The bacterial translocon interacts with both SecA-bound preproteins and nascent chain-ribosome complexes during Sec and signal recognition particle-dependent protein translocation, respectively. In their inactive state, translocans are saturated with ribosomes and SecA protein, reflecting the inherent affinity of these components for one another. We found that SecA and ribosomes are bound simultaneously and noncompetitively to a common set of inactive translocans. Furthermore, we demonstrate that at a later stage in binding, SecA possesses a ribosome-translocon dissociation activity that is coupled to its ATP-dependent membrane insertion and retraction cycle that drives protein translocation. This novel activity is presumably important in the commitment of the translocon to the Sec-dependent pathway. These results also provide a rationale for the compatibility and regulation of multiple protein translocation pathways that each makes distinct demands on a common translocon core.

Organisms contain multiple protein translocation pathways for targeting proteins into and across their membrane systems. In bacteria, signal recognition particle (SRP) and its receptor and SecA and its partner chaperone SecB comprise two major pathways for the insertion of membrane proteins and the translocation of secretory proteins (see Refs. 1 and 2 for recent reviews). These two pathways converge at the translocon, which consists of the heterotrimeric SecYEG protein that appears to form a protein-conducting channel (3–9). SecYEG is homologous to the Sec61p complex that is present in the endoplasmic reticulum of eukaryotes (10). Bacterial SRP and SRP receptor consist of Ffh (P48)/4.5 S RNA and FtsY components, respectively; they are homologous to the 54-kDa subunit/7 S RNA and α-subunit of eukaryotic SRP and SRP receptor, respectively (11, 12). The SRP-dependent pathway promotes the co-translational targeting of nascent chain-ribosome complexes to the translocon, where the ribosome can form a tight seal with the translocon by alignment of the peptide exit tunnel of the large ribosomal subunit with the translocon channel (13–15). By contrast, SecA and SecB promote both co-translational and post-translational protein translocation, where SecB transfers its bound protein substrate to SecA, which then undergoes multiple ATP-driven cycles of membrane insertion and retraction at SecYEG to promote the stepwise translocation of preproteins across the plasma membrane (16–18). Analysis to date suggests that the SRP and SecA-dependent pathways transport membrane and secretory protein substrates, respectively (14, 19). However, certain membrane proteins also require SecA for their biogenesis, and the two pathways may act consecutively or in concert with one another, making the rules that distinguish such differential targeting unclear presently (20–22).

Both purified SecA and ribosomes display high affinity binding activity for the translocon. Studies with proteoliposomes indicated that no additional components were necessary for this interaction, since purified SecYEG protein was sufficient to restore high affinity translocon-binding activity for both SecA and ribosomes (23, 24). In the case of SecA, this activity resides within its N-domain, which is homologous to DEAD helicase motor domains and consists of two nucleotide-binding domains that flank a substrate protein-binding site (25–28). The high affinity nucleotide-binding domain of SecA has been shown to regulate both its substrate protein-binding and release cycle along with its membrane insertion and retraction cycle (29, 30). By contrast, the C-domain of SecA mediates dimerization, low affinity binding to anionic phospholipids, and SecB association (31, 32). In the case of ribosomes, high affinity translocon-binding activity has been shown to be a highly conserved property, since ribosomes from Escherichia coli, yeast, and mammalian cells all translocate with similar affinities (24). Interestingly, binding was found to reside within 28 or 23 S rRNA of eukaryotes or prokaryotes, respectively.

Since both SecA and ribosomes have nanomolar affinity for the translocon and are considerably in excess of translocons in vivo (10-fold for SecA and >10-fold for ribosomes (33, 34)), a number of interesting questions arise. (i) Are SecA and ribosome binding at the translocon mutually exclusive or not? (ii) Has a mechanism evolved to remove potentially interfering ribosomes or SecA from translocons being targeted for protein translocation by another pathway? (iii) Do translocon-bound ribosomes or SecA serve any significant function when the protein-conducting channel is not occupied? We have performed a biochemical analysis of this system for the SecA-dependent pathway in order to address these questions.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Chemicals—MC4100 (F− ΔargF-lacU169 araD136 relA1 rpsL150 flbB5301 ptsF25 deoC1 thi-1) and its isogenic derivative, SE6004 (pRLA4 rpsEΔ) were obtained from Jon Beckwith. The MC4100 derivative, CK1801 (ΔuncB-uncC), was obtained from Carol Kumasoto. pBER200secYEG, which overproduces SecYEG protein, has been described previously (27). Unless otherwise noted, most chemicals were reagent grade or better and were obtained from Sigma.

Preparation of SecA, Ribosomes, IMF, and Preprotein—E. coli SecA protein was purified as described previously (27). 70 S E. coli MRE6000 ribosomes, a kind gift of Dr. H. Noller and co-workers, were purified on sucrose gradients as described previously (35). SecA and ribosomes were labeled with Na125I (Amersham Biosciences), utilizing the IODOGEN direct method, to a specific activity of 0.5 × 106 to 1.0 × 107 cpm/μg.
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cpm/pmol or 0.8 × 10^6 to 1.2 × 10^6 cpm/pmol, respectively, and filtered through a Micro BioSpin P-6 column (Bio-Rad) as described previously (27). Urea-treated IMV were prepared as previously described (36). The chimeric preprotein PSN, composed of the E. coli alkaline phosphatase signal sequence and mature portion of Staphylococcal nuclease with K97C and W140H substitutions, was prepared as described previously (37). Pro-OmpA was prepared as described previously (38).

**Membrane Binding Assays**—Binding assays (100 μl) containing 10-μg membrane protein equivalents of urea-treated IMV and the indicated concentrations of 125I-SecA or 125I-ribosomes in buffer A (50 mM Hepes-KOH, pH 7.2, 30 mM KCl, 50 mM MgCl₂, 0.5 mM Mg(OAc)₂, 0.5 mg/ml bovine serum albumin, 1 mM dithiothreitol) were incubated on ice for 15 min, when they were overlaid onto an equal volume of buffer A containing 0.2 mM sucrose and sedimented at 320,000 × g in an SW 28 rotor at 4 °C. The supernatant was removed, and the pellet was rinsed with 200 μl of buffer A followed by quantification of pellet-bound radioactivity with a γ-counter (Beckman 5500).

**In Vitro Protein Translocation and SecA ATPase Assays**—For analysis of pro-OmpA translocation, 100-μl reactions contained 10 μl of 10× TL buffer (500 mM Tris-HCl, pH 8.0, 500 mM KCl, 50 mM MgCl₂), SecA (50 μg/ml), SecB (66 μg/ml), bovine serum albumin (200 μg/ml), ATP (2 mM), NADH (5 mM), CK81014 urea-treated IMV (100 μg/ml), and 70 S E. coli ribosomes (100 nt) where indicated. Reactions were initiated by the addition of a mixture of 125I-pro-OmpA (5 × 10⁶ cpm/ml) and pro-OmpA (32 μg/ml) in 50 mM Tris-HCl, pH 8.0, 6 μM urea, and incubated for 20 min at 37 °C. Samples were transferred to ice and digested with proteinase K (1 mg/ml) in the presence or absence of Triton X-100 (0.1%) for 15 min. Protein was precipitated by the addition of 75 μl of 30% trichloroacetic acid, washed with acetone, and resuspended in 50 μl of SDSC-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue) and heated for 5 min at 100 °C. Samples were analyzed by 12.5% SDSC-PAGE followed by autoradiography. For SecA ATPase activity assays, 25-μl reactions contained 50 mM Hepes-KOH, pH 7.5, 30 mM KCl, 50 mM NaCl, 0.5 mM Mg(OAc)₂, 1 mM dithiothreitol, SecA dimer (0.2 μM), CK8101 IMV (50 μg of membrane protein/ml) (for membrane and translocation ATPase activity), PSN (20 μM) (for translocation ATPase activity), and ribosomes (100 nt) where indicated. Reactions were initiated by the addition of ATP (4 mM) and incubated at 37 °C for 15 min. ATPase activity was determined using the malachite green colorimetric method (39) with the modifications described previously (40).

**RESULTS AND DISCUSSION**

**Noncompetitive Binding of SecA and Ribosomes to SecYEG Protein**—Purified 125I-labeled SecA and 70 S ribosomes were prepared and bound to IMV that had been previously treated with urea in order to remove and inactivate endogenous SecA protein (25). Our results were consistent with previous studies both from our laboratory and others with respect to SecA and ribosome affinity and maximum binding capacity of IMV for these components (Fig. 1, A and B) (24, 25, 27). Variation of our incubation and sedimentation conditions indicated that the binding reaction had reached equilibrium and that IMV separation was specific with less than 2% contamination with unbound SecA or ribosomes (data not shown). In order to demonstrate that binding was specific for SecYEG protein, IMV were prepared from a strain that overproduced SecYEG (IMV-SecYEG) activate the SecA ATPase cycle (41). Analysis of SecA and protein content. Translocation ATPase activity requires binding of SecA or the ribosome engaged in “piggyback” binding to one another rather than in direct translocon association. However, in order to rule against this possibility, we determined whether SecA and ribosomes interact with each other in solution under mild conditions. For this purpose, 125I-labeled ribosomes were incubated with biotinylated SecA, and the mixture was specifically separated utilizing NeutraAvidin beads that were subjected to several washing and sedimentation steps. These studies indicated that greater than 95% of ribosomes were not associated with SecA, the latter of which was bound specifically and quantitatively to the NeutraAvidin beads under our experimental conditions (Table I and data not shown). Taken together, these results strongly suggest that both SecA and ribosomes bind independently to a common set of translocons in order to have equivalent maximal binding capacities. Additional studies described below strongly support this conclusion.

**SecA Possesses a Ribosome-Translocon Dissociation Activity**—It seemed probable to us that stable association of the ribosome with the translocon would interfere with the SRP-dependent protein translocation pathway, since it would ultimately block targeting of nascent chain-ribosome complexes to the translocon. Likewise, such a circumstance could also interfere with the Sec pathway, since a bound ribosome could limit the conformational flexibility of the translocon that is needed to promote SecA function particularly during its insertion and retraction cycle (30). However, since both pathways function well in vitro despite such theoretical concerns, it seemed likely that the cell may possess a mechanism(s) for dealing with the potential difficulty. In order to investigate whether translocon-bound ribosomes interfere with SecA function, we made use of a simple in vitro protein translocation system consisting of purified preprotein, SecA, ribosomes, and IMV. Pre-binding of ribosomes to IMV at a 3-fold excess over saturation did not interfere with SecA-dependent protein translocation (Fig. 3, compare lanes 4 and 7). Furthermore, the presence of such translocon-bound ribosomes did not interfere with either SecA-dependent membrane or translocation ATPase activities (Fig. 4). These two activities require SecA association with SecYEG or SecYE and an export-competent preprotein, respectively, in order to activate SecA ATPase activity (43).

It seemed implausible to us that ribosomes would remain bound to translocons during SecA membrane cycling, since...
both entities would need access to the translocon channel. Accordingly, these preliminary experiments suggested to us that SecA might possess a novel ribosome-translocon dissociation activity. In order to investigate this hypothesis, we measured whether SecA could dissociate translocon-bound ribosomes during protein translocation conditions. For this purpose, \(^{125}\text{I}\)-labeled ribosomes were prebound to IMV, followed by the addition of the components required to promote SecA-dependent protein translocation. In addition, we also included a 10-fold excess of unlabeled ribosomes in this mixture to act as a ribosomal “sink” to prevent the rebinding of \(^{125}\text{I}\)-labeled ribosomes to IMV. Our results show that fully 73% of ribosomes were released from the translocon during protein translocation conditions, and such release was dependent on
the absence of any competitor was defined as 100%.

**Experimental Procedures**

that such release may be mediated by the so called ribosomes in the presence of SecA and ATP alone, suggesting we also noted that there was a minor but significant release of SecA-dependent protein translocation and ribosomal release. It is conceivable that such translocons were poorly active for IMV leaves inactive SecA bound to a portion of translocons (44), a state can be further stabilized by inclusion of sodium azide, an inhibitor of SecA ATPase activity and the membrane deinsertion reaction (30, 44, 45). Therefore, 125I-labeled ribosomes were prebound to IMV, followed by the addition of SecA under conditions that promote its stable membrane insertion, and the amount of membrane-bound ribosomes was determined. Our results indicate that up to 70% of translocon-bound ribosomes were released under these conditions, and such release was dependent on SecA, AMP-PNP, and azide, conditions that stabilize translocon-inserted SecA protein (Fig. 5A). This level of dissociation was equivalent to that observed under conditions of normal SecA-dependent protein translocation. Neither AMP-PNP nor azide alone in the presence of SecA promoted a significant level of ribosome release. The poor release activity observed in the presence of SecA and AMP-PNP suggested to us that ribosome dissociation might not occur immediately upon SecA insertion, so that quantitative release of ribosomes required stabilization of the inserted state of SecA that only occurs in the presence of azide (44). Significant deinsertion of SecA has been observed previously when only AMP-PNP is present as indicated by the exchangeability of this translocon-associated species with exogenously added SecA (e.g. see Fig. 6

**TABLE I**

| MPB label | SecA Fraction | Ribosomes % |
|-----------|---------------|-------------|
| -         | Low salt      | 84          |
| -         | Medium salt   | 12          |
| -         | High salt     | 2.7         |
| -         | Pellet        | 1.3         |
| -         | Low salt      | 83          |
| -         | Medium salt   | 12          |
| -         | High salt     | 3.1         |
| -         | Pellet        | 1.5         |
| +         | Low salt      | 88          |
| +         | Medium salt   | 10          |
| +         | High salt     | 2.2         |
| +         | Pellet        | 1.5         |

**Fig. 2.** Noncompetitive binding of SecA and ribosomes to the translocon. Binding assays were performed as described under “Experimental Procedures” with the following modifications. The indicated concentration of unlabeled SecA protein (A) or ribosomes (B) was preincubated with wild-type IMV on ice for 30 min, when 10 μM 125I-ribosomes or 50 μM 125I-SecA protein, respectively, was added and incubated for an additional 30 min followed by quantification of membrane-bound radioactivity. The amount of 125I-labeled species bound in the absence of any competitor was defined as 100%.

**Fig. 3.** Ribosomes do not inhibit SecA-dependent protein translocation of pro-OmpA. Protein translocation reactions were performed as described under “Experimental Procedures.” Where indicated, ribosomes were added to the reactions to 100 nM. To assess the degree of protein translocation, appropriate samples were treated with proteinase K (PK) or proteinase K in the presence of Triton X-100. Lanes 9 and 10, 50 and 25% of input 125I-pro-OmpA, respectively.

![Image](image-url)
Endogenous, membrane, and translocation ATPase activity assays were performed as described under “Experimental Procedures.” Assays were conducted in the absence (open bars) or presence (filled bars) of 100 nM ribosomes. Each bar represents the average of triplicate assays.

The combined action of AMP-PNP and azide was shown to greatly increase the level of steady state translocon-inserted SecA protein (44). The presence of multiple attachment points between the ribosome and the translocon, which was recently observed for the eukaryotic system, may also enhance ribosomal stability at the translocon (46).

We were able to assess the kinetics of the dissociation reaction, since translocon-bound ribosomes were stable in the presence of bound SecA protein as long as AMP-PNP and azide were not present, thus allowing for synchronization of the reaction (Fig. 2A). We found that SecA-dependent ribosomal dissociation occurred at a relatively rapid rate (Fig. 5B), similar to that observed previously for AMP-PNP-promoted SecA membrane insertion at this temperature ($t$ of ~0.5 min) (30). A more elaborate assay methodology than that employed here would need to be devised in order to more accurately compare the kinetics of these two processes. However, as we speculated above, ribosome release may not occur immediately upon SecA insertion, since such release requires stabilization of the inserted state by azide. We have not tested whether the SRP-dependent targeting of nascent chain-ribosome complexes to the translocon also results in ribosome dissociation, since such experiments were beyond the present scope of our study.

**Isolation of SecA-Ribosome-Translocon Complex**—In order to directly demonstrate that both SecA and ribosomes bind simultaneously to a common set of translocons, we attempted to isolate the triple complex by utilizing conditions that allow for solubilization and selective isolation of SecA-bound translocons (47). His-tagged SecA and 125I-labeled ribosomes were bound to IMV at saturating levels, and IMV were subsequently isolated by sedimentation. After washing and resuspension, IMV were recovered in the specifically bound fraction (Fig. 6, compare lanes 11 and 12). SecY protein was positively identified by its aggregation behavior and inability to enter the gel after boiling in SDS (Fig. 6, compare lanes 4 and 5). Significantly, over 60% of the 125I-labeled ribosomes that had been previously bound to IMV were recovered with SecA and SecY proteins in the eluate fraction. No additional attempt was made at optimization of these conditions in order to further stabilize ribosome-translocon association. Taking all of our results together, we conclude that SecA and ribosomes are simultaneously bound to a common set of translocons, and such bound ribosomes can be released by the SecA insertion reaction.

The dual occupancy of both SecA and the ribosome at the translocon is somewhat remarkable given the large size of both species in comparison with the translocon. Whereas SecA dimer is ~100 x 80 x 60 Å (48), only the 68-kDa N-domain of SecA contains the high affinity SecYE-binding determinant that interacts with the translocon as a monomer (27). Structural analysis of the bacterial translocon has been difficult to date, given the tendency of SecYE protein to nonspecifically oligomerize in detergent solutions or within membrane systems (6–8). Recent electron microscopic analysis of two-dimen-

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**Fig. 4. Ribosomes do not inhibit SecA ATPase activities.** SecA endogenous, membrane, and translocation ATPase activity assays were performed as described under “Experimental Procedures.” Assays were conducted in the absence (open bars) or presence (filled bars) of 100 nM ribosomes. Each bar represents the average of triplicate assays.

**Fig. 5. SecA-dependent protein translocation or membrane insertion dissociates translocon-bound ribosomes.** Binding assays were performed as described under “Experimental Procedures” with the following modifications. A, 100 nM 125I-ribosomes were incubated with IMV on ice for 15 min, when a mixture of the indicated components was added to the concentration given, and incubation was continued for 15 min at 37 °C followed by quantification of membrane-bound radioactivity. The ATP-regenerating system contained 0.5 μM each of phosphocreatine and creatine kinase. B, similar to A, except that SecA was added to the prebound 125I-ribosome-IMV mixture to 500 nM followed by incubation on ice for an additional 15 min. A mixture of AMP-PNP and sodium azide (final concentrations of 3 and 20 mM, respectively) was then added, and incubation was continued at 37 °C for the indicated time period, when samples were removed and quenched in ice water to terminate the reaction.

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Two-stage Binding of SecA to the Bacterial Translocon

Fig. 6. Isolation of a SecA-ribosome-translocon complex. Binding assays were conducted as described under “Experimental Procedures” with the following modifications. SecA protein (125 nM) and 125I-ribosomes (100 nM) were incubated with wild-type IMV on ice for 30 min followed by sedimentation. Membrane pellets were washed with 200 μl of buffer A, and IMV (containing 23,176 cpm of bound 125I-ribosomes) were solubilized on ice in 100 μl of 50 mM Hepes-KOH, pH 7.2, 30 mM KCl, 2.5 mM Mg(OAc)2, 1 mM diithiothreitol, 1% digitonin, a 3.4 mg/ml concentration of a 1:1 (w/v) ratio of 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine and 1,2-dioleoyl-sn-glycerol-3-phospho-rac-(1-glycerol) (Avanti Polar Lipids), and 35% glycerol. The solubilized material was applied to a Micro-BioSpin column (Bio-Rad) packed with 50 μl of His-Bind Resin (Novagen) and incubated on ice for 15 min. The column was spun at 1000 × g for 1 min at 4°C, and wash and elution steps were performed as described by the manufacturer with the exception that 1% digitonin, a 1.5 mg/ml concentration of a 1:1 (w/v) ratio of 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine and 1,2-dioleoyl-sn-glycerol-3-phospho-rac-(1-glycerol), and 35% glycerol were included in the bind, wash, and elution buffers. A, Western blot of fractions probed with a mixture of INDIA His-probe-horseradish peroxidase conjugate (Pierce) and anti-peptide antisera to SecY protein followed by development by ECL (PerkinElmer Life Sciences). Lanes 1–3, 20, 50, and 100% input SecA, respectively. Lanes 4 and 5, 100% input IMV either boiled at 100°C for 5 min (lane 4) or unboiled (lane 5). Lanes 6–11, fractions collected from the Ni2+ column that included flow-through (lane 6), four consecutive washes (lanes 7–10), and the specifically bound eluate (lane 11). Lane 12, digitonin-solubilized sample prior to fractionation. B, quantification of 125I-ribosomes by fraction. Background counts (209 cpm) were subtracted from each sample.

Our results demonstrate an important new activity for SecA protein, whereby it is able to dissociate translocon-bound ribosomes that could potentially interfere with the Sec pathway at a later stage in the translocation cycle. Although we cannot currently conclude that ribosomal release is mandatory for the functioning of the Sec pathway, this inference appears likely and should be testable with appropriate secA mutants or inhibitors that block the ribosomal release step. Such an activity presumably evolved relatively early in the origin of the Sec system, given the universality of the SRP system and ribosome-translocon association in the three domains of life and the restriction of SecA to eubacteria and assimilated plastids (10). Such dissociation may occur via a SecA insertion-promoted conformational change of the translocon that results in a dramatically decreased affinity of the ribosome for this altered state of the translocon. Alternatively, SecA could play a more direct role in the dissociation reaction (e.g., by utilizing its RNA helicase activity to directly interact with 23 S rRNA in order to change ribosomal affinity for the translocon). Analysis of this system with two helicase-negative SecA proteins that possess normal protein translocation activity, SecA-A498C and SecA-G508S (50), showed that both proteins displayed normal ribosome dissociation activity, indicating that SecA-dependent RNA helicase activity is probably not involved in this process (data not shown).

Translocon-bound SecA protein is active in binding a substrate protein either directly or in complex with SecB chaperone, and in the presence of ATP, it undergoes the membrane insertion reaction that is coupled to protein translocation and ribosomal release (17, 25, 51, 52) (this study). By contrast, the function of translocon-bound ribosomes remains less clear. A function of ribosomes or the large ribosomal subunit in sealing of inactive translocons appears unlikely, however. Although a previous study indicated that urea-treated IMV (presumably depleted of ribosomes or 50 S ribosomal subunits as well as SecA protein) were defective in the generation of a membrane potential, this defect was attributed to the removal of the F1 portion of proton ATPase (53, 54). Measurement of the membrane potential of urea-treated IMV genetically lacking proton ATPase indicated that such membranes were properly sealed and energetically normal and that the addition of ribosomes or SecA had no effect on the membrane potential (data not shown). Recent analysis of purified ribosome-Sec61 complexes indicated that they were capable of initiating protein synthesis and that complexes bearing signal peptides matured to form tight ribosome-translocon junctions (55). This result suggests between the ribosome and translocon as well as binding of larger regions of SecA to peripheral portions of the translocon that are not sterically hindered by the bound ribosome (e.g., see Figs. 2 and 5 of Ref. 49). It seems likely that, since the ribosome-translocon interaction is more ancient than that of SecA protein (based on its conservation across the three domains of life and the restriction of SecA to eubacteria and assimilated plastids (10)), the initial SecYE-binding determinants of SecA protein would have evolved so as not to overlap with those of the ribosome. Although it is conceivable that the simultaneous binding of SecA and the ribosome to the translocon might cause the other species to reorient itself with respect to its initial binding site, such logic is not supported by our studies, since the observed binding affinities were equivalent, whether or not they were done in the presence of a molar excess of the other species. Clearly, this situation changes; however, when SecA becomes committed to preprotein insertion and ribosome-translocon association is perturbed in order to accommodate SecA-dependent protein translocation and SecA membrane cycling.
that, like SecA, translocon-bound ribosomes represent a functional staging area for initiation of the protein translocation cycle, although this inference requires further investigation, particularly in a prokaryotic system. Presumably, SRP and its receptor evolved to lend additional speed and specificity to this basal system, consistent with the importance but ultimate dispensability of SRP in certain organisms like yeast (56). This logic suggests that translocon-bound ribosomes may represent an impediment to SRP-dependent targeting of nascent chain. Recent comparison of 80 S ribosome-Sec61p complexes in the absence or presence of translocating polypeptide chains found similar connecting structures, supporting this logic (46). Thus, like SecA, the SRP-dependent pathway may have also evolved a ribosome-translocon dissociation activity. Investigation of this and other points relating to the regulation of protein translocation pathways that intersect and share common components should prove fruitful ground for future studies.

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