Recent work has demonstrated that the signal recognition particle (SRP) is required for the efficient insertion of many proteins into the *Escherichia coli* inner membrane (IM). Based on an analogy to eukaryotic SRP, it is likely that bacterial SRP binds to inner membrane proteins (IMPs) co-translationally and then targets them to protein transport channels ("translocons"). Here we present evidence that SecA, which has previously been shown to facilitate the export of proteins targeted in a post-translational fashion, is also required for the membrane insertion of proteins targeted by SRP. The introduction of SecA mutations into strains that have modest SRP deficiencies produced a synthetic lethal effect, suggesting that SecA and SRP might function in the same biochemical pathway. Consistent with this explanation, depletion of SecA by inactivating a temperature-sensitive amber suppressor in a *secA* mutant strain completely blocked the membrane insertion of AcrB, a protein that is targeted by SRP. In the absence of substantial SecA, pulse-labeled AcrB was retained in the cytoplasm even after a prolonged chase period and was eventually degraded. Although protein export was also severely impaired by SecA depletion, the observation that more than 20% of the OmpA molecules were translocated properly showed that translocons were still active. Taken together, these results imply that SecA plays a much broader role in the transport of proteins across the *E. coli* IM than has been previously recognized.

Proteins that are destined to be translocated across or inserted into the bacterial inner membrane (IM) are targeted to transport sites by multiple mechanisms. In *Escherichia coli*, many secreted proteins are targeted to the IM by molecular chaperones such as SecB, which keep them in a loosely folded, translocation-competent conformation (1, 2). The chaperone-based targeting pathways promote the translocation of fully synthesized proteins in vitro and probably also function in a post-translational fashion at least to some extent in vivo (3, 4). By contrast, recent studies have suggested that a variety of inner membrane proteins (IMPs) are targeted to the membrane by an essential ribonucleoprotein complex that is closely related to the eukaryotic signal recognition particle (SRP) (5–7). In mammalian cells, SRP is a complex composed of six polypeptides and a single RNA that targets proteins to the secretory pathway in a strictly co-translational fashion (reviewed in Ref. 8). The 54 kDa subunit of SRP (SRP54) binds to signal sequences of nascent polypeptides and guides ribosome-nascent chain complexes to transport sites in the endoplasmic reticulum (ER) via an interaction with the membrane-bound SRP receptor. Although the SRP found in *E. coli* and many other bacterial species contains only a single protein (a homolog of SRP54 called "Ffh") and a small RNA ("4.5 S RNA") (9), many aspects of its function appear to be conserved, including co-translational binding to substrates (10) and a specific interaction with a homolog of the SRP receptor ("FtsY") (11).

All of the different targeting pathways appear to converge at the IM. Both exported proteins and IMPs are transported by a common translocation channel or "translocon" composed of a phylogenetically conserved heterotrimer called the SecYEG complex, which is closely related to the Sec61p complex found in the eukaryotic ER (reviewed in Ref. 12). Bacteria differ from eukaryotes, however, in that they have a unique peripheral membrane protein called SecA that interacts with SecY (13, 14) and that plays an essential role in protein export (15, 16). SecA acts as a molecular motor that binds to SecB-preprotein complexes in the cytoplasm (17) and then uses the energy of ATP hydrolysis to catalyze post-translational translocation across the cytoplasmic membrane (18). Following translocation, SecA is released from the membrane (19). Relatively little is known about the role of SecA in IM insertion, and its role in transporting proteins targeted by the SRP pathway is particularly unclear. The insertion of some IMPs, but not others, has been proposed to be SecA-dependent (e.g. Refs. 20–24), but the SecA dependence does not correlate well with SRP dependence. Moreover, in all of the studies on IMP insertion, SecA activity has been blocked by adding sodium azide, a weak inhibitor of the SecA ATPase, or by shifting strains containing the temperature-sensitive *secA51Ts* allele to high temperature. Because ATP binding and hydrolysis regulates the affinity of SecA for different components of the system, azide treatment may interfere with the normal cycling of SecA and may therefore inhibit IMP insertion by an indirect mechanism. Likewise, the SecA51(Ts) protein becomes trapped on the IM above 33 °C (25) and may block IMP insertion by simply interfering with the docking of ribosomes. Indeed recent studies strongly suggest that the use of sodium azide and temperature-sensitive *sec* alleles can produce misleading results (22, 26).

To circumvent the problems associated with conditional alleles and inhibitors of SecA function, we used a novel approach to investigate the role of SecA in the membrane insertion of SRP substrates. Initially we tested for a genetic interaction between SRP and SecA using a synthetic lethality assay. The results from these experiments raised the possibility that SRP and SecA function in the same biochemical pathway. To test this idea directly, we examined the effect of SecA depletion on the membrane insertion of an SRP substrate, AcrB. In these experiments we used a strain that contains a *secA*-mut mutation.

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‡ To whom correspondence should be addressed: National Institutes of Health, Bldg. 10, Rm. 9D-20, Bethesda, MD 20892-1810. Tel.: 301-402-4770; Fax: 301-402-0387; E-mail: harris_bernstein@nih.gov.

‡ The abbreviations used are: IM, inner membrane; AP, alkaline phosphatase; CAT, chloramphenicol acetyltransferase; IMP, inner membrane protein; IPTG, isopropylthiogalactoside; OM, outer membrane; RBP, ribose-binding protein; SRP, signal recognition particle; ER, endoplasmic reticulum.

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and a temperature-sensitive amber suppressor. At low temperature full-length SecA protein is synthesized, but at high temperature only a small unstable fragment of the protein is produced (16). We found that after SecA depletion AcrB was quantitatively retained in the cytosol, where it was eventually degraded. Surprisingly, we found that although SecA depletion also had a profound effect on protein export, a fraction of at least one protein was still properly translocated. These results demonstrate that SecA function is at least as important for the insertion of IMPs targeted by SRP as for protein export and suggest that SecA plays a role in the transport of both cotranslationally and post-translationally targeted proteins.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Manipulations**—Strains HDB90 (secA Supplementary Fig. 3A, zab: Tn10), HDB91 (secA450 zab: Tn10), and HDB92 (secA51ts zab: Tn10) were constructed by introducing the secA mutations from MM52 and E4540 (27, 28) into HDB45 (MC4100 pHDB45 [fliB:kan]) by P1 transduction. Strains BA13 (MC4100 supF trp secA13ts zab: Tn15) and BA251 (MC4100 supF trp secA13ts zab: Tn15) were obtained from Dr. Don Oliver. Media preparation and bacterial manipulations were performed according to standard methods (28). Selective media contained 100 µg/ml ampicillin or 40 µg/ml chloramphenicol.

**Plasmid Construction**—Amino acids 266–1049 were deleted from AcrB by excising a NruI-SalI fragment from plasmid S215 (6) and repairing the ends with DNA polymerase (Klenow fragment) to generate plasmid S215Δ2. To create a fusion of alkaline phosphatase (AP) with AcrB at amino acid 265 (pJN4), S215Δ2 was digested with SalI and ligated to a BstHKAI-HelI fragment of pH1-1 containing the AP gene (30) using the oligonucleotide adapters 5′-CCGCCTGTCGACGTAATATCGCCT-3′ and 5′-TGCAGATATTACTTGACGGCGCGTTGCT-3′. A derivative of pJN4 in which the AP fusion was subcloned into pACYC184 (pJN8) was used for the experiments described here. A pACYC184 derivative containing the AcrB 576-AP fusion (pNU88) and plasmid pAP-1 have been described (6, 31).

**Pulse-Chase Labeling, Immunoprecipitations, and Western Blots**—Cells were grown overnight at 30 °C in M9 medium containing 0.4% glucose and 40 µg/ml l-amino acids, excluding methionine and cysteine. Cultures were then diluted into fresh medium at an optical density (A660) of 0.02 and grown to an OD of 0.05 at 30 °C. Each culture was then divided in half. One-half was maintained at 30 °C while the other was shifted to 41 °C. At various times after the temperature shift, cells were subjected to pulse-chase labeling and processed essentially as described (6). In some experiments, spheroplasts were divided into two portions, one of which was treated with proteinase K. Proteins were collected by trichloroacetic acid precipitation, and immunoprecipitations were performed as described (6, 31). To provide an internal standard, cells were labeled with [35S]methionine and [35S]cysteine for 30 min at 37 °C and of HDB92 cells at 30 °C was observed on LB plates containing this concentration of inducer (Fig. 1 A and B). When the level of inducer and therefore the level of Ffh was slightly reduced, however, strong growth defects were observed; HDB91 and HDB92 did not grow at all on plates containing 2 µg IPTG and no IPTG, respectively. HDB90 cells grew about as well on the plates containing reduced levels of inducer as on plates containing 10 µg IPTG. These results demonstrate that reduction in SRP concentration in cells that contain SecA mutations produces a synthetic lethal effect and therefore raise the possibility that SecA participates in the insertion of SRP substrates.

**SecA Mutations Are Lethal in Strains That Have SRP Deficiencies**—Although cells can generally tolerate slight deficiencies in essential biochemical pathways, it has often been observed that combining two deficiencies in the same pathway has a synergistic effect that leads to cell death (33). Thus, if SRP and SecA are required in successive steps of IMP insertion, then reduction in the activity of each of these factors might produce similar physiological effects that together would be lethal. To test the effect of reducing SRP and SecA activity simultaneously, we constructed strains HDB90 (secA13ts), HDB91 (secA450), and HDB92 (secA51ts) in which the expression of ffh is regulated by the trc promoter. Cells were plated on LB agar containing various amounts of IPTG and incubated at temperatures that are permissive for growth. Based on previous studies performed with the parent strain (HDB45), it was expected that a near wild-type level of Ffh would be produced in the presence of 10 µM IPTG (6). Growth of HDB91 cells at 37 °C and of HDB92 cells at 30 °C was observed on LB plates containing this concentration of inducer (Fig. 1 A and B). When the level of inducer and therefore the level of Ffh was slightly reduced, however, strong growth defects were observed; HDB91 and HDB92 did not grow at all on plates containing 2 µg IPTG and no IPTG, respectively. HDB90 cells grew about as well on the plates containing reduced levels of inducer as on plates containing 10 µg IPTG. These results demonstrate that reduction in SRP concentration in cells that contain SecA mutations produces a synthetic lethal effect and therefore raise the possibility that SecA participates in the insertion of SRP substrates.

**SecA Depletion Abolishes the Insertion of AcrB**—We next used a protease protection assay to study the effect of SecA depletion on the insertion of the SRP substrate AcrB. In this assay, protease treatment releases the AP domain from IMP-AP fusion proteins. Cells were grown under inducing conditions in the presence of 10 µM IPTG. These results suggested that reduction in the activity of each of these factors might produce similar physiological effects that together would be lethal. To test the effect of reducing SRP and SecA activity simultaneously, we constructed strains HDB90 (secA13ts), HDB91 (secA450), and HDB92 (secA51ts) in which the expression of ffh is regulated by the trc promoter. Cells were plated on LB agar containing various amounts of IPTG and incubated at temperatures that are permissive for growth. Based on previous studies performed with the parent strain (HDB45), it was expected that a near wild-type level of Ffh would be produced in the presence of 10 µM IPTG (6). Growth of HDB91 cells at 37 °C and of HDB92 cells at 30 °C was observed on LB plates containing this concentration of inducer (Fig. 1 A and B). When the level of inducer and therefore the level of Ffh was slightly reduced, however, strong growth defects were observed; HDB91 and HDB92 did not grow at all on plates containing 2 µg IPTG and no IPTG, respectively. HDB90 cells grew about as well on the plates containing reduced levels of inducer as on plates containing 10 µg IPTG. These results demonstrate that reduction in SRP concentration in cells that contain SecA mutations produces a synthetic lethal effect and therefore raise the possibility that SecA participates in the insertion of SRP substrates.

**Fig. 1.** Ffh deficiencies are lethal in secA mutant strains. HDB90 (secA13ts), HDB91 (secA450), and HDB92 (secA51ts) cells, which contain the ffh gene under control of the trc promoter, were streaked on LB agar containing the indicated concentration of IPTG. At IPTG concentrations below 10 µM, the cells contain less Ffh than isogenic ffh+ strains. Plates were incubated at 37 °C for 22 h (A) or 30 °C for 28 h (B).

**RESULTS**

** SecA Mutations Are Lethal in Strains That Have SRP Deficiencies**—Although cells can generally tolerate slight deficiencies in essential biochemical pathways, it has often been observed that combining two deficiencies in the same pathway has a synergistic effect that leads to cell death (33). Thus, if SRP and SecA are required in successive steps of IMP insertion, then reduction in the activity of each of these factors might produce similar physiological effects that together would be lethal. To test the effect of reducing SRP and SecA activity simultaneously, we constructed strains HDB90 (secA13ts), HDB91 (secA450), and HDB92 (secA51ts) in which the expression of ffh is regulated by the trc promoter. Cells were plated on LB agar containing various amounts of IPTG and incubated at temperatures that are permissive for growth. Based on previous studies performed with the parent strain (HDB45), it was expected that a near wild-type level of Ffh would be produced in the presence of 10 µM IPTG (6). Growth of HDB91 cells at 37 °C and of HDB92 cells at 30 °C was observed on LB plates containing this concentration of inducer (Fig. 1 A and B). When the level of inducer and therefore the level of Ffh was slightly reduced, however, strong growth defects were observed; HDB91 and HDB92 did not grow at all on plates containing 2 µg IPTG and no IPTG, respectively. HDB90 cells grew about as well on the plates containing reduced levels of inducer as on plates containing 10 µg IPTG. These results demonstrate that reduction in SRP concentration in cells that contain SecA mutations produces a synthetic lethal effect and therefore raise the possibility that SecA participates in the insertion of SRP substrates.

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AcrB-AP fusion proteins. A partial protected AP fusion protein that remained was immunoprecipitated with protease, and the released AP plus any protease-

The cells were harvested, spheroplasts were generated and treated with proteinase K (lanes 1–2) due to rapid degradation of the protein in the cytoplasm. Quantitative insertion of the AcrB 265-AP fusion was observed in BA13 cells grown at 30 °C and in DO251 cells at both high and low temperature (Fig. 3A, lanes 5–16), and the membrane-bound form of the protein was stable (Fig. 3B, lanes 3–8).

To confirm that the protease resistance of the AcrB-AP fusion proteins was due to retention in the cytoplasm, we examined their localization in more detail by cell fractionation. BA13 and DO251 cells were grown at 30 °C as described above and shifted to 41 °C for 3 h. The cells were then pulse-labeled and harvested following a 10-min chase. Cell proteins were prepared by sonication, and membranes were isolated by high speed centrifugation. About 80–90% of the AcrB 265-AP fusion protein in BA13 cells was found in the high speed supernatant and the released AP plus any protease-protected AP fusion protein that remained was immunoprecipitated with anti-AP antibodies.

We found that depletion of SecA had a profound effect on the membrane insertion of the AcrB-AP fusion proteins. A partial block of AcrB insertion was observed by 1.5 h after the shift (data not shown), but by 3 h a virtually complete block was observed. All of the pulse-labeled AcrB 265-AP fusion protein in BA13 cells was protease-protected following a 2-min chase, indicating that the protein was retained in the cytoplasm (Fig. 3A, lanes 1–2). Even after a 30-min chase, little or no fusion protein was detected in the membrane fraction. About 80–90% of the AcrB 265-AP fusion protein was inserted efficiently into the IM of BA13 cells grown continuously at 30 °C and DO251 grown at either 30 or 41 °C (Fig. 3A, lanes 5–16) as indicated by the complete susceptibility of the AP domain to proteolysis at all time points. Similar results were obtained in experiments in which the insertion of the AcrB 576-AP fusion was examined. At 41 °C, only protease-protected pulse-labeled fusion protein was immunoprecipitated from BA13 cells following a 2-min chase (Fig. 3A, lanes 1–2). After longer chase times, very little fusion protein was isolated from either untreated or protease-treated spheroplasts (Fig. 3B, lanes 1–2) due to rapid degradation of the protein in the cytoplasm. Quantitative insertion of the AcrB 576-AP fusion was observed in BA13 cells grown at 30 °C and in DO251 cells at both high and low temperature (Fig. 3A, lanes 5–16), and the membrane-bound form of the protein was stable (Fig. 3B, lanes 3–8).

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SecA Depletion Inhibits but Does Not Completely Abolish Protein Export—Although the effect of inhibiting SecA activity on protein export has been studied extensively in vitro, most experiments have involved treating cells with sodium azide or shifting strains containing the secA allele to the nonpermissive temperature. To test the effect of SecA depletion on protein export, BA13 and DO251 cells were first transformed with a plasmid expressing an AcrB-AP fusion or with plasmid pAP-1 (31). Cells were then grown and radiolabeled as described above, and AP, RBP, and OmpA were immunoprecipitated from samples that were not treated with proteinase K. Consist-
Role of E. coli SecA in the Insertion of SRP Substrates

FIG. 4. Intracellular localization of AcrB 265-AP and OmpA after SecA depletion. BA13 and DO251 transformed with plasmid pJN6 were grown at 30 °C as described in the legend to Fig. 2. When the $A_{600}$ reached 0.05, cultures were shifted to 41 °C and incubated for 3 h. Aliquots were pulse-labeled and subjected to a 10-min chase. Cell fractions were obtained as described under "Experimental Procedures." Panel A, AcrB 265-AP and CAT were immunoprecipitated from the total cell extract (T, lane 1), the cytoplasm (C, lane 2) and total membranes (IM, lane 3). Panel B, OmpA and its precursor (pro-OmpA) were immunoprecipitated from the total cell extract (T, lane 1), the cytoplasm (C, lane 2), inner membranes (IM, lane 3) and outer membranes (OM, lane 4).

ent with previous studies on SecA function, the export of AP and RBP was completely blocked by SecA depletion. None of the radiolabeled precursor was converted to the mature form in BA13 cells grown at 41 °C even after a 30-min chase (Fig. 5, lanes 1–3). Like the AcrB-AP fusion proteins, pre-AP retained in the cytoplasm (but not pre-RBP) was slowly degraded during the long chase period. By contrast, rapid export of both AP and RBP from BA13 grown at 30 °C or from DO251 cells grown at either high and low temperature was indicated by the complete processing of the radiolabeled precursors within 2 min (Fig. 5, lanes 4–12). Export of OmpA was also significantly inhibited by SecA depletion, but surprisingly a small fraction of the protein appeared to be slowly translocated across the inner membrane (Fig. 5, lanes 1–3). Only about 12% of the protein was processed after a 2-min chase, but after 30 min, more than 20% of the pro-OmpA was converted to OmpA. The remainder of the pro-OmpA was gradually degraded in the cytoplasm.

We next performed cell fractionation experiments to confirm that the processing of OmpA observed after SecA depletion was due to proper translocation and insertion of the protein into the outer membrane (OM). Pulse-labeled BA13 and DO251 cells that had been incubated at 41 °C for 3 h were harvested after a 10-min chase. Cell membranes were separated from the cytoplasm by centrifugation and then further divided into IM and OM fractions. The AcrB-AP fusion protein was used as a marker to validate the membrane fractionation method (data not shown). OmpA was then immunoprecipitated from each cell fraction. In BA13 cells, all of the processed OmpA was correctly localized to the OM (Fig. 4B, lanes 1 and 4). Most of the pro-OmpA remained in the cytoplasm, although a small portion was isolated in the IM fraction (Fig. 4B, lanes 1–3). As expected, all of the OmpA in DO251 cells was properly processed and inserted into the OM (Fig. 4B, lanes 1 and 4). Taken together, these results show that OmpA is accurately transported across the IM (albeit with reduced efficiency) even after a severe reduction in the level of SecA. Thus the IMP insertion defects described above are not due to a nonspecific inactivation of translocons following SecA depletion.

To determine whether SecA depletion affected translocon stability, we measured the SecY levels in BA13 and DO251 cells grown at 30 °C and 41 °C by Western blot. A similar amount of SecY was present in each strain at both temperatures (Fig. 6, lanes 1). This result strongly suggests that the AcrB insertion block observed in the absence of SecA was not caused by a loss of protein transport capacity.

DISCUSSION

In the experiments reported here we have obtained strong evidence that SecA plays an essential role in the membrane insertion of proteins targeted by SRP in E. coli. In an initial genetic test, we found that slight Ffh deficiencies are lethal in SecA mutant strains. This observation indicated that SecA and SRP are likely to function in either the same or in parallel biochemical pathways. To distinguish between these two possibilities, we analyzed the insertion of a model SRP substrate, AcrB, in cells that had normal levels of SRP but reduced levels of SecA activity. To avoid possible artifacts associated with conditional alleles and metabolic inhibitors in these experi-
Role of E. coli SecA in the Insertion of SRP Substrates

E. coli SecA, a member of the ATPase superfamily, has been shown to be essential for the insertion of SRP substrates into the ER membrane (1). SecA appears to function as a motor protein that facilitates the translocation of nascent polypeptide chains across membranes. The observation that inhibition of SecA blocks the insertion of SRP substrates suggests a role for SecA in this process (2, 3). The finding that SecA is required for the insertion of SRP substrates in E. coli is surprising in light of previous studies on the eukaryotic SRP pathway. In mammalian cells, a tight seal between the ribosome and the translocon is generally formed after nascent chains are targeted to the ER by SRP (36). Continual elongation of the nascent chain is thought to be sufficient to push the polypeptide through the translocon. If the molecular mechanisms of translation are conserved in bacteria, then the insertion of SRP substrates may be required to ensure continuous transport of nascent SRP substrates. Thus, our results, together with the observation that the SecA-dependent insertion of SRP substrates is not prevented by the translocation of OmpA observed after SecA was nearly completely depleted, suggest that SecA is required for the insertion of a wide range of proteins that have been previously suggested to participate in the translocon. In both E. coli and mammalian cells, the insertion of SRP substrates is dependent on SecA (16). The relative insensitivity of OmpA export to reduced SecA concentrations suggests that it has an atypical ability to persist in a transport-competent form or that it has a much higher affinity for SecA than most other peripheral membrane proteins. In either case, the unexpected behavior of OmpA in these experiments, together with the observation that only the core components of the translocon are evolutionarily conserved, suggest that there are significant differences between eukaryotic and prokaryotic translocation systems. It is possible that the composition of the translocon or of the interaction between the ribosome and the translocon may be more predominant in bacterial cells. If so, then the motor function of SecA may be required to ensure continuous transport of nascent chains across the membrane.

An alternative explanation of our results that would be consistent with the formation of a tight ribosome-translocon junction is that SecA promotes the insertion of SRP substrates by a mechanism that does not depend on its motor activity. Extensive analysis of protein translocation in vitro has clearly shown that SecA uses the energy of ATP hydrolysis to facilitate post-translational translocation, but the data do not rule out the possibility that SecA has additional functions. Recently it has been shown that the ER luminal protein BiP maintains the permeability barrier of the ER by sealing nontranslocating Sec61p complexes (38). It is conceivable that inactive translocons in bacteria are sealed by a different mechanism (no BiP equivalent has yet been identified) and that SecA triggers conformational changes that are required to initiate both protein translocation and IMP insertion. The observation that SecY is stable after substantial SecA depletion suggests that SecA is not required, however, to maintain the structural integrity of the translocon. In addition to providing evidence that SecA is required for the insertion of SRP substrates, our experiments also yielded several unanticipated results. Given that inhibition of the SRP pathway only partially blocks the insertion of all substrates that have been tested (5, 6, 26), the observation that SecA depletion completely abolished the insertion of AcrB is striking. This result suggests that the insertion of AcrB is SecA-dependent regardless of the mechanism by which it is targeted to the translocon. Furthermore, although our data are consistent with other types of evidence indicating that SecA is essential for protein export, the slow translocation of OmpA observed after SecA was nearly completely depleted was very surprising. OmpA is often used as a model protein for in vitro translocation studies, and its transport into membrane vesicles is strictly dependent on SecA (16). The relative insensitivity of OmpA export to reduced SecA concentrations suggests that it has an atypical ability to persist in a transport-competent form or that it has a much higher affinity for SecA than most other peripheral membrane proteins. In either case, the unexpected behavior of OmpA in these experiments, together with the finding that SecA is required for the transport of a wide range of proteins, suggests that many aspects of SecA function remain to be elucidated.

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