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Review

Structure and function of SecA, the preprotein translocase nanomotor

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Abstract

Most secretory proteins that are destined for the periplasm or the outer membrane are exported through the bacterial plasma membrane by the Sec translocase. Translocase is a complex nanomachine that moves processively along its aminoacyl polymeric substrates effectively pumping them to the periplasmic space. The salient features of this process are: (a) a membrane-embedded "clamp" formed by the trimeric SecYEG protein, (b) a "motor" provided by the dimeric SecA ATPase, (c) regulatory subunits that optimize catalysis and (d) both chemical and electrochemical metabolic energy. Significant recent strides have allowed structural, biochemical and biophysical dissection of the export reaction. A model incorporating stepwise strokes of the translocase nanomachine at work is discussed.

Keywords: Protein translocase; SecA; SecYEG; ATPase; Motor protein; Membrane transporter

Contents

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1. Introduction

Membrane and secreted polypeptides comprise more than 30% of the proteome in any given organism. More than seven distinct routes for exporting proteins have been described in the bacterial Domain of life alone. Of these, the Sec (for secretion) pathway is essential and ubiquitous and is responsible for the vast majority of protein export work. Protein export through the Sec pathway is responsible for the biogenesis of cell membranes and cell walls, the secretion of polypeptides like hydrolytic enzymes, toxins, signaling molecules, attachment and mobility appendages. Over the past 30 years, biochemical and genetic dissection allowed the isolation of all the genes and proteins involved in the process and the generation of several experimental tools. The pinnacle of these efforts was undoubtedly the complete functional in vitro reconstitution of the protein secretion reaction from purified components [1–4]. These achievements culminated in the first rough sketches of a reaction mechanism [3,5] and beckoned the molecular era. Following suit came three-dimensional structures of the periplasmic domain of leader peptidase [6], SecB [7,8], SecA [9,10] and SecYEG [11]. Structural information and the development of biophysical tools started delivering an understanding of the translocase at atomic resolution.

Export of bacterial secretory proteins is a three-stage reaction that appears to occur largely posttranslationally. Stage I: nascent preprotein chains are recognized by the cellular machinery as being "secretory" because they carry amino-terminal extentions, termed signal or leader peptides [12–14]. For some secretory proteins these "address tags" are recognized by secretion-specific chaperones like the Signal Recognition Particle [15]. For others, signal peptides appear to delay folding of the mature part of the chain thereby allowing other secretion-pathway chaperones like SecB [16] to bind to their mature domains. These events target the preprotein/chaperone complex specifically to the membrane. Stage II: One specific recognition event is with translocase, a nanomachine that operates as a sophisticated export pump. Translocase traps the substrate in a membrane-embedded "clamp" built of the SecY/SecE/SecG polypeptides, while at the same time it pushes it forward using a peripheral, highly flexible motor-chaperone device, the SecA ATPase. Additional translocase subunits optimize these cyclic events [17–20]. Stage III chemistries involve the proteolytic removal of the signal peptide by leader peptidases [6,14] and the proper folding of the chain on the *trans* side of the membrane [21].

Here we will focus on Stage II events and particularly in what involves the structure, mechanics and energetics of the translocase motor SecA and the vast network of intermolecular interactions that underlie its function. For a discussion of the SecYEG domain, see Ref. [22]. We will synthesize the currently available information and propose a working model of the events that govern export of secretory proteins in a SecA-dependent manner through the membrane.

2. SecA protomer structure and domain organization

The domain organization of SecA was revealed through biochemical dissection [23–26]. These data were later confirmed and enriched by crystallographic structures of SecA from *B. subtilis* [9] and *M. tuberculosis* [10] (Fig. 1). Each protomer (102 kDa) comprises three primary structural units: a central DEAD motor (blue; Fig. 1) and two appendages that protrude from it, substrate specificity domain (SSD; purple) and the C-domain (green). These domains and their subdomains will be discussed in detail below.

The amino-terminal DEAD motor domain (Figs. 1 and 2A,B) [24,25] is homologous to corresponding ATPase domains of "DEAD box" or "DExD/H" nucleic acids helicases that comprise five superfamilies [27,28]. The SecA DEAD motor is particularly homologous to Supefamily II DEAD and DExH helicases [9,10,25,27–29] like UvrB and RecG. Although helicase DEAD motors do not share extensive conservation at the level of primary sequence they are very well conserved at the level of tertiary structure. DEAD motors contain two "RecA-like" subdomains that form between them a mononucleotide cleft [9,10,25,28,30,31]. In SecA these subdomains were termed NBD (nucleotide binding domain; Fig. 2A and B) [24,25] and IRA2 (intramolecular regulator of ATPase; Fig. 2A and B) [25].

The ubiquitous helicase DEAD motor acquires enzymatic specificity for different substrates (DNA, RNA, polypeptides), through nonhomologous structures that we term "substrate specificity domains". These can be fused amino-terminally or carboxy-terminally to the DEAD motor or even protrude from different linker regions within the

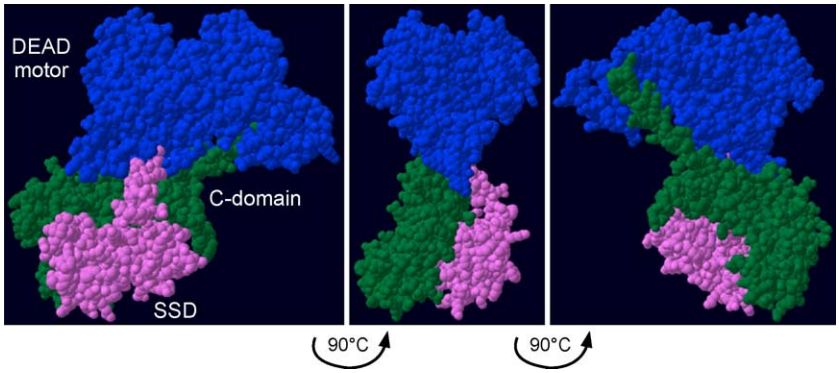

Fig. 1. Domain structure of the SecA protomer. Three views of a space-filling model of the *B. subtilis* SecA monomer (pdb code: 1M6N) [9]. Structures were visualized in SwissPDB viewer.

DEAD motor sequence. In all cases they leave the overall structure of the two "RecA-folds" unscathed [27,28,31]. In SecA, substrate specificity is provided by two appendages unique to SecA (i.e. not present in any other helicase) and were termed substrate specificity domain (SSD; Fig. 2A–C, purple shades) [26] and C-domain (Figs. 1 and 2B,C) [24]. These "specificity domains" can be seen as "levers" that establish conformational cross-talk with the different DEAD motor states (Fig. 6; see below) [33].

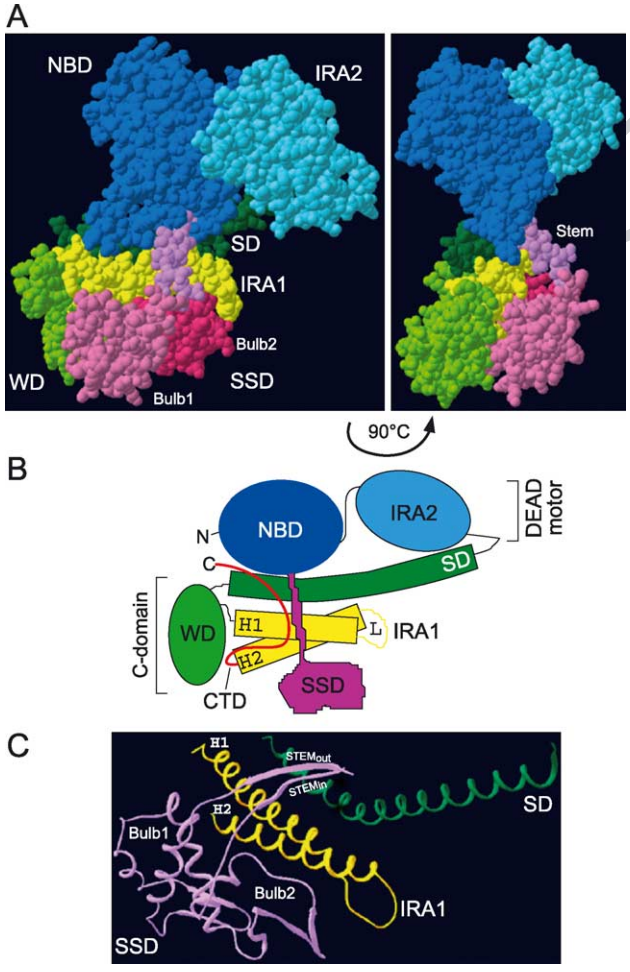

Fig. 2. Subdomain structure of the SecA protomer. (A) Two views of a space-filling model of the *B. subtilis* SecA monomer. CTD is not shown. (B) Schematic representation of the SecA domains from A. (C) Ribbon model of the SD–IRA1–SSD interface [9,33].

SSD "sprouts out" of NBD between helicase Motifs II and III (residues 220 and 373 in *E. coli* SecA; Fig. 3A) and has been implicated in preprotein binding through nonspecific cross-linking and surface plasmon resonance [26,34] and mutational studies [26,35]. SSD contains a "Stem" (Fig. 2A) with two anti-parallel beta strands (Stem "in" and "out") and a globular bilobate "Bulb" domain (Fig. 2C; Ref. [33]). $Stem_{out}$ is a site essential for signal peptide binding to SecA [26]. The 'Bulb', the major constituent of SSD, contains two subdomains Bulb1 and 2 (Fig. 2A and C), varies significantly in length between SecA proteins (~ 100–150 aa) and contains at least one large cavity [9]. A part of it (aa 267–340) has been chemically cross-linked with substrates [34], while mutation of Tyr326 was proposed to affect interaction with preproteins [35].

The exclusively α-helical C-domain is fused C-terminally to the IRA2 subdomain of the DEAD motor (Figs. 1 and 2B) and contains four sub-structures (Fig. 2) [9,10]: (a) the Scaffold domain (SD) is a 46-aa-long bent α-helix that extends all over the length of the DEAD motor. SD acts as a "molecular staple" binding both NBD and IRA2 with one surface of its helix and IRA1 with another. SD is the only contact interface between the DEAD motor and the C-domain [9,10,24]. (b) The Wing domain (WD) that is flexible and loosely linked with the rest of SecA [9,36,37]. (c) The conserved helix-loop-helix (H1-L-H2) IRA1 switch [24] that is a global regulator of protein translocase activities [33] and (d) the extreme C-terminal region (CTD) that is not well conserved in SecA proteins and is dispensible for SecA catalysis [38]. CTD is largely crystallographically unresolved and binds lipid and SecB [38,39]. The first β-strand of CTD forms a β-sheet with the two anti-parallel β-strands of the stem of SSD [9], while a peptide from its extreme carboxy-terminal end that binds both SecB, Zn^{2+} and lipids [38,40,41] was

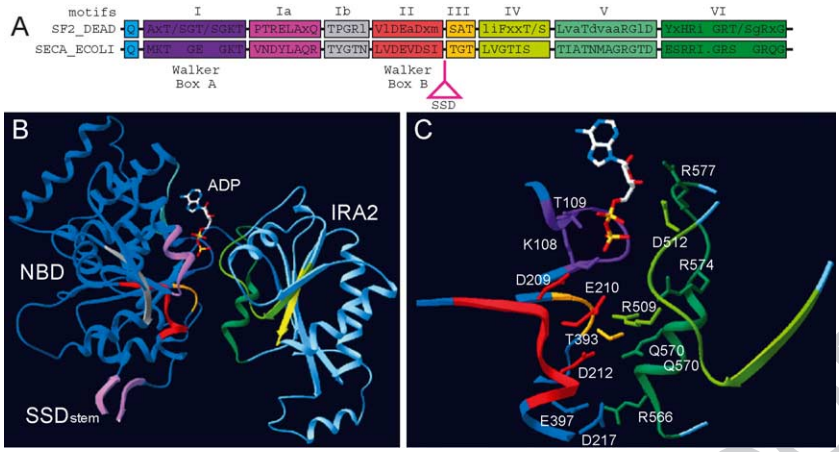

Fig. 3. Molecular features of the SecA DEAD motor. (A) Schematic map of signature motifs of DEAD helicases of Superfamily 2 [27,96] aligned with those from the DEAD motor *E. coli* SecA. The site of insertion of SSD is indicated. The dot in Motif VI of SecA indicates a five-residue insertion in the SecA proteins. Capital letters in the DEAD motifs indicate greater than 80% homology; small case letters indicate 50–79% conservation [27]. (B) Ribbon diagram of the DEAD motor from *B. subtilis* SecA with the helicase motifs indicated and coloured according to (A). Only the Stem structure of SSD is shown. The data were derived from the protein solved after soaking with ADP (pdb access code: 1M74) [9]. (C) Detail of the nucleotide cleft of the DEAD motor with ADP.

recently analyzed crystallographically in complex with SecB [39].

3. The SecA dimer and higher order structures

Native SecA is isolated chromatographically as a stable dimer [42–45] that is seen by small-angle X-ray scattering (SAXS) to be elongated (8 × 15 nm) [45,46]. The protein also unfolds with a distinct dimeric intermediate [47]. The rate-limiting step of this reaction is not monomerization but rather dissociation of the various domains of each protomer.

In solution SecA was proposed to exist in a monomer to dimer equilibrium ($K_D = 0.5–1$ μM) that depends on temperature, ionic strength and protein concentration [44]. Increased temperature and concentration and reduced ionic strength stabilize the dimer. Dimeric SecA should be the prevalent form (80–90%) in the *E. coli* cytoplasm where SecA concentration reaches 5 μM [44]. Surprisingly, *B. subtilis* SecA [9] was crystallized as a monomer in the unit cell. An anti-parallel dimeric species formed due to crystal contacts was hypothesized to represent the physiological dimer (Fig. 4). FRET analysis led to the proposal that a similar dimer forms in solution [48]. *M. tuberculosis* SecA has two monomers in the asymmetric unit (Fig. 4) [10]. The two protomers of *M. tuberculosis* SecA interact only through a few residues in a very limited dimerization interface. Unexpectedly, the organization of the two SecA dimers (Fig. 4) and the residues involved in dimerization are completely different in the two structures. This conundrum raises the possibility that the crystallized forms of the two SecA proteins represent different possible conformational states of an unusually dynamic enzyme. Alternatively, the physiological SecA dimer may dissociate during crystallization and reassemble occupying different possible but physiologically irrelevant arrangements in the crystal lattice.

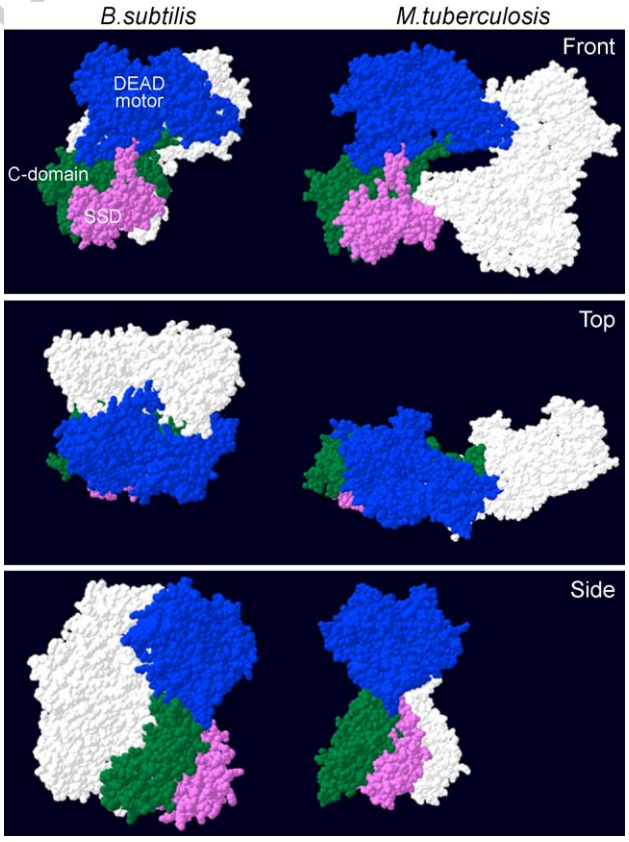

Fig. 4. Structure of the SecA dimer. Three views of a space-filling model of the *B. subtilis* (pdb code: 1M6N) [9] and *M. tuberculosis* (pdb code: 1NKT) [10] SecA dimers. One protomer is shown coloured (as in Fig. 1), while the other is in white. The *B. subtilis* dimer was generated after imposition of crystallographic symmetry.

SecA dimerization has been attributed to the C-terminal domain, since when this region is isolated from the rest of the protein it forms dimers in solution detectable by cross-linking and size exclusion chromatography [24,49]. In vague agreement with this assertion, both the crystallographic dimer [9] and the unit cell dimer [10], form mainly along the C-domain of SecA albeit along different interfaces are substantially different and in *M. tuberculosis* SecA a C-domain/C-domain dimer does not form [10]. In a SecA mutant derivative missing its CTD and carrying six amino-acyl residue substitutions in its IRA1 subdomain, the equilibrium appears to shift to the monomer in nonquantitative assays [50]. However, several other single mutations [33] or deletions and insertions [24] in IRA1 do not lead to monomerization. An alternative oligomerization interface was implied using SAXS analysis of the amino-terminal N68 domain of SecA (DEAD motor plus SSD). Using biophysical tools the amino-terminal N68 was shown to have a relatively weak (monomer to tetramer $K_D = 63$ μM) but clear tendency to form dimers and tetramers at high concentrations [46]. DEAD motor tetramers prevail at concentrations of 10 μM or higher and form a distended two lobe structure of $13.5 \times 9.0 \times 6.5$ nm dimensions, with a central pore.

Fluorescence resonance energy transfer (FRET) occurs continuously from a fluorescein-tagged to a coomarin-tagged protomer of a SecA heterodimer, in the presence or absence of translocation ligands and during ongoing protein translocation [43]. This led to the proposal that SecA functions during protein translocation as a dimer. Attesting to this in a small angle neutron scattering (SANS) study, liposome-bound SecA was found to remain dimeric and to have the same radius of gyration [51] and the «monomeric» SecA mutant [50] loses most (>95%) of its translocation capacity. Nevertheless, some experiments have demonstrated SecA dimer to monomer conversion in the presence of phospholipids and detergents [9,50] and have led to the isolation by detergent extraction of monomeric as well as dimeric SecA in complex to SecYEG [52]. These observations have led to the proposal that SecA may monomerize during part of its catalytic cycle.

Tetramer and higher order oligomeric forms of SecA have also been detected in subcellular extracts [52] and cross-linking studies [24]. More recently, in the presence of charged phospholipids two characteristic higher order SecA structures, a dumbbell shell extended form and a ring with 3–6-nm hole, were observed by negative staining electron microscopy [53]. The role of these oligomeric forms remains unknown.

4. SecA conformational plasticity

SecA is a particularly flexible enzyme. This property is presumably tantamount to its ability to interact with unfolded proteins, to insert into membranes, to "move" processively along polypeptides and to interact sequentially with numerous ligands (Fig. 5). Characteristically, 8-anilinonaphthalene-1-sulfonic acid (ANS) fluorescence studies have suggested that at 37 °C SecA may acquire a partial "molten globule state" [54].

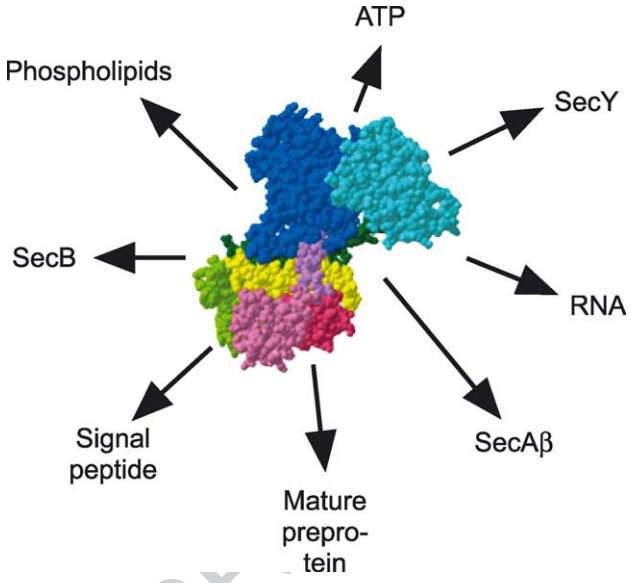

Fig. 5. Intermolecular interactions involving SecA. The only ligand binding site to have been precisely mapped is that of the nucleotide. SecAβ = second protomer.

Biophysical analyses revealed that most of the structural subdomains of SecA are flexible. IRA2 looses 75% of its helical content at the physiological temperature of 37 °C [25]. At least one region in IRA2 was shown by NMR studies to be highly mobile [55]. SSD appears to be also mobile as suggested by its high temperature factors in the solved structures [9,10] and limited proteolysis experiments [26,33]. Intrinsic fluorescence, anisotropy and trypsinolysis experiments have shown the SD, WD and IRA1 subdomains to be mobile [9,33,36,37,55–58] with WD proposed to move out of the main body of the protein [9] and IRA1 moving laterally towards and away from SD [9,33,57]. Proteolysis [54] and NMR studies [55] indicated the extreme carboxy-terminal region (CTD) is also flexible.

5. SecA in a network of intermolecular contacts

5.1. SecA and anionic phospholipids

Anionic phospholipids are a significant component of the *E. coli* membrane (20% phosphatidylglycerol (PG) and 5% cardiolipin (CL) [59] and are necessary for protein secretion both in vivo and in vitro [60]. Translocation is enhanced as the amount of anionic phospholipids in membranes is increased [61,62]. Anionic phospholipids are important for SecA binding to the membrane although at elevated temperature SecA undergoes conformational changes that allow

it to interact and insert into lipid bilayers even in the absence of anionic phospholipids [56]. Initial binding is poor due to ionic interactions of acidic SecA with the negatively charged acidic phospholipids head-groups. This interaction leads to a "Lipid ATPase" activity (in low Mg^{2+} conditions) that is enhanced over "basal ATPase" [61], while it is apparently inhibited by the presence of ATP [63]. Using spin-labeled phospholipids, mobility changes in 15 molecules of DOPG [64] were observed, in the presence of SecA, by electron spin resonance (ESR) [64].

5.2. SecA and nonlamellar-prone lipids

Phospholipids like PG and PC give rise to organized bilayers. Nevertheless, some physiological lipids cannot form bilayer structures in vitro, but promote the reverse hexagonal phase (H_{II}) [65]. One such lipid is phosphatidylethanolamine (PE), the major (75%) lipid of the *E. coli* membrane [66]. Membrane vesicles from a strain that does not produce PE are functional only with highly increased amounts of PG, CL and bivalent cations like Mg^{2+}, Ca^{2+}, and Sr^{2+} [67]. These cations together with the anionic phospholipids promote a non-bilayer structure playing the role of PE. Reconstituted SecYEG-proteoliposomes reach a maximum efficiency only when they contain the same kind and percentage of lipids as the wild-type *E. coli* membrane [68]. Nonlamellar-prone lipids specifically enhance SecA binding to the bilayer [69] and are thought to activate its ATPase activity indirectly by promoting phase separation of bilayer-forming lipids [70].

5.3. SecA–SecYEG interaction

SecA binds to the membrane with low affinity at acidic phospholipids and with high affinity (20–40 nM) at SecYEG leading to the formation of the translocase holoenzyme [2,4,71,72]. Numerous studies including biochemical solubillization and co-purification [52,73], in vivo cross-linking, and extragenic suppressor analysis [74–77] suggest that this is indeed the functional state of the enzyme.

The SecA dimer is four times as long as the diameter of a SecYEG monomer [11,45]. The extended nature and dimensions of dimeric SecA, as determined from SAXS [45,46], SANS [51], electron microscopy [53] and crystallography [9,10] studies, make it difficult to envision how the enzyme deeply penetrates across the membrane [5,78–84] in a way that shields it from phospholipids [83,85]. A high-resolution structure of an Archaeal SecYEG reveals the protein to be a monomer that contains only a narrow 8-Å "pore" that would "exclude" SecA [11]. One possibility is that only limited regions of SecA penetrate into the membrane so deeply as to become actually exposed to the periplasmic phase [80,81,84] in a way reminiscent of the ABC transporter ATPases [86]. Another possibility is that SecA monomerizes upon SecY binding [52] and hence a significantly reduced surface would need to be accommodated within or at SecYEG. Another possibility is that SecYEG could form dimers [87] or other multimers (perhaps tetramer) in the presence of SecA [88]. Finally, the specific interaction of SecA with phospholipids raises the possibility that parts of it insert in the phospholipid bilayer but they are not detectable by the nonspecific cross-linking probes that have been tested [83,85].

The region(s) of SecA that interacts with SecYEG remains controversial. Extragenic genetic suppressors of SecY *prl* (protein localization) and thermosensitive mutations map all over SecA [76,89,90] and do not provide precise topological information. In one study the C-domain of SecA was proposed to provide the interaction interface [91], while other studies attribute this property to the DEAD motor [33,92]. Clearly, although an isolated C-domain polypeptide has no detectable binding to SecYEG [33,92], DEAD motor affinity for SecYEG increases up to four times in the presence of the C-domain of SecA [33] indicating some synergistic contribution.

5.4. SecA and nucleotides

Initial biochemical mapping of nucleotide binding to SecA proposed the presence of one [93,94] to three ATP molecules bound per SecA monomer. Subsequently, sequence alignments lead to the proposal that each SecA protomer contains two nucleotide binding sites [95]: a high affinity site (0.13 μM) in NBD and a very low affinity (340 μM) for nucleotide in IRA2. The high affinity site was experimentally confirmed by mutagenesis [58,95], [γ^{32}P]ATP photolabelling [93], and thermal melting and enzyme kinetic experiments [25,26]. This high affinity site is fully contained within the amino-terminal 227 residues of NBD (Fig. 3A and B) [25]. As is common with ATPases, ATP does not bind to a buried cleft on NBD. Rather, ATP binds superficially to NBD and is in effect "sandwiched" between the two "RecA-like" structural repeats (NBD and IRA2; Fig. 3B) [9,10]. The nine Superfamily II motifs [27,28,96] line the walls of this crevice (Fig. 3A). Helicase motifs I and II correspond to the characteristic Walker box A and B of all known ATPases (Fig. 3A) [97]. Walker box B in the helicases contains the characteristic Asp-Glu-Ala-Asp (hence the "DEAD" monicker for this class of enzymes) sequence or variations thereof (e.g., Asp-Glu-Val-Asp in most SecAs). IRA2 does not exhibit ATP binding or hydrolysis properties and does not get stabilized by ADP and is therefore unlikely to represent an independent low affinity nucleotide binding site [25].

DEAD motor residues responsible for various aspects of ATP binding and catalysis have been identified experimentally [25,93,95,98,99]. The majority of interactions with the nucleotide are performed with the helicase motifs (Fig. 3A). Adenine nucleotide binding to NBD of *B. subtilis* and *M. tuberculosis* SecA happens with the same geometry as in the F_1ATPase [100]. Motif I (Lys108 and T109 in *E. coli* SecA) forms the 'P loop' that binds the β and γ phosphates of the

nucleotide, while Motif II contains Asp209 which chelates Mg^{2+} ions and Glu210, a potential catalytic base that forms a hydrogen bond with a water molecule suitably positioned so as to hydrolytically attack the γ-phosphorus of ATP. Other direct interactions with the nucleotide involve helicase motifs V (Gly510 and Asp512) with the β-phosphorus and the ribose) and VI (Arg577 with the ribose; Asp512 and Gln570 with the γ-phosphorus) residues. Other residues of the cleft do not interact directly with the nucleotide but ensure tight communication between the walls NBD–IRA2. Thus, Gln570 (Motif VI) forms hydrogen bonds with NBD residues like Asp212 (Motif II) and Thr393 (Motif III), while Arg566 (Motif VI) interacts with Glu397 and Asp217 located downstream of Motifs III and II, respectively. As is the norm with DExD/H helicases, several residues from the juxtaposed IRA2 domain (Fig. 3B) do not make contact with the nucleotide but are essential for catalysis (e.g., R509, R574) [25,95]. This suggests that side-chain rearrangements may take place during catalysis.

5.5. SecA and preprotein signal peptides

Signal peptides have distinct functional roles and physicochemical properties but do not share a consensus sequence [11–14]. They usually contain approximately 20 amino acyl residues, with a 6–10-residue hydrophobic core flanked by small polar regions; they acquire α-helical structures [101,102] and they do not contain bulky amino acids at the −1 and −3 positions preceding the cleavage site [103].

Signal peptides bind to SecA in solution [26,61,104–107] and in the presence of liposomes leading to an increased "lipid ATPase" activity [61]. Signal peptides bind to an amino-terminal fragment of SecA that contains the DEAD motor and SSD (Fig. 2C) [26,108]. Using an immobilized signal peptide biosensor and chemical cross-linking, it was demonstrated that the 234 amino-terminal residues of SecA (1–220 from the NBD subdomain of the DEAD motor and 14 residues from SSD) are necessary and sufficient for low-level signal peptide binding [26]. Full binding of the signal peptide is observed to the amino-terminal 263 residues (1–220 from NBD and 43 residues from SSD) [26]. In contrast, signal peptide binding to a SecAΔ220–240 or to a fragment containing the amino-terminal 227 residues is practically abolished [26]. Therefore, residues 228–234 (that include the $Stem_{out}$ beta strand of SSD; aa 221–227 in *E. coli* SecA; Papanikolau et al., in preparation) are likely to be essential for signal peptide binding. In addition, residues 235–263 of the SSD Bulb 1 appear important for optimal signal peptide binding, either because they are bona fide residues of the binding pocket or because they contribute indirectly to its structural integrity [26]. In view of the close proximity of the SSD $Stem_{out}$ and NBD, a signal peptide binding site may be formed by surfaces provided from both NBD and the SSD $Stem_{out}$ [26]. In *B. subtilis* SecA, this interface is conserved and largely hydrophobic and was proposed as a possible signal peptide binding site [9].

5.6. SecA and preprotein mature regions

Interaction of SecA with the mature part of preproteins is a very important process that is poorly understood. Proteins can translocate through membranes without signal peptide sequences [109–111], while signal peptide additions at the beginning of cytoplasmic proteins does not always ensure their secretion [112]. M13 procoat, a small (73 aa) protein that normally inserts in the membrane through the YidC pathway, can be diverted to the Sec pathway by introduction of a mutation in its mature domain. The mutant M13 protein has developed the ability to bind to SecA [113].

Dependence of preprotein translocation on Proton Motive Force (PMF) and on SecB relies on mature preprotein regions [114,115]. Translocation is blocked if positively charged amino acids are inserted at the beginning of the mature region [112,116–118], even in the absence of a signal peptide [111], suggesting that the interaction of the mature region of the protein substrate is the same with or without signal peptide. The positively charged residues affect loop formation between the signal peptide and approximately 20 amino acids of the mature protein, and consequently the start of translocation reaction [118].

The preprotein mature domain binding site is expected to have three fundamental characteristics for: (a) it can accommodate only a limited portion of the preprotein substrate each time (approximately 20–30 aa), (b) it has no sequence substrate specificity, since SecA binds to a variety of substrates, even cytoplasmic enzymes with engineered signal peptide sequences, (c) affinities for substrates are varied and the energetic barrier between the bound and unbound form remains low but sufficient for transfer of the chain with the help of SecA. It seems unlikely that a lock–key mechanism is in effect, since there are several *prl* (protein localization) mutant SecAs spread all over the protein that suppress signal peptide defects [90,110,119]. A preprotein substrate has been cross-linked within residues 267–340 [34] of the SSD Bulb. This site may be a mature domain binding site. Mutation of the conserved Tyr326 of Bulb2 resulted in lower apparent affinity for preproteins and somewhat compromised secretion in vivo [35].

5.7. SecA and chaperones

In Gram-negative bacteria SecB works early in the translocation process by recognizing preproteins, keeping them unfolded [15] and directing them to the translocation pathway, through its specific interaction (K_D = 10–30 nM) with membrane-bound SecA [71,120]. As the translocation of preproteins starts and SecA binds ATP, SecB-bound substrates are relocated to the membrane and SecB is released from SecA [41]. One SecB binding site on SecA is located within the 22 amino acids of its C-terminus. The

C-terminal part is highly conserved in a subset of SecAs, it is positively charged, and binds a zinc ion [40]. A chemically synthesized 27-residue SecA C-terminal peptide folds in a well-structured CysCysCysHis zinc-binding motif and interacts with SecB primarily with electrostatic (salt bridges) and hydrogen bonding interactions [39]. One SecB tetramer binds two SecA peptides. Additional SecB-binding sites on SecA have also been proposed to exist [41,121,122]. Residues important for interaction with SecA have also been found in SecB [121], and all these are clustered around a flat, solvent-exposed and negatively charged surface, on both sides of the tetrameric SecB molecule [7,39].

One study proposed that SecB modulates the ATPase activity of SecA by stabilizing the ATP state [123]. However, such an effect would have to be transient since SecB is expelled from SecA once translocation initiates [41]. The incorporation of sequence motifs in the mature region, which confer SecB dependence in vivo, had no impact on SecA activation in vitro [124].

In Gram-positive bacteria that lack SecB, another chaperone, CsaA, was proposed to contribute to preprotein substrate targeting. CsaA is a dimeric protein with two large hydrophobic cavities on its surface [125], binds preproteins and was shown by immunoprecipitation experiments to also bind to SecA [126].

5.8. SecA and mRNA

In *E. coli*, but not in other bacteria, SecA regulates its own production, through translational control [127] that does not involve its helicase activity [128]. It is expected consequently that SecA possesses also a single-stranded nucleic acid binding site. From the crystallographically solved structures of SF-I (e.g., PcrA [30]; Rep [129]), and SF-II helicases (e.g., NS3 [130]), with DNA, a conservative binding cleft of single-stranded DNA has been proposed to exist in the interface of domains interfering with the binding of nucleotides. A cleft of analogous geometry exists in SecA between NBD and IRA2 domains and could interact with the adenine rich loop of single-stranded RNA (ssRNA) [9]. SecA synthesis is regulated by the upstream gene SecM (secretion monitor) upon sensing of the secretion status of the cell. This mechanism was proposed to involve control of the melting of the Shine–Dalgarno of the SecA mRNA [131].

6. SecA plasticity modulated by ligands

Understanding catalysis of a multi-liganded enzyme like SecA necessitates a complete description and ordering of the conformational changes invoked by its substrates. Binding of nucleotides [24,57,81,132], acidic phospholipids [54,56,60,132], inverted membrane vesicles [132], signal peptides [26,107], and complete preproteins [132] all affect SecA conformation. These changes are expected to reflect functional steps of the translocation process.

Nucleotides cause several movements in the SecA molecule: (a) Changes in the NBD–IRA2 interface, conformation and stability [24,25,55]. Nucleotides thermally stabilize SecA by 10 °C [25,57]. (b) Changes in SSD conformation [33]. (c) Tighter DEAD motor/C-domain assembly and enhanced C-domain stabilization [24,133]. Isothermal titration calorimetry (ITC) [133] studies indicated that SecA complexed with ADP acquires a more compact conformation. Intrinsic tryptophan fluorescence studies confirmed that nucleotide-driven conformational changes of the SecA DEAD motor are transferred to the C-domain [9,33,57]. (c) IRA1-SD binding and release [33]. Binding and release of IRA1 to SD appears a central mechanism of SecA activation and is reflected in enhanced nucleotide-modulated mobility of Trp775, one of the bulky residues that allow IRA1 communication with the Scaffold domain [9,57]. (e) WD dissociation/reassociation from the compact core of the enzyme [9]. (f) CTD conformation [55,132].

The crystal structures of other helicases [28,30,134,135,] and far-UV CD and biochemical studies [25] all suggest that substantial nucleotide-regulated conformational changes may occur. Nevertheless, no nucleotide-driven changes in the radius of gyration of SecA are detectable by SAXS analysis [45] and far-UV CD scan experiments failed to detect widespread changes in secondary structure [24,25,33]. Moreover, nucleotide soaked into SecA crystals does not yield any measurable structural change [9,10]. Crystallization and/or NMR experiments in the presence of ligands will be necessary to determine precisely the nature and extent of SecA conformational states.

Other translocation ligands also affect SecA conformation. Phospholipids and IMVs bind to SecA [54,56,132] and alter the conformation of both the DEAD motor and the C-domain [36]. Upon binding to anionic phospholipids SecA undergoes a conformational change [56,132], which possibly exposes hydrophobic surfaces for more strong interactions with lipids and allows it to insert all the way across the lipid bilayer [56] so that it even becomes exposed to the lumen [81,136,137]. Chemical cross-linking, analytical ultracentrifugation and fluorescence anisotropy [50,138] suggested that acidic phospholipids can cause SecA monomerization. Membrane phospholipids are sufficient for this effect and do not require SecYEG, while they can be replaced by nonionic detergents [52,138]. In contrast, determination of SecA shape and dimensions in liposomes using SANS was proposed to reflect a dimeric state [51]. Nevertheless, this lipid-bound SecA dimer may monomerize when nucleotides are added [51].

SecA undergoes cyclic conformational changes at SecYEG during translocation [5,18,81,139]. These invoke reciprocal conformational changes to the SecG subunit [17] and to SecY [140–143].

Preproteins and signal peptide binding exert various conformational changes on both the DEAD motor and the C-domain [26,36,106–108]. One effect is on the conformation of the SSD "Bulb" and this was proposed to activate

binding of mature preprotein domains [26]. These long range changes may explain the isolation of signal peptide suppressor mutants to regions far from the presumed binding site [90,119]. Moreover, they are likely to control an allosteric regulatory mechanism by which signal peptides control ATP hydrolysis by the DEAD motor [26]. Signal peptides were also proposed to re-dimerize lipid-monomerized SecA, albeit to a different apparent conformational state [138]. However, another study found signal peptide to have the exact opposite effect [50].

7. Model of SecA-mediated protein translocation catalysis

Synthesis of the biochemical, structural and biophysical data leads to a stepwise sequential model of SecA-dependent secretory protein export through the bacterial Sec translocase.

7.1. Preassembly

SecA in the cytoplasm hydrolyzes ATP rapidly and converts it to ADP [24]. The generated ADP becomes tightly bound to SecA and thermally stabilizes it significantly [25,57]. ADP release is the main rate-limiting step of SecA catalysis and hence SecA·ADP is a biochemically inert enzyme [25] that exhibits a compact conformation with local conformational changes in the SSD and C-domains [24,57], while its overall shape is not changed [9,10,45]. While in the cytoplasm SecA can interact with preprotein–SecB complexes [26,41,113,132,143–145], these are low affinity interactions and therefore are only transient.

7.2. Membrane assembly

Cytoplasmic SecA binds to the membrane due to the affinity of its DEAD motor domain for SecYEG [33,71,92]. SecA affinity for acidic phospholipids [56,71] may also facilitate collisions with SecYEG receptors by restricting SecA diffusion to two dimensions. Translocase holoenzyme assembles at the membrane.

7.3. Translocase priming

Binding of the SecA DEAD motor to SecYEG drives a conformational change that could involve loosening of the dimeric interface [52] and is 'sensed' by the IRA1 hairpin [24,26]. IRA1 changes its conformation and/or interaction with SD and SSD [33] (Figs. 2C and 6). These events 'prime' the translocase for the translocation reaction by affecting: (I) the conformation of the SD helix and reduce SD association to NBD-IRA2 [33]; (II) the conformation of the anticipated preprotein binding site SSD; (III) the conformation of the SecB binding site CTD [39,55]; and (IV) the enhanced rates of nucleotide release from SecA [146].

7.4. Preinitiation complex formation

Through translocase "priming" events, SD detaches from the DEAD motor and this leads to relaxation of IRA2–NBD association [25,33]. As a result, ADP release from the nucleotide 'pocket' is somewhat favoured and this leads to a marginal increase in ATP hydrolysis (membrane ATPase) [61]. More importantly, alterations in the IRA1–SSD–CTD interface vastly enhance SecA affinity for preprotein substrates and SecB (>20–30-fold; 40–60 nM; Refs. [26,71,144]). Preprotein–SecB complexes bind to

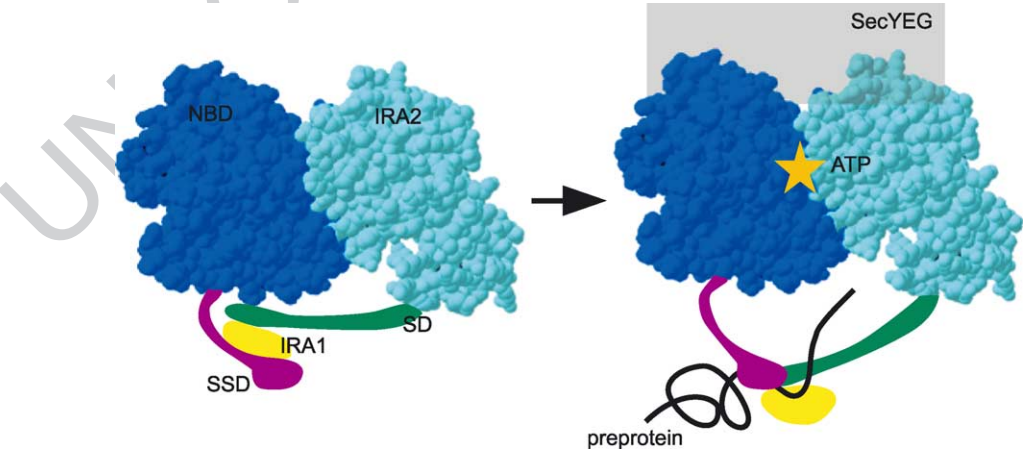

Fig. 6. Model of the SecA as a machine comprising the ATP energy utilizing DEAD motor connected to two specificity "levers" SSD and SD (see text for details). IRA1 that is attached to SD is the sole physical link between the two levers. Substrates bind to SSD and could get trapped within or on the levers. SecYEG, which binds to the DEAD motor, and ATP regulate the conformational states of the DEAD motor and these are transmitted to the specificity "levers" that move along the substrate during processive catalysis. In the reciprocal event, binding of the preprotein to the specificity levers signals back to the DEAD motor and affects ATP catalysis and conformation [33]. A second protomer (not drawn) may be involved in the process. WD and CTD domains have been omitted for simplification.

SecA at the membrane. Binding of the signal peptide to the SSD–NBD interface [26] and of SecB to CTD [39,41,120] change the conformation of the SSD bulb and this facilitates binding/trapping of the mature domain by SSD.

7.5. Preprotein-triggered nucleotide cycling

Preprotein binding to SecA leads to a substantial increase of SecA ATPase (Translocation ATPase) [61]. Obviously, substrate binding, probably at SSD [26,34], and SecB binding to CTD [40,41] alleviate IRA1-mediated ATPase suppression. One likely mechanism for this is detachment of IRA1 from SD leading to subsequent dissociation of SD from the NBD–IRA2 surface [33] (Fig. 6).

7.6. SecA-preprotein co-insertion

Membrane and substrate/chaperone binding promote substantial ADP release. SecA binds ATP and acquires a more loose extended conformation [24,25,56,57,132], which drives SecA membrane insertion at SecYEG [5,18,78,82]. Significant portions of SecA become membrane-embedded [5,79,82–84] to the point of exposure to the periplasm of CTD [80,81], SSD and IRA2 residues [81]. Despite deep membrane penetration, inserted SecA appears to be excluded from the lipid bilayer [83,85]. SecG [17,19,20] and the SecDF complex [18,20] appear to favour SecA membrane insertion. How the SecA dimer or monomer is accommodated in the membrane is difficult to envision considering the dimensions (15 × 8 nm) and shape of SecA dimer [9,10,45], the width of the membrane (5 nm) and the cavity, 1.6 × 2.5-nm wide and 2.2-nm deep, of the dimeric SecYEG [11,87].

As ATP binding-driven SecA membrane insertion initiates, the SecB chaperone hands over the preprotein chain to SecA [41,120] and preprotein segments of 20–30 aminoacyl residues co-insert [3,5,139,147]. The translocated substrate enters the translocation pathway and is found in the vicinity of both SecA and SecY [147]. As translocase translocates a variety of substrates, it is expected to recognize the polypeptide backbone rather than side chains.

7.7. Pre-protein dissociation from SecA

If the SecA-bound ATP is not hydrolyzed, the translocation reaction stalls, after insertion into the membrane of 20–30 amino acids of the preprotein [3]. Indeed, multiple cycles of ATP hydrolysis are needed for complete transfer of the preprotein substrate through the membrane [3,5,114,148,149]. When ATP hydrolysis is allowed, preprotein is partially dislodged from SecA [3]. This removal may be favoured also by the topology changes of SecG [17] and by SecDF [19,20,150]. Even after preprotein is released, it stays in the vicinity of SecA [147], physically trapped in SecYEG and cannot slip backwards [3,147].

7.8. SecA de-insertion

When ATP gets hydrolyzed to ADP and Pi localized conformational changes occur to the DEAD motor and are transmitted to the specificity appendages SSD and C-domain [9,24,26,33]. Interaction between NBD and IRA2 becomes tighter [25,57] and SecA acquires a more compact structure enabling it to de-insert from the membrane [5,151]. SecG topology reversal could also help SecA membrane de-insertion [17]. De-inserted SecA remains engaged to the translocase-threaded preprotein and stays at or near SecY, ready to attach to the subsequent fragment of the polypeptide with a "hand over hand" mechanism and continue processive translocation. De-inserted SecA can exchange with the cytoplasmic SecA pool [5] or remain tightly membrane-bound [79].

7.9. PMF-driven translocation

In later stages of the translocation reaction, after significant portions of the substrate have been translocated, when the substrate is released from SecA, the electrochemical proton motive force (PMF) can complete translocation [3,152]. PMF may act through promoting SecA de-insertion [153] by favouring hydrolysis product release [154] and SecY conformational changes [155,156]. In the absence of PMF and SecA, backward slippage of the preprotein can occur leading to uncoupled ATP over-consumption during translocation [3].

7.10. Multiple catalytic turnovers

ATP binding-driven SecA membrane insertion can occur even during advanced stages of translocation [18] and leads to threading of another 20–30 amino acyl residues of the preprotein through the membrane ([3,147–149]. In order for the elongated (i.e. when extended it is many times as long as the membrane is wide) aminoacyl polymer to translocate through the lipid bilayer, SecA must perform multiple catalytic turnover cycles [3,5,18]. The preprotein polymer remains embraced by the translocase as it moves step by step processively (Fig. 6) [3,18,139]. At this stage the mature preprotein domain is able to move freely inside the translocation channel. The "molecular ruler" of 20–30 amino acyl residues translocated per/SecA insertion cycle [3,148] may be attributed either to the recognition mechanism of the preprotein substrate [149] or to the physical distance covered by the SecA domains during their conformational changes [18] or to the distance from one SecA protomer to the other.

7.11. Signal peptide cleavage

After completion of the translocation reaction, signal peptide cleavage of the preprotein substrate takes place through signal peptidase [6,14].

7.12. Periplasmic release

As the translocated polypeptide appears at the *trans* side of the inner membrane, folding may initiate allowing acquisition of the native tree-dimensional structure. This process may be accelerated with the help of SecDF [150] or via periplasmic chaperones of the periplasmic space [157]. The fully matured periplasmic protein is released to the periplasmic space of Gram-negative bacteria, where it either resides, or from where it is directed to the outer membrane. In Gram-positive bacteria, the released protein resides in the outer cellular space.

8. Conclusion

In recent years we are witnessing a steady and welcoming maturation of bacterial protein secretion studies. Preprotein translocase is recognized as a highly dynamic nanomachine that moves processively on its multiple polymeric substrates. At the centre of it lies the SecA ATPase that powers translocation. A bevy of bimolecular interactions provides energy conversion by the SecA DEAD motor to mechanical work, through ordered defined conformational steps that involve the two specificity levers, SSD and C-domain (Fig. 6). Understanding these interactions necessitated the passing of the torch from genetic and initial biochemical studies over to advanced enzymology, biophysics and structural biology. This combination of quantitative tools with atomic resolution structures and real time conformational studies promise to enlighten us on the inner workings of this essential, fascinating and unique cellular machine.

9. Uncited reference

[32]

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