, we found that even the parental N95 derivative, which contains the 11 N-terminal residues, is monomeric in cells unless overexpressed. Thus, the equilibrium between dimers and monomers, which in purified N95 preparations is on the side of dimers, appears to be shifted towards monomers under the conditions in vivo. Given that the total concentration of SecA in E. coli cells is about 5 μM and that the dissociation constant of the dimer is 0.3-0.5 μM, one would have expected SecA to be mostly dimeric. Possible explanations for the discrepancy are that the effective concentration of SecA is lowered by its interaction with other cellular components or membranes (39,40), or that the dissociation constant is higher at the ionic conditions in the cell (20,41). Full-length SecA dimers may dissociate less readily than the N95 dimers because the C-terminal 70 residues seem to contribute slightly to the interaction between subunits7, but our results clearly show that the translocation mechanism of SecA does not require the presence of dimers.

The deletion of the first 11 residues from the parental SecA derivative N95 was not entirely without effect on its translocation activity. In vitro, it was significantly less efficient in the translocation of proOmpA into proteoliposomes (16% compared with wild type SecA), perhaps because it dissociates more readily from the SecY channel5. However, when tested with mutant SecY(prlA4), which binds SecA with higher affinity (42), its efficiency reached 74%. In vivo, the monomeric derivative supported a growth rate close to that of wild type SecA, both in wild type and in SecY(prlA4) cells, although some precursor accumulation was observed. Thus, the N-terminal residues appear to be less important in vivo, perhaps due to the presence of other factors.

Our conclusion that the monomeric state of SecA is the active species is consistent with a new X-ray structure of B. subtilis SecA in which the protein crystallized as a monomer (43). Compared with the structure of the SecA dimer (7) the monomeric structure shows a drastic conformational change, likely corresponding to the opening of the peptide binding groove (43). Chemical modification and cross-linking experiments show that conditions that lead to the dissociation of the dimer also result in opening of the groove. An active SecA monomer is also consistent with the demonstration that a detergent-solubilized translocation intermediate contains only one copy of SecA (44).

The ATPase domain of SecA is structurally-related to super-family I and II helicases (7). One of them, PcrA, functions like SecA as a monomer and is proposed to use an “inchworm” mechanism to move along a single-stranded nucleic acid strand (45); the two RecA-like nucleotide binding folds move relative to each other during the nucleotide hydrolysis cycle. SecA might undergo similar conformational changes with its nucleotide binding folds while bound to the SecY complex, thereby moving the polypeptide chain through the channel.

The reason why SecA can form dimers and even higher oligomers (17,22,46,47) is unclear. Our results make it unlikely that dimerization is an obligatory step during the translocation cycle. It is even possible is that the dimer would never occur under physiological conditions. Alternatively, however, the dimer may be generated in certain situations, for example, when secretion is blocked and SecA is upregulated (48,49). Dimerization could prevent SecA from interacting with unfolded cytoplasmic proteins. In this scenario, the dimer would only dissociate upon binding to the membrane in preparation for protein translocation.

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FOOTNOTES

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1 Abbreviations used: EDC, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide; IPTG, Isopropyl β-D-thiogalactoside; TCA, Trichloroacetic acid; OmpA, outer membrane protein A; MBP, maltose binding protein.

2 Data not shown.
Data not shown.

The activity of N95 in these assays was the same as that of full-length SecA; data not shown.

These crosslinks do not, however, react with anti-SecY antibodies; data not shown.

Data not shown.

Data not shown.

E.O and T.A.R.- unpublished results.

**FIGURE LEGENDS**

**Figure 1.** Intersubunit contacts within the SecA dimer of B. subtilis. The two subunits in the crystal structure of B. subtilis SecA (7), considered to represent the physiological dimer, are colored green and cyan. The first nine residues of each subunit are shown in yellow and blue, respectively. Glycine 587 and Arginine 750 are colored red. Figures were generated with Molscript (50) and Raster3D (51).

**Figure 2.** Deletion of the 11 N-terminal residues converts dimeric N95(SecA) into a monomer. Purified N95(SecA) and Δ11/N95 (90 μg each) were subjected to sucrose gradient centrifugation. Twenty-four 520 μl fractions were collected and 60 μl-aliquots from fractions 1-20 were analyzed by SDS-PAGE followed by Coomassie blue staining. The arrows point to the peak positions of ‘SecA’ and ‘BSA’ analyzed under the same conditions.

**Figure 3.** Δ11/N95 retains significant activity in vitro. A, Binding of [125I]SecA (5 nM, 38,000 cpm) to proteoliposomes containing reconstituted SecY complex was performed in the presence of increasing concentrations of unlabeled SecA or Δ11/N95. Binding of [125I]SecA in the absence of a competitor protein was taken as 100%. The curves are the best fit to the equation: Bound (as % of initial) = (100 - Offset) * K_i/(K_i + [I]) + Offset. B, ATPase activity of SecA (filled symbols) and of Δ11/N95 (open symbols) was measured in presence of proteoliposomes containing reconstituted SecY complex (circles) or reconstituted SecY(prlA4) complex (squares) or in the absence of any liposomes (triangles). The concentrations of SecA and Δ11/N95 were 0.25 μM. C, proOmpA (0.25 μg), spiked with [35S-Met]proOmpA, was incubated for 15 minutes with SecA or with Δ11/N95 in the presence of proteoliposomes containing either wild type SecY or SecY(prlA4) complex. The numbers below are the fraction of total proOmpA that was translocated and protected from protease. D, proOmpA was translocated by SecA or Δ11/N95 into proteoliposomes containing SecY(prlA4) complex. Raw data (inset) were quantified by phosphor-imaging and normalized to the value obtained by SecA after 12 minutes. ‘-ATP’- 15 minutes incubation without ATP. ‘TX’- Triton X-100 present during proteinase K treatment. E, Δ11/N95 or SecA were treated with EDC in the presence or absence of 2 μl proteoliposomes containing reconstituted SecY(prlA4). Samples containing 0.5 μg were resolved on a 5% gel and immunoblotted with antibodies against SecA.

**Figure 4.** Δ11/N95 can replace SecA in supporting the growth of bacteria. A, Exponentially growing EO527 cells were diluted 20-fold into LB containing either 2 mM arabinose (filled circles) or 2 mM glucose (open circles) and grown at 30°C for three hours, diluted again and grown for additional three hours. Cell growth was monitored at the indicated times by measuring absorbance at 600 nm. B, Samples from each time point in ‘B’ were resolved by SDS-PAGE and subjected to immunoblotting with antibodies against SecA. Samples correspond to 50 μl culture of O.D.600=0.2. C, EO527 or EO528 cells harboring the empty plasmid pDSW204 (‘-’) or expressing either ‘N95’ or ‘Δ11/N95’ from the IPTG-inducible trc-promoter, were streaked on LB plates containing either 2mM arabinose, 2mM glucose or 2mM glucose plus 100 μM IPTG and incubated at 30°C for 36 hours. The plates also contained 50 μM MnCl2, 200 μM CuCl2, 15 μg/ml kanamycin and 100 μg/ml ampicillin. D, ‘IPTG’- EO527 cells
expressing Δ11/N95 in the presence of 150 μM IPTG. ‘Ara’- EO527 cells expressing wild type SecA in the presence of 2mM arabinose. Samples corresponding to 200 μl culture of O.D. 600=0.5 were resolved on a 5% gel and immunoblotted with antibodies against SecA.

Figure 5. SecA and Δ11/N95 expressed at similar levels support comparable growth rates. A, Strain EO527 containing the SecA gene under the arabinose promoter was grown either in the absence or presence of 100 μM arabinose. The parental strain, DY378, was grown in parallel as a reference. Cells were grown in LB at 30ºC for three hours and then diluted into fresh media to O.D. 600=0.07. Growth was continued for three hours and monitored at the indicated time points by measuring absorbance at 600 nm. B, Strain EO527 expressing Δ11/N95 was grown in the absence or presence of 150 μM IPTG. Growth was monitored as in ‘A’. C, Samples from each time point of ‘A’, corresponding to 80 μl culture of O.D.600=0.2, were resolved by SDS-PAGE and immunoblotted with SecA antibodies. D, Samples of each time point of ‘B’ were analyzed by immunoblotting with SecA antibodies as in ‘C’. E-H, As in ‘A’-‘D’, but with strain EO528 instead of EO527.

Figure 6. Δ11/N95 reduces accumulation of precursor proteins. A, EO527 or EO528 cells having wild type SecY or SecY(prlA4) background, respectively, were incubated in the absence (none) or presence (SecA) of arabinose for five hours. In parallel, cells expressing Δ11/N95 from a plasmid were grown in the presence of 150 μM IPTG for five hours (Δ11/N95). Samples corresponding to 25 μl culture of O.D.600=0.2 were resolved by SDS-PAGE and blotted with antibodies against DegP, maltose binding protein (MBP) or outer membrane protein A (OmpA). B, EO527 cells were grown in glycerol M63 minimal medium in the absence (none) or presence of arabinose (SecA). EO527 cells expressing Δ11/N95 from a plasmid were grown in the presence of 150 μM IPTG. Cells were pulsed with [35S]methionine and chase-incubated for the indicated times. The processed and unprocessed forms of radio-labeled OmpA were immunoprecipitated and the samples were resolved by SDS-PAGE and analyzed by phosphor-imaging. ‘p’ – proOmpA, ‘m’ – mature OmpA.

Figure 7. Probing the oligomeric state of N95(CC) and Δ11/N95(CC) by disulfide crosslinking in vivo. A, The following purified proteins were treated with diamide and samples (0.5 μg) were resolved on a 5% gel and immunoblotted with SecA antibodies (lanes 2-5): N95(CC), N95(C636) plus N95(C801), N95(C636) and N95(C801), respectively; lane1- in vivo cross-linked N95(CC). Lanes 6-8: Same samples as in lanes 1-3, treated with 50 mM DTT. B, Strains EO529 and EO527, containing an oxidizing and reducing cytoplasm, respectively, and expressing N95(CC) from a plasmid, were grown in the absence or presence of IPTG, as indicated. The monomeric derivative Δ11/N95(CC) was expressed by EO529 cells in the presence of IPTG. Where indicated, the cells were treated with diamide. The samples were resolved on a 5% SDS gel under non-reducing conditions and blotted with SecA antibodies. The sample volumes were adjusted so that equal amounts of the SecA derivatives were loaded: lanes 1, 2, 5, 6 – 40 μl; lanes 3, 4, 11 – 6 μl; lanes 7, 8 – 20 μl; lanes 9, 10 – 3 μl. The sample in lane 11 is identical to that in lane 4, except that 50mM DTT was added. The sample in lane 12 contained purified N95/SecA (0.5 μg) treated with the cross-linker EDC. ‘Dimers’ indicates the positions of the crosslinked bands. ‘**’- Cross-links with unidentified proteins.
| Plasmids            | Description                             | Source or ref. |
|---------------------|-----------------------------------------|----------------|
| pT7N95-SecA         | pT7SecA(Met1-Val831)-6His               | (21)           |
| pET21A11N95         | SecA(Met1-Val831)-6His cloned in pET21 | This work      |
| pDSW204             |                                        | (25)           |
| pDSW204N95          | SecA(Met1-Val831)-6His cloned in pDSW204 | This work      |
| pDSW204A11N95       | SecA(Ser12-Val831)-6His cloned in pDSW204 | This work      |
| pDSW204N95(C636)    | pDSW204N95 (C98S, S636C)                | This work      |
| pDSW204N95(C801)    | pDSW204N95 (C98S, Q801C)                | This work      |
| pDSW204N95(CC)      | pDSW204N95 (C98S, S636C, Q801C)         | This work      |
| pDSW204A11N95(CC)   | pDSW204A11N95 (C98S, S636C, Q801C)      | This work      |

| Strains            | Genotype                               | Source or ref. |
|--------------------|----------------------------------------|----------------|
| DY378              | W3110 λcl857 Δ(cro-bioA)               | (24)           |
| EO527              | DY378 SecM<>kan-araC-P_{BAD}           | This work      |
| EO528              | DY378 prlA4..Tn10 SecM<>kan-araC-P_{BAD} | This work      |
| SMG96              | DHB4 ΔtrxB Δgor ahpC*                  | (52)           |
| EO529              | SMG96 SecM<>kan-araC-P_{BAD}           | This work      |

**Table 1: Bacterial Strains and plasmids used in this work.**
Figure 2

Sedimentation

Fraction #:  2  4  6  8  10  12  14  16  18  20

SecA

N95

Δ11/N95

BSA
Figure 3

A: Graph showing the binding of 125I-SecA (%) of initial with competitor (nM) for SecA and Δ11/N95.

B: Graph showing ATP hydrolyzed (%) over time (min) for +YEG(prlA4) and +YEG(wt).

C: Western blot with 15% 9.1% 1.5% 7.7% 5.7% of SecY prlA4, with SecA, Δ11/N95, and X-links.

D: Scatter plot showing the translocation of proOmpA (%) over time (min) for SecA and Δ11/N95.

E: Western blot with SecA, Δ11N95, SecY prlA4, X-links, and Mr markers 207kDa, 116kDa, and 98kDa.
Figure 4
Figure 5

A. EO527 + Arabinose

B. EO527-Δ11/N95 + IPTG

C. EO527
- Arabinose
- none
- Hours: 0 1 2 3

D. EO527-Δ11/N95
- IPTG
- none
- Hours: 0 1 2 3

E. EO528 + Arabinose

F. EO528-Δ11/N95 + IPTG

G. EO528
- Arabinose
- none
- Hours: 0 1 2 3

H. EO528-Δ11/N95
- IPTG
- none
- Hours: 0 1 2 3
Figure 6

A

| Strain       | EO527 (Wt SecY) | EO528 (prlA Δ SecY) |
|--------------|-----------------|----------------------|
| none        |                 |                      |
| SecA        |                 |                      |
| Δ11/N95     |                 |                      |

Strain: EO527 (Wt SecY)  EO528 (prlA Δ SecY)

B

Chase (min): 0 1 2 3.5 5 7.5 10

SecA

none

Δ11/N95

Figure 6
Figure 7

A

N95(C636): + + + +
N95(C801): + + + +
N95(CC): + + + +

in vivo

Dimers

Monomer

1 2 3 4 5 6 7 8

Mr

207kDa

116kDa

98kDa

B

Strain: EO529 EO527

construct: N95(CC) Δ11/N95(CC) N95(CC)

IPTG: – + +

Diamide: – + – + – + – +

DTT EDC

Mr

207kDa

116kDa

98kDa

Figure 7
The bacterial ATPase SecA functions as a monomer In protein translocation
Eran Or, Dana Boyd, Stephanie Gon, Jonathan Beckwith and Tom Rapoport

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