The SecA protein deeply penetrates into the SecYEG channel during insertion, contacting most channel transmembrane helices and periplasmic regions

Tithi Banerjee, Zeliang Zheng, Jane Abolafia, Shelby Harper, and Donald Oliver

From the Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, Connecticut 06459

Edited by Thomas Sollner

The bacterial Sec-dependent system is the major protein-bio- genic pathway for protein secretion across the cytoplasmic membrane or insertion of integral membrane proteins into the phospholipid bilayer. The mechanism of SecA-driven protein transport across the SecYEG channel complex has remained controversial with conflicting claims from biochemical and structural studies regarding the depth and extent of SecA insertion into SecYEG during ongoing protein transport. Here we utilized site-specific in vivo photo-crosslinking to thoroughly map SecY regions that are in contact with SecA during its insertion cycle. An arabinose-inducible, rapidly folding OmpA-GFP chimera was utilized to jam the SecYEG channels with an arrested substrate protein to “freeze” them in their SecA-inserted state. Examination of 117 sites distributed throughout SecY indicated that SecA not only interacts extensively with the cytosolic regions of SecY as shown previously, but it also interacts with most of the transmembrane helices and periplasmic regions of SecY, with a clustering of interaction sights around the lateral gate and pore ring regions. Our observations support previous reports of SecA membrane insertion during in vitro protein transport as well as those documenting the membrane penetration properties of this protein. They suggest that one or more SecA regions transiently integrate into the heart of the SecY channel complex to span the membrane to promote the protein transport cycle. These findings indicate that high-resolution structural information about the membrane-inserted state of SecA is still lacking and will be critical for elucidating the bacterial protein transport mechanism.

This work was supported by National Institutes of Health Grant GM110552 (to D. O.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

1 Present address: BioMed: Molecular Biology, Cell Biology, and Biochemistry, Brown University, Box G-J364, 185 Meeting St., Providence, RI 02912.
2 Present address: Duke University School of Medicine, Durham, NC 27710.
3 To whom correspondence should be addressed. E-mail: doliver@wesleyan.edu.
the reciprocating piston model that utilizes both conformational changes during the dynamic SecA monomer–dimer cycle as well as the reciprocating action of the two-helix finger to drive SecA-bound substrate into the channel in two successive steps (19, 20), or (iv) some combination of the above models that are not mutually exclusive (21). Thus the current models of SecA action no longer call for deep penetration of SecA into the membrane or the channel proper or expansion of the channel in the process. However, measurements of the pore size of the SecYEG channel with engineered substrates indicate that it can accommodate structures of at least 22–24 Å in diameter, which is significantly larger than predictions of the channel dimensions based on molecular dynamic simulations of existing crystal structures (22, 23). In addition, a number of genetic and biochemical studies of protein transport point to the relevance of the original SecA insertion assay as a measure of transport function within the system (e.g., see Ref. 24).

Beyond the work that led to the original SecA power stroke model (14), the literature is replete with reports of SecA membrane penetration either alone or in the context of SecYEG protein. For example, studies with purified SecA indicate that it penetrated deeply into lipid monolayers or bilayers or spanned them in an acidic phospholipid-dependent fashion and that the associated conformational changes were modulated by adenosine nucleotides (25–27). Electron microscopic studies of SecA in phospholipid monolayers showed that it forms ring-like structures with a 2-nm central pore (28), whereas the Xenopus oocytes system has been utilized to detect ion channel activity elicited by SecA incorporated into liposomes (29).

Subcellular fractionation studies of Escherichia coli have previously detected soluble, lipid, and SecYEG-bound forms of SecA, where the latter form was found to be integral to the membrane as defined by extractability with standard chaotropic reagents (30–32). Several studies have investigated the topology of integral membrane SecA in right-side out membrane vesicles, outer membrane-permeabilized cells, or normal cells utilizing membrane-impermeable biotinylation reagents or probe/te probing. Integral membrane SecA was found to reveal multiple periplasmically-exposed regions under these conditions with its CTL4 region accessible to trypsinolysis (31, 33–35). Unfortunately in such studies it is impossible to distinguish whether the observed labeling pattern was due to the existence of authentic periplasmic regions of SecA or whether such labeling occurred utilizing the SecY channel to gain access to internal portions of SecA protein.

Given the foregoing analysis and its equivocal nature, we decided to directly readdress the issue of whether SecA insertion into the SecYEG translocon occurs as part of protein transport, and more specifically to probe the depth of SecA channel insertion. Given the biochemical and structural approaches that have been utilized previously, we undertook a site-directed in vivo photo-crosslinking approach to map the SecA interaction sites throughout the SecY protein in a more physiological manner. Our results are described below, and they provide convincing evidence of extensive integration of SecA into the SecYEG channel complex, consistent with the original power stroke model as described by Economou and Wickner (14).

**Results**

We have previously utilized site-directed in vivo photo-crosslinking to characterize the SecY-interactive face of SecA protein and to validate the in vivo relevance of the Thermotoga maritima SecA-SecYEG crystal structure for the E. coli model system (16, 36). In addition, this technique has previously been utilized to characterize the SecA-interactive residues on the cytosolic loops of the SecY protein (37), which mediate, in part, SecY-dependent SecA receptor activity (38). In the present study we have extended this analysis to include an additional 7 residues within the six cytosolic loops of SecY, 86 residues within the 10 transmembrane helices of SecY, and 24 residues within the five periplasmic loops of SecY (Fig. 1A and Table 1). In general we engineered amber mutations approximately every third residue in the latter two regions of SecY to achieve good coverage of these previously uncharacterized regions. Residues on both the interior and exterior surface of the channel were selected to probe for SecA interaction within the pore region or on the lipidic side of the channel complex, respectively. We also made use of an araBAD-inducible OmpA-GFP chimera that contains the OmpA signal peptide and early mature portion fused to a rapidly folding domain of GFP to jam the channel with this substrate and “freeze” SecA in its membrane-inserted state (Fig. 1B). The efficacy of this system to achieve rapid channel jamming and inhibition of protein transport has been documented previously (39, 40).

We constructed strains that contained the appropriate plasmids for the production of amino terminally c-Myc-tagged SecY with the relevant amber allele along with the orthogonal amber suppressor tRNA and pBpA-inserting tRNA synthetase as well as the ara-BAD-inducible OmpA-GFP chimera. We first titrated our system with the SecY434 mutant to ensure that sensitive detection of the relevant photo-crosslinked SecA-SecY product could be readily detected (Fig. 2A). This SecY allele contains the amber mutation within the C6 cytosolic loop, and it has been shown previously to strongly photo-crosslink to SecA (37). Titration of our T7-driven SecYEG system with inducer indicated that c-Myc-tagged SecY was overproduced moderately at 30 μM IPTG (~7-fold) (Fig. 2B), and this concentration was chosen for further study. In our pilot photo-crosslinking experiment, the formation of the SecA-SecY434 cross-linked product was dependent on UV exposure and was greatly enhanced by translocon jamming (Fig. 2, C–E), consistent with previous results indicating that SecY C6 association with SecA occurs mainly during the translocation reaction itself (37). The build-up of pre-MBP within the cytoplasm–membrane fraction as translocon jamming progressed and plateaued ~45 min after arabinose induction demonstrated that jamming was effective under these conditions (Fig. 2F). Fur-
thermore, we demonstrated efficient insertion of the OmpA-GFP chimera to the trans side of the channel by monitoring in vivo disulfide cross-linking of Cys\textsuperscript{21} at the end of the OmpA signal peptide of the chimera to Cys\textsuperscript{68} within the plug domain of SecY as demonstrated previously (Fig. 2, G and H) (39). Of course this method underestimates the extent of translocon jamming, because it is limited to the efficiency of disulfide cross-linking for particular cysteine pairs.

We next screened our collection of 117 SecY amber mutants under these established conditions by in vivo photo-crosslinking. This resulted in 4, 33, and 8 cross-linking-positive SecY residues within the cytosolic loops, transmembrane helices, or periplasmic loops of SecY, respectively (Fig. 3). Two or more electrophoretically distinct, SecA-SecY cross-linked products were observed in many of the SecY mutants (e.g. see P100 or K434 in Fig. 3A) consistent with earlier studies (37, 40, 41). This result arises because of the broad chemical reactivity of pBpA and its ability to cross-link to two or more neighboring residues of the target to create products of differing axial radii when analyzed by SDS-PAGE. In certain cases it appeared that some cross-linked SecA-SecY products also underwent limited proteolysis based on their smaller size despite the presence of multiple protease inhibitors during sample preparation (e.g. see starred bands in Fig. 3). Alternatively, such species may represent cross-linking of smaller membrane proteins to SecY along with nonspecific immunodetection by our polyclonal SecA antisera (examined in more detail below). The intensity of the various SecA-SecY cross-linked products varied considerably within and between mutants, which presumably was dependent on several features: (i) the stability of the various SecA-SecY contacts, (ii) their dependence on OmpA-GFP jamming, and (iii) the geometry of the UV-induced pBpA diradical relative to

---

**Figure 1. Experimental strategy.** A, Sec61αβγ/SecYEG crystal structures from M. jannaschii (PDB code 1RHZ) (6) (upper row) or T. maritima (PDB 3DIN) (16) (lower row) highlighting the residues selected (depicted as colored spheres) for probing for SecA interaction within the SecY cytosolic loops as viewed from the top (i and iv), transmembrane helices as viewed from the side (ii and v), and periplasmic loops as viewed from the side (iii and vi). Sec61αβγ/SecYEG polypeptide chains are colored in gray, green, and orange, respectively. B, schematic diagram of SecA binding (left-hand side) and insertion (right-hand side) into the SecYEG channel during jamming caused by OmpA-GFP chimera production after arabinose induction.
### Table 1

List of SecY mutations used in this study

| Residue in E. coli | Equivalent residue in M. jannasch | Equivalent residue in T. maritima | Position in T. maritima | In vivo photo-crosslinking result |
|-------------------|-----------------------------------|-----------------------------------|------------------------|---------------------------------|
| P100              | S91                               | P100                              | C2 loop                | +                               |
| R181              | Y164                              | K181                              | C3 loop                | +                               |
| I183              | H166                              | I183                              | C3 loop                | −                               |
| G273              | S255                              | G269                              | C4 loop                | −                               |
| V274              | N273                              | V270                              | C4 loop                | −                               |
| I275              | E273                              | I271                              | C4 loop                | −                               |
| K434              | A431                              | K423                              | C4 loop                | ++f                             |
| K20               | K18                              | K24                               | TM1                    | −                               |
| R21               | E25                               | D16                               | TM1                    | −                               |
| L26               | K28                               | I19                               | TM1                    | +                               |
| V26               | T30                               | T21                               | TM1                    | +                               |
| V32               | L36                               | V27                               | TM1                    | +                               |
| F35               | Y37                               | F28                               | TM1                    | +                               |
| I35               | I39                               | A30                               | TM1                    | −                               |
| E62               | E57                               | S62                               | TM2a                   | −                               |
| f                 |                                   |                                   |                        |                                 |
| S68               | T63                               | T68                               | TM2a                   | −                               |
| G70               | S65                               | G70                               | b/w TM2a-TM2b          | ++f                             |
| L72               | S67                               | L72                               | b/w TM2a-TM2b          | −                               |
| A74               | N74                               | R74                               | b/w TM2a-TM2b          | R74                              |
| S76               | S69                               | S76                               | b/w TM2a-TM2b          | +                               |
| f                 |                                   |                                   |                        |                                 |
| F78               | I71                               | F78                               | b/w TM2a-TM2b          | −                               |
| L80               | L73                               | M80                               | TM2b                   | −                               |
| I82               | I75                               | V82                               | TM2b                   | ++f                             |
| P84               | P77                               | P84                               | TM2b                   | ++f                             |
| I86               | V79                               | I86                               | TM2b                   | ++f                             |
| f                 |                                   |                                   |                        |                                 |
| S87               | T80                               | T87                               | TM2b                   | −                               |
| f                 |                                   |                                   |                        |                                 |
| A88               | A81                               | A88                               | TM2b                   | +                               |
| S89               | G82                               | S89                               | TM2b                   | −                               |
| I90               | I83                               | I90                               | TM2b                   | +                               |
| f                 |                                   |                                   |                        |                                 |
| I28               | M85                               | L92                               | TM2b                   | −                               |
| Q93               | I86                               | Q93                               | TM2b                   | −                               |
| Q131              | I127                              | Q131                              | TM3                    | −                               |
| G134              | I125                              | F134                              | TM3                    | −                               |
| f                 |                                   |                                   |                        |                                 |
| L139              | A140                              | A140                              | TM3                    | +                               |
| N141              | S142                              | S142                              | TM3                    | −                               |
| f                 |                                   |                                   |                        |                                 |
| T159              | L142                              | L159                              | TM4                    | −                               |
| T166              | F149                              | A166                              | TM4                    | −                               |
| F170              | I153                              | F170                              | TM4                    | −                               |
| I179              | I150                              | I179                              | TM4                    | −                               |
| I189              | I172                              | I189                              | TM5                    | ++f                             |
| I191              | I174                              | I191                              | TM5                    | −                               |
| f                 |                                   |                                   |                        |                                 |
| V196              | S179                              | S196                              | TM5                    | −                               |
| f                 |                                   |                                   |                        |                                 |
| L199              | I182                              | V199                              | TM5                    | −                               |
| L218              | E207                              | L214                              | TM6                    | −                               |
| f                 |                                   |                                   |                        |                                 |
| V225              | E214                              | A221                              | TM6                    | −                               |
| F232              | L221                              | F228                              | TM6                    | −                               |
| F236              | V224                              | I231                              | TM6                    | −                               |
| A277              | V259                              | I273                              | TM7                    | +                               |
| I278              | V260                              | I274                              | TM7                    | +                               |
| f                 |                                   |                                   |                        |                                 |
| F279              | L261                              | F275                              | TM7                    | +                               |
| S282              | A264                              | A278                              | TM7                    | −                               |
| I283              | L265                              | I279                              | TM7                    | −                               |
| f                 |                                   |                                   |                        |                                 |
| F286              | N268                              | I282                              | TM7                    | −                               |
| P287              | I269                              | P283                              | TM7                    | −                               |
| T289              | L274                              | A285                              | TM7                    | ++f                             |
| A291              | G273                              | A287                              | TM7                    | +                               |
| F294              | L276                              | I289                              | TM7                    | −                               |
| L316              | V318                              | L305                              | TM8                    | +                               |
| S323              | I325                              | L312                              | TM8                    | −                               |
| I325              | C327                              | V314                              | TM8                    | ++f                             |
| F327              | M329                              | E316                              | TM8                    | +                               |
| C329              | G331                              | T318                              | TM8                    | −                               |
| Y332              | W334                              | Y321                              | TM8                    | −                               |
Table 1—continued
List of SecY mutations used in this study

| Residue in E. coli<sup>a</sup> | Equivalent residue in M. jannaschii<sup>b</sup> | Equivalent residue in T. maritima<sup>c</sup> | Position in T. maritima SecYEG structure<sup>d</sup> | In vivo photo-crosslinking result<sup>e</sup> |
|-----------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| T333                        | V335                            | S322                            | TM8                             | −                               |
| L335                        | T337                            | V324                            | TM8                             | −                               |
| R372                        | R372                            | R361                            | TM9                             | −                               |
| L375                        | P375                            | P364                            | TM9                             | −                               |
| L379                        | V379                            | V368                            | TM9                             | −                               |
| M391                        | I391                            | V380                            | TM9                             | ++                              |
| T404                        | T402                            | T393                            | TM10                            | +                               |
| L406                        | V404                            | A395                            | TM10                            | +                               |
| I408                        | L406                            | I397                            | TM10                            | ++                              |
| D415                        | R413                            | D404                            | TM10                            | ++                              |
| F416                        | M414                            | I405                            | TM10                            | ++                              |
| M417                        | Y415                            | I406                            | TM10                            | −                               |
| Q421                        | L419                            | E410                            | TM10                            | −                               |
| M424                        | K422                            | M413                            | TM10                            | ++                              |
| V48                         | I52                             | A43                             | P1 loop                         | +                               |
| A50                         | NA                              | G45                             | P1 loop                         | −                               |
| L52                         | NA                              | I47                             | P1 loop                         | −                               |
| E54                         | NA                              | R50                             | P1 loop                         | −                               |
| Q56                         | NA                              | T54                             | P1 loop                         | −                               |
| G58                         | P53                             | A58                             | P1 loop                         | ++                              |
| I60                         | I55                             | I60                             | P1 loop                         | −                               |
| P143                        | A131                            | P144                            | P2 loop                         | −                               |
| Q146                        | I134                            | V147                            | P2 loop                         | −                               |
| L148                        | T136                            | P149                            | P2 loop                         | −                               |
| V149                        | P137                            | G150                            | P2 loop                         | −                               |
| F154                        | NA                              | L154                            | P2 loop                         | ++                              |
| I207                        | L197                            | NA                              | P3 loop                         | ++                              |
| H216                        | P205                            | N212                            | P3 loop                         | ++                              |
| G299                        | I281                            | NA                              | P4 loop                         | −                               |
| W300                        | G290                            | NA                              | P4 loop                         | −                               |
| W302                        | A292                            | T294                            | P4 loop                         | −                               |
| P312                        | I314                            | A301                            | P4 loop                         | ++                              |
| R392                        | A392                            | Q381                            | P5 loop                         | −                               |
| A394                        | F394                            | A383                            | P5 loop                         | −                               |
| V397                        | A397                            | V386                            | P5 loop                         | ++                              |
| P398                        | L398                            | N387                            | P5 loop                         | −                               |
| Y400                        | NA                              | W389                            | P5 loop                         | −                               |
| G402                        | G400                            | G391                            | P5 loop                         | −                               |

<sup>a</sup> The residue of E. coli SecY that was mutated to amber is indicated.
<sup>b</sup> The residue of M. jannaschii SecY that is equivalent to its E. coli SecY homolog is indicated using the alignment given in Zimmer et al. (16).
<sup>c</sup> The residue of T. maritima SecY that is equivalent to its E. coli SecY homolog is indicated using the alignment given in Zimmer et al. (16).
<sup>d</sup> The location of the SecY residue selected in the T. maritima SecA-SecYEG structure is given and grouped according to the SecY cytosolic loops, transmembrane helices or periplasmic loops. b/w indicates “between.”
<sup>e</sup> The intensity of the resulting SecA-SecY crosslinked product for a given mutant is indicated on a graded scale of plus signs using SecY434, which was always included as the standard in the various Western blots that were performed. − indicates no cross-linked SecA-SecY product was observed.

Indicates that the listed SecY mutant was constitutively-positive for crosslinking. All other SecY mutants missing this superscript were jamming dependent for cross-linking.

NA, not applicable.

The available C-H acceptors that require a 108.9 angle for an optimal reaction to occur (42). Regarding the overall selectivity of this methodology, we note that most of our mutants were negative for SecA-SecY cross-linking (see Fig. 3 for representative examples), and positive cross-linking was entirely dependent on the presence of the orthogonal tRNA-tRNA synthetase pair and pBpA within the media (data not shown).

Our data fell into two basic patterns: we found a smaller subset of SecY residues that were positive for cross-linking with or without OmpA-GFP jamming (i.e. constitutively positive), and a larger subset of SecY residues whose cross-linking was largely or entirely dependent on OmpA-GFP jamming (i.e. jamming dependent). One would expect a lower level of cross-linking in the normal (i.e. non-jammed) state for this later group due to a modest level of membrane-inserted SecA during normal protein transport. Both types of patterns were found for mutants within the cytosolic loops of SecY (Fig. 3A), consistent with Mori and Ito’s (37) hypothesis that certain regions of SecA bound to SecY in a static fashion (e.g. SecY275), whereas other regions were recruited for binding during the conformation changes associated with ongoing protein translocation (e.g. SecY100, SecY181, and SecY434). Remarkably, SecY mutants for residues within the TM2a and TM2b region (Phe<sup>64</sup>–Ala<sup>88</sup>) around the lateral gate were also found to be constitutively positive (Fig. 3B), indicative of at least transient SecA insertion into this deeper and critical region of the channel that was independent of substrate occupancy (Fig. 4). The remainder of the positive SecY residues within the transmembrane segments and all of those that were positive within the periplasmic loops were found to be jamming dependent (Fig. 3, B and C), consistent with the proposal that SecA undergoes membrane insertion that is dependent on substrate binding and ongoing protein transport. Combining our findings with those of Mori and Ito (37), the percentage of positive SecY residues within these three regions was 45% (cytosolic loops), 35% (transmembrane helices), and 33% (periplasmic loops) from a total of 47, 94, and 24 residues examined, respectively. It is notable that these three cross-linking frequencies are not so dissimilar given the very different topological environments sampled here. Thus our results indicate that SecA interacts extensively with all three
regions of SecY in its membrane-inserted (jammed) state. SecA interaction was detected in five of six cytosolic loops (C2, C3, C4, C5, and C6), eight of 10 transmembrane segments (TM1, TM2a, TM2b, TM3, TM6, TM7, TM8, TM9, and TM10), and all five periplasmic loops (P1, P2, P3, P4, and P5) of SecY protein.

Given the unorthodox nature of these results, we performed a number of critical control experiments to check the validity of these findings. Because the basis for detection of the relevant SecA-SecY cross-linked product was our rabbit hyper-immune SecA antisera, we first explored the possibility that the antisera detected SecY interaction with another protein(s) other than SecA given its polyclonal nature. For this purpose we performed an immunoadsorption experiment where a constant amount of antisera was preincubated with increasing amounts of highly purified SecA protein prior to its usage in Western blots. This experiment demonstrated that the loss of the major cross-linked product(s) occurred readily and commensurately with that of SecA protein itself, indicating that our antisera was detecting authentic SecA-SecY cross-linked products (Fig. 5A).

In fact this approach allowed detection of a minor UV and jamming independent species (indicated next to the diamond in Fig. 5A) migrating just above the dominant SecA-SecY band, which was unrelated to SecA, because its intensity was rela-
细化地了解SecA-SecY跨膜事件。我们确定了SecA-SecY相互作用是否依赖于具有功能信号肽的OmpA-GFP突变体。我们通过Western blot分析SecA和SecY蛋白，已知SecA-SecY产物（见图2A）。该方法允许通过在不同膜层中分离蛋白来进行这一试验以确定膜特异性来源的观察到的SecA-SecY产物（图7A）。这种方法允许通过在不同膜层中分离蛋白来进行这一试验以确定膜特异性来源的观察到的SecA-SecY产物（图7A）。根据这些结果，我们观察到的跨膜连接产物（图7B）存在于内腔部分（图7C），其中SecY蛋白几乎完全位于内腔膜和内外混合膜部分（图7C），与优选的组装的内腔膜在光通道之间存在光通道（43）。这一结果支持了我们将观察到的SecA-SecY跨膜连接事件仅限于SecY的事实，其正确嵌入于细胞膜内。

作为最终控制对功能局部化在我们的实验系统中的确定，我们确定了在不同的条件下是否观察到跨膜连接依赖的SecA-SecY跨膜连接事件。SecY434突变体的SecA整合入SecY通道依赖性。细胞在抑制剂添加后在环中被移除并被分成两个等分（图5B），表明这种特定的SecA抗体的活性在细胞膜内被发现。

We screened another two SecY mutants, SecY207 and SecY216, by this approach and obtained similar results in both cases (Fig. 5B), indicative of the appropriate specificity of our SecA antisera.

We next explored the possibility that cell lysis may be occurring during the translocon jamming and/or photo-crosslinking step(s), thereby causing the artificial association of soluble SecA with portions of SecY on the trans-side of the membrane. We utilized the SecY58 mutant for this purpose, because the SecA interaction site is within the P1 periplasmic domain of SecY. Accordingly, after the standard growth, induction, and UV treatment regimen, cells were spun down, and both the supernatant and cell pellet were assayed for the cytoplasmic enzyme β-galactosidase (Fig. 6). The results indicated that there was little or no cell lysis occurring during our procedure, because less than 1% of β-galactosidase activity was found in the cell-free supernatants under the various experimental conditions tested.

We also explored the possibility that modest overexpression of SecYEG might result in internal aggregates of this protein that were not properly integrated into the plasma membrane, which could expose non-physiological sites for SecA interaction. For this purpose we utilized the SecY391 mutant within TM9, which showed a robust interaction with SecA during translocon jamming (Fig. 3B). We isolated membranes after our standard experimental regimen and performed a membrane flotation experiment to determine the membrane-specific source of the observed SecA-SecY product (Fig. 7A). This approach allows separation of the different membrane fractions or protein aggregates by their differing density. The results showed that the relevant cross-linked product (Fig. 7B) was within the inner membrane fraction (Fig. 7C), whereas SecY protein was found almost entirely within the inner membrane and inner/outer hybrid membrane fractions (Fig. 7C), consistent with the preferred assembly of the most active translocons within zones of light inner membrane (43). This result supports the conclusion that the observed SecA-SecY cross-linking occurs exclusively with SecY that is correctly embedded within the plasma membrane.

As a final control for proper localization within our experimental system, we determined whether the observed jamming dependence of our SecA-SecY cross-linking was dependent on a functional signal peptide or not. We utilized an OmpA-GFP chimera that contained arginine substitutions at positions 8 and 10 of the signal peptide for this purpose, which has previously been shown to be non-functional for jamming because of a transport defect (39). Comparison of four jamming-dependent SecY mutants, SecY58, SecY207, SecY216, and SecY391, with the relevant cross-linking site located within the P1, P3, P3, and TM9 domains of SecY, respectively, demonstrated that the relevant SecA-SecY cross-linked product was only detected when the OmpA-GFP chimera contained a functional signal peptide (Fig. 8A). Appropriate controls showed that a strain containing the signal peptide defect resulted in little or no build-up of pre-MBP within the cytoplasm–membrane fraction compared with a strain containing a functional signal peptide (compare Fig. 8B with Fig. 2F) even though both OmpA-GFP chimeras were produced at approximately equal levels (Fig. 8C). It was demonstrated previously that the OmpA-RR-GFP chimera containing Cys21 is also unable to efficiently form the disulfide cross-link with SecY Cys38 (see scheme in Fig. 2G), indicative of its inability to effectively contribute to translocon jamming (39). We conclude that the appearance of jamming-dependent SecA-SecY cross-linked products require a functional signal peptide on the OmpA-GFP chimera. Thus this experiment demonstrates that productive photo-crosslinking captures SecA-SecY complexes exclusively during active jamming, which relies on a functional signal peptide interacting with a functional translocon.

Discussion

Recent biochemical and structural approaches toward elucidating the SecA-SecY interaction have mainly focused on regions of SecY that lie at the surface of the plasma membrane or are contained within the upper portion of the mouth of the channel (Refs. 16–18 and 44; also see references in Ref. 36). However, such approaches would miss interactions that may occur deeper within the channel as part of the normal protein translocation cycle. We sought to address this bias as well as to re-investigate the original SecA power stroke model that called for deeper SecA membrane insertion at SecYEG (14). Toward this end we made use of an unbiased physiological approach by performing in vivo photo-crosslinking of 117 SecY mutants
with potential cross-linking sites located throughout the SecY protein along with conditional translocon jamming to freeze SecA in its membrane-inserted state. A series of controls were done to establish the validity of this approach, including demonstrating the authenticity of the identified SecA–SecY products by their (i) dependence on the orthogonal tRNA-tRNA synthetase pair and pBpA in the growth media as well as UV irradiation (Figs. 2 and 3), (ii) reliance on SecA-specific immuno-detection (Fig. 5), (iii) verifying that these interactions did not occur in vitro as a result of cell lysis (Fig. 6), and (iv) checking for their formation with properly assembled SecY protein (Fig. 7). In the case of jamming-dependent SecA-SecY cross-linking, we also showed that such interactions rely on the production of the OmpA-GFP chimera with a functional signal peptide that rapidly and effectively inhibited ongoing protein transport and associated with the trans-side of the channel complex (Figs. 2 and 8). These last controls indicate that the productive site of cross-linking occurs within previously functional SecYEG channels that receive the jamming-proficient form of the chimera.

Our data indicates that SecA makes extensive interactions with all three regions of the SecY protein: the cytosolic loops, the transmembrane segments, and the periplasmic loops (Fig. 9). Its analysis leads to a number of important implications regarding the mechanism of SecA-dependent protein transport and SecYEG channel dynamics. First, comparison of our cross-linking data in the normal and translocon-jammed states shows that none of the SecY mutants that were positive in the former
case became negative for cross-linking in the latter case. This result suggests that regions of SecA that initially bind to the cytosolic loops of SecY are relatively static and are maintained throughout the protein translocation cycle, indicative of an initial SecY receptor function that properly positions SecA for its various distal functions. In assessing the higher density of SecA interaction sites within the cytosolic loops of SecY, it is important to remember that the two halves of the SecA substructure (NBD-1/NBD-2 and PPXD/HSD/HWD/CTL) interact with both surfaces of the SecYEG dimer (36).

Second, among the constitutively positive class of SecY mutants, we identified a group of residues that lie deep within the cytosolic loops of SecY.

**Figure 3.—continued**

|                  | F279 (TM7) | P287 (TM7) | T289 (TM7) | A291 (TM7) | L316 (TM8) | I325 (TM8) |
|------------------|------------|------------|------------|------------|------------|------------|
| -Ara +Ara        | -          | -          | -          | -          | -          | -          |
| +Ara             | +          | +          | +          | +          | +          | +          |
|                  |            |            |            |            |            |            |
|                  | F327 (TM8) | M391 (TM9) | T404 (TM10)| L406 (TM10)| I408 (TM10)| D415 (TM10)|
| -Ara +Ara        | -          | -          | -          | -          | -          | -          |
| +Ara             | +          | +          | +          | +          | +          | +          |
|                  |            |            |            |            |            |            |
|                  | F416 (TM10)| M417 (TM10)| M424 (TM10)| L24 (TM1)| F170 (TM4)|            |
| -Ara +Ara        | -          | -          | -          | -          | -          | -          |
| +Ara             | +          | +          | +          | +          | +          | +          |
|                  |            |            |            |            |            |            |
|                  | V48 (P1)   | G58 (P1)   | L148 (P2)  | F154 (P2)  | I207 (P3)  |            |
| -Ara +Ara        | -          | -          | -          | -          | -          | -          |
| +Ara             | +          | +          | +          | +          | +          | +          |
|                  |            |            |            |            |            |            |
|                  | H216 (P3)  | P312 (P4)  | V397 (P5)  | G299 (P4)  | R392 (P5)  |            |
| -Ara +Ara        | -          | -          | -          | -          | -          | -          |
| +Ara             | +          | +          | +          | +          | +          | +          |

Anti-SecA
Figure 4. Location of constitutively positive SecA interaction sites within the lateral gate region of SecY. The *M. jannaschii* Sec61/H9251/H9253/H9252/SecYEG crystal structure is shown highlighting the *E. coli* SecY homologous residues Phe64, Gly70, Ala75, Ile82, Pro84, and Ala88 (red spheres) that were constitutively positive for cross-linking to SecA. The TM2b and TM7 helices within the lateral gate region are colored in pink, whereas the Sec61/H9251/H9253/H9252/SecYEG polypeptide chains are colored in gray, green, and orange, respectively.

Figure 5. Specificity of SecA antisera. *A*, the SecY434 mutant was grown with or without arabinose induction and subjected to UV irradiation as indicated followed by cell breakage, membrane isolation, and Western blotting with SecA antisera as described under “Experimental procedures.” For pre-adsorption of the antisera, 1 μL of SecA antisera was added to a 99-μL reaction containing 20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 0.25% Tween 20 and the indicated amount of purified SecA protein, which was incubated on ice for 1 h prior to being used for Western blotting. The band highlighted by the diamond represents a nonspecific interaction given its behavior during immunoabsorption. *B*, comparison of the in vivo photo-crosslinking pattern of three SecY mutants by Western blotting utilizing SecA antisera or SecA antisera that was pre-adsorbed with 10 ng of purified SecA protein as indicated above.

Figure 6. Evaluation of cell lysis during the translocon jamming and in vivo photo-crosslinking procedure. The SecY58 mutant was grown with or without arabinose induction and subjected to UV irradiation as indicated under “Experimental procedures,” when samples were separated into supernatant and cell pellet fractions by sedimentation at 12,000 × g for 2 min at 4 °C. β-Galactosidase assays of the supernatant (S) and cell pellet (P) fractions were done in triplicate as described by Miller (55), and the standard deviation is shown.
the membrane at the TM2a and TM2b lateral gate region (Fig. 4). This result implies that some portion of SecA must at least transiently bind to this region of SecY both prior to and after the insertion event. A logical function for this binding event would be the initial delivery of the SecA-bound signal peptide to the lateral gate of SecY given the eventual position of the signal peptide within this region in its SecY-bound state (10). Alternatively, this region of SecA may be involved in expansion of the lateral gate region for channel activation. This latter event could lead to the creation of a composite channel consisting of both SecA and SecY proteins that could allow for the creation of a larger yet properly sealed channel, which would be consistent with current estimates of channel dimensions under in vitro protein transport conditions (22). Such a channel-expanding activity of SecA could explain its inherent ability to form channels and ring-like pore structures in lipid monolayers and bilayers in the absence of its physiological SecYEG binding partner (28, 29, 45).

Third, we found that the jamming-dependent SecY mutant residues lie in residues that are located throughout all three regions of SecY, indicative of extensive SecA–SecY interaction in the SecA-inserted state. The existence of such residues within the cytosolic loops of SecY indicates the importance of this region in facilitating the actual translocation steps. This finding would fit models of SecA action that require cross-talk between its ATP hydrolysis cycle and ongoing translocation steps within regions of SecY that lie deeper within the membrane and can be “sensed” through these more superficial contact sites between the two proteins (18).

Mapping of the jamming-dependent SecY mutant residues within the transmembrane helices and periplasmic loops to the SecYEG structure shows that most of them cluster to the lateral gate and pore ring regions of SecY (Fig. 9, see dotted rectangle and oval, respectively). Another SecA-interactive residue of this class, SecY64, lies within the TM2a plug domain. Because these three regions are critical for channel opening/activation and substrate entry/accommodation as well as maintenance of a properly sealed channel, these results implicate SecA in these important activities as well. We did not find obvious SecA-interaction sites on the exterior (i.e. lipidic) side of SecY, consistent with an earlier study that reached a similar conclusion utilizing phospholipids containing a photo-crosslinking group within the acyl chain region (46). The generation of an extensive SecA interaction network within these critical interior portions of SecY would require the insertion of at least one or more membranous regions of SecA. Thus this portion of our study supports the original SecA power stroke model positing a dramatic rearrangement of the membrane interior portions of SecY to accommodate the SecA membrane insertion event (14). The direct interaction of SecA with the pore ring and plug regions of SecY where signal sequence suppressor (prLA) alleles cluster (47) would also provide a potentially simple structural solution for the cooperation of these two proteins in maintaining a signal sequence proofreading activity for the translocon.

We note that our new findings are also consistent with the presence of integral membrane SecA within normal E. coli cells (30–32), the existence of trypsin-resistant domains within integral membrane SecA (48, 49), and reports of periplasmically

---

**Figure 7. Evaluation of the assembly state of SecY during in vivo photo-crosslinking.** A, schematic diagram of a three-step sucrose density gradient indicating the floatation centrifugation positions of different membrane or protein species. B and C, the SecY391 mutant was grown with or without arabinose induction and subjected to UV irradiation as indicated followed by cell breakage and membrane isolation as described under “Experimental procedures.” B, samples were subjected to Western blotting with SecA antibody to assess the degree of SecA-SecY cross-linking. C, the isolated membrane fraction from the arabinose induced and UV-irradiated culture was adjusted to 1.74 M sucrose, and a 55-μl aliquot was loaded at the bottom of a centrifuge tube, and consecutively overlaid with 95 μl of 1.6 M sucrose followed by 75 μl of 1.25 M sucrose buffered with 50 mM Tris-HCl, pH 8, 50 mM KCl, 5 mM MgCl₂. The sample was centrifuged at 98,000 rpm in a Sorvall SS120AT3 rotor for 18 h at 4 °C. Eight equal fractions were collected from the top to bottom of the tube and analyzed by Western blotting using SecA (top), c-Myc (middle), or OmpA (bottom) antibody. The SecY391 mutant cells utilized for this experiment were the same as utilized for Fig. 3, and thus the image in B is the same as that of the corresponding image in Fig. 38.
exposed regions of SecA (31, 33–35). The reported inherent ability of SecA to insert into phospholipid monolayers and bilayers likely underlies the basis of its ligand-gated membrane insertion activity at SecYEG (25–27). Collectively our study suggests that SecA must now be considered a membrane integral part of the SecYEG—SecDF–YajC holo-translocon complex at least during the membrane-insertion portion of the protein translocation cycle. The provocative nature of our findings leaves ample room to now address the precise roles of membrane-inserted SecA within this complex and dynamic system.

In this regard we note that high-resolution structural information regarding the membrane-inserted state of SecA is lacking and will be critical to continue to elucidate the bacterial protein transport mechanism.

**Experimental procedures**

**Chemicals, media, strains, and plasmids**

LB (Miller) broth, IPTG, and arabinose were purchased from Fisher Scientific. pBpA and maltose were purchased from Bachem and Difco, respectively. QuikChange™ and Wizard Plus SV Miniprep DNA Purification System kits were obtained from Stratagene and Promega, respectively. The Western Bright™ Sirius enhanced chemiluminescence kit was obtained from Advanta. Protease inhibitor mixture was obtained from Sigma. Most other common chemicals were obtained from the latter supplier or Fisher Scientific and were laboratory grade or better. SecA, MBP, and OmpA antisera were prepared in rabbits with highly purified preparations of the relevant protein by Cocalico Biologicals (Reamstown, PA). SecA and OmpA purification have been described previously (50, 51), whereas MBP purification was done according to the protocol provided by New England Biolabs (Beverly, MA) for the pMAL Protein Fusion and Purification System™. Mouse anti-c-Myc monoclonal antibody and HRP-conjugated goat anti-mouse antibody were obtained from GenScript, whereas chicken anti-GFP antibody and HRP-conjugated goat anti-rabbit antibody were obtained from Abcam. HRP-conjugated goat anti-chicken antibody was obtained from Jackson ImmunoResearch Laboratories. Peptide affinity-purified SecY antisera was prepared by hyper-immunizing rabbits to a peptide identical to the carboxyl terminus of SecY (CYESALKKANLKGYGR) conjugated to keyhole limpet hemocyanin by maleimide chemistry utilizing Tana Laboratories, LC (Houston, TX) for peptide synthesis, protein carrier conjugation, immunization, and peptide affinity purification of the antisera. *E. coli* BLR(ADE3) [F<sup>-ompT hsdS</sup> (r<sup>B</sup> <sup>-</sup> m<sup>B</sup> <sup>-</sup>) gal dcm Δ(srl-recA)306:Tn10 (Tet<sup>+</sup>)] (52) was obtained from Stratagene. MC4100.2(ADE3) is a recA1 srl::Tn10 derivative of MC4100 (53) that has been lysogenized with ADE3, which has been previously described (52). The pSup–pBpARS-6TRN plasmid encoding the *Methanococcus jannaschii* amber suppressor tRNA·tRNA synthetase pair that efficiently incorporates pBpA at an amber codon has been described previously (54). The

**Figure 8. Jamming-dependent SecA-SecY cross-linking requires a functional signal peptide within the OmpA-GFP chimera.**

A, strains containing an amber mutation in the indicated SecY residue and an OmpA-GFP chimera with a functional (left-hand side) or non-functional (right-hand side) signal sequence were grown with arabinose induction and subjected to UV irradiation as indicated followed by cell breakage, membrane isolation, and Western blotting with SecA antibody as described under “Experimental procedures.” B, cells of the SecY58 mutant carrying the OmpA-RR-GFP chimera with a non-functional signal sequence were removed after arabinose addition at the indicated times and divided into cytoplasm–membrane (C) or periplasm (P) fractions and compared with the total cell (T) input by Western blotting using MBP antibody as described under “Experimental procedures.” C, comparison of the expression levels of the OmpA-GFP and OmpA-RR-GFP chimeras 45 min after arabinose induction for the SecY58 mutant by Western blotting using GFP antibody. The four mutant cultures utilized in this experiment were the same as utilized in Fig. 3, and thus the image in A is the same as the corresponding image in Fig. 3.
pCDFT7secYEG plasmid with secYEG under control of the T7 promoter with an amino-terminal c-Myc tag on secY has been described (36). The pBAD-OmpA-GFP plasmid carrying the arabinose-inducible, OmpA-GFP jamming chimera was obtained from Tom Rapoport (39). A control version of this plasmid was created that contains a defective OmpA signal sequence by changing codons 8 and 10 to incorporate arginine using QuikChange™ mutagenesis resulting in pBAD-OmpA-RR-GFP plasmid, which was verified by DNA sequence analysis utilizing the University of Pennsylvania DNA Sequencing Facility. All codon substitutions within the plasmid-borne secY gene were made by QuikChange™ mutagenesis and also verified by DNA sequence analysis.

**In vivo photo-crosslinking**

In general a freshly struck out single colony of MC4100.2/H9261DE3 containing pSup-BpARS-6TRN, pBAD-OmpA-GFP, and pCDFT7secYmycEG plasmids with a given secY amber mutation was inoculated into LB media supplemented with appropriate antibiotics (25 μg/ml of chloramphenicol, 100 μg/ml of ampicillin, and 50 μg/ml of spectinomycin) and grown overnight at 37 °C with shaking at 250 rpm. The overnight culture was diluted 1:100 into LB media supplemented with appropriate antibiotics, 1 mM pBpA, and 30 μM IPTG and grown until A₆₀₀ of 0.15, when OmpA-GFP jamming was induced for a portion of the culture by addition of arabinose to a final concentration of 0.2% for 45 min. All subsequent steps were done at 4 °C or on ice. To adjust for somewhat different cell densities, 4.8 A₆₀₀ cell equivalents of each culture were harvested by sedimentation in a microcentrifuge at 14,000 rpm for 5 min, washed with 5 ml of PBS (10 mM sodium phosphate, pH 7.5, 140 mM NaCl), and resuspended in 4 ml of PBS buffer. 2-ml samples were UV irradiated on ice at 365 nm for 20 min using a Rayonet 2000 UV cross-linker (Southern New England Ultraviolet Co.), whereas 2 ml of non-irradiated samples served as negative controls. Each sample was sedimented, resuspended in 1 ml of breakage buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.25 mM PMSF, 1 mM DTT, 100 μg/ml of RNase, 100 μg/ml of DNase, 60 μg/ml of lysozyme, 1× Protease inhibitor mixture), and cells were placed in a polycarbonate tube and disrupted with 30-s bursts of a cup horn sonicator (Heat Systems) until near clarity. Unbroken cells were removed by sedimentation in a microcen-
trifuge at 14,000 rpm for 5 min. Supernatant was isolated and sedimented in a Sorvall S120 AT2 rotor at 82,000 rpm for 30 min at 4 °C to isolate the membrane fraction. Each membrane pellet was solubilized in 60 μl of ABB buffer (5% SDS, 10 mM Tris-Cl, pH 8, 1 mM EDTA) with constant stirring for 1 h at 37 °C, when 20 μl of 4× sample buffer (8% SDS, 500 mM Tris-HCl, pH 6.8, 20% 2-mercaptoethanol, 60% glycerol, 0.02% bromphenol blue) was added, and stirring continued for an additional 10 min. 15-μl (for SecA or GFP westerns) or 30-μl (for SecY Western blots) samples were loaded onto a 10% (for SecA Western blots) or 15% (for SecY or GFP Western blots) SDS-PAGE gel, which was run at 100 V at 4 °C until the dye front reached the bottom. Western transfers were performed at 100 V for 1 h, and nitrocellulose membranes were blocked overnight with 10 ml of TBS buffer (20 mM Tris-Cl, pH 7.5, 140 mM NaCl, 0.25% Tween 20) supplemented with 10% nonfat dry milk. The membrane was probed with appropriate primary antibodies at 1:5,000 dilution except for SecA antibody that was used at a 1:10,000 dilution, followed by probing with the appropriate HRP-conjugated secondary antibody that were all used at 1:5,000 dilution. Proteins were visualized using a Western Bright™ Sirius kit as described by the manufacturer.

Assessment of translocon jamming

The kinetics of translocon jamming was assessed by removal of 0.6 A400 cell equivalent of culture at each time point, which was harvested by sedimentation in the microcentrifuge at 14,000 rpm for 5 min, and resuspended in 200 μl of 20% sucrose, 0.03 mM Tris-HCl, pH 8. The supernatant (i.e. periplasmic fraction) was removed, whereas the cell pellet (cytoplasm–membrane fraction) was resuspended in 200 μl of ABB buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgSO4). All samples were boiled for 10 min in the presence of 1× sample buffer, and 5-μl aliquots were analyzed by SDS-PAGE and Western blotting with rabbit anti-MBP antibody and HRP-conjugated goat anti-rabbit antibody, both at 1:5,000 dilution.

References
1. du Plessis, D. J., Nouwen, N., and Driessen, A. J. (2011) The Sec translocon. Biochim. Biophys. Acta 1808, 851–865
2. Park, E., and Rapoport, T. A. (2012) Mechanisms of Sec61/SecY-mediated protein translocation across membranes. Annu. Rev. Biophys. 41, 21–40
3. Ng, D. T., Brown, J. D., and Walter, P. (1996) Signal sequences specify the targeting route to the endoplasmic reticulum membrane. J. Cell Biol. 134, 269–278
4. Lee, H. C., and Bernstein, H. D. (2001) The targeting pathway of Escherichia coli presecretory and integral membrane proteins is specified by the hydrophobicity of the targeting signal. Proc. Natl. Acad. Sci. U.S.A. 98, 3471–3476
5. Randall, L. L., and Hardy, S. J. (2002) SecB, one small chaperone in the complex milieu of the cell. Cell Mol. Life Sci. 59, 1617–1623
6. Van den Berg, B., Clemons, W. M., Jr., Collinson, I., Modis, Y., Hartmann, E., Harrison, S. C., and Rapoport, T. A. (2004) X-ray structure of a protein-conducting channel. Nature 427, 36–44
7. Tsukazaki, T., Mori, H., Fukui, S., Ishitani, R., Mori, T., Doehma, N., Perederina, A., Sugita, Y., Vassylyev, D. G., Ito, K., and Nureki, O. (2008) Conformational transition of Sec machinery inferred from bacterial SecYc structures. Nature 455, 988–991
8. Egrea, P., and Stroud, R. (2010) Lateral opening of a translocon upon entry of protein suggests the mechanism of insertion into membranes. Proc. Natl. Acad. Sci. U.S.A. 107, 17182–17187
9. Tanaka, Y., Sugano, Y., Takemoto, M., Mori, T., Furukawa, A., Kusakizako, T., Kumaizaki, K., Kashima, A., Ishitani, R., Sugita, Y., Nureki, O., and Tsukazaki, T. (2015) Crystal structures of SecYEG in lipidic cubic phase elucidate a precise resting and a peptide-bound state. Cell Rep. 13, 1561–1568
10. Li, L., Park, E., Ling, J., Ingram, J., Ploegh, H., and Rapoport, T. A. (2016) Crystal structure of a substrate-engaged SecY protein-translocation channel. Nature 531, 395–399
11. Sachelaru, I., Petriman, N. A., Kudva, R., Kuhn, P., Welte, T., Knapp, B., Drepper, F., Warscheid, B., and Koch, H.-G. (2013) YidC occupies the lateral gate of the SecYEG translocon and is sequentially displaced by a nascent membrane protein. J. Biol. Chem. 288, 16295–16307
12. Tsukazaki, T., Mori, H., Echizen, Y., Ishitani, R., Fukai, S., Tanaka, T., Perederina, A., Vassylyev, D. G., Kohno, T., Maturana, A. D., Ito, K., and Nureki, O. (2011) Structure and function of a membrane component SecDF that enhances protein export. Nature 474, 235–238
13. Lill, R., Cunningham, K., Brundage, L. A., Ito, K., Oliver, D., and Wickner, W. (1989) SecA protein hydrolyzes ATP and is an essential component of the protein translocation ATPase of Escherichia coli. EMBO J. 8, 961–966
14. Economou, A., and Wickner, W. (1994) SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. Cell 78, 835–843
15. van der Does, C., Manting, E. H., Kaufmann, A., Lutz, M., and Driessen, A. J. (2011) Probing the SecYEG translocation pore size with synthetic membranes. Nature 477, 201–210
16. Zimmer, J., Nam, Y., and Rapoport, T. A. (2008) Structure of a complex of the ATPase SecA and the protein-translocation channel. Nature 455, 936–943
17. Erlandson, K. J., Miller, S. B., Nam, Y., Osborne, A. R., Zimmer, J., and Rapoport, T. A. (2008) A role for the two-helix finger of the SecA ATPase in protein translocation. Nature 455, 984–987
18. Allen, W., Corey, R., Oatley, P., Sessions, R., Baldwin, S., Redford, S., Tuma, R., and Collinson, I. (2016) Two-way communication between SecY and SecA suggest a Brownian ratchet mechanism for protein translocation. Elife 5, 15598
19. Kusters, I., van den Bogaart, G., Kedrov, A., Krasnikov, V., Fulyani, F., Poolman, B., and Driessen, A. (2011) Quaternary structure of SecA in solution and bound to SecYEG probed at the single molecule level. Structure 19, 430–439
20. Lycklama, A., Nijeholt, J., and Driessen, A. J. (2012) The bacterial Sec-translocon: structure and mechanism. Philos. Trans. R. Soc. Lond. B Biol. Sci. 367, 1016–1028
21. Bauer, B. W., Shemesh, T., Chen, Y., and Rapoport, T. A. (2014) A "Push and Slide" mechanism allows sequence-insensitive translocation of secretory proteins by the SecA ATPase. Cell 157, 1416–1429
22. Bonardi, F., Halza, E., Walko, M., Du Plessis, F., Nouwen, N., Feringa, B. L., and Driessen, A. J. (2011) Probing the SecYEG translocation pore size with preproteins conjugated with sizable rigid spherical molecules. Proc. Natl. Acad. Sci. U.S.A. 108, 7775–7780
23. Tian, P., and Andricioaei, I. (2006) Size, motion, and function of the SecY translocon revealed by molecular dynamics simulations with virtual probes. *Biophys. J.* **90**, 2718–2730

24. Matsumoto, G., Mori, H., and Ito, K. (1998) Roles of SecG and ATP- and SecA-dependent protein translocation. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 13567–13572

25. Breukink, E., Demel, R. A., de Korte-Kool, G., and de Kruijff, B. (1992) SecA insertion into phospholipids is stimulated by negatively charged lipids and inhibited by ATP: a monolayer study. *Biochemistry* **31**, 1119–1124

26. Ulbrandt, N. D., London, E., and Oliver, D. B. (1992) Deep penetration of a portion of *Escherichia coli* SecA protein into model membranes is promoted by anionic phospholipids and by partial unfolding. *J. Biol. Chem.* **267**, 15184–15192

27. Ahn, T., and Kim, H. (1994) SecA of *Escherichia coli* traverses lipid bilayer of phospholipid vesicles. *Biochem. Biophys. Res. Commun.* **203**, 326–330

28. Wang, H.-W., Chen, Y., Yang, H., Chen, X., Duan, M.-X., and Sui, P. F. (2003) Ring-like pore structures of SecA: implications for bacterial protein-conducting channels. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 4221–4226

29. Hsieh, Y.-H., Zhang, H., Lin, B.-R., Cui, N., Na, B., Yang, H., Jiang, C., Sui, S.-F., and Tai, P. C. (2011) SecA alone can promote protein translocation and ion channel activity. *J. Biol. Chem.* **286**, 44702–44709

30. Cabelli, R. J., Dolan, K. M., Qian, L. P., and Oliver, D. B. (1991) Characterization of membrane-associated and soluble states of SecA protein from wild-type and secA51(Ts) mutant strains of *Escherichia coli*. *J. Biol. Chem.* **266**, 24420–24427

31. van der Does, C., den Blauwen, T., de Wit, J. G., Manting, E. H., Groot, N., Fekkes, P., and Driessen, A. J. (1996) SecA is an intrinsic subunit of the *Escherichia coli* prepore translocase and exposes its carboxyl terminus to the periplasm. *Mol. Microbiol.* **22**, 619–629

32. Eichler, J., Rinard, K., and Wickner, W. (1998) Endogenous SecA catalyzes prepore translocation at SecYEG. *J. Biol. Chem.* **273**, 21675–21681

33. Ramamurthy, V., and Oliver, D. (1997) Topology of the integral-membrane protein-conducting channel. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 4221–4226

34. Eichler, J., and Wickner, W. (1998) Endogenous SecA catalyzes prepore translocation at SecYEG. *J. Biol. Chem.* **273**, 21675–21681

35. Hsieh, Y.-H., Zhang, H., Wang, H., Yang, H., Jiang, C., Sui, S.-F., and Tai, P. C. (2013) Reconstitution of functionally efficient SecA-dependent protein-conducting channels: transformation of low-affinity SecA-liposome channels to high-affinity SecA-SecYEG-SecDF-YajC channels. *Biochem. Biophys. Res. Commun.* **431**, 388–392

36. van Voorst, F., van der Does, C., Brunner, J., Driessen, A. J., and de Kruijff, B. (1998) Translocase-bound SecA is largely shielded by the phospholipid acyl chains. *Biochemistry* **37**, 12261–12268

37. Flower, A. (2007) The SecY translocation complex: convergence of genetics and structure. *Trends Microbiol.* **15**, 203–210

38. Chen, X., Xu, H., and Tai, P. (1996) A significant fraction of functional SecA is permanently embedded in the membrane. *J. Biol. Chem.* **271**, 29698–29706

39. Chen, X., Brown, T., and Tai, P. (1998) Identification and characterization of protease-resistant SecA fragments: SecA has two membrane-integral forms. *J. Bacteriol.* **180**, 527–537

40. Ding, H., Hunt, J. F., Mukerji, I., and Oliver, D. (2003) *B. subtilis* SecA ATPase exists as an antiparallel dimer in solution. *Biochemistry* **42**, 8729–8738

41. Crooke, E., Guthrie, B., Lecker, S., Lill, R., and Wickner, W. (1988) ProOmpA is stabilized for membrane translocation by either purified *E. coli* trigger factor or canine signal recognition particle. *Cell* **54**, 1003–1011

42. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**, 60–89

43. Casadaban, M. J. (1976) Transposition and fusion of the lac genes to selected promoters in *Escherichia coli* using bacteriophage λ and μ. *J. Mol. Biol.* **104**, 541–555

44. Wang, L., Xie, J., and Schultz, P. (2006) Expanding the genetic code. *Annu. Rev. Biophys. Biomol. Struct.* **35**, 225–249

45. Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY