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Review

Traffic Jam at the Bacterial Sec Translocase: Targeting the SecA Nanomotor by Small-Molecule Inhibitors

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The rapid rise of drug-resistant bacteria is one of the most serious unmet medical needs facing the world. Despite this increasing problem of antibiotic resistance, the number of different antibiotics available for the treatment of serious infections is dwindling. Therefore, there is an urgent need for new antibacterial drugs, preferably with novel modes of action to potentially avoid cross-resistance with existing antibacterial agents. In recent years, increasing attention has been paid to bacterial protein secretion as a potential antibacterial target. Among the different protein secretion pathways that are present in bacterial pathogens, the general protein secretory (Sec) pathway is widely considered as an attractive target for antibacterial therapy. One of the key components of the Sec pathway is the peripheral membrane ATPase SecA, which provides the energy for the translocation of preproteins across the bacterial cytoplasmic membrane. In this review, we will provide an overview of research efforts on the discovery and development of small-molecule SecA inhibitors. Furthermore, recent advances on the structure and function of SecA and their potential impact on antibacterial drug discovery will be discussed.

Bacterial Resistance and the Need for New Antibiotics

Improper use of antibiotics has led to a widespread increase in the occurrence of drug-resistant and multidrug-resistant bacteria (Chen et al., 2009). Consequently, physicians are increasingly being faced with infections caused by bacteria for which limited or no effective antibiotics exist. In terms of high incidence of infection, morbidity, mortality and resistance factors undermining standard antibiotic therapy, the most problematic bacterial pathogens include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci*, and extended-spectrum β-lactamase–producing gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, and *Klebsiella pneumonia*, among others) (Nordmann et al., 2007; Shorr, 2009; Woodford and Livermore, 2009). Infections caused by antibiotic-resistant bacteria are not only associated with higher mortality rates, but also result in prolonged hospital stays and increased healthcare costs (Slama, 2008).

Paradoxically, as the problems accompanying antibiotic resistance increase, the number of different antibiotics available to treat serious infections is dwindling. In the last 30 years, only three completely new classes of antibiotics have been introduced: the oxazolidinones (linezolid) (Herrmann et al., 2008), the cyclic lipopeptides (daptomycin) (Robbel and Marahiel, 2010), and a natural product isolated from *Streptomyces platensis* (platensimycin) (Lu and You, 2010). The reasons behind the decreasing number of antibiotics reaching the market are complex, but are partly a result of decreased research and development activities in the pharmaceutical industry (Spellberg et al., 2008). As a consequence, there are only a handful of antibiotics in the drug development pipeline, most of them being analogs of existing classes of antibacterials with improved properties (Devasahayam et al., 2010). Hence, there is an urgent need for new antibacterials, preferably from new structural classes and with novel modes of action to avoid cross-resistance with existing antibiotics (Payne, 2008).

In the framework of the search for new antibacterial therapies to combat infections caused by multidrug-resistant bacteria, new antibiotic targets are currently being proposed and evaluated, including components of the bacterial protein secretion pathways such as SecA (Baron, 2010; Economou, 2001; Marra, 2006) and signal peptidase type I (Rao et al., 2009; Smith et al., 2011). In this review, we will focus on SecA, a key protein of the general secretory (Sec) pathway.

The Sec Pathway

General Features of Sec-Dependent Preprotein Translocation

The Sec pathway, together with the twin-arginine translocation (Tat) pathway, accounts for the majority of protein transport across the bacterial cytoplasmic membrane (Holland, 2010). Although the Tat pathway has mostly been implicated in the transport of folded proteins, the Sec pathway is involved in the translocation of unfolded preproteins and the insertion of membrane proteins across or into the cytoplasmic membrane (Natale et al., 2008). In contrast to the Tat pathway, the Sec machinery is essential for bacterial viability, making it an attractive target for antibacterial therapy (Economou, 2001). Moreover, several components of the Sec pathway are highly conserved among gram-negative and -positive pathogens but do not have close structural homologs in humans, making them attractive candidates for antibacterial drug discovery (Cao and Saier, 2003; Hand et al., 2006).

Secretory proteins that are exported via the Sec pathway are synthesized at the ribosome as unfolded precursors with

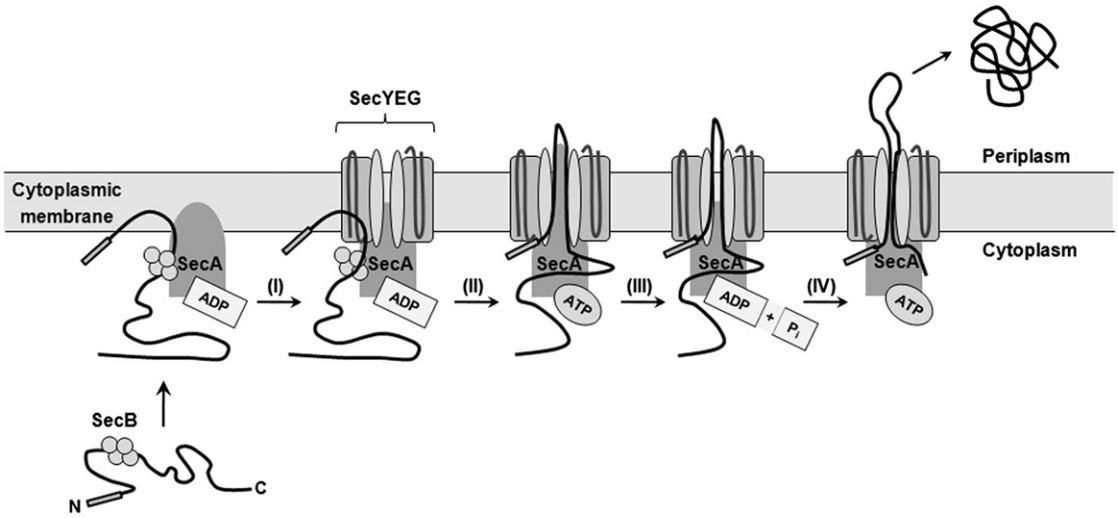

Figure 1. Schematic Model of Sec-Dependent Preprotein Translocation in Gram-Negative Bacteria
Sec-dependent secretory preproteins (thick black line; the signal peptide is represented as a gray rectangle) are targeted to SecA by the molecular chaperone SecB (I). Binding of the SecA-preprotein complex to the SecYEG translocation channel (II) and subsequent rounds of ATP binding and hydrolysis (III–V) result in large conformational changes in SecA that are coupled to the stepwise translocation of the preprotein. When translocation is complete, the signal peptide is cleaved off by signal peptidase that is present at the periplasmic side of the membrane, leading to the release and folding of the mature protein.

a cleavable amino-terminal signal sequence (Figure 1). After a secretory preprotein has emerged from the ribosome, it can be targeted to the SecYEG preprotein-conducting channel in the cytoplasmic membrane via different pathways. A first targeting route involves the molecular chaperone SecB, a secretion-dedicated chaperone that is present in many gram-negative bacteria but is absent in gram-positive organisms (Muller et al., 2000; van Wely et al., 2001). SecB acts by binding to partially folded precursor proteins, either free in the cytosol or bound to cytoplasmic SecA (Bechtluft et al., 2010; Crane et al., 2005; Randall et al., 2005; Randall and Henzl, 2010; Suo et al., 2011; Zhou and Xu, 2003). The interaction with SecB keeps the newly synthesized preproteins in a more or less unfolded conformation that allows them to pass through the translocation channel of the Sec pathway. However, not all secretory proteins depend on SecB. These preproteins are targeted to the Sec translocase by SecA or by general cytosolic chaperones such as GroEL and/or DnaK, which can substitute partially for SecB under certain conditions (Bochkareva et al., 1998; Lecker et al., 1989). A second, SecB-independent targeting route is mediated by the signal recognition particle (SRP). Secretory preproteins that are targeted by the SRP pathway are transported to the Sec translocase in a nascent, ribosome-associated state, which does not necessarily require an interaction with SecA (Kuhn et al., 2011; Luirink and Sinning, 2004).

Irrespective of the targeting route, all preproteins eventually reach the cytoplasmic membrane, where they are transferred to the translocase. The translocase is a multisubunit membrane protein complex that consists of the peripheral membrane ATPase SecA and a preprotein-conducting channel composed of the integral membrane proteins SecY, SecE, and SecG (du Plessis et al., 2011). After docking of the preprotein to the translocase complex, the preprotein is translocated through the SecYEG channel in a stepwise manner, using the energy from SecA-catalyzed ATP hydrolysis as well as the proton motive force. Following preprotein translocation, the signal peptide is cleaved off by an exocytoplasmic signal peptidase, which results in the release and folding of the mature protein.

SecA, a Central Component of the Sec Pathway

Structure and domain organization. The domain organization of SecA has been revealed by biochemical and biophysical analyses and by the elucidation of several SecA structures from different species. Of the six published SecA crystal structures, five are dimeric (Hunt et al., 2002; Papanikolau et al., 2007; Sharma et al., 2003; Vassylyev et al., 2006; Zimmer et al., 2006), and one is monomeric (Osborne et al., 2004). The protomers of each dimer all have a similar structure, but surprisingly the position of each protomer relative to one another is unique (Sardis and Economou, 2010). Although most dimers have an antiparallel orientation, the structure from *Thermus thermophilus* revealed a parallel conformation. At the moment it is unclear which of these structures, if any, represents a physiological relevant state. One possibility raised by the structural studies is that some of the dimeric structures are the result of artifacts arising from crystal packing and the harsh crystallization conditions used (Hunt et al., 2002; Papanikolau et al., 2007; Sardis and Economou, 2010; Sharma et al., 2003; Vassylyev et al., 2006; Zimmer et al., 2006). However, cross-linking and directed cysteine mutagenesis studies have demonstrated that the parallel and two antiparallel forms also exist in solution (Jilaveanu and Oliver, 2006; Or et al., 2005; Vassylyev et al., 2006; Zimmer et al., 2006). Moreover, it has been demonstrated that unliganded SecA exists in solution in a monomer-dimer equilibrium with a K_D in the nanomolar or micromolar range, depending on the salt concentration (Das et al., 2008; Woodbury et al., 2002; Wowor et al., 2011). This finding suggests that SecA is mostly dimeric in vivo, because the cellular SecA concentration is estimated to be up to 8 μM (Or et al., 2002). However, the precise oligomeric state of SecA as it functions during the translocation cycle is still a matter of debate. Although some lines of evidence

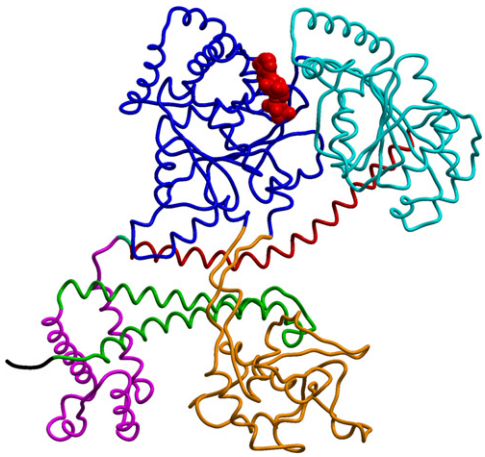

Figure 2. Three-Dimensional Structure of the *E. coli* SecA Monomer
Color coding of SecA domains: NBD, dark blue; IRA2, cyan; PBD, orange; IRA1, green; SD, red; HWD, pink; and CTL (first 4 residues), black. The bound ATP molecule is shown in ball representation (red). The figure was created with ICM-browser (Molsoft LLC), using the coordinates of the ecSecA NMR structure (Gelis et al., 2007) (PDB code 2VDA).

support the notion that monomeric SecA can function in protein export (Alami et al., 2007; Benach et al., 2003; Duong, 2003; Or et al., 2005; Or et al., 2002; Or and Rapoport, 2007; Zimmer et al., 2008), several studies indicate that complete dissociation of the two protomers is not essential for translocation (Akita et al., 1991; Bu et al., 2003; Das et al., 2008; de Keyzer et al., 2005; Jilaveanu and Oliver, 2006; Jilaveanu et al., 2005; Karamanou et al., 2005; Mao et al., 2009; Randall et al., 2005; Shin et al., 2006; Wang et al., 2008).

In each of the SecA crystal structures, the protomer structures are very closely related. Each SecA protomer can be subdivided into several structural domains (Figure 2). The "DEAD" motor, the catalytic core of SecA, consists of two domains: the nucleotide binding domain (NBD) and the intramolecular regulator of ATPase activity 2 domain (IRA2). Both domains are structurally homologous to the RecA-like nucleotide binding folds that are also found in DNA/RNA helicases (Tanner and Linder, 2001). At the interface of the NBD and the IRA2 domain is a large cleft, which constitutes the active site of SecA.

The substrate specificity of SecA is conferred by two unique domains that distinguish SecA from other helicases: the preprotein binding domain (PBD) and the C-terminal domain. The PBD protrudes from the NBD and contains an antiparallel β strand (stem) and a bilobate globular domain (bulb). The stem and bulb region are located at the bottom of a large cleft that has been implicated in preprotein binding (Cooper et al., 2008; Papanikou et al., 2005). The C-terminal domain is composed of four substructures: the helical wing domain (HWD), a long α-helical scaffold domain (SD), the intramolecular regulator of ATP hydrolysis (IRA1), and a C-terminal linker (CTL) (Figure 2).

Model for SecA-mediated preprotein translocation. On the basis of the currently available biochemical, structural, and biophysical data, a model for SecA-mediated preprotein translocation has been proposed. In this model, SecA is a soluble protein that partitions equally between cytoplasmic and membrane-associated states (Cabelli et al., 1991). The ATPase activity of SecA is regulated by a complex allosteric mechanism that controls the intramolecular interaction of the NBD and IRA2 domains, thereby regulating the opening and closure of the nucleotide-binding cleft and modulating the ATPase activity (Lill et al., 1990; Robson et al., 2009; Sianidis et al., 2001). In the absence of translocation ligands, the release of ADP is the main rate-limiting step of SecA catalysis (Fak et al., 2004). Consequently, cytoplasmic SecA is predominantly in an ADP-bound conformation with low ATPase activity. This compact, high-affinity ADP state is characterized by strong intramolecular interactions between the NBD and IRA2 domains and has low affinity for membranes and the SecYEG complex (Fak et al., 2004; Hunt et al., 2002). Binding to SecYEG and the membrane incurs a loosening of these interactions, resulting in an increased rate of nucleotide exchange and a marginal stimulation of the intrinsic ATPase activity ("membrane ATPase"). In addition, SecYEG binding to the DEAD motor and C-terminal domain of SecA triggers conformational changes at the interface of the IRA1, PBD, and CTL domains, leading to an increased affinity for signal peptides. Binding of the signal peptide to the PBD-NBD interface changes the conformation of the bulb region, which facilitates trapping of mature preprotein domains (Baud et al., 2002; Gouridis et al., 2009; Zimmer and Rapoport, 2009). This binding relieves the C-terminal domain–mediated suppression, which in turn results in a substantial increase of the ATPase activity ("translocation ATPase") and opening of a highly conserved salt bridge (termed Gate 1) at the base of the DEAD motor (Karamanou et al., 2007). This leads to IRA2 detachment from the NBD, which renders the DEAD motor in a loose state with decreased affinity for ADP (Karamanou et al., 2007; Keramisanou et al., 2006). The release of ADP affects the conformation of the PBD (with its preprotein bound) (Ding et al., 2003; Keramisanou et al., 2006; Papanikou et al., 2005) and results in SecB release (Fekkes et al., 1997). Subsequent binding of ATP at the nucleotide-binding cleft induces additional conformational changes that facilitate deeper insertion of SecA into the membrane and drive the coinsertion of preprotein segments of 20–30 amino acids (Schiebel et al., 1991). Next, hydrolysis of ATP causes a partial release of the preprotein into the SecYEG channel (Osborne and Rapoport, 2007) and tightens the NBD-IRA2 interaction. These local conformational changes in the DEAD motor domain are transmitted to the C-terminal domain and trigger reassociation of both domains (Vrontou et al., 2004). This compact, ADP-bound state enables SecA to de-insert from the membrane, which allows a new round of ATP binding and catalysis (de Keyzer et al., 2003).

SecA as an Antibacterial Target

A potential drug target should fulfill several criteria in order to be considered for successful chemotherapeutic intervention. In addition to being essential for bacterial growth, it should be conserved across a range of relevant pathogens and ideally should have no close human homologs. Other desirable features of the drug target include the presence of ligand-binding pockets that are druggable by small molecules and the availability of functional assays that allow high-throughput screening of large compound libraries. As will be illustrated in the following paragraphs, SecA meets each of these criteria, making it an attractive target for the development of novel antibacterials.

SecA is essential for cell viability. An important criterion for the selection of a gene product as an antibacterial target is that it is

essential for the survival of the pathogen in the host (Payne et al., 2004). Several studies have identified SecA as an essential gene in both gram-negative and -positive organisms. First, a number of conditionally lethal SecA amber mutants have been isolated in a variety of bacterial species, including the gram-negative and -positive model organisms *E. coli* and *Bacillus subtilis* (Kang and Shapiro, 1994; Oliver and Beckwith, 1981; Sadaie and Kada, 1985). Further characterization of these mutant strains has demonstrated that the SecA amber mutation is suppressed by temperature-sensitive amber suppressors, which restore bacterial growth at the permissive temperature (30°C). At the nonpermissive temperature (42°C), however, the SecA mutation leads to defective protein secretion and cell division, ultimately resulting in cell death (Oliver and Beckwith, 1981; Sadaie and Kada, 1985).

The essentiality of the SecA protein was further demonstrated via a protein knockout technique (Benson et al., 2003). Peptides that bind specifically to purified *E. coli* SecA (ecSecA) were isolated from phage display libraries and subsequently were expressed in *E. coli*. Expression of the selected peptides resulted in bacterial growth inhibition, whereas the expression of scrambled peptides had no effect. Moreover, growth inhibition could be relieved by concurrent overexpression of the SecA gene but not by coexpression of an irrelevant gene, indicating that the observed inhibition was due to a specific interaction between SecA and the expressed peptide.

The SecA gene was also found to be essential in *Corynebacterium glutamicum* (Rigel and Braunstein, 2008) and several pathogens, such as *Mycobacterium* species (Braunstein et al., 2001), *Streptococcus pneumoniae* (Akerley et al., 1998), *Staphylococcus aureus* (Forsyth et al., 2002), and *Burkholderia pseudomallei* (Moir et al., 2008).

SecA is a conserved protein with no close human homolog. The availability of the human genome sequence and the increasing number of complete microbial genome sequence data has led to the development of new approaches for the identification of promising bacterial drug targets. One such strategy is based on a subtractive genomics approach, in which the subtraction dataset between the host and pathogen genome provides information for a set of genes that are likely to be essential to the pathogen but absent in the host (Barh et al., 2009). Thus, comparison of essential bacterial genes against the human genome permits the identification of bacterial gene products that are not present or are nonhomologous to the human host. Such gene products can be considered as attractive antibacterial targets because drugs targeting these proteins will likely have a reduced toxicity to the host. Subtractive genomics approaches have been successfully used to identify drug targets in many bacterial species, such as *Pseudomonas* (Sakharkar et al., 2004), *Mycobacterium* (Anishetty et al., 2005), *Clostridium* (Chhabra et al., 2010) and *Burkholderia* (Chong et al., 2006). In these in silico studies, the SecA gene was found to be essential and unique to bacteria, suggesting that it may represent an attractive therapeutic target.

Is SecA a druggable target?. In addition to being essential for bacterial growth and having no close functional human homolog, an antibacterial drug target must also be druggable—that is, it should possess the propensity to bind druglike small molecules with high affinity. It is estimated that ~60% of small molecule drug discovery projects fail in the hit-to-lead stage because the biological target is found to be not druggable (Brown and Superti-Furga, 2003).

One approach for evaluating protein druggability is on the basis of sequence and structural homology to known therapeutic drug targets (Watson et al., 2005). Several inhibitors of superfamily 2 RNA helicases are currently in clinical development (Dropulic and Cohen, 2010), indicating that it is feasible to target members of this enzyme class by small molecules.

Another approach to predict protein druggability relies on the identification of binding pockets of suitable size, shape, and hydrophobicity to accommodate druglike molecules (Hajduk et al., 2005). Many such methods have been developed and validated using training sets of ligand-binding sites extracted from the Protein Data Bank (PDB) (Perot et al., 2010). To identify putative ligand-binding pockets on SecA, we have analyzed the molecular surface of the ecSecA NMR structure (Gelis et al., 2007) using PocketFinder (An et al., 2005). The PocketFinder algorithm, which is based on a transformation of the Lennard-Jones potential, not only detects the location of the binding pocket but also predicts envelopes representing the shape and size of putative ligand-binding pockets. The size and character of the predicted cavities can help to estimate the druggability of a pocket, because druggable pockets tend to be deep, hydrophobic, and of limited size (typically between 200 and 600 $Å^3$). Several ligand-binding pockets with appropriate shape descriptors (surface area and volume) were identified on the SecA surface (data not shown). This finding not only indicates that SecA is amenable to targeting by small molecules but also suggests that different strategies can be pursued for the design of potent and selective SecA inhibitors. For example, it is conceivable that SecA inhibitors targeting regions other than the active site can be discovered, which may serve as lead compounds for the development of antibacterials with a completely novel action mechanism.

One of the factors affecting the druggability of a bacterial target is its accessibility for small molecules. This can be a particular problem with cytoplasmic targets such as SecA, because antibiotics that target these proteins have to cross at least one bacterial membrane. Cell impermeability issues are more often encountered with gram-negative bacteria, because gram-positive organisms possess a permeable cell wall that usually does not restrict the penetration of antibacterials (Lambert, 2002).

The outer membrane of gram-negative bacteria is an additional barrier that excludes large molecules from entering the cell. However, small polar molecules, including many antibiotics, can enter the cell through aqueous protein channels formed by transmembrane proteins (porins) (James et al., 2009). Furthermore, most antibiotics show some hydrophobicity, which allows them to diffuse across the lipid bilayers of the cytoplasmic membrane.

Screening assays for the discovery of SecA inhibitors. An important aspect of the early drug discovery process is the design and implementation of high-throughput functional assays that allow the cost-effective screening of large compound libraries to identify novel drug candidates. Therefore, it is of crucial importance that the activity of the target protein can be monitored using assays that are compatible with high-throughput screening formats. Several in vitro assays with purified components have

been developed to study the function of SecA. A commonly used assay to measure the intrinsic, membrane, and preprotein-stimulated ATPase activities of SecA is based on the malachite green colorimetric method for the detection of free inorganic phosphate (Gouridis et al., 2010; Lanzetta et al., 1979; Mitchell and Oliver, 1993). Although malachite green assays have proven to be useful for high-throughput screening, the SecA ATPase activity assays are not readily adaptable to automated high-throughput screening for several reasons. A major disadvantage of the membrane and translocation ATPase assays is the complexity of the assay system, which requires the use of membrane preparations (inner membrane vesicles or proteoliposomes containing functional SecYEG complexes) and unfolded preproteins. Because many compounds in small-molecule libraries tend to be hydrophobic, it can be expected that a large proportion of the screened molecules will bind to the membrane and possibly also affect membrane integrity. This would result in many false positives, similar to the high number of membrane-damaging molecules among the hits that were selected in an in vivo high-throughput screen to detect secretion inhibitors (Alksne et al., 2000). An alternative to circumvent the difficulties associated with the use of membrane components is the use of a high-throughput screen assay to measure the inhibition of the intrinsic SecA ATPase activity. However, because the ATPase activity of SecA is suppressed by several intramolecular mechanisms in the absence of translocation ligands, such screening assays would suffer from a poor signal-to-noise ratio and reduced sensitivity. To address this problem, we have developed an automated high-throughput screen assay in 384-well format using an ecSecA mutant with elevated intrinsic ATPase activity (Segers et al., 2011). The mutated residue, Trp^{775}, is located at the interface of the SD and IRA1 domains, which are important for the intramolecular regulation of the SecA ATPase activity (Vrontou et al., 2004). Mutation of the bulky, hydrophobic Trp^{775} side chain for a short alanine side chain results in a weakened IRA1/SD interaction, which partially relieves the IRA1-mediated suppression and causes an approximately 5-fold increase in intrinsic ATPase activity compared to wild-type ecSecA (Keramisanou et al., 2006; Vrontou et al., 2004). Hopefully, our developed high-throughput screen assay is only a first progression in the development of detection methods that will facilitate the screening of large chemical libraries to discover SecA inhibitors. With respect to this, it should be noted that the SecA HTS assay monitors the inhibition of intrinsic ATP hydrolysis and therefore essentially active site inhibitors will be identified. In addition to nucleotides, however, SecA interacts with many other ligands such as the cytoplasmic membrane, the SecYEG translocation channel, molecular chaperones, and unfolded preproteins. This offers additional opportunities for the development and design of SecA inhibitors with different action mechanisms. Nevertheless, high-throughput screen methods to discover inhibitors that target the interaction of SecA with its nonnucleotide ligands still have to be developed. Fluorescence and surface plasmon resonance (SPR) techniques have already been applied to study the interaction of SecA with signal peptides, preproteins, SecYEG, and membranes (de Keyzer et al., 2003; Kusters et al., 2010; Musial-Siwek et al., 2005). Because both methods are compatible with high-throughput screening, they may prove promising tools for the development of next-generation high-throughput screen assays that are optimized to discover SecA inhibitors with different action mechanisms.

Targeting the Preprotein Translocase Motor

Small-Molecule Inhibitors of SecA

To date, only a few small-molecule inhibitors of SecA have been described. The antibacterial activity of sodium azide, the first known SecA inhibitor, was already reported at the end of the 19^{th} century (Loew, 1891). One hundred years after this discovery, it was demonstrated that sodium azide effectively inhibits Sec-dependent protein translocation in vitro and in vivo (Oliver et al., 1990). Mutations conferring sodium azide resistance in *E. coli* and *B. subtilis* were found to occur primarily in the SecA gene, indicating that SecA is the major cellular target of sodium azide (Klein et al., 1994; Nakane et al., 1995; Oliver et al., 1990). Sodium azide is known to inhibit the translocation ATPase activity of *E. coli* and *B. subtilis* SecA (IC_{50}, ~5 mM for ecSecA), but it does not affect the intrinsic, preprotein-independent ATPase activity (Nakane et al., 1995; Oliver et al., 1990). Binding of sodium azide to SecA does not block nucleotide binding at the active site and has also no effect on the formation of the compact, protease-resistant form of SecA in the presence of ATP or the poorly hydrolyzable ATP analog ATP-γ-S (van der Wolk et al., 1997). Furthermore, azide promotes the formation of a protease-resistant 30-kDa fragment in limited trypsinolysis experiments (Eichler et al., 1998; van der Wolk et al., 1997). The formation of this fragment is induced by ATP and preproteins and has been correlated with SecA membrane insertion. On the other hand, it was demonstrated by pulse-chase experiments that azide blocks membrane de-insertion of SecA, which requires the hydrolysis of ATP (Economou et al., 1995; van der Wolk et al., 1997). Taken together, it can be concluded that azide traps SecA in a transitional state during the translocation reaction by stabilizing its membrane-inserted, ADP-bound state.

In addition to SecA, azide also inhibits a number of other ATPases, including mitochondrial F-ATPases. In the crystal structure of the ternary complex of F_1-ATPase with ADP and azide, the azide anion interacts with the β-phosphate of ADP and several active site residues (Bowler et al., 2006). The presence of the azide anion brings the side chains of two catalytically essential amino acids closer to the nucleotide, creating a tighter binding interface for the ADP molecule and thereby stabilizing the ADP-bound state. Because SecA binds adenine nucleotides with a very similar geometry to F_1-ATPase (Hunt et al., 2002), it can be expected that azide inhibits SecA via a comparable mechanism. This would also explain how azide stabilizes the membrane-inserted, ADP-bound conformation of SecA.

An in vivo, high-throughput screen using a SecA-LacZ fusion reporter construct in *E. coli* was used by researchers from Wyeth to discover Sec-dependent secretion inhibitors (Alksne et al., 2000). The assay is based on the observation that SecA translation is autogenously regulated in response to changes in secretion levels (Oliver and Beckwith, 1982). Under normal secretion conditions, SecA inhibits its own expression by binding to SecA mRNA. In contrast, inhibition of secretion leads to dissociation of the SecA-mRNA complex and subsequent up-regulation of SecA translation (Schmidt and Oliver, 1989). Although this whole-cell screening system has resulted in the identification of several molecules that induced SecA expression (Figure 3,

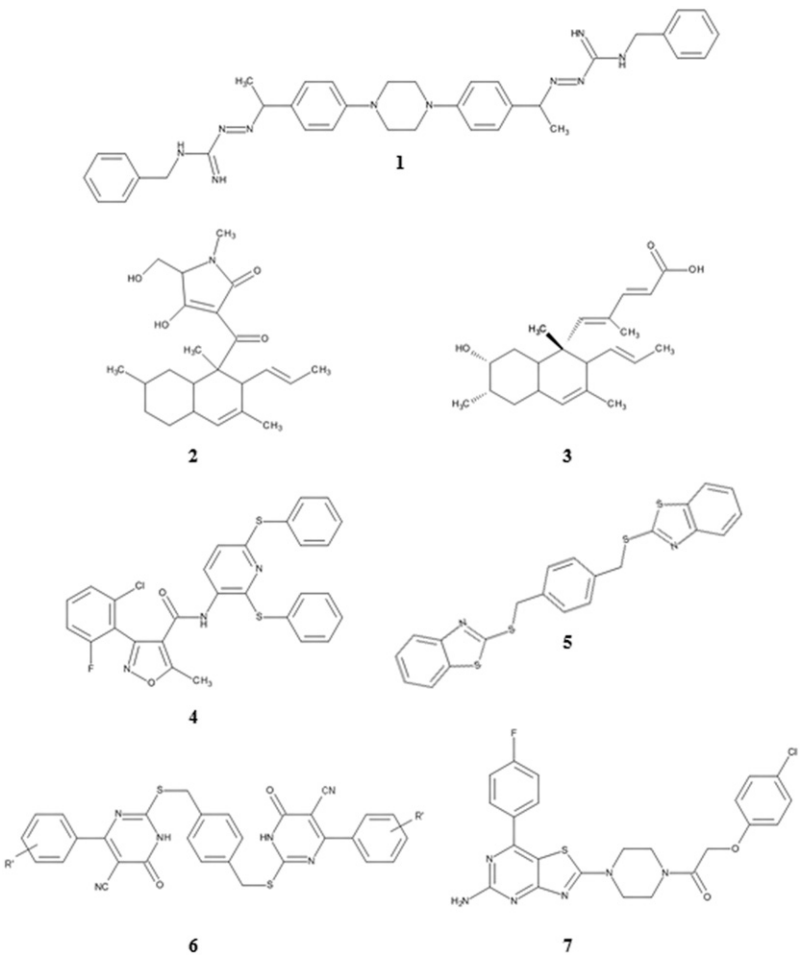

Figure 3. Selected Examples of SecA Inhibitors
1: 2-benzyl-1-[1-[4-[4-[4-[1-[(N'-benzylcarbamimidoyl)diazenyl]ethyl]phenyl]piperazin-1-yl]phenyl]ethylimino]guanidine (Alksne et al., 2000); **2**: CJ-21058 (Sugie et al., 2002); **3**: pannomycin (Parish et al., 2009); **4**: SEW-05929 (Li et al., 2008); **5**: HTS-12302 (Li et al., 2008); **6**: 2,2′-(α,α′-xylene) bis(sulfanediyl)bis-4-oxopyrimidine (Chen et al., 2010); **7**: 1-(4-(5-amino-7-(4-fluorophenyl)thiazolo[4,5-*d*]pyrimidin-2-yl)piperazin-1-yl)-2-(4-fluorophenoxy)ethanone (Jang et al., 2010).

compound **1**), indicating that they cause inhibition of protein secretion, all hit molecules were found to have deleterious effects on membranes (Alksne et al., 2000).

The first natural product inhibitor of SecA, CJ-21058 (Figure 3, compound **2**), was isolated from the fermentation broth of an unidentified fungus (Sugie et al., 2002). CJ-21058 was found to be an analog of the fusarium toxin equisetin, a fungal metabolite first isolated from the white mold *Fusarium equiseti* (Burmeister et al., 1974). Equisetin derivatives, which fall into the acyl tetramic acid class of natural products, exhibit a wide range of biological properties, including antibiotic and HIV inhibitory activity, cytotoxicity, and mammalian DNA binding (Schobert and Schlenk, 2008). CJ-21058 was found to inhibit the translocation ATPase activity of SecA (IC_{50}, 15 μg/ml) and also showed good inhibitory activity against multidrug-resistant *S. aureus* and *Enterococcus faecalis* (MIC, 5 μg/ml), but not against *Streptococcus pyogenes* and *E. coli* (Sugie et al., 2002).

A second natural product inhibitor of SecA was discovered by an antisense screening strategy, using an *S. aureus* strain with an inducible SecA antisense construct (Parish et al., 2009). A library containing over 115,000 natural product extracts was screened against this SecA antisense strain and a vector control strain using a high-throughput two-plate whole-cell differential sensitivity screen. By this strategy, a new cis-decalin secondary metabolite from *Geomyces pannorum*, a fungal strain isolated from leaf litter, was identified as a SecA inhibitor. The isolated compound, pannomycin (Figure 3, compound **3**), showed very weak antibacterial activity against the gram-positive organisms *S. aureus* (MIC, 1.4 mM), *E. faecalis* (MIC, 1.4 mM), and *B. subtilis* (MIC, 0.4 mM), but no detectable activity against *S. pneumoniae*, *Haemophilus influenzae*, *E. coli*, and *Candida albicans* (Parish et al., 2009). The structure of pannomycin is closely related to that of cissetin, a tetramic acid derivative with activity against MRSA (MIC, ~10 μM) (Boros et al., 2003). Remarkably, both molecules contain a quaternary carbon atom at position C-6, a feature that is also present in the SecA inhibitor CJ-21058. The more potent activity of cissetin and CJ-21058 may be ascribed to the presence of a tetramic acid in both structures. Altogether, the structural similarity between these natural products suggests that the decalin-tetramic acid scaffold with a quaternary carbon center at position C-6 could provide a starting point for the development of more potent SecA inhibitors.

The first structure-based approach to discover SecA inhibitors was based on virtual ligand screening against the *E. coli* SecA crystal structure (Li et al., 2008). Initially, around 60,000 commercially available compounds were screened in silico for predicted binding to the SecA active site. The 31 best-ranked molecules were then tested for in vitro inhibition of the intrinsic ATPase activity of the isolated N68 domain of SecA, which contains the DEAD motor and PBD. Using this approach, two SecA inhibitors were identified with modest activity (IC_{50}, ~100 μM) (Figure 3, compounds **4** and **5**) (Chen et al., 2010; Li et al., 2008). Chemical optimization of these two hits has resulted in the discovery of substituted thiouracils with improved inhibitory activity (IC_{50}, 20–60 μM for inhibition of the intrinsic ATPase activity of full-length SecA; Figure 3, compound **6**) (Chen et al., 2010).

A high-throughput screening assay using a SecA mutant with elevated intrinsic ATPase activity was used to screen a diverse compound library of ~27,000 molecules (Segers et al., 2011). Several inhibitors of the intrinsic, membrane and translocation ATPase activity of SecA were identified with IC_{50} values between 50 and 150 μM. Furthermore, it was shown that these hit molecules did also block the in vitro translocation of the model

preprotein AlkproPhoA into *E. coli* inner membrane vesicles containing overexpressed SecYEG. The inhibitors identified in this HTS study belong to different chemical classes, including the pyrrolo-pyrimidines and nipecotic acid derivatives.

Finally, synthesis and screening of a library of thiazolo[4,5-d] pyrimidine derivatives has identified several ATPase inhibitors of *E. coli* and *S. aureus* SecA1 (Jang et al., 2010). Kinetic analysis of the most potent inhibitor (Figure 3, compound **7**) indicated a mixed-type inhibition, with an inhibition constant of 60 μM for inhibition of the translocation ATPase activity of saSecA1.

SecA Inhibitors and the Bacterial Cellular Response

Because SecA is the essential motor protein that provides the energy required for the Sec-dependent translocation of preproteins across the cytoplasmic membrane, it can be anticipated that SecA inhibition will result in the accumulation of unfolded preproteins in the bacterial cytoplasm. Several studies have indeed demonstrated the intracellular accumulation of preproteins upon SecA inhibition (Doerrler and Raetz, 2005; Newitt and Bernstein, 1998; Oliver and Beckwith, 1982; Wild et al., 1993). It has been demonstrated that the accumulation and aggregation of unfolded proteins in the bacterial cytoplasm triggers a cellular stress response (Sabate et al., 2010; Wild et al., 1993). This response involves an increase in the levels of heat-shock proteins and chaperones, which are capable of repairing protein damage by degrading the unfolded, toxic protein aggregates or by preventing protein misfolding. However, if these rescue systems fail, for example because of an overload of unfolded or misfolded proteins, the accumulation of insoluble protein aggregates ultimately leads to cell death (Sabate et al., 2010).

Additional evidence that inhibition of SecA will have a bactericidal effect comes from a recent study on the action mechanism of chloramphenicol and tetracycline. It is widely accepted that both antibiotics target protein synthesis by specifically binding to the bacterial ribosome (Pioletti et al., 2001; Schlunzen et al., 2001), but recently another action mechanism of these molecules has been proposed (Breukink, 2009; van Stelten et al., 2009). Both antibiotics stop the translation of messenger RNA, leaving incomplete polypeptides that are firmly attached to ribosomes. Ribosomes bearing incomplete proteins with a Sec signal peptide are targeted to the cytoplasmic membrane, where they cause a physical jamming of the SecYEG translocation channel. Obstruction of the SecYEG channel results in the cytoplasmic accumulation of unfolded preproteins, which triggers a stress response (van Stelten et al., 2009). This cell response is mediated by the two-component Cpx regulatory system and leads to the proteolytic digestion of SecY by the protease FtsH. The suicidal nature of SecY destruction reflects the observation that SecY is required for the incorporation of newly synthesized SecY in the membrane (Akiyama and Ito, 1989), and further supports the notion that blocking Sec-dependent protein translocation is a valid strategy for antibacterial intervention.

Future Strategies for the Discovery of SecA Inhibitors

The majority of the SecA inhibitors identified so far are able to block the intrinsic ATPase activity, indicating that these molecules compete with ATP binding or prevent ATP hydrolysis by binding to an allosteric site on SecA. However, considering the fact that SecA plays a pivotal role in protein translocation and therefore has to interact with a plethora of ligands, additional strategies for the development of novel SecA inhibitors may be explored. Recently, a crystal structure of SecA in complex with the SecYEG translocation channel and an NMR solution structure of a SecA-signal peptide complex became available (Gelis et al., 2007; Zimmer et al., 2008). These high-resolution structures do not only provide a basis for determining the mechanistic details of the preprotein translocation process, but also offer opportunities for the rational design of inhibitors that target the interaction of SecA with the SecYEG complex or with preproteins.

The SecA-SecYEG Interaction

Despite the fact that SecA has been extensively studied and crystal structures of SecA in various conformational states are available (Hunt et al., 2002; Sharma et al., 2003; Vassylyev et al., 2006; Zimmer et al., 2006), structural information about its association with the SecYEG translocation channel is limited. In recent years however, substantial progress has been made toward a better understanding of the SecA-SecYEG interaction.

The first detailed view of a bacterial core translocase was provided by the high-resolution crystal structure of the archaeal SecYEβ complex of *Methanococcus jannaschii* (Van den Berg et al., 2004). The structure reveals that the 10 transmembrane segments (TM) of the main subunit of the complex, SecY, are arranged like a clamshell in which the two halves (TM 1–5 and TM 6–10) are connected by a periplasmic loop between TM5 and TM6 (Figures 4A and 4B). The second subunit of the complex, SecE, has a single highly tilted TM domain that tracks across one face of the complex and makes extensive contacts with SecY (Figure 4B). A surface-exposed amphiphatic helix of SecE runs parallel to the cytoplasmic face of the membrane. The small β subunit was identified as a single helix on the outside of the complex, consistent with the observation that it is not essential for the major activity of the protein channel.

Different techniques have been used to define the interaction of SecA with the SecYEG complex and to map the relevant binding sites (Karamanou et al., 2008; Mori and Ito, 2006; Robson et al., 2007; van der Sluis et al., 2006). By a combination of peptide scanning and cysteine-directed cross-linking, two functionally important regions in SecY that are involved in the SecA interaction were identified (van der Sluis et al., 2006). The first region comprises several residues in the cytoplasmic loop between TM8 and TM9, whereas the second interaction site is part of TM4 of SecY that is buried in the membrane region of SecYEG (Figures 4A and 4B). On the basis of an in vivo site-directed cross-linking approach, a dynamic and function-related interaction between SecA and the C-terminal cytoplasmic region (Figures 4A and 4B) of SecY was proposed (Mori and Ito, 2006). In the same study, cytoplasmic loops 4 and 5 of SecY were put forward to provide constitutive binding sites for SecA. A synthetic peptide-binding array based on the cytosolic regions of SecY and SecE identified regions in the C4 and C5 loops of SecA as binding partners for SecA (Robson et al., 2007). An extensive study using peptide binding arrays, thermodynamic quantification, mutagenesis, and functional assays have mapped the SecA interaction sites to five major binding regions in SecYEG (Karamanou et al., 2008). With the exception of two minor sites, all the identified SecA-binding sites in this study were localized at the cytoplasmic side of SecY and SecE.

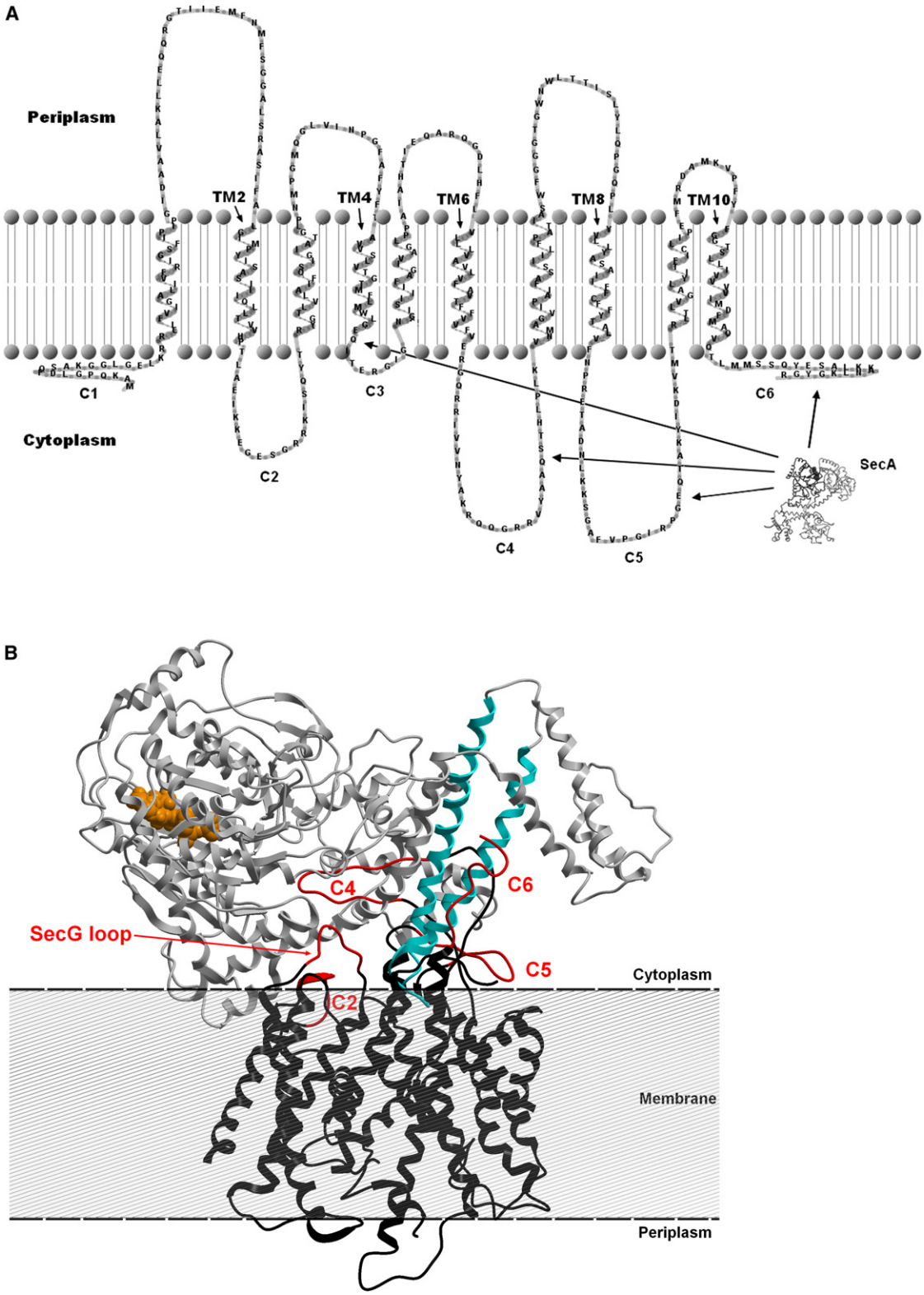

Figure 4. Membrane Topology of *E. coli* SecY and Interaction of SecA with the SecYEG Complex
(A) Transmembrane domains (TM) and cytoplasmic loops (C) are numbered starting from the N-terminal end of the SecY protein. Transmembrane regions and cytoplasmic loops interacting with SecA are indicated with arrows.
(B) The crystal structure of *Thermotoga maritima* SecA (gray) in complex with SecYEG (black) is shown (PDB code 3DIN). Cytoplasmic loops making contacts with SecA are indicated in red. The SecA helix-two-helix finger is shown in cyan, ATP in ball representation (orange).

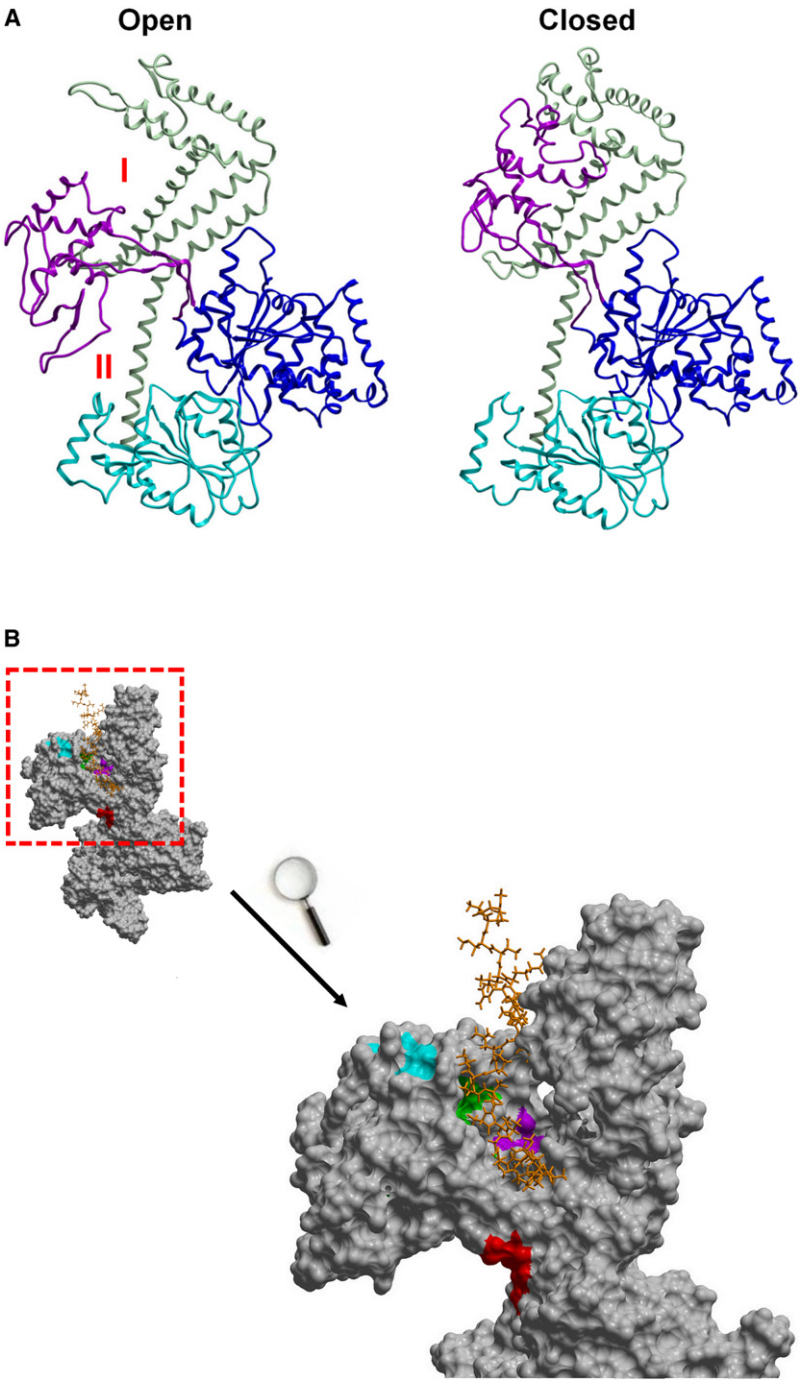

Figure 5. Open and Closed Conformation of SecA and Potential Preprotein-Binding Sites in SecA
(A) The open (PDB code 1TF5) and closed (PDB code 1M6N) conformation of *B. subtilis* SecA are shown in ribbon representation. Color coding of SecA domains: NBD, dark blue; IRA2, cyan; PBD, purple; and IRA1, SD, and HWD, green. Preprotein binding grooves 1 and 2 are indicated by the Roman numerals I and II, respectively.
(B) The NMR structure of *E. coli* SecA (gray surface) in complex with the LamB signal peptide (orange ball-and-sticks) is shown (PDB code 2VDA). The two hydrophobic pockets in groove 1 that are discussed in the text are represented as green and purple surfaces. The potential signal peptide binding region proposed by Musial-Siwek et al. (2007) is shown as a cyan surface. The β strand region in groove 2 that has been implicated in preprotein binding is represented as a red surface.

Most of the findings of these studies on the SecA-SecYEG interaction were confirmed recently by the elucidation of the crystal structure of the SecA-SecYEG complex from *Thermotoga maritima* (Figure 4B) (Zimmer et al., 2008). The structure shows that the flat SecA molecule is oriented approximately parallel to the plane of the membrane, creating an extensive binding interface with SecYEG. In line with the available functional data, the most crucial interactions occur between the C4 and C5 loops of SecY and the PBD of SecA. Other important contacts are made by the SD of SecA, which interacts with the SecG loop, the C2 loop, and the C-terminal tail of SecY. Minor interactions also occur between NBD1 of SecA and SecG, whereas IRA2 is not involved in SecYEG binding. Remarkably, the two-helix finger of the SD is inserted deeply into the cytoplasmic funnel of SecY, suggesting an important role of this substructure in protein translocation. This was confirmed later by mutagenesis and cross-linking studies, which have demonstrated that the tip of the two-helix finger interacts with the preprotein during translocation (Zimmer and Rapoport, 2009). On the basis of these functional and structural data, it was proposed that the helix-finger of SecA moves up and down during the ATP hydrolysis cycle and pushes the polypeptide into the SecY channel (Zimmer et al., 2008; Zimmer and Rapoport, 2009).

The SecA-Preprotein Interaction

The available high-resolution structures of SecA show that SecA can adopt at least two distinct conformational states (Hunt et al., 2002; Osborne et al., 2004; Papanikolau et al., 2007; Sharma et al., 2003; Vassylyev et al., 2006; Zimmer et al., 2006; Zimmer et al., 2008). In the closed conformation, the PBD and HWD are in direct contact with each other, whereas in the open conformation, the PBD has been rotated toward IRA2 by about 80° (Figure 5A) (Osborne et al., 2004). This rigid body movement of the PBD creates two deep grooves on the SecA surface. The first groove is formed between the PBD, HWD, and HSD, whereas the second groove is created at the interface of the PBD and IRA2. Both grooves have been proposed as potential preprotein-binding sites on the basis of functional and structural data.

Groove 1 was initially suggested as a potential polypeptide-binding site on the basis of the crystal structure of *B. subtilis* SecA in a monomeric, open conformation (Figure 5A) (Osborne et al., 2004). Two large pockets in the groove have been proposed to accommodate the side chains of the bound

polypeptide (Figure 5B), whereas charged and polar residues lining the groove have been put forward to interact with the polypeptide backbone. Selective photoaffinity labeling using a photolabeled alkaline phosphatase signal peptide has identified a potential signal peptide-binding site in tandem with groove 1 (see also Figure 5B) (Musial-Siwek et al., 2007). It has been speculated that the mature region of the preprotein may extend C-terminally into groove 1, making backbone contacts with the charged and polar residues lining the groove. Further evidence for the involvement of groove 1 in preprotein binding is provided by an NMR solution structure of ecSecA in complex with a functional signal peptide derived from the LamB porin (Gelis et al., 2007). The structure shows that the α-helical signal peptide binds into a large hydrophobic groove that is surrounded by polar and charged residues. The importance of both hydrophobic and electrostatic interactions for strong peptide binding was confirmed by site-directed mutagenesis and functional and thermodynamic studies (Gelis et al., 2007).

The second groove, often referred to as "the clamp," is lined by two β strands that connect the PBD and NBD1 (Figure 5B). Several functional studies have suggested that both β strands play an important role in peptide binding (Auclair et al., 2010; Baud et al., 2002; Bauer and Rapoport, 2009; Chou and Gierasch, 2005; Osborne et al., 2004; Papanikou et al., 2005). Further support that the clamp is important for preprotein binding is found in a cocrystal structure of bsSecA and a hydrophilic peptide that does not resemble a signal peptide (Zimmer and Rapoport, 2009). The structure shows that the peptide augments the β sheet at the back of the clamp. The extension of the β sheet would be sequence-independent, consistent with the fact that SecA needs to bind a broad range of polypeptide segments.

Targeting the Interaction of SecA with Its Diverse Ligands

The increasing number of high-resolution structures of SecA in complex with its different ligands offers opportunities for the rational design of inhibitors that target the interaction of SecA with its various binding partners.

A first strategy is to identify inhibitors that prevent ATP binding and/or hydrolysis. Crystal structures with bound nucleotide show that the active site of SecA is located at the interface of NBD and IRA2. The main interactions with the nucleotide take place in a narrow pocket that contains the classic mononucleotide-binding motif, known as the P loop or Walker motif. Most of these water-mediated or direct interactions with the nucleotide are electrostatic in nature. In these structures, the adenine ring points toward the solvent and makes only a limited number of contacts with the protein (Hunt et al., 2002; Papanikolau et al., 2007). Altogether these structural features are not very appealing for drug discovery. It has been reported that the strength of the interaction between a protein and a small molecule is highly correlated with the apolar surface area that is buried upon the interaction (Olsson et al., 2008). Hence, it may be difficult to find potent and selective active site inhibitors of SecA, which is also reflected by the fact that no such inhibitors have been discovered so far.

An alternative strategy would be to identify compounds that inhibit protein translocation by preventing the interaction of SecA with the SecYEG translocation channel or with preprotein. The identification or design of protein-protein interaction (PPI) inhibitors is a far more complicated process than the development of molecules targeting active sites or ligand-binding pockets. First, the contact surfaces involved in PPIs are much larger compared with those in protein-small molecule interactions (Wells and McClendon, 2007). Another major challenge in the search for potent PPI inhibitors is the flatness of most protein interfaces, which implies that only a few cavities are available for small-molecule binding. Finally, many protein-binding sites turn out to be structurally adaptive, suggesting that the best binding site for a small-molecule inhibitor will not always be visible in a single crystal structure (Wells and McClendon, 2007). Despite the many challenges associated with the development of PPI inhibitors, significant progress has been made in the field, as illustrated by the fact that several candidate PPI inhibitors have entered clinical trials in recent years (Gao et al., 2010; Richardson and Kaye, 2008). This success is mainly due to the existence of "hot spots," which are compact regions at many protein-protein interfaces that are crucial for the high affinity of the interaction (Arkin and Wells, 2004). The binding of a small molecule to a subset of hot spot residues is sufficient to interfere with the interaction of two proteins, making hot spots promising drug targets (Arkin and Wells, 2004; Wells and McClendon, 2007).

Hot spots on the SecA surface that are important for SecYEG or preprotein binding have not been identified yet, but the structures of SecA bound to nucleotide (Hunt et al., 2002), SecYEG (Zimmer et al., 2008), or a preprotein peptide (Gelis et al., 2007; Zimmer and Rapoport, 2009) reveal the presence of several pockets at the SecA surface that may represent attractive drug targets.

First, computational analysis of the molecular surface of *B. subtilis* SecA has revealed the presence of a number of methionine-rich cavities at the interface of NBD1, IRA2, and SD that may represent a potential binding site for a phospholipid ligand or a preprotein (Hunt et al., 2002). Although the cavity is only wide enough to accommodate a hydrocarbon moiety, the high backbone B factors in this region of the crystal structure suggest that the cavity could expand upon ligand binding. If this region is indeed crucial for ligand binding, it may represent an attractive drug target site because this region is conserved and hydrophobic in nature (and therefore likely suited to bind small-molecules with high affinity).

Another potential drug target is a deep pocket that is formed by residues of the PBD and the C-terminal domain of SecA. In the SecA-SecYEG crystal structure, this pocket is occupied by the C4 loop of SecY, which makes one of the most crucial interactions with SecA (Figure 4B) (Zimmer et al., 2008).

Other candidate drug target sites on the SecA surface are the three grooves that were proposed to be involved in preprotein binding (Gelis et al., 2007; Musial-Siwek et al., 2007; Zimmer and Rapoport, 2009). The crystal structure of SecA in complex with a hydrophilic peptide bound to groove 2 indicates that the two β strands lining the groove interact with the peptide backbone by inducing a β strand conformation (Zimmer and Rapoport, 2009). The absence of a well-defined, hydrophobic pocket suggests that groove 2 may not represent a very attractive target for drug discovery. The two other regions in SecA that have been implicated in preprotein binding, the large groove 1 (Gelis et al., 2007) and a smaller groove adjacent to it (Musial-Siwek et al., 2007), appear to be more druggable. Both sites contain several

hydrophobic cavities that are buried enough to bind small molecules with high affinity. If these sites are indeed crucial for preprotein binding, they may represent promising targets for the structure-based rational design of small molecules that inhibit the SecA-preprotein interaction.

Future Perspective

Several high-resolution structures of SecA in its apo form or in complex with nucleotide or protein ligands have become available in recent years. These structures have not only increased our understanding of the molecular mechanism of Sec-dependent protein translocation, but also provide a starting point for structure-based drug discovery.

The cocrystal structures of SecA with bound nucleotide show that the adenine ring points toward the solvent and there is little interaction with the protein. Together with the fact that the ATP-binding Walker motifs in the active site of SecA are present in many ATP-binding proteins, it is obvious that it might be difficult to design selective and potent active site inhibitors of SecA. An appealing alternative to active site inhibitors is to target the interaction of SecA with the SecYEG complex or with preprotein. The recently determined high-resolution structures of SecA with bound preprotein peptide or in complex with the translocase channel will certainly aid in the development of such inhibitors. A future challenge is to obtain high-resolution structures that provide representative snapshots of the different conformational states of SecA that are adopted during the ATP hydrolysis cycle. In combination with additional functional data that provide detailed information on the mechanism by which the dynamics and the ATPase activity of SecA are regulated, such structures may provide the framework for the structure-based design of allosteric SecA inhibitors. Allosteric binding sites have not faced the same evolutionary pressure as active sites to accommodate an endogenous ligand and are therefore more diverse. Consequently, it can be anticipated that more selective SecA inhibitors may be obtained by targeting allosteric sites.

Finally, the crowning glory of SecA research would be the elucidation of the nanomotor in action—that is, bound to the SecYEG channel and a translocating preprotein polypeptide. Such structure will undoubtedly offer new opportunities for drug design and will further advance our understanding of the molecular mechanism of SecA-dependent preprotein translocation.

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