A signal peptide is required for entry of a preprotein into the secretory pathway, but how it functions in concert with the other transport components is unknown. In *Escherichia coli*, SecA is a key component of the translocation machinery found in the cytoplasm and at membrane translocation sites. Synthetic signal peptides corresponding to the wild type alkaline phosphatase signal sequence and three sets of model signal sequences varying in hydrophobicity and amino-terminal charge were generated. These were used to establish the requirements for interaction with SecA. Binding to SecA, modulation of SecA conformations sensitive to protease, and stimulation of SecA-lipid ATPase activity occur with functional signal sequences but not with transport-incompetent ones. The extent of SecA interaction is directly related to the hydrophobicity of the signal peptide core region. For signal peptides of moderate hydrophobicity, stimulation of the SecA-lipid ATPase activity is also dependent on amino-terminal charge. The results demonstrate unequivocally that the signal peptide, in the absence of the mature protein, interacts with SecA in aqueous solution and in a lipid bilayer. We show a clear parallel between the hierarchy of signal peptide characteristics that promote interaction with SecA in *vitro* and the hierarchy of those observed for function in *vivo*.

Many proteins that are synthesized in the cytoplasm of cells are ultimately found in noncytoplasmic locations. It is well documented that the correct targeting and transport of these proteins requires several proteinaceous components that make up the cellular transport pathway (1–3) and a signal peptide at the amino terminus of the secreted protein to direct entry into the secretory pathway, but how it functions in concert to achieve the overall transport process. Consequently, a fundamental issue involves identifying the key interactions between the signal peptide and transport machinery and the conditions that promote these associations.

In *Escherichia coli*, SecA is a large, multifunctional protein that is a critical component of the general secretion pathway; depletion of SecA results in loss of cell viability (4, 5). SecA is found in both cytoplasmic and membrane-associated forms (6), and this may reflect a role in targeting preproteins to membrane translocation sites. Upon binding ATP (7) and lipid (8), SecA undergoes a substantial conformational change (9) and is deeply inserted into the membrane bilayer (8). This membrane association, in particular with anionic lipids (6, 10), results in stimulation of SecA ATPase activity and preprotein translocation activity (10–12). SecA has been shown to interact with preproteins (10, 12, 13), and recently, our laboratory established that the signal peptide region alone can bind SecA in liposomes and stimulate SecA-lipid ATPase activity (14). However, it remains unclear whether signal peptides alone specifically interact with SecA in aqueous solution. Addressing this issue is fundamental to any translocation model that employs SecA to facilitate targeting of the preprotein from the cytoplasm to membrane translocation sites. Furthermore, the substrate specificity of SecA in any environment is unknown. If SecA is a key component of the sorting mechanism that discriminates secreted proteins from nonsecreted ones, then it must be able to distinguish between functional signal peptides and all other sequences, including some that may be marginally hydrophobic.

The features of signal peptides that promote the transport process overall have been delineated (15, 16). The importance of the hydrophobic core region, the hallmark of a signal sequence, is underscored by the observation that a variety of mutations in this domain produce transport-incompetent signal peptides (17). Titration of signal peptide hydrophobicity and *in viv*o analysis has demonstrated that the ideal signal sequence will meet a threshold “hydrophobic density” to ensure rapid translocation (18). Furthermore, there is a narrow range of signal peptide hydrophobicity that supports transport of the protein to which the signal peptide is attached yet does not have such a high affinity for the transport pathway that it disrupts the appropriate balance of other secreted proteins (19). In addition, the positive charge at the amino-terminal end of signal peptides has been suggested to enhance protein translocation efficiency (13, 20). Using signal sequence mutants and *in vivo* analysis, no functional differences are observed for signal peptides with one or three positively charged residues and with core regions rich in leucine residues (21). Yet for those signal peptides that are less hydrophobic, a clear correlation between amino-terminal charge and transport activity is found (22, 23). Thus, the signal peptide core region could be an important recognition element for interaction with the transport machinery and its affinity may be attenuated by flanking charged residues. That one such interaction involves SecA has been inferred by the graded response of signal sequence mutants involving titration of amino-terminal charge and core region hydrophobicity to SecA inhibition *in vivo* (22). Previous studies involving *in vitro* cross-linking of purified SecA in solution with proOmpA variants do not help resolve this issue because the cross-linking agent utilized depends on the presence of basic residues in forging cross-links (13, 24). Elucidating the extent to which SecA can discriminate different properties of the signal peptide is fundamental to understanding its role in protein transport.

In this study, the direct binding of synthetic signal peptides...
with SecA, in the absence of the mature protein domain, was established using three different assays involving: peptide-stimulation of SecA-lipid ATPase activity, SecA sensitivity to V8 protease, and a peptide competition displacement binding assay. In each case, binding was shown to require a hydrophobic signal peptide corresponding to a transport competent signal sequence. Stimulation of the SecA-lipid ATPase activity became dependent on amino-terminal charge as well when the core region was of intermediate hydrophobicity. Furthermore, binding of synthetic peptides corresponding to functional signal sequences was shown to occur for SecA in both aqueous solution and a lipid bilayer.

**EXPERIMENTAL PROCEDURES**

**Materials**—All media and culture conditions for the overproduction and purification of SecA from strain BL21.14 pCS1 have been described (7). Staphylococcus aureus V8 protease (EC 3.4.21.19), reactive blue 4 agaroze, ADP, ATP and its nonhydrolyzable analog AMP-PNP, 1 dioleoylphosphatidylcholine (C18:1), dioleoylphosphatidylethanol (C18:1), and peptide T (ASSTTNTYT) were purchased from Sigma. Acetone-precipitated and ether-extracted E. coli phospholipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). All lipid stocks were kept at CHCl3 at −20 °C until needed. The cross-linker, APDP, was purchased from Pierce.

**Peptide Synthesis and Purification**—Synthetic signal peptides used in this study (Tables I and II) were synthesized using an ABI model 431A automated peptide synthesizer and FastMoc chemistry as reported earlier (21). The crude peptides were purified using either isocratic or gradient reverse-phase high pressure liquid chromatography and further analyzed to confirm identity and purity by analytical high pressure liquid chromatography, amino acid analyses, and matrix-assisted laser desorption ionization/time of flight mass spectrometry. Stock solutions (3 mM) were prepared in either Me2SO (100%) or 1 mM acetic acid, respectively, 2 μm for the V8 protease studies and the SecA ATPase assays, aliquoted (10 μl) to avoid repeated freezing and thawing, and stored at −70 °C until needed. In all experiments, control reactions with the appropriate solvent, in the absence of peptide, were run. No solvent induced effects were observed.

**SecA Purification**—SecA protein was isolated and purified from E. coli BL21.14 pCS1 S300 extracts by affinity chromatography on reactive blue 4 agaroze as described previously (7) with minor modifications. The SecA concentration was determined by the Bradford assay (25) using bovine serum albumin as a standard. Working stock solutions (1–2 mg/ml) were stored at 4 °C, aliquoted, and frozen at −70 °C until analyzed by SDS-PAGE (29). Gel densitometric scanning was accomplished using an LKB Ultrascan XL laser densitometer. All samples at each time point were immediately boiled for 3 min and stored at −70 °C until analyzed by SDS-PAGE (29). Gel densitometric scanning was accomplished using an LKB Ultrascan XL laser densitometer. All experiments were repeated independently at least three times.

**RESULTS AND DISCUSSION**

In order to evaluate the interaction between SecA and signal peptides, and the signal sequence features that promote this interaction, the series of synthetic signal peptides shown in Table I was made. In addition to a peptide corresponding to the wild type signal peptide for alkaline phosphatase, three pairs of model peptides were generated. Each pair varies from the others by the ratio of alanine and leucine residues in the core region and consequently the hydrophobicity of this domain. The 1K7L and 3K7L peptides are rich in leucines and correspond to functional signal peptides, whereas the 1K2L and 3K2L are rich in alanines and correspond to nonfunctional signal peptides. The two peptides of intermediate hydrophobicity were also synthesized, 1K4L and 3K4L. Signal peptides with comparable sequences function in vivo but with reduced efficiency (18). Within each pair, the peptides differ in the number of basic residues in the amino-terminal segment, providing peptides with either a +1 or +3 net charge (excluding the charge of the α-amino group at the amino terminus).

In Table II, some of the properties of the peptides are summarized. The effective hydrophobicity of each peptide is given based on retention time on a C4 column during reverse-phase high pressure liquid chromatography elution. For the entire series, the rank order for retention time correlates very well with the overall hydrophobicity calculated using various hydrophobicity scales (30–32). The close agreement in the hydrophobicity of the 3K7L and the wild type peptide serves to verify that the sequence design of the former is a reasonable model of the latter, although the two share little primary sequence similarity. Interestingly, the 1K7L peptide represents the extreme in hydrophobicity for the series except when evaluated by the Fauchère et al. (32) hydrophathy scale. Because the latter considers proline significantly more hydrophobic relative to other
The effects of the synthetic signal peptides on SecA ATPase activity in the presence of *E. coli* phospholipids are illustrated in Fig. 1A. In general, the extent to which the synthetic peptides stimulated SecA-lipid ATPase activity correlates very well with the extent to which the corresponding signal sequences function *in vivo* (18). Maximum stimulation of SecA-lipid ATPase by the wild type, 3K7L, and 1K7L peptides (about 175, 165, and 160 pmol of P_i released/min/μg SecA, respectively) occurred at a peptide concentration of 10 μM. Half-maximal stimulation was in the 1.5–2.0 μM range. No significant increases in ATPase activity were observed for the nonfunctional peptides, 3K2L and 1K2L, or for the unrelated peptide T. The peptides with intermediate hydrophobicity, 3K4L and 1K4L, stimulated the SecA ATPase to intermediate levels with maximal stimulation of 125 and 54 pmol of P_i released/min/μg SecA, respectively. Strikingly, for these peptides, the extent of ATPase stimulation is not similar; rather, it is dependent on amino-terminal charge in addition to core region hydrophobicity. For example, at a peptide concentration of 10 μM, the ATPase activity levels for 3K4L and 1K4L were 71 and 31%, respectively, relative to the wild type peptide (Fig. 1B).

The graded response of SecA-lipid ATPase to stimulation by peptides of varying hydrophobicities agrees well with the extent of transport *in vivo* observed previously for signal peptide mutants of varying hydrophobicity (18). Maximum stimulation of SecA-lipid ATPase by the wild type, 3K7L, and 1K7L peptides (about 175, 165, and 160 pmol of P_i released/min/μg SecA, respectively) occurred at a peptide concentration of 10 μM. Half-maximal stimulation was in the 1.5–2.0 μM range. No significant increases in ATPase activity were observed for the nonfunctional peptides, 3K2L and 1K2L, or for the unrelated peptide T. The peptides with intermediate hydrophobicity, 3K4L and 1K4L, stimulated the SecA ATPase to intermediate levels with maximal stimulation of 125 and 54 pmol of P_i released/min/μg SecA, respectively. Strikingly, for these peptides, the extent of ATPase stimulation is not similar; rather, it is dependent on amino-terminal charge in addition to core region hydrophobicity. For example, at a peptide concentration of 10 μM, the ATPase activity levels for 3K4L and 1K4L were 71 and 31%, respectively, relative to the wild type peptide (Fig. 1B).

The graded response of SecA-lipid ATPase to stimulation by peptides of varying hydrophobicities agrees well with the extent of transport *in vivo* observed previously for signal peptide mutants of varying hydrophobicity (18). This strong parallel suggests that it is the interaction of the signal peptide with SecA that plays a fundamental role in dictating the efficiency of transport overall. The finding that amino-terminal charge enhances the SecA-lipid ATPase activity for signal peptides of varying hydrophobicities agrees well with the extent to which the corresponding signal sequences function *in vivo* (18).

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shown in Table III, the two functional peptides, unlabeled wild type peptide and 3K7L, displaced 62 and 84% of the labeled ligand, respectively. To have achieved such high levels of displacement is remarkable considering that the theoretical displacement expected from an optimal competition between identical species is 90%. Nevertheless, the experiment is somewhat less than ideal because the labeled peptide is more hydrophobic than its unlabeled counterpart and because the illumination and separation of bound ligand from free is not instantaneous (14). These data indicate that the specific binding to SecA of the synthetic peptides corresponding to functional signal sequences occurs in the absence of membranes and that the binding step is not dependent on ATP hydrolysis. The nonfunctional peptide, 3K2L, displaced only 9% of the radiolabeled ligand, whereas the unrelated control, peptide T, had no effect. The binding specificity observed here is comparable to what we observed for SecA in lipid (14), suggesting that although much of SecA undergoes a conformational change upon lipid binding (8), the signal peptide binding pocket must not substantially change.

The above binding assay also allows us to assess the relative affinity of synthetic signal peptides for SecA and for lipid. When liposomes in 300-fold excess of SecA were added to the binding reaction, also in the presence of excess wild type peptide, 68% of the radiolabeled ligand was displaced. However, in a parallel reaction in which liposomes but no unlabeled peptide were added, only 3% displacement of the radioligand occurred (Table III). These data indicate that the affinity of the labeled wild type peptide for SecA is substantially stronger than for the lipid bilayer. This suggests that even if the signal peptide of the preprotein is exposed to lipid during transport, it will preferentially bind SecA. Furthermore, the data argue that the stimulation of the SecA-lipid ATPase activity observed with functional signal peptides (Fig. 1) is a result of a direct interaction with SecA and not an indirect effect due to lipid partitioning of the peptides.

Further evidence that functional signal peptides specifically bind SecA in both aqueous solution and a lipid environment is provided by an assay based on the sensitivity of SecA to V8 protease (9, 28). In general, digestion of the 102-kDa SecA by V8 involves the initial loss of a carboxyl-terminal fragment, leaving a 95-kDa truncated form of SecA (9), which is subsequently completely digested to low molecular weight fragments after 2–3 h of proteolysis (data not shown). Because V8 protease can only cleave carboxyl-terminal to aspartic acid and glutamic acid residues, none of the synthetic signal peptides serve as substrates. The effects of nucleotides and signal peptides on the V8 proteolysis of SecA in aqueous solution are shown in Fig. 2. In the presence of ATP, its nonhydrolyzable analog AMP-PNP, or ADP, the 95-kDa fragment of SecA that contains the nucleotide binding sites (7) and the proposed peptide binding site (33), became more resistant to V8 hydrolysis, in agreement with earlier work (9). However, the addition of wild type peptide markedly increased the V8 sensitivity of SecA, either in the absence or the presence of ATP. The presence of the functional signal peptide overrides the nucleotide effect, apparently by inducing its release from SecA. The V8 protease had no such effect on SecA digestion in the presence of the nonfunctional peptide, 1K2L, whereas the presence of the 1K4L peptide produced an intermediate effect. No apparent difference was observed between 3K4L and its 1K4L counterpart (data not shown), which may be because electrostatic interactions do not play a role in binding SecA in solution or simply because this assay is not sensitive enough to detect such differences.

As shown in Fig. 3, a clear dose-dependent response of the wild type peptide on SecA V8 protease sensitivity was observed. Maximal V8 proteolysis of SecA occurred within the SecA to peptide molar ratio range of 1:30–1:75. This is comparable to the SecA to peptide molar ratio of 1:25–1:50 (and peptide concentrations of 10–20 μM) required for the maximal peptide-stimulated SecA-lipid ATPase activity shown in Fig. 1A.

The above data confirm our findings with the competition displacement binding assay; namely, that a functional signal peptide can specifically bind SecA in solution and that this binding event is not dependent on the energy derived from ATP hydrolysis. Even in the absence of lipid, the peptide induces a significant conformational change in SecA that exposes additional protease-sensitive sites and seems to result in the release of nucleotide.

The effects of phospholipids in the presence and absence of AMP-PNP and signal peptides on the sensitivity of SecA to V8 digestion are shown in Fig. 4A. In the presence of liposomes, an increased sensitivity of SecA to V8 proteolysis was observed. This is consistent with previous studies (9) and the notion that anionic lipids induce a partial unfolding of SecA (8). Unlike in aqueous solution, the protease sensitivity of SecA in liposomes is not appreciably affected by the further addition of AMP-PNP (Fig. 4A) or ADP (data not shown). However, the addition of the wild type signal peptide plus or minus AMP-PNP to SecA in liposomes resulted in a SecA molecule that was substantially more resistant to V8 digestion. No such effect was observed with the nonfunctional signal peptide, 1K2L and an intermediate effect was seen for the peptides of intermediate hydrophobicity, 3K4L and 1K4L. Densitometric scanings of some of the gels shown in Fig. 4A, converted to relative percentage of

### Table III

| Peptide            | Displacement*  |
|--------------------|----------------|
| wild type          | 62%            |
| 3K7L               | 84%            |
| 3K2L               | 9%             |
| Peptide T          | 0%             |
| +Lipid             | 3%             |
| +Lipid + wild type | 68%            |

* Relative radioactivity of 3H Ac-wild type-APDP displaced by 10-fold unlabeled peptide.
Multiple lines of evidence presented here demonstrate unequivocally that functional signal peptides bind to SecA, whereas nonfunctional ones do not. Furthermore, the hierarchy of signal peptide characteristics that best promote transport activity in vivo parallels the hierarchy of those that enhance interactions with SecA in vitro. A priori it was not known whether the SecA-signal peptide interaction was central to the kinetics of transport overall. By comparing the series of peptides in vitro that we have analyzed in vivo (18, 22), this issue can be addressed. The strong correlation observed argues that the signal peptide-SecA interaction could be the rate-limiting step for accomplishing transport.

Interestingly, the 3K2L and 1K2L peptides had little effect on SecA by any of the assays employed. The core region of these peptides are, nonetheless, weakly hydrophobic and certainly more hydrophobic than most polypeptide sequences found in water-soluble proteins. A broader substrate specificity would be expected if SecA interacts with successive segments of the translocating polypeptide. Consequently, if regions of the mature protein, in addition to the signal peptide, do interact with SecA during membrane translocation, they must be very weak interactions. If such interactions do occur they must be enhanced by virtue of the extremely high concentration of polypeptide in the local environment of the translocon. It is also possible that the hydrophobic regions within some transmembrane proteins interact with SecA. Nevertheless, our data suggest that the outcome of proOmpA-SecA interactions observed in many studies (9, 10, 12) must be largely derived from interaction with the signal peptide portion of the preprotein.

We further show that functional signal peptides interact with both soluble and membrane-associated forms of SecA. This is consistent with the notion that SecA may have a targeting function directing preproteins from the cytoplasm to the membrane. The V8 protease sensitivity experiments suggest that the conformations of the signal peptide-bound forms of SecA are quite different in the two environments. It is particularly satisfying that complex formation with the membrane-bound form of SecA results in a species that is more resistant to proteolysis. Although we can not rule out the possibility that the signal peptide simply masks sites of recognition by V8, the
formation of a more stable complex would be consistent with the need to dock with SecY in the translocation channel. Unfortunately, the proteolysis assay was not able to detect a nucleotide-induced conformational change in SecA in liposomes, and consequently the relationship between signal peptide and nucleotide binding to membrane-bound SecA could not be addressed. Although we observed a clear correlation between signal peptide binding and stimulation of SecA-lipid ATPase activity, it is not known whether this simply reflects an enhancement in the ATP hydrolysis step or whether binding serves to facilitate ADP-ATP exchange. In any case, it appears that the core region hydrophobicity and amino-terminal charge either independently or collectively contribute to one of the functions of signal peptides: the modulation of SecA binding and SecA-lipid ATPase activity. Because these studies employ model signal peptides, the findings should have global application to preprotein transport in general and circumvent the limitations of studying only one particular preprotein or signal peptide.

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