Complexes between Protein Export Chaperone SecB and SecA

EVIDENCE FOR SEPARATE SITES ON SecA PROVIDING BINDING ENERGY AND REGULATORY INTERACTIONS*

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During localization to the periplasmic space or to the outer membrane of Escherichia coli some proteins are dependent on binding to the cytosolic chaperone SecB, which in turn is targeted to the membrane by specific interaction with SecA, a peripheral component of the translocase. Five variant forms of SecB, previously demonstrated to be defective in mediating export in vivo (Gannon, P. M., and Kumamoto, C. A. (1993) J. Biol. Chem. 268, 1590–1595; Kimsey, H. K., Dagarag, M. D., and Kumamoto, C. A. (1995) J. Biol. Chem. 270, 22831–22835) were investigated with respect to their ability to bind SecA both in solution and at the membrane translocase. We present evidence that at least two regions of SecA are involved in the formation of active complexes with SecB. The variant forms of SecB were all capable of interacting with SecA in solution to form complexes with stability similar to that of complexes between SecA and wild-type SecB. However, the variant forms were defective in interaction with a separate region of SecA, which was shown to trigger a change that was correlated to activation of the complex. The region of SecA involved in activation of the complexes was defined as the extreme carboxyl-terminal 21 aminoacyl residues.

Export of protein from the cytosol of Escherichia coli to the periplasm and to the outer membrane is mediated by a membrane-bound translocase, which comprises a peripheral ATPase, SecA, and two integral membrane heterotrimeric protein complexes: SecYEG and a complex of SecD, SecF, and YajC. Some of the precursor proteins translocated by this apparatus are targeted to the membrane by specific interaction with SecA, a peripheral component of the translocase. Five variant forms of SecB, previously demonstrated to be defective in mediating export in vivo (Gannon, P. M., and Kumamoto, C. A. (1993) J. Biol. Chem. 268, 1590–1595; Kimsey, H. K., Dagarag, M. D., and Kumamoto, C. A. (1995) J. Biol. Chem. 270, 22831–22835) were investigated with respect to their ability to bind SecA both in solution and at the membrane translocase. We present evidence that at least two regions of SecA are involved in the formation of active complexes with SecB. The variant forms of SecB were all capable of interacting with SecA in solution to form complexes with stability similar to that of complexes between SecA and wild-type SecB. However, the variant forms were defective in interaction with a separate region of SecA, which was shown to trigger a change that was correlated to activation of the complex. The region of SecA involved in activation of the complexes was defined as the extreme carboxyl-terminal 21 aminoacyl residues.

forms of SecB that are altered in oligomeric structure and in interaction with precursor proteins (4) or in the interaction with SecA (5). Here we have analyzed variants that are defective in binding SecA. We have demonstrated that wild-type SecB forms two types of complexes with wild-type SecA in solution and binds the membrane translocase in a functional manner. Each of the SecB variants was capable of making only one of these complexes, and that complex did not bind to membrane. A truncated form of SecA, missing the 21 carboxy-terminal residues and identified previously as the sole binding site for SecB (6), was shown to retain the ability to bind both wild-type and variant forms of SecB, but all the complexes formed were nonfunctional. Thus there are at least two separate regions of SecA that are involved in the formation of active complexes with SecB. The interactions at sites as yet unidentified but common to the wild-type and variant forms of SecB provide sufficient energy to stabilize the complexes. The interaction between SecB and the extreme carboxyl-terminal region of SecA, which is defective for the variants of SecB, plays a regulatory role in conversion of complexes to an active state.

Experimental Procedures

Bacterial Strains and Plasmids—E. coli strain HB1215 is an OmpT− host (7) harboring plasmids that overexpress SecE (pMAN809) and SecY (pMAN510) (8) under the control of the tac promoter. Strain HB1590 was constructed by transformation of the same plasmids into HB1187 (MC4100, Δabc-C) (9). E. coli strain HB1732 is JM109 (New England BioLabs) harboring plasmid pAL81, which overexpresses SecAN880, a truncated SecA consisting of the first 880 aminoacyl residues. To construct plasmid pAL81, the SecA expression vector pMAN400 (10) was used as template in a polymerase chain reaction with the primer 5′-AGTCGCTGAAGAAATGTGG-3′ and mutagenic primer 5′-ACCTTGTAGCTTATCCATTTTGCG-3′. The mutagenic primer places a stop codon after codon 880 of the secA gene followed by a SacI restriction site. After MunI/SacI (MBI Fermentas) digestion, the polymerase chain reaction product was ligated into pMAN400 cut with the same enzymes. The secA gene was sequenced to verify the change.

Protein Purification—Mature galactose-binding protein (11), precursor galactose-binding protein (12), SecB, and the five variants of SecB (4, 13) were purified as described. SecA and SecAN880 were purified from strains RR1/paAN400 (10) and HB1732, respectively. Cells were harvested, washed, and suspended in 20 mM KOAc, potassium acetate; MgOAc, magnesium acetate; GdnHCl, guanidine hydrochloride; PGPB and mgGBP, precipitous and mature galactose-binding protein, respectively.

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SecB Binding and Regulatory Interaction Sites on SecA

nitrogen and stored at -70 °C. The cell suspension was thawed in twice its volume of 30 mM Tris-Cl, pH 7.5, 2 mM DTT, 1 mg/ml lysozyme, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), sonicated, and centrifuged at 178,000 × g for 10 min at 4 °C (Beckman). The supernatant was loaded onto a Microprep DEAE (Bio-Rad) column eluted with 50 mM Tris-Cl, pH 8.0. The eluted activity in the first column was then concentrated by centrifugal filtration using a Centricon, and then 1 column volume of the same buffer with 0.2 mM NaCl. The SecA was eluted with a gradient of 0.2 to 1.0 mM NaCl in the same buffer. Fractions containing SecA were pooled and dialyzed against 10 mM sodium phosphate, pH 6.5, 2 mM DTT at 4 °C. The dialyzed sample was applied to a cellulose phosphate P11 column (Whatman) pre-equilibrated with 10 mM sodium phosphate, pH 6.5, 2 mM DTT. The column was washed with 10 mM sodium phosphate, pH 6.5, 2 mM DTT, and the SecA was eluted with a gradient of 0.01 to 0.5 mM sodium phosphate, pH 6.5 in 2 mM DTT. Fractions containing pure SecA were pooled, concentrated, dialyzed against 10 mM HEPES (KOHi), pH 7.6, 300 mM potassium acetate (KOAc), 2 mM DTT, and stored at -70 °C.

Concentrations of purified proteins were determined spectrophotometrically at 280 nm using coefficients of extinction as follows: SecB and SecB variants, 47,600 M -1 cm -1 for the tetramer; denatured precursor and mature galactose-binding proteins, 37,410 M -1 cm -1; SecA and SecA(N880), 157,800 M -1 cm -1 for the dimer. All SecA concentrations are expressed as tetramer, and all SecA concentrations are expressed as the dimer.

Purification of Radiolabeled SecB—14C-SecB was purified from strain BL21(DE3)pGEX26 (15). 14C-SecBET77K, and 14C-SecB(L75Q) were purified from strain CK2212BL21(DE3) secB::Tn5 srl:Tr10 recA1 containing two plasmids. One plasmid contains the SecB variant gene under the natural promoter, and the second plasmid contains the SecB variant gene under control of the T7 promoter. Cells were grown in 24 ml of M9 minimal medium (14) with 0.4% glycerol and 50 µg/ml ampicillin at 30 °C. When the cell density reached an absorbance of 560 nm of 0.6, 0.1 mM IPTG was added; 90 minute later, 0.4 µCi of 14C-labeled amino acid mixture (ICN) per milliliter of culture was added. After 10 min of incorporation, the cells were harvested and washed with 10 mM Tris-Cl, pH 7.6, 30 mM NaCl, and the resulting pellet was frozen at -70 °C. The pellet was thawed and suspended in 20 mM Tris-Cl, pH 7.6, 2 mM EDTA, 0.1 mM PMSF, 100 µg/ml lysozyme and incubated on ice for 20 min. The suspended cells were sonicated and centrifuged 15 min at 16,000 × g. The supernatant was collected and centrifuged at 356,000 × g in a TL100.1 rotor (Beckman) for 20 min at 4 °C. The supernatant was loaded onto a Q-Sepharose (0.8-ml bed volume; Amersham Pharmacia Biotech) column equilibrated in 20 mM Tris-Cl, pH 7.6. The column was washed with the same buffer, and the bound proteins were eluted with 0.2, 0.3, 0.35, 0.4, 0.45, 0.5, and 0.6 M NaCl steps. The fractions containing SecB as determined by SDS-polyacrylamide gel electrophoresis were pooled and concentrated using a Centricon 30 centrifugal filter (Millipore), and the buffer was exchanged for 10 mM HEPES (KOHi) pH 7.0, 150 mM KOAc by repeated dilution and concentration in the Centricon. The concentration of SecB was determined by Coomassie Blue-staining bands on a SDS-polyacrylamide gel and comparing its intensity to the intensity of bands generated from known amounts of SecB.

Size-exclusion Chromatography—Chromatography was carried out using a TSK G3000SW (Tosohaas) size-exclusion chromatography column (7.5-mm inner diameter × 60 cm) equilibrated in 10 mM HEPES, 1 mM EDTA, 300 mM KOAc, 5 mM MgOAc, 2 mM DTT, pH 7.5. Samples of 200 µl were prepared in the same buffer, separation was carried out at 8 °C at either 0.9 or 1 ml/min, and absorbance was monitored at 280 nm. The slight variation of the elution positions of the proteins that is seen upon comparison of the Figures is the result of using two different TSK G3000SW columns during the course of this work. The profiles shown in Figs. 1, 3B, 3C, 4, and 5B were obtained using one column, and those in Figs. 2, 3A, and 9A were obtained using the other column.

Preparation of Inverted Cytosplasmic Membrane Vesicles—Bacterial cultures (HB1215 or HB1590) were grown in Luria broth (14) in the presence of appropriate antibiotics to an absorbance of 0.7 at 560 nm, induced with 1.5 mM IPTG, and harvested 2 h later. Vesicles were prepared as described (16), suspended in 10 mM HEPES, pH 7.6, 150 mM KOAc, 1 mM DTT, 250 mM sucrose to give a protein concentration of 6–9 mg/ml as determined by the bicinchoninic acid assay (Pierce), and stored at -70 °C.

14C-SecB Binding Assays—Binding assays were carried out in 125 µl of 10 mM HEPES, pH 7.6, 300 mM KOAc, 2 mM DTT, 1 mM EGTA, 50 mM guanidine hydrochloride (GdnHCl), 20 mM sucrose, HB1590 vesicles containing 75 µg of protein, and where indicated, 5 mM MgOAc, and 2 mM ATP. The concentration of metabolically labeled 14C-SecB (wild-type, L75Q, or E77K) was 1 µM. When present, the concentration of SecA and precursor galactose-binding protein was 1 µM each unless indicated otherwise. Mixtures were incubated on ice for 15 min. Vesicles were then pelleted by centrifugation (Beckman L8–70 ultracentrifuge) in a 42.2 Ti rotor at 14,000 rpm for 21 min (α = 2.4 × 105 rad/s) from the total reaction mixture, the supernatant, and the suspended pellet was quantified by liquid scintillation counting. The amount bound was calculated as the percentage of the total radioactivity that was recovered in the pellet after subtracting the radioactivity that pelleted in the absence of vesicles (--3%). Recovery of radioactivity was typically 95–100%.

In Vitro Processing of Precursor Galactose-binding Protein—Processing of precursor galactose-binding protein by vesicles prepared from HB1215 was performed in a 30-µl reaction mixture containing 10 mM HEPES, pH 7.0, 300 mM KOAc, 2 mM DTT, 0.7 mM EGTA, 6.25 mM magnesium acetate (MgOAc), 2.5 mM ATP, and 2 µM SecB unless indicated otherwise. Unfolded precursor galactose-binding protein was diluted 15-fold from a 30 µM stock (the final GdnHCl concentration varying from 50 to 70 mM) into the mixture. SecA (2 µM) was added, and the translocation reaction was initiated by the addition of vesicles containing 19–38 µg of proteins. Samples were incubated at 37 °C for the indicated times and transferred to ice, and an equal volume of 2-fold-concentrated electrophoresis sample buffer was added. After boiling for 5 min, the samples were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting. The amount of mature galactose-binding protein in each sample was determined by quantifying the immunoblot using a densitometer (Molecular Dynamics, Inc.) and relating the intensity of the band representing mature galactose-binding protein to a standard curve of pure authentic mature galactose-binding protein on the same immunoblot.

Gel Electrophoresis and Immunoblotting—SDS-polyacrylamide gel electrophoresis was performed as described (17). Immunoblotting was performed as described (4) using an antisemir to galactose-binding protein.

RESULTS

Mutational studies of SecB have led to the identification of substitutions that result in defects in protein export (2,3). The substitution mutations cluster in two regions, one near the amino terminus at residues Asp-20 and Glu-24, and a second region between aminoacyl residues 73 and 81. The altered forms of SecB have been classified into two groups based on the functional defect exhibited (3). One group, comprising those with substitutions at even-numbered residues between 73 and 81, was originally characterized as having a reduced ability to form complexes with precursor proteins (3) and recently has been shown to be altered in the oligomeric state of SecB (4). The second group, comprising substitutions at Asp-20, Glu-24, Leu-75, and Glu-77, was shown to interact normally with precursor protein ligands as assessed by coimmunoprecipitation of SecB with precursor maltose-binding protein (Asp-20 and Glu-24 (3)) as well as by direct binding assays (Leu-75 and Glu-77 (4)). Two members of that group, those having substitutions at Leu-75 and Glu-77, were further investigated and shown to be defective in interaction with membrane-associated SecA (5). To determine the molecular basis of the inability to sustain protein export, which is reflected in the defective membrane interaction, we have extended the analysis of the two previously characterized variants of SecB to include examination of their interactions with SecA free in solution. We have also investigated three additional variant forms of SecB. It is important to examine the interactions in solution as well as at the membrane, because under physiological conditions in vivo soluble complexes are expected to form. There is an excess of SecA relative to its binding sites at the membrane translocase, and the concentrations of both the cytosplasmic SecA and SecB (estimated to be approximately 4 and 20 µM, respectively) are above the dissociation constant that has been determined for the SecA-SecB complex in solution (Kd approximately 1.6 µM).

2 J. M. Crane and L. L. Randall, unpublished results.
of SecB and precursor galactose-binding protein at molar ratio 3:1. Immediately following mixing, the sample was analyzed by chromatography using a TSK G3000SW column. The samples applied were mixtures of SecB and precursor galactose-binding protein at molar ratio 3:1. Purified unfolded galactose-binding protein in 1.0 M GdnHCl, 10 mM Hepes (KOH), pH 7.0, 1 mM EGTA, 300 mM KOAc was diluted into a solution containing pure SecB to final concentrations of 3 mM SecB, 3 mM precursor galactose-binding protein, 50 mM GdnHCl, 10 mM Hepes (KOH), pH 7.6, 300 mM KOAc, 5 mM MgOAc, 1 mM EGTA, 2 mM IPTG. Immediately following mixing, the sample was chromatographed using a TSK G3000SW column. The samples applied were mixtures of SecB and precursor galactose-binding protein at molar ratio 3:1. SecB:3 mM precursor galactose-binding protein as follows: wild-type SecB, dotted line; SecBE24A, solid line; and SecBE24K, short-dashed line. SecB only is represented by the long-dashed line and unfolded precursor galactose-binding protein by the dotted-dashed line. Lower panel, SDS-polyacrylamide gel electrophoresis of trichloroacetic acid precipitates of successive 0.33-ml fractions collected during chromatography of the SecBE24A:pGBP complex. Lane S contains 2.5% of the quantity of sample applied. The positions of precursor galactose-binding protein (pGBP) and SecB are indicated.

(18)). Furthermore, soluble ternary complexes of SecA, SecB, and precursor proteins have been isolated from cellular lysates (19). It is also of interest in this context to note that the membrane-associated SecA has been shown to exist in vivo in two different conformations, one of which is exchangeable with soluble SecA (20, 21). Therefore, although it is not clear whether or not formation of complexes between SecA and SecB in the cytoplasm is a necessary step in delivery of SecB and its precursor protein ligand to the translocase, characterization of the soluble complexes should help to elucidate the nature of the interaction at the membrane.

**Analyses of Ligand Binding by SecB Variants**—The ability to bind precursor galactose-binding protein, a natural ligand of SecB, has been assessed previously (4) using purified proteins for two of the variant forms of SecB under study here: SecBL75Q (the leucine at position 75 is replaced by glutamine) and SecBE77K (the glutamate at position 77 is replaced by lysine). The binding was shown to be indistinguishable from that between precursor galactose-binding protein and wild-type SecB by two methods: analysis of complex formation by size-exclusion chromatography and by titration calorimetry. Here we demonstrate that the three previously uncharacterized variants that have aminoacyl substitutions in a different region, SecBD20A, SecBE24A, and SecBE24K, also show normal interaction with precursor galactose-binding protein. SecB only binds proteins as ligands if they are in a non-native state (22). Therefore, to assess interaction precursor galactose-binding protein was unfolded in guanidinium chloride and refolding was initiated by dilution or the denaturant in the presence of SecB. Equimolar mixtures of SecB and precursor galactose-binding protein were analyzed by size-exclusion chromatography. In all cases whether the SecB were wild-type (Fig. 1, dotted line) or one of the variants, SecBE24A (Fig. 1, solid line), SecBE24K (Fig. 1, short-dashed line), or SecBD20A (data not shown) the galactose-binding protein coeluted with SecB, ahead of the positions of free SecB (15.8 ml) and free precursor galactose-binding protein (17.2 ml). We conclude that these variants, like SecBL75Q and SecBE77K, are unaltered in their oligomeric state (they elute at 15.8 ml as does wild-type) and with respect to binding of ligand. We next investigated all five variants for their ability to bind SecA.
the fact that SecB stains less well than does SecA. The relative intensity difference of the SecA and SecB is a result of the large difference in molecular weight of the two proteins and indicated. The positions of SecA and SecB are described under "Experimental Procedures." Samples applied were 4 μM SecA only, dotted line; 4 μM SecB only, short-dashed line; 1 μM SecA:1 μM SecB, dotted-dashed line; 4 μM SecA:1 μM SecB, solid line; and 6 μM SecA:6 μM SecB, long-dashed line. Lower panel, SDS-polyacrylamide gel electrophoresis of trichloroacetic acid precipitates of successive 0.33-ml fractions collected during chromatography of the 4 μM SecA:4 μM SecB sample. Lane S contains 0.5% of the quantity of sample applied to the column. The positions of SecA and SecB are indicated. The relative intensity difference of the SecA and SecB is a result of the large difference in molecular weight of the two proteins and the fact that SecB stains less well than does SecA.

![Fig. 2. Complexes between SecA and wild-type SecB.](image)

**Upper panel.** Absorbance profiles of complexes between SecA and SecB observed by size-exclusion chromatography. HPLC was carried out as described under “Experimental Procedures.” Samples applied were 4 μM SecA only, dotted line; 4 μM SecB only, short-dashed line; 1 μM SecA:1 μM SecB, dotted-dashed line; 4 μM SecA:1 μM SecB, solid line; and 6 μM SecA:6 μM SecB, long-dashed line. Lower panel, SDS-polyacrylamide gel electrophoresis of trichloroacetic acid precipitates of successive 0.33-ml fractions collected during chromatography of the 4 μM SecA:4 μM SecB sample. Lane S contains 0.5% of the quantity of sample applied to the column. The positions of SecA and SecB are indicated. The relative intensity difference of the SecA and SecB is a result of the large difference in molecular weight of the two proteins and the fact that SecB stains less well than does SecA.

at least two species of complex as revealed by the distinct shoulder discernable between the earliest eluting peak and the latest (representing uncomplexed SecA and/or SecB).

We examined the ability of variant forms of SecB to make complexes with SecA. Two of the variants, SecBL75Q and SecBE77K have been reported to be defective in binding to membrane vesicles through interaction with SecA (5), but they had not been tested for binding free in solution. The interaction of SecA with the other three variants, SecBD20A, SecBE24A, and SecBE24K, has not been examined either in solution or at the membrane translocase. All five species formed complexes with SecA in solution as assessed by size-exclusion column chromatography. Fig. 3A shows that the complex formed with SecBD20A differed from that formed between SecA and wild-type SecB in that only one species of complex appeared to be present and it eluted at 14.7 ml, a position that corresponds to the shoulder in the elution pattern of the complexes formed between SecA and wild-type SecB (Fig. 3A; compare the solid (SecBD20A) and dotted lines (SecB wild-type)). Examination of the interaction of SecA with SecBE24A, SecBE24K, SecBL75Q, and SecBE77K (Fig. 3, B and C) showed that each variant formed one species of complex eluting at the same position as that seen for SecBD20A (Fig. 3A). We have designated the complex that is formed by both the wild-type SecB and the variants as complex 1 (eluting at 14.7 ml), and the species that is formed only with wild-type SecB (eluting earlier, at around 13.5 ml when applied at 4 μM) as complex 2. We are justified in considering the complexes designated complex 1 as closely related whether formed by wild-type or variant SecB species, because two of the SecB variants, SecBL75Q (Fig. 4) and SecBE77K (data not shown), were shown to compete with radioabeled wild-type SecB for the SecA in complex 1 but not in complex 2.

Addition of precursor galactose-binding protein to the mixtures of SecA and SecB resulted in formation of ternary complexes, which contained the precursor and eluted even earlier from the column. However, the pattern observed in the absence of precursor was maintained: the complexes containing wild-type SecB eluted earlier than did those formed by the variants (data not shown).

**Binding of SecB Variants to Membrane Vesicles via Interaction with SecA**—The published studies demonstrating that SecBL75Q and SecBE77K were defective in binding to inverted vesicles via association with SecA were carried out with SecA at 250 nM (5) (the Km for the interaction of SecB and membrane-bound SecA is approximately 30 nM (6, 23)). Thus, because this concentration is too low to allow formation of the soluble complexes under study here, it was necessary to re-examine the binding to inverted vesicles. In our assay both SecA and SecB were present in the micromolar concentration range that would allow complexes to form in solution so that we could determine whether these complexes would bind the translocase. Under these conditions, the binding of wild-type SecB (1 μM) to inverted vesicles that contained the SecYEG translocase was dependent on the addition of SecA (Fig. 5). Approximately 16% of radioabeled SecB (corresponding to 0.16 μM SecB) pelleted with vesicles when SecA was added. In the absence of SecA only
vesicles of metabolically labeled 14C-SecB (wild-type, L75Q, or E77K, at The binding to inverted cytoplasmic membrane
membrane vesicles.

SecA in complex with wild-type SecB. Each mixture was ana-
yzed by size-exclusion chromatography as described under “Experimental Proce-
dures” except that the buffer was pH 7.0 and did not contain MgOAc. The absorb-
ance at 280 nm was monitored (dashed
and solid lines), fractions were collected by drop (approximately 0.2 ml), and the
radioactivity in each fraction was deter-
mined by liquid scintillation counting (C), ■. The recovery of the radioactivity was
greater than 90% of that applied.

3% of the SecB was membrane-associated. Addition of precur-
sor galactose-binding protein to the system increased the bind-
ing of SecB to the membrane to 30% (Fig. 5), whereas addition of
mature galactose-binding protein did not affect the binding
data not shown). This is consistent with previously published obser-
vations that the presence of a precursor protein in the
complex enhanced binding at the membrane translocase rela-
tive to the binding of the SecB-SecA complex alone (5, 23). Assessed under the same conditions (Fig. 5), the two variant
SecB species showed very little SecA-dependent binding in the
absence of precursor galactose-binding protein. Addition of
precursor galactose-binding protein stimulated binding of
SecBL75Q but not of SecBE77K. The lack of effect of the pre-
cursor on the binding of SecBE77K was unexpected, because
both SecBL75Q and SecBE77K show normal binding of precur-
sor galactose-binding protein (4), and in the previous study by
Fekkes et al. (5) the ability of SecBE77K to compete with
wild-type for membrane-bound SecA was enhanced by
proOmpA, a natural ligand of SecB. If the defect in binding to
membrane were one of affinity, then it would be overcome by
increasing the concentration of variant forms of SecB. Fig. 6
shows that this is not the case. Although binding to membrane
vesicles of complexes containing wild-type SecB was saturated
at 2.0 μM, there was no detectable binding of either of the
variants even at 10 μM SecB. These results show that the defect
in binding of the variant forms of SecB to the membrane via
SecA persists even at concentrations of the proteins that should
result in formation of soluble complexes.

Translocation and Processing of Precursor Galactose-bind-
ing Protein—In vivo SecB is required for efficient export of its
ligands, because it modulates a kinetic partitioning of the
precursor polypeptides between folding in the cytosol and
entering the export pathway (24). The binding to SecB is
rapidly and readily reversible and thus the ligand continu-
ously samples the free state (22). Because the variant forms
of SecB bind their ligands normally and thereby maintain
them in an export competent state, it was possible that the
ligands could be passed to SecA directly without the necessity
for a complex containing SecA and SecB to bind to the mem-
brane. To eliminate this possibility, it was necessary to dem-
strate that the variant forms of SecB in the soluble com-
plexes could not mediate translocation, whereas the wild-
type SecB could. The first step toward this goal was a
demonstration that a kinetic partitioning between proteolytic
maturation and folding does occur in our in vitro protein
translocation system and accurately mimics SecB-dependent
export in vivo. It should be noted that, just as in the binding
assay used in this study, the translocation system described
here differs from published systems that are used to trans-
locate radiolabeled precursors present at very low concentra-
tions. Our assay contains the SecA, SecB, and precursor
galactose-binding protein at concentrations in the micromo-
lar range so that soluble complexes would be present. The
amount of precursor galactose-binding protein that is proteo-
lytically processed to the mature form is sufficiently high
that it can be detected by staining with Coomassie Blue and
can be quantified by immunoblotting using an antiserum
raised to galactose-binding protein. We have previously
shown that in the absence of Ca2+
the folding of precursor
galactose-binding protein is so slow that, with each cycle of
dissociation from SecB, the precursor galactose-binding protein
rebinds before it folds (12). However, if Ca2+
is present
the rate of folding is increased sufficiently so that the prob-
ability of precursor galactose-binding protein refolding is
higher than the probability of its rebinding to SecB (12). Thus,
in the presence of Ca2+ the precursor partitions to the
The presence of Ca\(^{2+}\) SecB, indicating that when the protein folds rapidly in the translocation reaction was carried out without addition of SecB. The processing was completely eliminated if the folding, processing was greatly diminished even in the presence of SecB. However, if the translocation buffer contained Ca\(^{2+}\), a low level of processing occurs even in the absence of SecB, whether the SecB were wild-type or a variant. Fig. 9 shows that this is the case. Truncated SecA incubated with wild-type SecB or with SecBL75Q binds to yield complex 1. We conclude that interaction between SecB and the carboxyl terminus of SecA is crucial to trigger conversion of the inactive complex 1 to complex 2. To further correlate the inactive state with complex 1 we demonstrated that wild-type SecB in complex with SecAN880 does not bind to the membrane (data not shown) even at the micromolar concentrations used in our assay as described for the variant forms of SecB and wild-type SecA.

**Discussion**

As discussed in Murén et al. (4), substitutions in SecB at positions 76 and 78 disrupt the quaternary structure so that SecB, which is normally tetrameric, exists in solution as a dimer. The variants studied here with substitutions at positions 20, 24, 75, and 77 have no detectable alteration in quaternary structure or in binding to precursor polypeptides (4, and this report) but are affected in the interaction of SecB with SecA. These variant forms of SecB interacted with SecA in solution with an affinity similar to that of wild-type SecB, but the species of complex formed, designated complex 1, was defective in binding to inverted membrane vesicles and in mediating processing of a natural ligand, precursor galactose-binding protein to the matured form. The wild-type SecB, which formed complex 2, was capable of binding to inverted membrane vesicles and mediating processing of precursor. Because the truncated SecA formed the inactive complex (complex 1) with all species of SecB tested, including the wild-type SecB, we conclude that the carboxyl-terminal region of SecA is not involved in interactions that provide the majority of energy to stabilize that complex; rather, because the complex is inactive, the interaction between SecB and the carboxyl terminus of SecA has a role in triggering conversion of the inactive complex to an active state. It was previously concluded (5, 6, 25) that the sole interaction between SecA and SecB is confined to this carboxyl-terminal 22 aminoacyl residues of SecA and that the variant forms of SecB are defective in this interaction and therefore do not bind to SecA. These conclusions were based on two experimental approaches. In one approach (5, 6), a protein comprising glutathione S-transferase fused to the 22 aminoacyl residues from the carboxyl terminus of SecA was shown to bind to wild-type SecB but not the variant forms. This result indicates only that this aminoacyl sequence is a site of interaction but it need not be the only binding site. Therefore, this is in conflict with our results, which implicate this region as a site of regulatory interaction. Another study (5) assessed the binding of SecB to membrane vesicles via SecA but did not include investigations of interactions in solution. Thus, that work is not inconsistent with our conclusions that the variant species do not form a complex with SecA in solution and that the SecA in those complexes is in a state that is not capable of interaction with the membrane.
Soluble complexes would be expected to exist in vivo, because the binding sites at the membrane-associated translocase are saturated and the excess SecA and SecB are present in the cytoplasm at concentrations well above the $K_d$ level for the interaction in solution (18). Complexes between the defective variants of SecB and cytosolic SecA might trap SecA in a nonfunctional state. This could explain the dominant phenotype described for SecBL75Q (2) and the fact that the export defect caused by SecBL75Q can be suppressed by overproduction of SecA (26).

The apparent affinity of SecB for SecA bound to the membrane translocase is higher ($K_d$, 30 nM (6, 23)) than is the affinity of SecB for SecA free in solution ($K_d$, 1.6 μM (18, 23)). This suggests either that SecB makes contacts with other membrane-associated components in addition to SecA to provide increased binding energy or that the membrane-associated SecA is in a different conformational state than that free in solution. A change in conformation of SecA might be elicited by binding to SecY in the translocase, and this form could have high affinity for SecB. It is also possible that SecB itself could trigger a conformational change in SecA and induce the high affinity state through interaction with the carboxyl-terminal peptide of SecA.

The carboxyl-terminal peptide cannot simply be the site of high affinity binding itself, because if it were the complex would form in solution at submicromolar concentrations, which we have shown it does not. Presence of the carboxyl-terminal peptide is necessary for the creation of the high affinity site, but it is not sufficient. Interaction at other sites, which are present even in complexes between defective variants of SecB and SecA, are required to form a complex that then can be converted to an active form. The active form is likely to correspond to a complex that contains SecA in a state that has high affinity for wild-type SecB. Evidence for such a high affinity state is provided by the demonstration that the variant forms of SecB could compete with wild-type SecB for SecA in complex 1 but not for SecA in complex 2. It is possible that the SecA in the complex characterized here in solution as complex 2 in fact represents the high affinity membrane form of SecA. This SecA might well be in the conformational state that was shown by Tai and his co-workers (20) to be exchangeable with membrane-bound SecA.

It is of great interest to determine the difference between the

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**Fig. 7.** Demonstration of kinetic partitioning in vitro. Processing of pGBP was carried out immediately after dilution of the pGBP or after a 10-min incubation at 24 °C in the presence of 0.7 mM EGTA or 9 mM CaCl$_2$. After addition of SecB (lanes with "+") and inverted vesicles, processing was allowed to proceed for 54 min at 37 °C. The positions of pGBP and mGBP are indicated.

**Fig. 8.** Processing of pGBP in vitro as a function of the concentration of SecB. Processing of pGBP was carried out for 45 min as described under “Experimental Procedures” in the presence of wild-type SecB (●) or SecBE77K (×).

**Fig. 9.** Complexes between truncated SecA and SecB. A: upper panel, absorbance profiles of complexes separated by size-exclusion chromatography. Samples were: 4 μM SecAN880, dashed line; 4 μM SecA:4 μM SecB, dotted line; 4 μM SecAN880:4 μM SecB, solid line. Lower panel, SDS-polyacrylamide gel electrophoresis of fractions collected during chromatography of 4 μM SecAN880:4 μM SecB. B, absorbance profiles of complexes separated by size-exclusion chromatography. Samples were: 4 μM SecAN880:4 μM SecBL75Q, solid line; 4 μM SecAN880:4 μM SecB, dashed line; 4 μM SecA:4 μM SecB, dotted line.
active and inactive complex. It seems likely the difference is one of conformation. However, it is a nontrivial task to distinguish definitively differences in stoichiometry and mass from differences in conformation in such a complicated system, because both components of the complex are themselves oligomeric and the complexes continuously re-equilibrate during column chromatography. Whatever the differences are between the complexes, it is clear that SecA has at least two regions that interact with SecB. Interactions between regions of SecA and SecB that are as yet undefined provide sufficient energy of binding to stabilize a complex that has a $K_d$ value in the micromolar range. That complex is inactive in mediating translocation of precursors unless there is interaction between SecB and the region at the carboxyl terminus of SecA. The region of SecB that interacts with the carboxyl terminus of SecA includes residues from two widely separated stretches along the primary sequence of 155 aminoacyl residues of SecB. The residues 20, 24, 75, and 77 are likely to be in close proximity in the quaternary structure of the SecB tetramer so that they can interact with the carboxyl-terminal 21 residues of SecA.

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