SecA insertion and integration into the *Escherichia coli* inner membrane is a critical step for the catalysis of protein translocation across this layer. To understand this step further, SecA topology was investigated. To determine which regions of SecA are periplasmically exposed, right-side out membrane vesicles were prepared from strains synthesizing monocysteine SecA variants produced by mutagenesis and probed with a membrane-impermeant sulfhydryl-labeling reagent. To determine which regions of SecA contain membrane-integration determinants, inverted inner membrane vesicles were subjected to proteolysis, and integral-membrane fragments of SecA were identified with region-specific antibodies. The membrane association properties of various truncated SecA species produced *in vivo* were also determined. Our analysis indicates that the membrane topology of SecA is complex with amino-terminal, central, and carboxyl-terminal regions of SecA integrated into the membrane where portions are periplasmically accessible. Furthermore, the insertion and penetration of the amino-terminal third of SecA, which includes the proposed preprotein-binding domain, is subject to modulation by ATP binding. The importance of these studies to the cycle of membrane insertion and de-insertion of SecA that promotes protein translocation and SecA's proximity to the preprotein channel are discussed.

Considerable progress has been made during the past decade in our understanding of the basic pathways of protein trafficking within cells. In microorganisms a combination of genetic and biochemical strategies have allowed identification of the components of the secretion apparatus and a detailed study of their mechanism of action (1–3). In *Escherichia coli*, the ease of genetic analysis combined with the ability to reconstitute a microorganism's protein translocation and SecA's proximity to the preprotein channel are discussed.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, Media, and Chemicals—**BL21.19 (secA13 (Am) supF (Ts) trp (Am) zch::Tn10 recA::CAT clpA::KAN) (27) is a derivative of BL21 (DE3) (28) that was used as the host strain for plasmids
containing the T7 promoter. SecA or its monocysteine derivatives were produced from pT7secA2 or its derivatives (29), and SecA75 and SecA30 were produced from pT7secA75 (16) and pT7secA30, respectively. pT7secA30 was constructed from pT7secA2 by introducing an ochre mutation at codon 236 of SecA utilizing primer 5'-ATTGAGCGTTTAT-CATATTTCCG-3' and PCR mutagenesis. Cj236 (F'cat rela1 ung dut-1 spoT1 thi1 mecA) and DH5α (F'endA1 hasdR17 supE44 thi-1 recA1 gyrA relA1 ΔlacZYA-argF)U169 deor (Δ80tdac1/ΔacZ1M15) were used for preparation of single-stranded DNA for oligonucleotide-directed mutagenesis and subsequent transformation, respectively, as described previously (27). The growth media employed in these studies have been described previously (30). The concentration of ampicillin used in media was 100 μg ml⁻¹. 4-Acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS),³ 3-N-maleimido-propionyl) biotin (MBP), and (tris-2-carboxyethyl)phosphine (TCEP) were purchased from Molecular Probes Inc., and N-hydroxysuccinimide 6-biotinylamidohexanoate (NHS-X-biotin) was purchased from Pierce.

*SecA Membrane Topology*

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**TABLE I**

**Oligonucleotides utilized for cysteine-scanning mutagenesis**

| Mutation | Oligonucleotide |
|----------|-----------------|
| Ser-896  | 5'-GGC GGC ATG GGA CTG CTT GTA TT-3' |
| Ser-885/887 | 5'-ACC AGC ACC GGA CGG AGA AGC ATC GTT AGC-3' |
| Ser-98   | 5'-TTC GGC GAT GGA TTC GTC GTT AGG 3' |
| Cys-858  | 5'-CAT CTC GAT GGA AAA GCT GCT G 3' |
| Cys-827  | 5'-ACC TGA ACT TCT CAC GAC GTA CT 3' |
| Cys-600  | 5'-GGG TAC TCG GTC GCA AGC AAA AAT AGC 3' |
| Cys-530  | 5'-ATT TGC TCT GCG GAC GAT CCC AGC TG 3' |
| Cys-518  | 5'-GCC TGC CAG CAA CCA CCG AGC 3' |
| Cys-470  | 5'-ATT TGC TCT GCG GAC GAT CCC AGC TG 3' |
| Cys-402  | 5'-GCA GAC CAT CAG ACC AGC GAG CTC 3' |
| Cys-350  | 5'-TTG TGG CCG GAG AGC ACC GC 3' |
| Cys-264  | 5'-TTT CTT CCA CCG AGA GGTGCC CTG 3' |
| Cys-47   | 5'-AAA CTC TGG CCA GTA CCC CTT CAG 3' |

³ The abbreviations used are: AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; DTT, dithiothreitol; ECL, enhanced chemiluminescence; FrD-A, fumarate reductase A subunit; IMV, inverted inner membrane vesicles; MBP, maltose-binding protein; MBP, 3-N-maleimido-propionyl) biotin; NHS-X-biotin, N-hydroxysuccinimide 6-biotinylamidohexanoate; PAGE, polyacrylamide gel electrophoresis; RSO, right-side out membrane vesicles; TCEP, (tris-2-carboxyethyl)phosphine.

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30 min at 0 °C. RSO were collected by sedimentation and resuspended in 200 μl of 50 mM Tris-HCl, pH 7.5, 5 mM EDTA. MBP dissolved in dimethyl sulfoxide (50 mg ml⁻¹) was added to RSO to a final concentration of 0.05 mM, followed by incubation at 0 °C for 3 min. The reaction was quenched by addition of 1 ml of TDT to 100 mM or by addition of 1 mM N-ethylmaleimide to 10 mM and incubation at 0 °C for 10 min. RSO were collected by sedimentation and resuspended in 200 μl of lysis buffer (10 mM Tris-HCl, pH 8.0, 1% SDS, 1 mM EDTA), followed by immunoprecipitation with either 2 μl of anti-SecA antiserum or 2 μl of anti-FrD-A antiserum as described previously (36). Samples were analyzed by SDS-PAGE and immunoblotting utilizing previously described methods (7). Visualization of biotinylated proteins employed streptavidin-conjugated horseradish peroxidase and ECL, and visualization of other proteins utilized rabbit anti-rabbit secondary antibody and ECL as described by the manufacturer (Amersham Corp).

**Production of Region-specific SecA Antiserum and Affinity Purification**—The construction of strains over-producing various MBP-SecA fusions and the purification of the protein chimeras has been described previously (16). MBP-SecA1, MBP-SecA2, MBP-SecA3, MBP-SecA4, MBP-SecA5, and MBP-SecA6 containing SecA amino acid residues 1–209, 211–350, 351–509, 519–664, 665–820, and 822–901, respectively, fused to MBP were used to make region-specific SecA antisera. For this purpose 1 mg of each protein in complete Freund’s adjuvant was injected intradermally and intermuscularly into New Zealand White rabbits, who were boosted at 2-week intervals with 1 mg of each protein in incomplete Freund’s adjuvant until high titer antisera were obtained. Region-specific SecA antibody was isolated by affinity purification utilizing chemically cross-linked SecA protein as described previously (34).

**Purification of SecA Protein, Biotinylation, and ATPase Assays**—SecA proteins were purified from S300 extracts of over-producing strains by affinity chromatography on Cibacron Blue-agarose as described previously (27). SecA and its cysteine derivatives (5 μg) in 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA were reduced by the addition of 5 mM TCEP followed by incubation on ice for 2 h. MBP dissolved in dimethyl sulfoxide (50 mg ml⁻¹) was added to the proteins to a final concentration of 0.05 mM, followed by incubation for 5 min. The reaction was quenched by the addition of 1 mM N-ethylmaleimide to 10 mM, and samples were analyzed by SDS-PAGE and immunoblotting followed by ECL as described above. Assays for SecA-dependent endogenous, membrane, and translocation ATPase activities utilizing the colorimetric reagent malachite green were performed as described previously (27).

**RESULTS**

**Construction and Characterization of Monocysteine Derivatives of SecA**—SecA, a 901-amino acid residue protein, has Cys residues located at position 98, 885, 887, and 896. A strain producing a form of SecA protein lacking any Cys residues, BL21-19 (pT7secA4), was constructed (see “Experimental Procedures”) and tested for SecA functions. It was found that this strain could grow on LB ampicillin plates at 42 °C and gave rise to colonies of normal size, indicating that secA4 would allow complementation of the unsuppressed secA13(Am) allele under conditions of modest over-expression (approximately 5-fold). Cell fractionation experiments utilizing sodium carbonate revealed that the distribution of SecA4 between cytosolic,
FIG. 1. MPB labeling of RSO containing monocysteine derivatives of SecA. A, RSO (200 μg of membrane protein) prepared from strains producing the indicated monocysteine variant of SecA protein, wild-type SecA protein (WT), or SecAC4 were subjected to MPB labeling as described under “Experimental Procedures.” After solubilization of membrane protein, SecA protein was immunoprecipitated, resolved by SDS-PAGE, transferred to nitrocellulose, and MPB-labeled protein was detected by ECL using streptavidin-conjugated horseradish peroxidase. SecA indicates purified SecA protein treated with NHS-X-biotin. The MPB Accessibility Studies of RSO—

peripheral, and integral membrane fractions was similar to that found for wild-type SecA ([20] data not shown). Similar to SecAC3 containing Ser substitutions at positions 885, 887, and 896 of SecA, SecAC4 showed normal SecA-dependent endogenous and membrane ATPase activities and approximately 50% higher translocation ATPase activity (31). Having satisfied ourselves that SecAC4 was biologically active and similar to wild-type SecA protein in its subcellular distribution, we constructed a series of strains that each produced a monocysteine derivative of SecA protein (see “Experimental Procedures”). The positions of the Cys residues were chosen with regard to maintaining conservative amino acid substitutions as well as achieving an even distribution of Cys residues across the entire SecA protein. We tested the biological function of each secA allele by complementation and the subcellular localization behavior of each SecA variant by cell fractionation studies. All monocysteine SecA derivatives used in this study showed normal biological function as assessed by these two criteria (results not shown).

MPB Accessibility Studies of RSO—Previous studies have shown that integral membrane SecA protein is periplasmically accessible to trypsin or labeling by NHS-X-biotin and that at least one of the carboxyl-terminal Cys residues can be labeled with MPB ([20], [26]). In an effort to develop a map of the topology of integral-membrane SecA protein we prepared RSO from strains under conditions where only a monocysteine variant of SecA was produced in moderate amounts (i.e. growth at 41 °C without isopropyl-β-D-thiogalactopyranoside induction), and we probed the accessibility of a given Cys residue of SecA to labeling by MPB. Previous studies have shown that at low concentrations MPB is membrane-impermeable so that it can be used to map the external portions of membrane proteins ([37], [38]) also shown below). The result of these studies is shown in Fig. 1. Whereas wild-type SecA was strongly labeled by this procedure, SecAC4 was not labeled (Fig. 1A), demonstrating both the specificity of MPB for sulfhydryl labeling as well as the depletion of chromosomally derived SecA protein under our experimental conditions. Western analysis indicated that the SecA content of the different RSO preparations did not differ substantially (Fig. 1C), indicating that the pattern of MPB labeling could be viewed directly as a specific activity measurement. SecAC858 and SecAC896 were labeled by this procedure indicating that at least the carboxyl-terminal 45 amino acid residues of SecA are periplasmically accessible. In addition, we found strong labeling of SecAC530, moderate labeling of SecAC300 and SecAC350, and weak labeling of SecAC47. All of the other monocysteine derivatives were not labeled in RSO by this procedure. It’s possible that some of the Cys residues that were not labeled may be buried within the interior of SecA or some other Sec protein rather than being shielded from labeling by the membrane. To investigate this point further, MPB labeling was carried out in the presence of 0.1% Triton, where MPB can cross the membrane (see below for verification of this point). All of the SecA proteins that were previously labeled (SecAC896, SecAC858, SecAC530, SecAC350, and SecAC300) were labeled more strongly by this condition, and in addition, SecAC470 was also labeled (Fig. 1B). All of the other monocysteine variants of SecA showed very little labeling under this condition, suggesting that their Cys residues are located within the interior of SecA or some other Sec protein. These results suggest that the topology of integral membrane SecA protein is complex and that at least three different regions of SecA span the membrane or are located within a channel that is periplasmically accessible to molecules the size of MPB.

To demonstrate the quality and specificity of our results, we performed several control experiments that are shown in Fig. 2. We first monitored MPB labeling of FrD-A as a control for the integrity and homogeneity of our RSO preparations. FrD-A is a 602 amino acid residue protein containing 10 Cys residues located throughout the protein that resides on the inner face of the inner membrane (39), and thus it should not be labeled by our procedure. In accordance with this prediction none of the RSO preparations examined (Fig. 2A, SecAC300, SecAC530, SecAC858, SecAC896, and wild-type) showed MPB labeling of FrD-A unless the integrity of the membrane was compromised.

FIG. 2. Specificity of MPB-labeling conditions. A, RSO were subjected to MPB labeling and analysis as described in Fig. 1 legend except that FrD-A protein was immunoprecipitated instead of SecA protein. Biot. FrD-A (lane 1), purified FrD-A labeled with NHS-X-biotin; 0.1% Triton (lane 7), identical to wild type (WT) (lane 6) except that RSO were pretreated with 0.1% Triton X-100 prior to labeling with MPB. B, RSO were subjected to MPB labeling and analysis as described in Fig. 1 legend. C, similar to B except that RSO were first incubated with 0.05 mM AMS for 30 min at 0 °C, when DTT was added to 100 mM for 10 min at 0 °C, RSO were sedimented 12,000 × g for 10 min; washed in 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM DTT, resuspended in 200 μl of 50 mM Tris-HCl, pH 7.5, 5 mM EDTA and labeled with MPB.
by addition of 0.1% Triton X-100. This indicates that the observed MPB labeling of SecA in these cases was not due to contamination with IMV or to the loss of integrity of the inner membrane of RSO. Next, to ensure that the different pattern of MPB labeling of SecA was not due to different rates or extents of MPB penetration in the various RSO preparations, we first blocked surface-exposed Cys residues in RSO with the highly water-soluble, membrane-impermeant maleimide, AMS (38), followed by labeling with MPB. The results demonstrate that MPB labeling of SecA can be blocked by prior treatment with AMS (Fig. 2, compare B with C), strongly suggesting that such labeling does not occur within the membrane bilayer.

**MPB Accessibility Studies of Purified SecA Protein**—To study the exposure of Cys residues on soluble SecA protein, monocysteine derivatives of SecA were purified, reduced with the non-thiol reagent TCEP, and subjected to MPB labeling under different conditions. The results are shown in Fig. 3. SecAC530, SecAC858, and SecAC896 as well as wild-type SecA were labeled at both 0 and 37 °C under these conditions (Fig. 3, A and B). The inability to label SecAC4 here demonstrates the purity of our SecA mutant protein preparations as well as the specificity of labeling. Interestingly, when labeling was done at 37 °C in the presence of ATP, SecAC300 was moderately labeled and SecAC350 was weakly labeled (Fig. 3C). These results suggest that temperature and ATP binding may promote a conformational change in SecA that allows exposure of this particular region of SecA and that this alteration may promote SecA membrane insertion and periplasmic exposure of this region of SecA in RSO. Unfolding reactions of SecA in response to nucleotide and membrane binding have been described previously (19, 41).

**Membrane Association of Tryptic Fragments of SecA Protein**—To locate regions of SecA protein that are peripherally or integrally associated with the membrane, we conducted limited trypsinolysis of SecA in IMV and determined the resistance of any tryptic fragments of SecA to extraction by urea. These results are shown in Fig. 4. Under conditions of mild trypsinolysis, 95-, 80-, 64-, 47-, 41-, and 38-kDa fragments of SecA were obtained as well as lower levels of 30- and 28-kDa SecA fragments (Fig. 4A; also see Fig. 5). A number of these proteolytic fragments of SecA in IMV have been noted previously (24, 41, 42). Trypsin did not penetrate into IMV under these conditions, since TolA, an inner membrane protein with a large periplasmic domain (43), was resistant to even the highest concentrations of trypsin utilized here (Fig. 4B). By contrast, TolA in RSO was readily degraded at even the lowest concentration of trypsin employed here (results not shown), similar to results with EDTA-permeabilized whole cells (43). This result further verifies the correct topology and homogeneity of our membrane preparations. Following digestion with trypsin, IMV were extracted with urea and isolated to distinguish peripheral versus integral portions of the SecA substructure. The 95-, 80-, 64-, 41-, and 38-kDa tryptic fragments of SecA were found to be urea-resistant (Fig. 3A), indicating that they were associated with the membrane by at least one membrane-anchoring determinant. By contrast, the 47-kDa fragment of SecA was found to be urea-sensitive (Fig. 4A), indicating that it is only peripherally associated with the inner membrane. In addition, the 47-kDa fragment of SecA was found to be the most resistant to digestion by trypsin (data not shown), suggesting that it consists of a tightly folded domain(s) that lacks any highly exposed Lys or Arg residues.

To identify the origin of the various tryptic fragments on the primary structure of SecA, region-specific antibodies to SecA were made using six different MBP-SecA chimeras, and the antisera were affinity purified using SecA protein (see “Experimental Procedures”). To characterize the affinity and specificity of these antibodies, a Western dot-blot was performed. Antibody to each specific region of SecA reacted strongly only to the appropriate MBP-SecA chimera (result not shown), demonstrating the utility of these antibodies for mapping purposes. The antibodies were used to map the various tryptic fragments of SecA, and the result is shown in Fig. 5. All of the urea-resistant fragments of SecA (95, 80, 64, 41, and 38 kDa) represent an overlapping series of fragments which all share a common amino-terminal portion of SecA, since they reacted to anti-SecA1–210 and anti-SecA211–350 antibodies along with the appropriate subset of carboxy-distal antibodies (Fig. 5, A
and B). This indicates that at least one region within the first 350 amino acid residues of SecA contains a membrane-integrated region that resists urea treatment. This result is entirely consistent with the MPB labeling studies shown above, where SecAC300 and SecAC350 were labeled in RSO. Further analysis showed that the urea-sensitive 47-kDa tryptic fragment was derived from a region somewhere between amino acid residues 350 and 820, based on its immuno-reaction profile. Given the fact that our IMV preparations do possess some peripherally bound SecA protein, we cannot conclude that the 47-kDa SecA fragment is derived from integral membrane SecA. Finally, the absence of the carboxyl-terminal portion of SecA in any of these larger tryptic fragments is consistent with a trypsin-sensitive site distal in SecA protein but which is prior to amino acid residue 858. A similar analysis of the proteolysis pattern of soluble SecA revealed that most tryptic fragments were of similar size and location to their corresponding SecA fragments in IMV in any of these larger tryptic fragments is consistent with a trypsin-sensitive site distal in SecA protein but which is prior to amino acid residue 858. A similar analysis of the proteolysis pattern of soluble SecA revealed that most tryptic fragments were of similar size and location to their corresponding SecA fragments in IMV, with the exception that the 80-kDa fragment was not detected, and the 47-kDa fragment was derived from the amino-terminal portion of SecA (data not shown). These data suggest that there is considerable conservation of protein structure between the soluble and integral membrane forms of SecA protein.

Fractionation Behavior and Topology of Recombinant SecA Fragments—The foregoing studies strongly suggested that the amino-terminal portion of SecA represents an important determinant for integration of SecA protein into the inner membrane. In an effort to confirm this result and determine whether this portion of SecA can integrate into the membrane independently of the remainder of the molecule, we used several strains that produce truncated forms of SecA protein and determined the subcellular fractionation properties of each SecA fragment. BL21.19 (pT7secA95) produces a 95-kDa portion of SecA lacking the last 66 amino acid residues, SecA95, which can complement conditional lethal secA mutants when over-produced severalfold (31). Purified SecA95 protein displays normal endogenous and membrane ATPase activities, but it possesses approximately 50% higher translocation ATPase activity (31). BL21.19 (pT7secA75) produces a non-complementing 75-kDa portion of SecA (amino acid residues 1–665), SecA75, which includes both ATP-binding domains (27). Purified SecA75 protein has very high endogenous ATPase activity, half the normal level of membrane ATPase activity, and no translocation ATPase activity (data not shown). BL21.19 (pT7secA30) produces a 30-kDa portion of SecA (amino acid residues 1–235), SecA30, which complements a secA51(Ts) mutant by mixed oligomerization (7, 44). SecA30 contains only the high affinity ATP-binding domain of SecA (27), and this protein has not been purified or characterized yet. Subcellular fractionation studies were performed on these three strains as well as BL21.19 (pT7secA2) producing wild-type SecA protein after depletion of chromosomally encoded SecA protein, and the result is shown in Fig. 6. Consistent with our previous results, wild-type SecA protein was found distributed equally between cytosolic and membrane fractions, and membrane-bound SecA was distributed between peripheral and integral forms as defined by resistance to extraction with sodium carbonate (7, 20). The distribution of the cytosolic protein, 6-phosphogluconate dehydrogenase, and the integral membrane protein, OmpA, confirmed the quality of our frac-
tation procedure. The fractionation patterns of SecA95 and SecA75 were grossly similar to wild type, whereas SecA30 showed an increase in the integral membrane form, very little peripheral membrane form, and a reduction in soluble SecA. These results are consistent with the data presented above, and they suggest that a membrane integration determinant lies within the first 235 amino acid residues of SecA protein, although artifacts due to misfolding of these truncated protein cannot be ruled out here. The increased level of integral membrane SecA30 may be due to truncation of the high affinity ATP-binding domain of SecA, since mutations in this domain led to defects in SecA membrane cycling and increased levels of integral membrane SecA protein (21, 22). Furthermore, these results suggest that SecA95 and SecA75 may be able to cycle between membrane inserted and de-inserted states similarly to wild-type SecA protein, whereas SecA30 may be defective in this regard. To determine whether SecA75 can insert into the inner membrane with a topology similar to full-length SecA protein, Cys residues at position 350 or 530 were engineered into SecA75, and MPB labeling studies of the corresponding RSOs were performed. The results are shown in Fig. 7. SecA75C350 and SecA75C530 were labeled in RSO but with a somewhat reduced specific activity compared with SecAC350 and SecAC530 proteins (Fig. 7 compare A and B). These results indicate that SecA75 is capable of inserting into the inner membrane with a similar topology as full-length SecA protein but that the efficiency of this reaction is reduced significantly.

**FIG. 6. Fractionation behavior of recombinant SecA fragments.** BL21.19 (pT7secA2), BL21.19 (pT7secA95), BL21.19 (pT7secA75), and BL21.19 (pT7secA30) were grown in LB ampicillin at 30 °C until mid-logarithmic phase when the culture was shifted to 41 °C for 1 h. Cells were harvested, disrupted in the French pressure cell, and divided into total cell (T), cytosolic (C), peripheral membrane (P), and integral membrane (I) fractions by sedimentation, treatment of each membrane fraction with 0.2 M sodium carbonate, pH 11, followed by re-sedimentation as described previously (20). SecA or the truncated form of SecA produced by each strain is indicated at the left. The fractionation pattern of 6-phosphogluconate dehydrogenase and OmpA form of SecA produced by each strain is also given. Similar results were obtained in three different experiments.

**FIG. 7. MPB labeling of RSO containing cysteine derivatives of SecA75.** A, RSO (200 μg of membrane protein) prepared from strains producing the indicated SecA protein were subjected to MPB labeling as described under “Experimental Procedures” and analyzed as described in Fig. 1 legend. B, Western blot of RSO probed with SecA antisera.

**DISCUSSION**

While earlier studies regarded SecA as a peripheral subunit of the translocase complex that may have functioned more like an ATP-dependent chaperone when bound to SecYEG (45), later studies have shown that SecA penetrates deeply into the inner membrane and that such integration is essential for the initial penetration of the preprotein into this layer (7, 17, 20). Thus more recent views of SecA function suggest that it may be part of a proteinaceous surface that along with the SecY subunit comprises a protein-conducting channel (46). Furthermore, evidence for a mobile domain of SecA that undergoes membrane insertion and de-insertion cycles coupled to ATP hydrolysis and net movement of the translocating chain of the preprotein has been reported (23), in keeping with the co-insertion model for SecA function presented previously (47). Despite these advances in our thinking about SecA function, considerable uncertainty surrounds the issue of which portions of SecA are integrated into the membrane and what function such domains carry out during the translocation cycle. For example, one study found that a fraction of translocationally active SecA was permanently embedded within the inner membrane, where it did not appear to undergo membrane cycling (24). To shed light on such controversies and to gain insight into the topology of integral membrane SecA protein, we undertook the present study. Since SecA is not a conventional membrane protein, results of hydropathy or gene fusion analysis cannot reliably be utilized to provide useful information on SecA topology (48). This is particularly the case as portions of SecA may reside within the protein-conducting channel (46), and therefore, hydrophobicity per se may not correlate well with membrane residency. With this in mind we employed several different methods that should give useful information about SecA topology despite these complexities. Site-directed sulphydryl labeling, for example, has proven to be useful to resolve small loops at the surface of membranes that are normally resistant to proteolysis or immunodetection (49, 50).

Our results are summarized in the working model shown in Fig. 8. SecA can be divided into distinct regions based on the patterns of MPB labeling, trypsinolysis, and truncation. At least three noncontiguous regions of SecA, around amino acid residues 300/350, 530, and 858/895, appear to be periplasmically exposed, whereas the portions of the two ATP-binding domains of SecA that contribute to nucleotide binding (e.g. the Walker A and B motifs (27)) are assumed to be cytoplasmically localized. A previous study showed that the carboxy-terminal region of SecA is periplasmically accessible to MPB labeling and proteolysis (26), suggesting that it lies at the membrane exterior rather than being buried within a protein-conducting channel. Based on our data, the carboxy-terminal region of SecA must span the membrane if amino acid residues 858 and 895 are periplasmically accessible to MPB labeling in RSO, but a 95-kDa fragment of SecA can be generated by trypsinolysis of IMV. The function of the carboxy-terminal region of SecA is
currently unknown. Although it is dispensable, it does increase SecA activity significantly, and it has been suggested to bind SecB as well as anionic phospholipids (15, 31, 51).

Our data provide important new insight into the topology and potential function of the amino-terminal third of SecA protein. Its integral membrane nature is supported by our MPB labeling studies of RSO, the urea resistance of the 38-kDa tryptic SecA fragment in IMV, and by the carbonate resistance of SecA30. Of particular interest was the fact that a region around amino acid residues 300 and 350 was not accessible in soluble SecA unless ATP was included in the labeling conditions. This result suggests that ATP binding promotes a change in SecA conformation that normally allows this region to insert into the membrane to achieve a periplasmic exposure. This conclusion is entirely consistent with earlier biochemical studies that showed that ATP binding is required for SecA insertion into the inner membrane (23) and with biophysical studies indicating that ATP and membrane binding open up SecA structure somewhat to allow for membrane insertion (19, 41). It is also of particular interest that this periplasmically accessible region of SecA overlaps with the proposed preprotein-binding site of SecA at amino acid residues 267–340 (14). If this region were to bind preprotein in soluble or peripheral membrane SecA, then membrane insertion of SecA could translocate the bound preprotein segment to the periplasmic side of the membrane.

One of the surprising results of our topological studies was the labeling of SecAC530 and SecAC518 in RSO (Fig. 1, and results not shown). This region of SecA showed strong exposure in both soluble SecA and SecA in RSO. These amino acid residues are within the low affinity ATP-binding domain of SecA, and they are close to the Walker A motif at amino acid residues 503–511 that was shown previously to be important in low affinity ATP-binding activity by mutational studies (27). A phylogenetic inspection of this portion of SecA reveals that the region between amino acid residues 518 and 542 is highly variable and that certain species lack this portion of SecA, whereas others have retained it (Fig. 9). Deletion of this region of SecA protein resulted in a loss of biological function, indicating its importance in SecA activity in E. coli. Further studies are clearly needed to understand the role of this interesting and potentially important segment of SecA protein.

Our topological studies provide a basis for the structure of integral membrane SecA protein as well as for SecA function in protein translocation. It appears that ATP binding to SecA opens up a region distal to the high affinity ATP-binding domain and allows its insertion and integration into the membrane, where regions around amino acid residues 300 and 350 become periplasmically exposed. Normally, insertion of this domain is likely to be coincident with insertion of a preprotein bound to SecA at the putative preprotein-binding site on SecA (14). This feature explains the normal dependence of SecA insertion on preprotein and ATP binding, as well as the accumulation of integral membrane SecA protein in the presence of nonhydrolyzable ATP analogs or mutants defective in hydrolysis at ABC I (21–23). Together, the high and low affinity ATP-binding domains of SecA and the intervening region im-

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**Fig. 8. Working model of the topology of integral membrane SecA protein.** The position of amino acid residues that are periplasmically accessible to sulfhydryl labeling are indicated, along with the proposed preprotein-binding site (amino acids 267–340 (14)) and SecY-binding site (amino acids 665–820 (16)). ABC I or ABC II indicate the high or low affinity ATP-binding domain of SecA, respectively. The positioning of the SecYEG protein subunits is arbitrarily drawn.

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**Fig. 9. Alignment of SecA sequences corresponding to amino acid residues 518–542 of E. coli SecA.** SecA sequences from E. coli (M20791), Hemophilus influenzae (U32772, L42023), Bacillus subtilis (D10279, D90218), and Listeria monocytogenes (L32090) were aligned using the BLAST software. Thr530, corresponding to the position of Cys mutagenesis in this region, is shown enlarged. SecA from H. influenzae had an overall similarity of 92% when compared with E. coli. The position of the Walker A motif is indicated.

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2 M. Schmidt, V. Ramamurthy, and D. Oliver, unpublished results.
plicated in preprotein binding (14) appear to constitute a substructure of SecA that is capable of integrating into the inner membrane and undergoing membrane cycling, based on the normal distribution of cytosolic, peripheral, and integral membrane SecA75 and on the ability of this truncated SecA protein to integrate into the membrane in a topology similar to the corresponding region of full-length SecA protein. Our data and speculation on this point are consistent with the recent demonstration that both the amino-terminal 65-kDa and carboxy-terminal 30-kDa regions of SecA undergo membrane cycling at SecYEG during protein translocation (42). The carboxyl-terminal third of SecA protein appears to traverse the kind gift of FrD-A and TolA antisera, respectively, and Thavamani Rajapandi for construction of pT7secA75.

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