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COMMENTARY

Substrate Proteins Take Shape at an Improved Bacterial Translocon

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ABSTRACT Characterization of Sec-dependent bacterial protein transport has often relied on an *in vitro* protein translocation system comprised in part of *Escherichia coli* inverted inner membrane vesicles or, more recently, purified SecYEG translocons reconstituted into liposomes using mostly a single substrate (proOmpA). A paper published in this issue (P. Bariya and L. Randall, J Bacteriol 201:e00493-18, 2019, https://doi.org/10.1128/JB.00493-18) finds that inclusion of SecA protein during SecYEG proteoliposome reconstitution dramatically improves the number of active translocons. This experimentally useful and intriguing result that may arise from SecA membrane integration properties is discussed here. Furthermore, determination of the rate-limiting transport step for nine different substrates implicates the mature region distal to the signal peptide in the observed rate constant differences, indicating that more nuanced transport models that respond to differences in protein sequence and structure are needed.

KEYWORDS ATPase, SecA, SecYEG, bacterial secretion, membrane reconstitution, protein translocation

Our current understanding of the Sec-dependent bacterial protein transport system is based on a congruence of *E. coli* genetics and biochemistry during the 1980s and 1990s that led to the discovery and cloning of the relevant *sec* genes and the purification and characterization of their protein products (for a brief history, see reference 1; for a recent review, see reference 2). Much of the mechanistic insight into this pathway arose from *in vitro* protein translocation systems composed of inverted inner membrane vesicles (IMV) to which an extract of essential soluble proteins was added, including a freshly translated protein substrate (often the outer membrane protein A containing its signal peptide, proOmpA) (3). As purified Sec proteins became available, they were included in this system, which allowed for a reduction in its complexity to eventually include only its essential components. Thus, the current bare-bones posttranslational *in vitro* protein translocation system includes the SecYEG translocon channel complex that is purified and reconstituted into liposomes (4, 5), along with the SecA motor protein and ATP, which are added as soluble factors (6). Protein substrates typically need to be rapidly diluted out of high molar urea or other denaturants to start the transport reaction, since their folding and/or aggregation induces export incompetence (unlike some protein transport systems, the Sec pathway cannot transport folded protein substrates and has very limited unfolding capability) (7). Auxiliary factors have been added to this core: SecDF-YajC, which has been proposed to couple membrane proton-motive force to substrate protein folding on the *trans*-side of the membrane (8), YidC, which plays a role as a chaperone in membrane protein integration and assembly (9), and SecB, a SecA-specific chaperone for certain periplasmic and outer membrane proteins (10). The enzyme that cleaves off most signal peptides, signal peptidase I, is generally not included in such systems, although it is an essential component of the Sec-dependent pathway (11).

Until recently, this simplified *in vitro* protein translocation system was considered

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sufficient to study the mechanism of protein transport, but no one had bothered to assess how good it really is. This has changed due to a report by Bariya and Randall (12), who carefully compared the traditional IMV system with defined systems with SecA added either during SecYEG proteoliposome reconstitution or subsequent to it, when transport was being assessed. The results are dramatic. While approximately half of the *in vivo*-assembled translocons retain normal transport activity during the steps leading to IMV purification (which includes treatment with 4 M urea to remove and inactivate endogenous SecA), this number drops to only ~10% for SecYEG proteoliposomes reconstituted by the standard method (PLYEG). In contrast, proteoliposomes that are reconstituted with SecYEG and SecA (PLYEG·SecA) have a substantially higher number of active translocons: approximately half, similar to IMV. This difference cannot be explained simply by a dramatic increase in the correct orientation of translocons within the bilayer, since translocon orientation was close to random in both cases. The number of active translocons was measured by titration with radiolabeled substrate proteins that jam channels due to the presence of a large disulfide loop at the C terminus of the substrate (13). Furthermore, for a number of periplasmic or outer membrane protein substrates, the inherent rate constant of substrate transport and the maximal amplitude of transport per accessible translocon were carefully measured by Bariya and Randall (12), and they found that they were comparable in all three systems for a given substrate if the number of active translocons was taken into account. In short, while the number of active translocons differed among the three systems, those that were active appear to be functionally equivalent and, thus, homogenous.

These important findings should make the improved PLYEG·SecA system the preferred one going forward, not only because of its substantially higher transport activity, which should make certain types of ensemble and single-molecule experiments more tractable, but also because it should reduce the potential for off-signal data from nonfunctional, but not entirely "dead," translocons. Regarding the latter concern, the authors did find that the number of ATP molecules hydrolyzed per protein translocated (a measurement of ATP coupling by SecA within the system) was severalfold higher for the PLYEG system than the PLYEG·SecA or IMV system, indicative of translocon-bound substrate that was unproductively engaged with SecA ATPase in the former case.

Of course, we are left with the nagging question of what role SecA plays in potentiating the more efficient reconstitution of active translocons for the PLYEG·SecA system. While the answer to this question remains speculative, one hint comes from recent studies on the N-terminal region of SecA and its dual role in promoting SecA-lipid binding and subsequent SecA-SecYEG binding and activation. Recently, Koch et al. (14) utilized single copies of SecYEG reconstituted into nanodiscs of differing lipid content in order to show that SecA gains access to SecYEG translocons via a lipid-bound intermediate utilizing SecA protein's amphipathic lipid-binding N terminus. More recently, Findik et al. (15) showed that this region of SecA assumed different conformations in PLYEG and PLYEG·SecA liposomes, where the N terminus penetrated the lipid bilayer more deeply in the former case, while it was found in a more shallow state, parallel to the plane of the membrane at the interface between the polar and hydrophobic regions, in the latter case. These formative studies help to define the role of the SecA N-terminal ~20 residues as a molecular switch that alternates between two lipid-inserted conformations that turn off and on its high-affinity SecYEG binding and translocation ATPase activities (16, 17).

Is this the only difference between the PLYEG and PLYEG·SecA liposome systems that accounts for the remarkably more efficient assembly of functional translocons in the latter case? I think that the answer is probably no, but in order to engage in such educated speculation, we need to discuss the various properties of SecA, a remarkable multifunctional protein that has been the subject of intensive studies over the past 35 years. Such studies logically divide SecA function along three main lines that are discussed in turn below: (i) critical SecA binding interactions that sequentially bring substrate into topologically correct contact with the SecYEG channel complex in order to initiate the transport process, (ii) elucidation of SecA ATPase motor function that

drives the elongation phase of the translocation process, and (iii) SecA membrane interaction properties, many of which remain poorly understood on a functional level but which are likely to reveal important new insights into the nature of the transport mechanism.

A number of SecA binding interactions have been noted in the past, a list that includes the signal peptide and mature region of the protein substrate (18–21), multiple regions of SecB chaperone (22), and the SecY and SecG subunits of the translocon complex (23–25). Such interactions allow SecA to either directly or indirectly (via a SecB-substrate handoff step) capture the first loop of the protein substrate and correctly position it within the SecYEG complex to initiate protein transport. The *Thermotoga maritima* SecA-SecYEG cocrystal, as well as recent mapping that aligns the N-terminal substrate protein hairpin along the SecA two-helix finger (THF) subdomain that is positioned at the mouth of the translocon channel, represents our best structural understanding of this initiation complex (20, 24). However, recently it has been shown that SecA also interacts with ribosomes (26), where it binds to nascent chains of substrate proteins when they reach a critical length of ~100 residues long (27). This result then confounds the simple textbook bifurcation of the Sec pathway into cotranslational and posttranslational routes, and it places the signal recognition particle (which generally handles the transport of integral membrane proteins in bacteria, like *E. coli*) and SecA (which generally handles the transport of secretory proteins) in direct competition with one another for scanning more or less hydrophobic signal-anchor sequences, respectively, which then sort their respective substrate proteins into the correct pathway (28). In fact, mutations in SecA that disrupt its ribosome interaction also disrupt SecB-substrate binding, suggesting that a more physiological model of SecA-SecB action is that both proteins participate in substrate protein capture at the ribosome and that the subsequent handoff of the bulk of the bound substrate from SecB to SecA is not effectuated until SecA becomes appropriately situated at the translocon. Such speculation does fit prior *in vivo* and *in vitro* findings (29, 30). Unfortunately, most work with the *in vitro* protein translocation system has artificially compartmentalized the SecA pathway into posttranslational events in order to avoid the additional layer of complexity at the ribosome. Further complicating this matter is the observation that SecA is required for the biogenesis of integral membrane proteins that contain sufficient periplasmic domains (i.e., larger than ~30 amino acid residues) or even single spanning membrane proteins (31), indicating that it also plays a role in the mechanistically coupled, cotranslational pathway, where it would presumably alternate with the ribosome for translocon binding and transport steps. The latter area of SecA biology is very poorly understood.

Here, we come back to the posttranslational *in vitro* protein translocation system and the present work of Bariya and Randall (12), who clearly find that both the inherent rate constant and maximal amplitude of transport vary by as much as 10-fold based on the particular protein substrate employed or whether SecB is included in the assay or not. Such results obviously smack of the underappreciated role that substrate protein structural heterogeneity plays in the overall performance of such systems. While the positive effect of SecB in preventing the aggregation of outer membrane proteins that are high in β-structure (e.g., proOmpA) has been well appreciated, the greatest increase in amplitude was observed for the soluble pre-galactose-binding protein that contains a typical mixture of alpha and beta structures. Intriguingly, SecB had both positive and negative effects on the intrinsic rate constant of transport depending on the substrate protein, and these differences were determined by the mature sequences, not the signal peptide of a given protein, a result most easily interpreted as indicating that SecB is directly involved in the rate-limiting step (at initiation), where the bulk of the bound substrate is delivered from the SecB homotetramer to the SecA dimer, bound by one of its subunits to SecYEG protein (32–34). Indeed, this speculation is consistent with a recent single-molecule study that found that transport through the translocon channel was relatively rapid, but it was preceded by a relatively slow initiation step involving both SecB and SecA proteins (35). The complex choreography of this large substrate

protein-SecB-SecA-SecYEG ternary complex obviously leaves ample room for modulating the transport rate constant in either direction depending on the substrate contacts with SecB and their effects on the conformational dynamics of the delivery mechanism to SecYEG-bound SecA protein. Finally, it is important to recognize that the measured intrinsic rate constant of transport for the current posttranslational *in vitro* protein translocation system (in the range of $\sim$1 min^{-1}) is at least 10-fold slower than that for *in vivo* protein transport (based on bioinformatic analysis in reference 6). While there may be multiple reasons for the poor performance of the current *in vitro* system, it seems likely that protein substrate conformation is a major impediment to rapid initiation complex formation, and workers in our field need to go back to more complex coupled protein translation and translocation systems in order to assess what may have been lost in uncoupling them.

Another important number to come out of the current work by Bariya and Randall is the ATP consumption or coupling rate (12), which also varied substantially among the different protein substrates tested. Values of 1,000 to 7,000 mol ATP hydrolyzed per mol of substrate protein transported were obtained, similar to previous measurements (16, 36). The variation in this number once again reflects the importance of substrate protein sequence and structure in the efficiency at which the current *in vitro* protein translocation system operates. The authors were careful to collect these data during the first 15 s of the transport reaction, attempting to avoid the latter phase when export-incompetent substrate proteins accumulate but continue to promote unproductive ATP hydrolysis at the translocon. The continued difficulty in making this important measurement points out the need to continue to develop an improved posttranslational *in vitro* protein translocation system with appropriately engineered substrates that might avoid this problem. Collaborations between scientists working on the protein-folding problem and those working on protein transport might prove particularly fruitful in this regard.

The observed ATP coupling numbers are consistent with the elongation phase of the protein transport cycle, when ATP-driven conformational cycles of the SecA motor would facilitate repeated rounds of substrate transit through the translocon channel. Three different types of models have been proposed to explain SecA motor function. In one case the SecA THF subdomain acts as an ATP-dependent reciprocating piston to drive protein transport at the mouth of the SecY channel (the SecA power stroke model). This model is essentially a more limited version of the original SecA membrane cycling model of Economou and Wickner (37), which was formulated based on the membrane insertion and retraction property of a mobile 30-kDa domain of SecA that was coupled to ATP-dependent forward movement of substrate protein. This property has been suggested to be a biochemical artifact (38), and the original model has been further discounted based on the presumably limited size of the translocon channel from X ray structures (39, 40) (however, see below for the potential resurgence of this older model). The newer model was formulated based on the structure of the SecA-SecYEG complex in an intermediate state of ATP hydrolysis, where the THF subdomain is situated vertically over the mouth of the translocon channel and partially inserted into it (24). Unfortunately, the extent of movement of the THF during the translocation cycle remains unclear (e.g., see reference 41), but this point should be clearly addressable now using single-molecule approaches with appropriately positioned fluorescent probes. More difficult is the extent of SecA insertion into the translocon channel and its role in the elongation phase of protein transport, which has yet to be resolved (see below). A second model relies on two-way communication between SecA and SecY proteins in order to coordinate channel opening and ATP hydrolysis events (the Brownian ratchet model), thereby biasing Brownian motion of the substrate in one direction (42). Such communication would allow SecA to sense when substrate translocation has stalled because the channel aperture needs to be expanded, while such stalled structures would feed back on the SecA ATPase motor via its switch domains in order to perform the necessary conformational work of channel expansion. Finally, a third model utilizes both the conformational changes of the dynamic SecA monomer-

dimer cycle and the reciprocating action of the THF to drive SecA-bound substrate into the channel in two successive steps (the reciprocating piston model) (34, 43). Indeed, SecA monomer-dimer dynamics (44) appear to play a more proximal role during the substrate protein handoff step from SecB to SecA, since association of the N terminus of SecA with the C terminus of SecB would dissociate the 1M6N-like SecA homodimer, which has been shown to be the relevant form *in vivo* (45, 46). Later on, the SecA N terminus also contributes to SecYEG targeting through its amphipathic anionic phospholipid-binding activity, as discussed above. It is worth pointing out that these three different models are not mutually exclusive of one another, and indeed a recent publication found that substrate movement across the translocon channel was promoted by a mixture of SecA ATP-dependent "pushing" and Brownian-motion "sliding," and the latter component was critical to achieve a rapid transport rate when the process was modeled mathematically based on known or estimated rate constants (47).

Independent of one's favorite model, it is clear that the interactions that the protein substrate has with SecA and SecY affect the efficacy and rate of the transport reaction. In the case of SecA, several distinct substrate peptide-binding sites have been found: one on each side of the THF subdomain for the signal peptide and early mature region for substrate hairpin insertion (the latter site would presumably double in function for the elongation phase of protein transport), and a third one for the downstream segment of the substrate protein, termed the SecA clamp, which lies at the interface between SecA's preprotein cross-linking domain and nucleotide-binding domain 2 (19–21). In the case of SecY, after completion of initiation, the inserted signal peptide most likely resides just outside the lateral gate region based on a recent crystal structure of SecYEG with a substrate mimic (40), while the dimensions of the channel itself suggest multiple interactions with its walls, most particularly with the narrow pore ring region at the center of the membrane based on a previous cross-linking study (48).

While protein transport proceeds at a more or less constant rate on a macroscale during the elongation phase of transport (36), there are several reports on the effects of localized amino acid sequences slowing down or stalling Sec-dependent transport. Indeed, the translocon channel itself must distinguish between more or less hydrophobic signal anchor sequences to determine whether such sequences will exit via the lateral gate into the lipid bilayer in the case of integral membrane proteins or, alternatively, traverse the membrane in the case of secretory proteins. How such scanning is accomplished is largely unknown. Earlier *in vitro* studies found that proOmpA naturally stalls at runs of hydrophobic sequence as well as positive and negative charges (49, 50). This observation allowed investigators to skirt around the slow and asynchronous initiation phase of *in vitro* protein transport in order to directly study its elongation phase (51, 52). Recently, assays have been set up to measure SecA and SecY interaction with specific regions of the substrate using proOmpA substrates transiently stalled at translocation-arresting disulfide bridges that can be quickly released by addition of reductant (47). Amino acid sequences were engineered following the first cysteine residue in order to measure their effects on SecA- and SecY-dependent transport elongation rates. Such microscale experiments revealed decidedly different translocation rates depending on the amino acid composition of the sequence in question. Moreover, SecA interacted poorly with particular runs of amino acid residues, most notably glycine, which induced backsliding. Additional studies along these lines with a robust reporter system are now clearly warranted in order to discern the rules that govern SecA and SecY substrate recognition and their role in determining transport dynamics.

Finally, we come to the least understood and most controversial area of SecA biology: its membrane interaction properties. Early work by investigators demonstrated soluble, lipid-bound, and integral membrane forms of SecA protein and identified lipid-binding regions at both its N and C termini (53–55). Later work showed that SecA has the remarkable ability to bind and penetrate deeply into lipid monolayers and bilayers or span them in an acidic phospholipid-dependent manner, activities that can be modulated by various SecA ligands (56–58). Electron microscopic studies of SecA in such monolayers show that it forms ring-like structures with a 2-nm central pore (59);

furthermore, SecA channel-forming activity has been detected in a couple of different assays. While some investigators have reported that SecA alone can form active protein transport channels (60, 61), the relevance of this work for physiological protein transport remains unclear; thus, the bulk of the work in our field has focused on SecA function at channels comprised of SecYEG protein: the universal translocon channel complex found in all three domains of life. In particular, a number of studies suggest that in addition to binding SecYEG protein peripherally, SecA inserts into the translocon and in fact spans it. This conclusion comes from a variety of membrane topology studies utilizing right-side-out membrane vesicles, outer membrane-permeabilized cells, as well as untreated cells (62–65). More recently, Banerjee et al. (66) utilized site-specific *in vivo* photocrosslinking and translocon jamming with an arresting substrate to thoroughly map SecY regions contacting SecA during substrate protein insertion. Remarkably, this study found that SecA contacts most SecY transmembrane helices and periplasmic regions surrounding the channel during a translocation arrest. These results are consistent with a proteolysis mapping study of integral membrane SecA and its inaccessibility to phospholipid acyl chains based on cross-linking studies (67–69). Such results therefore appear to extend SecA's role in the transport process, putting it on more equal footing with SecY in contributing to channel structure and dynamics during substrate protein transit. Indeed, this result provides a rationale for the large discrepancy in the measured translocon pore size obtained utilizing the *in vitro* protein translocation system with engineered substrates versus predictions based on molecular dynamic simulations or measurements of a SecYEG crystal structure containing an inserted substrate mimic (40, 70, 71). Obviously, high-resolution structural information about the SecA membrane-inserted state within such arrested translocons is lacking and will be critical for elucidating the bacterial protein transport mechanism.

Given the foregoing discussion of the membrane dynamic properties of SecA protein and its interplay with SecYEG, perhaps it is no wonder that its inclusion during SecYEG reconstitution improves the efficiency of translocon assembly. Thus, we can add one more activity to the already lengthy list of SecA functions: that of a SecYEG translocon assembly chaperone, at least for the improved *in vitro* protein translocation system.

In sum, the new results of Bariya and Randall (12) provide us with an improved *in vitro* protein translocation system and a variety of new substrates to address the various shortfalls of the previous system, along with a growing number of next-generation questions for our field to explore in the coming days. In doing so, our field needs to be more quantitative in our studies so as to avoid observations that may bear little physiological truth. In that regard, linkage of *in vitro* studies with *in vivo* ones provides a powerful cross check by which to assess the relevance of new findings. In addition, a continuation of structural studies, including those at the single-molecule level, should provide new insights into the various steps of this complex process. While more crystal structure snapshots of the different stages of protein transport will be valuable, other structural approaches that offer dynamic information are sorely needed at this juncture for the further maturation of our field.

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