Identification of a Region of Interaction between *Escherichia coli* SecA and SecY Proteins*

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SecA ATPase promotes *Escherichia coli* protein translocation by its association with the preprotein or pre-protein-SecB complex, anionic phospholipids, and the other core component of translocase, integral membrane protein SecYEG. Using ligand affinity blotting we demonstrate a direct interaction of SecA with SecY protein. Proteolysis and gene truncation or fusion studies were used to further define this interaction. Our results demonstrate that the carboxyl-terminal third of SecA protein binds to the amino-terminal 107 amino acid residues of SecY protein. The direct demonstration of these interactions culminate studies that have inferred an interaction between SecA and SecYEG, and they are consistent with studies suggesting that this region of SecA interacts with the inner membrane.

Recent progress in defining the components of the bacterial protein secretion apparatus and studying their mechanism of action in an *in vitro* system has opened the way to a detailed analysis of this fundamental process (for reviews see Refs. 1 and 2). Such studies demonstrate that SecA plays a central role in protein export by binding to many of the components of the translocation complex. Interaction of SecA with the signal peptide and the mature portion of the preprotein, the SecB chaperone, anionic phospholipids, and integral membrane protein SecYEG has been inferred using a variety of approaches (3–8). Membrane-bound SecA inserts into and spans the inner membrane (9), an activity originally defined in lipid monolayers and bilayers composed of anionic phospholipids (10, 11). Once SecA nucleates formation of a translocation complex at the membrane periphery it appears to act as a molecular ratchet by undergoing ATP-driven cycles of membrane insertion and de-insertion, which have been proposed to promote productive translocation of bound preproteins (12–15). In addition, translocation of distal segments of preprotein can occur without bound SecA by using the proton motive force (12).

While evidence for this basic model of protein translocation has been accruing, individual steps in the overall reaction need further clarification. For example, the basis of preprotein recognition by SecA and SecB is still largely unknown as is the nature of SecA interaction with SecYEG. Although previous cross-linking of a translocating preprotein to export machinery components suggests that SecA and SecY may constitute a preprotein channel (16), consistent with previous electrophysiological studies (17), the nature of this channel and the functions of SecA and SecY proteins in channel activity remain ill defined. Furthermore, SecD and SecF proteins are essential for efficient protein secretion *in vitro* (18), but their role(s) *in vivo* has not been clearly defined yet, although certain activities have been proposed (9, 14, 19).

In this study we have investigated the nature of SecA interaction with its membrane receptor SecYEG. Previous studies suggested that SecA interacts with the SecY subunit based on (i) the ability of high concentrations of SecA to suppress the protein translocation defect found normally for heat-inactivated IMV derived from the secY24(Ts) mutant (20), (ii) the requirement of SecA-dependent translocation ATPase activity for functional SecY protein (3), (iii) the ability of SecY antibody to reduce SecA affinity for IMV by 3-fold (6), and (iv) the ability of SecA when added to IMV to afford a 2-fold protection of SecY to proteolysis (6). While compelling, none of these studies provide direct proof of SecA-SecY interaction nor do they provide any information about the potential regions of interaction of these two proteins. Further complicating this picture are recent proposals that (i) SecG facilitates the cycle of membrane insertion and de-insertion of SecA protein by itself undergoing a cycle of inversion of its membrane topology (21), and (ii) SecD and SecF proteins are required to stabilize the membrane-inserted state of SecA protein (14). To begin to clarify the different protein-protein interactions that facilitate the biochemical function of the bacterial translocon, and specifically those proteins that interact directly with SecA protein, we have used ligand affinity blotting. This method is particularly appropriate for defining interactions with integral membrane proteins where it is difficult to study the biochemical function of the protein out of the context of the membrane environment. Our results show that SecA interacts directly with SecY protein by this method, and they define specific regions of these proteins that are required for such association.

**EXPERIMENTAL PROCEDURES**

*Strains and Plasmids—*BL26 (ΔargF-lacU169) is a derivative of BL21(ΔDE3) used for protein overproduction utilizing the T7 promoter system (22). BL21.19 (secA13 (Am) supF (Ts) trp (Δm) zch::Tn10 recA::CAT clpA::KAN) (23) containing the SecA overproducing plasmid pTTsecA2 (24) has been described previously. BL21.19 containing pTTsecA95 (25) or pTTSecA75, which contains an ochre codon at amino acid residue 665 of SecA, was used for production of SecA95 or SecA75, respectively. pTTSecA75 was constructed from pTTSecA2 by oligonucleotide-directed mutagenesis employing 5′-CCACATGTTATTACGCGTG-3′ as described previously (23). W3110 M25 (pMan510, pMan809), an ompT strain containing plasmids for overproduction of SecE and SecY using the tac promoter, has been described previously (26). Strains BW313 (relA1 ungI dut1 spoT1 thi1) and XL1-Blue (endA1 hsdRI17 supE44 thi1 recA1 gyr96 relA1 ΔlacI (F′ proAB lacI9 lacZM15 Tn10) were used for preparation of single-stranded plasmid DNA for mutagenesis and subsequent transformation, respectively, as described...
previously (23). TB1, araD Δlac-proAB) rpsL ΔlacZ M15 hsdR (New England Biolabs), was used as a host for the plasmids overproducing MBP-SecA chimeras.

Cell Growth, SecA Purification and Biotinylation, and Membrane Preparation—Plasmid-bearing strains were grown in LB medium (27) with 100 µg ml⁻¹ ampicillin and/or 30 µg ml⁻¹ gentamycin, as appropriate. For SecA overproduction, the culture was grown at 30 °C to an Aₚₜ of 0.5 and shifted in a water bath to 42 °C (for SecA variants only) when 1 mM IPTG was added, and the culture was grown for an additional 2 h. SecA was purified using blue dextran-agarose (Sigma) as described previously (23). SecE protein was biotinylated using Immobilized photocleavable biotin (Pierce) as described by the manufacturer. For SecYE overproduction the culture was grown at 37 °C to an Aₚₜ of 0.6–0.7 and followed by induction with 1 mM IPTG for 2 h. Cells were harvested by sedimentation at 5000 × g, washed in 10 mM Tris, pH 7.5, 7.5–7.0 KCl, 10 mM magnesium acetate, 1 mM dithiothreitol (buffer TKMD), and then resuspended in 1 ml of buffer TKMD per g of cell paste containing 10 µg ml⁻¹ DNase I (Sigma). Cells were disrupted in a French pressure cell at 70 °C until further use. To prepare detergent-solubilized membranes, membranes were solubilized in 1.25% (w/v) n-octyl-β-D-glycopyranoside (Sigma), 2 mg ml⁻¹ Escherichia coli phospholipid (Avanti Polar Lipids), 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10% (w/v) glycerol at 0 °C for 30 min, and the supernatant was used after sedimentation at 300,000 × g for 3 h.

Ligand Affinity Blotting, Antibody Production and Purification, and Western Blotting—Detergent-solubilized membranes were separated on 15% SDS-PAGE gels using the discontinuous system of Laemmli (28). Proteins were transferred to nitrocellulose (Schleicher & Schuell) as described previously (29). Filters were blocked in 5% dried milk in TBS (20 mM Tris-HCl, pH 7.5; 140 mM NaCl, 0.25% Tween 20) at 4 °C for up to 24 h. Filters were washed with TBS and incubated with affinity-purified anti-SecA antibody at a 1:10,000 dilution at 4 °C for 2–4 h. Filters were washed with TBS and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody at a 1:10,000 dilution at 4 °C for 2 h. Filters were washed with TBS and detection of bound antibody was done using ECL (Amersham Corp.) as described by the manufacturer. Biotinylated SecA or biotinylated molecular weight markers (Bio-Rad) were detected using a streptavidin-horseradish peroxidase conjugate (Amersham Corp.) at a 1:10,000 dilution. Detection of the primary and secondary antibody was performed using horseradish peroxidase-conjugated anti-rabbit secondary antibody and a chemiluminescent substrate of horseradish peroxidase followed by autoradiography. The results are shown in Fig. 1. Membranes were resuspended in 50 mM Tris acetate, pH 7.5, 1 mM dithiothreitol (buffer TKMD), and then resuspended in 1 ml of buffer TKMD per g of cell pellet (P300). Membranes were resuspended in 50 mM Tris acetate, pH 7.5, 10 mM NaCl, 10% (w/v) glycerol, 8.5% sucrose, aliquoted, frozen in a dry ice/ethanol bath, and stored at −70 °C until further use. To prepare detergent-solubilized membranes, membranes were solubilized in 1.25% (w/v) n-octyl-β-D-glycopyranoside (Sigma), 2 mg ml⁻¹ Escherichia coli phospholipid (Avanti Polar Lipids), 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10% (w/v) glycerol at 0 °C for 30 min, and the supernatant was used after sedimentation at 300,000 × g for 3 h.

Detection of SecA-SecY Interaction—To define the interaction of SecA with SecYE we developed a ligand affinity blotting assay. Membranes were prepared from a strain, W3110 M25 (pMan510, pMan809), in which SecYE was conditionally overproduced by induction with IPTG (SecYE+ membranes) (26). While this strain overproduces SecY protein by approximately 40-fold and SecE by 300-fold, SecY still remains a minor component of total membrane protein (Ref. 26 and results not shown). SecYE+ membranes were solubilized in detergent, separated by SDS-PAGE, transferred to nitrocellulose, and probed with purified SecA protein. Bound SecA protein was detected using affinity-purified rabbit anti-SecA antibody, horseradish peroxidase-conjugated anti-rabbit antibody, and a chemiluminescent substrate of horseradish peroxidase followed by autoradiography. The results are shown in Fig. 1. SecA bound to a protein with an apparent molecular mass of 35 kDa (Fig. 1C), which was SecY protein based on the following criteria: (i) it reacted with anti-peptide antiserum directed against either the carboxyl or amino terminus of SecY (Fig. 1A and B, respectively); the latter antiserum also detected a species at approximately 55 kDa that may be a dimer of SecY (33); (ii) it was overproduced in an IPTG-dependent fashion (compare lanes 1 and 2 of the different panels); and (iii) it was not present when SecYE− membranes were boiled in the presence of SDS (lane 3), conditions that induce irreversible aggregation of SecY (34). SecY binding was not detected when SecA protein was omitted during a mock ligand blot, but instead only SecA protein (102 kDa) and a presumed proteolytic fragment of SecA of 48 kDa were detected by the anti-SecA antibody and secondary antibody employed during development of the ligand blot (panel E). As noted previously the level of membrane-bound SecA protein increased during SecYE overproduction (35). It was noted that detection of the endogenous Oligonucleotides 1–12

Polymerase chain reaction products were purified on agarose gels, digested with the appropriate restriction enzymes, and cloned into compatible restriction enzyme sites in pMal-C2 using the manufacturer’s protocol. malE-secA fusions were verified by sequence analysis, and the MBP-SecA chimeras were overproduced from TB1 containing the appropriate plasmid and purified according to the manufacturer’s instructions.

RESULTS

Detection of SecA-SecY Interaction—To define the interaction of SecA with SecYE we developed a ligand affinity blotting assay.
SecA protein during ligand blotting was somewhat variable depending on the level of exposure of the blot (Figs. 1–3).

To provide additional proof that we were specifically detecting SecA interaction with SecY protein using this assay we altered our detection method. In this case we used biotinylated SecA as the probe and streptavidin-conjugated horseradish peroxidase and a chemiluminescent substrate of horseradish peroxidase for detection. SecY protein was detected similarly by Western blotting, or with purified SecA (Fig. 1, panels A and B). Neither method appeared to interfere with SecA binding to IMV as well as SecA-dependent ATPase activity (3, 6, 37).

SecA-binding site on SecY we made use of the natural pattern of proteolysis of SecY protein that occurs during detergent solubilization of SecYE−− membranes (32, 33). Comparison of Western and ligand blots of these membrane samples showed that the pattern of proteolytic fragments of SecY detected was essentially identical when anti-peptide antibody to the amino terminus of SecY was used for Western blotting (Fig. 2, compare lanes 1 and 2 in panels A and B). Furthermore, an 27-kDa carboxyl-terminal fragment of SecY generated by trypsinolysis of these samples was not detected by ligand blotting (results not shown). These results suggested that the SecA-binding determinant resided in the early amino-terminal portion of SecY protein. To verify this presumption, we created a chimera in which the amino-terminal 107 amino acid residues of SecY were fused to LacZ (SecY107-LacZ), and we determined whether it contained SecA binding activity by ligand blotting. According to the predictions of Akiyama and Ito (36) this chimera should contain the first two transmembrane segments of SecY, TM1 and TM2, along with the first cytoplasmic and periplasmic domains, C1 and P1, respectively, fused to cytoscilically-disposed β-galactosidase. The overproduced 128-kDa chimera could be detected readily by Western blotting employing either anti-peptide antibody to the amino terminus of SecY or anti-β-galactosidase antisera (Fig. 2A, lane 3, and results not shown; note that wild-type levels of SecY protein in these samples are at or below our standard level of detection here). In addition, like SecY protein the chimera aggregated when membranes were solubilized by boiling in SDS (Fig. 2A, compare lanes 3 and 4). A ligand blot of the SecY107-LacZ chimera showed that it possessed strong SecA binding activity (Fig. 2, compare lanes 3 and 4 of panels A and B). These results indicate that there is a SecA-binding determinant located in the extreme amino-terminal portion of SecY protein. Our results are entirely consistent with previous reports that an anti-peptide antibody directed against the amino terminus of SecY interfered with SecA binding to IMV as well as SecA-dependent preprotein binding to IMV, and it blocked also translocation ATPase activity (3, 6, 37).

Location of the SecY-binding Site on SecA—To locate the SecY-binding site on SecA we utilized two truncated SecA

![Fig. 1. Detection of SecA-SecY interaction by ligand blotting.](image)

![Fig. 2. Location of the SecA-binding determinant on SecY.](image)
proteins, SecA95 and SecA75, that could be readily purified in soluble form and characterized. SecA95 lacks the carboxy-terminal 66 amino acid residues of SecA, and it shows reduced protein translocation activity but it can still complement conditional lethal secA mutants when overproduced severalfold (25). SecA75 contains the amino-terminal 665 amino acid residues of SecA protein including both ATP-binding domains (23). It is inactive in catalyzing both in vivo and in vitro protein translocation, but it displays an elevated endogenous ATPase activity, a reduced membrane-stimulated ATPase activity, and no translocation ATPase activity (2). The truncated SecA proteins were purified (Fig. 3A, lanes 2 and 3) and used for ligand blotting as shown in Fig. 3B. SecA95 displayed reduced SecY binding activity compared with wild-type SecA, while the activity of SecA75 was essentially undetectable (compare lanes 2–4). The faint SecA band in each ligand blot indicated that similar sensitivities were being detected by this procedure in each case. This result suggests that the carboxy-terminal third of SecA protein is important for SecY recognition. In an attempt to confirm and extend these results we constructed a series of six chimeras in which portions of SecA were fused to the carboxy-terminal 66 amino acid residues of MBP. These chimeras were designated MBP-SecA through MBP-SecA6, and they contained the following portions of SecA: amino acid residues 1–209, 211–350, 351–509, 519–664, 665–820, and 822–901, respectively. The chimeras were purified (Fig. 3A, lanes 4–9) and used for ligand blotting as shown in Fig. 3C. MBP-SecA5 showed strong SecY binding activity similar to wild-type SecA, while the other MBP-SecA chimeras showed little or no SecY binding activity (lane 5 versus lanes 1–4 and lane 6). MBP alone displayed no SecY-binding activity in this assay (results not shown). These results support our finding that the carboxy-terminal third of SecA protein is important for SecY recognition. They further suggest that a region between amino acid residues 665 and 835 of SecA contains an important SecY-binding determinant, although conformational effects due to truncation of these proteins cannot be ruled out as a cause of certain of the negative results obtained here. Our result is consistent with recent studies that show that the carboxy-terminal third of SecA protein inserts into the membrane during protein translocation and is protected against proteolysis (13, 38), as well as our recent finding that a region somewhere between amino acid residues 665 and 858 of integral membrane SecA traverses the membrane.2

DISCUSSION

As more of the details regarding the mechanism of protein translocation are revealed, there will be an increasing need to define subreactions promoted by individual protein-protein interactions among the different components of the secretion machinery. Genetic tools such as suppressor analysis or synthetic lethality (39) and biochemical tools such as cross-linking (16) or ligand affinity blotting can provide valuable information in this context. Ligand affinity blotting has been used extensively in the past to characterize membrane receptors for diverse ligands such as hormones, interleukins, or lipoproteins (e.g. see Refs. 40–42). Previous studies suggested that SecA interacts with the SecY subunit of SecYEG, but these studies were indirect in nature or were limited by interpretation since the large antibody molecules used could directly affect neighboring interactions (3, 6, 20). Our studies using ligand blotting provide a straightforward approach to this problem, and they have allowed assignment of SecA-binding activity to SecY protein. In particular, the amino-terminal 107 amino acid residues of SecY protein were shown to interact with the carboxy-terminal third of SecA protein. One major limitation of this approach, however, is that detergent solubilization of membranes prevents us from knowing whether this interaction takes place normally within the interior of the membrane or at its periphery, or both, nor have we gained insight into the physiological function of this interaction. Additional fine structure mapping of the binding determinants on SecA and SecY proteins along with appropriate mutant analysis and comparison to the known or emerging topology of these proteins in the membrane should allow clarification of these issues.

Of the ten transmembrane segments (TM1–TM10), six cytoplasmic domains (C1–C6, including both termini), and five periplasmic domains (P1–P5) contained in the proposed topology of SecY (36), specific functions have been suggested for several regions of SecY. These include a potential region of "catalytic function" (C5, TM9, and C6) (43, 44), a region that recognizes signal sequences and performs a signal sequence proofreading function (TM7) (45), and regions that interact with SecE protein (C4, TM10, and P1) (39, 46). Previous studies have proposed at least four functional regions of SecA protein:...
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(i and ii) high and low affinity ATP-binding domains at amino acid residues 100–230 and 500–665, respectively (23, 26); (iii) a preprotein-binding site at amino acid residues 267–340 (7); and (iv) lipid- and SecB-binding site(s) residing in the carboxy-terminal 70 amino acid residues of SecA (8). Recently, it has been shown that the carboxy-terminal third of SecA protein is at least one of the portions that is integrated into the membrane during protein translocation, whereby its carboxyl terminus is periplasmically exposed (35, 38). These findings are consistent with our recent studies of the topology of integral membrane SecA protein in right side out membrane vesicles that show that SecA traverses the membrane somewhere between amino acid residues 665 and 858.2 Remarkably this region corresponds to the SecY-binding site that has been mapped in this study. Therefore, it appears quite possible that the SecY-binding determinant in SecA may interact with portions of SecY that are within the interior of the membrane. Additional studies will be required to explore this point further and to define the function(s) of the integral membrane portions of SecA protein. By doing so the parallel between our work and SecA-SecY interaction within native inner membranes should be established.

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