SecA Specificity for Different Signal Peptides†

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ABSTRACT: SecA performs a critical function in the recognition, targeting, and transport of secretory proteins across the cytoplasmic membrane of *Escherichia coli*. In this study we investigate the substrate specificity of SecA, including the influence of the early mature region of the preprotein on SecA interactions, and the extent to which SecA recognizes targeting signals from different transport pathways. A series of fusion proteins were generated which involved the tandem expression of GST, signal peptide, and the first 30 residues from alkaline phosphatase. These were purified and evaluated for their ability to promote SecA ATPase activity. No significant difference in the stimulation of SecA−lipid ATPase activity between the synthetic wild-type alkaline phosphatase signal peptide and a fusion that also contains the first 30 residues of alkaline phosphatase was observed. The incorporation of sequence motifs in the mature region, which confer SecB dependence in vivo, had no impact on SecA activation in vitro. These results suggest that the early mature region of alkaline phosphatase does not affect the interactions between SecA and the signal peptide. Sec, Tat, and YidC signal peptide fusions were also assayed for their ability to stimulate SecA ATPase activity in vitro and further analyzed in vivo for the Sec dependence of the transport of the corresponding signal peptide mutants of alkaline phosphatase. Our results demonstrate that *E. coli* Sec signals give the highest level of SecA activation; however, SecA−signal peptide interactions in vitro are not the only arbiter of whether the preprotein utilizes the Sec pathway in vivo.

Multiple pathways exist for the insertion and translocation of proteins across the *Escherichia coli* inner membrane, namely, Sec, Tat, and YidC (*1*). The common denominator among the substrates for these pathways is the presence of an N-terminal signal peptide that plays an important role in directing the preprotein to its export pathway. In general, these signal peptides are similar; yet remarkably most, if not all, preproteins utilize a single pathway with high fidelity. To date, the molecular interactions between elements in the preprotein and the transport machinery that determine this specificity and bring about directionality to the transport process in *E. coli* remain poorly understood.

The majority of secretory proteins utilize the well-characterized Sec pathway, which involves a membrane-embedded SecYEG complex that is believed to function as the translocation channel, and the peripheral dimeric ATP-hydrolyzing SecA protein, which helps to thread proteins in an extended conformation through the channel (*2*−*5*). Most presecretory proteins are believed to be targeted to the SecYEG complex by the molecular chaperone, SecB, or the signal recognition particle (SRP) (*6*).

The Tat (*t*win *a*rginine *t*ranslocation) pathway exports folded proteins with an N-terminal signal peptide containing the conserved sequence motif S/TRRXFLK (*7*). Thus far, four genes have been identified that code for proteins involved in the Tat pathway, *tatA*, *tatB*, *tatC*, and *tatE*; however, the precise role that these proteins play in recognition of the Tat signals is not clear (*8*−*10*).

In the third pathway, YidC, a bacterial homologue of mitochondrial Oxa1p that is associated with the Sec translocase (*11*), mediates the insertion of proteins that were earlier thought to spontaneously insert in the *E. coli* inner membrane (*12*). YidC appears to play a role in translocation rather than in membrane targeting and partitioning of its substrate (*13*).

How is specificity maintained and mistargeting prevented among these three pathways? For the Sec pathway, numerous lines of evidence indicate that SecA performs a critical function in the recognition and targeting of secretory proteins to the membrane. Previous studies have demonstrated that SecA binds to precursor proteins and targets them to the translocation channel by binding to the inner membrane (*14*) and that there is a lack of productive binding of preproteins to plasma membrane vesicles in the absence of SecA (*15*, *16*). Cross-linking studies showed that SecA binds to the signal peptide (*17*), precursor protein (*18*), and interacts with the positive charges in the N-terminus of signal peptides (*19*). The importance of these interactions is further underlined by the isolation of *secA* alleles which relieve the export defect of signal sequence mutations in maltose-binding protein (*20*). In addition, biochemical studies (*21*) demonstrated that the signal peptide, in the absence of the mature protein, interacts specifically with SecA in aqueous solution and in a lipid bilayer.

In the present study our objective was twofold: first, to explore the possible impact of the early mature region of the preprotein on the signal peptide induced activation of SecA; second, to investigate the substrate specificity of SecA and to determine whether the interactions, or lack thereof, between SecA and the signal peptide dictate the transport

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pathway used by the secretory preprotein. To do so, we developed a system to rapidly examine interactions between SecA and a wide spectrum of different signal peptides in vitro. Using SecA−lipid ATPase activity as an earmark of productive SecA−signal peptide interactions, we find that the early mature region of alkaline phosphatase does not affect the stimulation of SecA by the signal peptide. No other class of signal peptides, as a group, activate SecA to the high degree that the *E. coli* Sec signals do; however, SecA−signal peptide interaction in vitro is not necessarily the only arbiter of whether the preprotein utilizes the Sec pathway in vivo.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Media. E. coli strain BLR/DE3 [F^-ompT $hsdS_B(r^-_Bm^-_B)$ gal dcm Δ(srl-recA)306::Tn10-(DE3)] was used for the generation of GST and GST fusion proteins. Cloning experiments were performed in *E. coli* strain XL1-blue [recA1 endA1 gyr96 thi1 hsdR17 supE44 relA1 Δlac (F^- proAB $lacI^q$ lacZΔM15 Tn10)]. *E. coli* strain AW1043 [Δlac galU galK Δ(leu-ara) phoA-E15 proC::Tn5] was used for all alkaline phosphatase transport experiments and activity assays. For the overexpression of GST and GST fusion proteins, BLR/DE3 was grown in 2× YTG medium (*22*) supplemented with 250 μg/mL ampicillin and 12.5 μg/mL tetracycline. For general propagation of AW1043, MOPS[1] (*23*) medium under low phosphate conditions (100 μM) was used, supplemented with 250 μg/mL ampicillin and 50 μg/mL kanamycin. Synthesis and purification of the synthetic peptide corresponding to the wild-type alkaline phosphatase signal peptide have been described previously (*21*).

Construction and Purification of the GST Fusion Proteins. PCR reactions were performed with Afl-WT-Nhe, a derivative of WT-Afl (*24*), or hcPC-AP (*25*) as templates and the primer combinations DBK456 and DBK457, and K059 and DBK457 (Table 1). The resulting PCR fragments, coding for the signal peptide plus the first 30 amino acids of alkaline phosphatase, and the M13 signal peptide fused to the first 30 amino acids of alkaline phosphatase, respectively, were digested with *Bam*HI and *Eco*RI and ligated into pGex-2T (Pharmacia, Piscataway, NJ), resulting in plasmids pA8 and pGex/M13, respectively. The *Bss*HII site in the $LacI^q$ gene of pA8 was removed using QuikChange (Stratagene, La Jolla, CA) with the primers K032 and K033 (Table 1), resulting in plasmid pA8B3; removal of this site was verified by restriction enzyme analysis. A plasmid carrying the dysfunctional mutant 10A (*26*) was digested with *Sal*I and *Bss*HII, and the insert coding for an alkaline phosphatase signal peptide with 10 alanines in place of the wild-type hydrophobic core was ligated to pA8B3, yielding pA8B3/10Ala. WT-K_5L_5, WT-$K_3N_2L_5$, and WT-N_5L_5 (*27*) were digested with *Eco*RI and *Nhe*I, and the resulting inserts were ligated to pA8B3, yielding pA8B3/K_5L_5, pA8B3/$K_3N_2L_5$, and pA8B3/N_5L_5, respectively. pGex/K,K was derived from pGex/M13 using QuikChange and the primers K119, GCK119, K120, and GCK120 (Table 1). PCR reactions were also carried out with pMal-P2 (New England Biolabs, Beverly, MA), pBR322, TorA/pDHB5700 (*28*), pNR14, pNR19 (*29*), and pGem-3z (*30*), as templates and the primer combinations K066 and K067, K068 and K069, K074 and K075, K076 and K077, and K106 and K107 (Table 1). The resulting PCR fragments coding for the signal sequences of *E. coli* maltose-binding protein (MBP), β-lactamase (β-lac), trimethylamine *N*-oxide reductase (TorA), SufI protein, Yack protein, and hydrogenase I small subunit (HyaA) were digested with *Bam*HI and *Bss*HII and ligated into pA8B3, resulting in pGST/MBP, pGST/β-lac, pGST/TorA, pGST/Suf, pGST/Yack, and pGST/HyaA, respectively. Variants of pA8B3, pA8B3/K_5L_5, pA8B3/$K_3N_2L_5$, and pA8B3/N_5L_5 were also constructed with a hexahistidine tag just C-terminal to the 30th alkaline phosphatase residue. This involved introduction of a *Kpn*I site just before the stop codon using QuikChange and the primers K149 and GCK149 (Table 1); the resulting plasmids were digested with *Eco*RI and *Kpn*I and ligated to the following oligonucleotides encoding six histidines: 5′-GCATCACCATCACCATCACTGAG-3′ and 5′-AATTCTCAGTGATGGTGATGGTGATGCGTC-3′. All GST constructs contained an in-frame *Bam*HI site that separated the coding region of GST from that of the signal peptide and a *Bss*HII site at the border of the signal peptide with the early mature region. The sequence of the signal peptide and early mature region for all GST constructs was verified by direct DNA sequencing (*31*). For the purification of GST fusion proteins, cultures were grown to mid-log phase (OD_{600} ~0.6) and induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) for 1−3 h. Cells were harvested by centrifugation at 7700*g* for 20 min, resuspended in PBS (phosphate-buffered saline) containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (pH 7.3),

Table 1: Primers Used in This Study

primer	sequence (5′−3′)
DBK456	GAGAGGATCCATGAAACAGTCGACTATTG
BDK457	CACGGAATTCTCACTGATCACCCGTTAAAC
K059	CGTGGATCCATGAAAAAATCGCTGGTACTGAAA-GAATCTGTAGCCGTTGC
K032	CGCTGGGCGCAATGCGTGCATTACCGAGTCCGG
K033	CCGGACTCGGTAATGGCACGCATTGCGCCCAGCG
K066	CGTGGATCCATGAAAATAAAAACAGGTGC
K067	CACGCGCGCGAGAGCCGAGGCGGAAAACATC
K068	CGTGGATCCATGAGTATTCAACATTTCCG
K069	CACGCGCGCAAAAACAGGAAGGCAAAATGC
K063	CGTGGATCCATGAACAATAACGATCTC
K064	CACGCGCGCTGCGCCGCAGTCGCACG
K074	CGTGGATCCATGTCACTCAGTCGGCGTCAG
K075	CAGGCGCGCACTGGCCTTCAGGGGAACAGC
K076	CGTGGATCCATGCAACGTCGTGATTTC
K077	CACGCGCGCAAATACTGCGCGGCTCCAC
K106	CGTGGATCCATGAATAACGAGGAAACATTTTACC
K107	TATAAAGCGCGCCCAGGCAATCTTTGGTGCC
K113	GTATTTGTACATGGAGAAGGATCCGTGAAACAG-TCGACTATTGC
K114	GCAATAGTCGACTGTTTCACGGATCCTTCTCCAT-GTACAAATAC
K119	CGCTGGTACTGGCCTCTGTAGCCGTTGC
GCK119	GCAACGGCTACAGAGGCCAGTACCAGCG
K120	GCGTGGATCCATGAAAAAATCGAAACTGGTACT-GGCCTCTG
GCK120	CAGAGGCCAGTACCAGTTTCGATTTTTTCATGGA-TCCACGC
K149	GCCGTTTAACGGGTGATCAGGGTACCTGAGAATT-CATCGTG
GCK149	CACGATGAATTCTCAGGTACCCTGATCACCCGTT-AAACGGC

[1] Abbreviations: BSA, bovine serum albumin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; MOPS, 4-morpholinepropanesulfonic acid.

and lysed by sonication on ice. The lysate was mixed with 1% Triton X-100 and 1 mM PMSF for 30 min on ice, followed by centrifugation at 12000*g* for 30 min. The resulting supernatant was then loaded at 4 °C onto a 10 mL glutathione−Sepharose column (Pharmacia, Piscataway, NJ) and equilibrated with buffer 1 (50 mM Tris, pH 7.6, 50 mM KCl, 20 mM $MgCl_2$). The column was washed twice with 20 bed volumes of 5 mM adenosine 5′-triphosphate (ATP) in buffer 1 to remove copurifying chaperones (*32*). Fusion proteins were eluted with 20 mM reduced glutathione in 100 mM Tris (pH 9), according to the manufacturer's protocol (Pharmacia, Piscataway, NJ). Variants incorporating a C-terminal His tag were isolated from a 1 mL Ni-NTA agarose (QIAGEN, Chatsworth, CA) column essentially as described in ref *33*. All purified products were dialyzed against 2.5 mM Tris (pH 7.5), 25 mM KCl, 0.5 mM EDTA, 0.5 mM PMSF, and 5 mM β-mecaptoethanol, and protein concentrations were determined by the Bradford assay at 595 nm (*34*) using BSA[1] as a standard. All fusions gave rise to species with electrophoretic mobilities corresponding to the full-length proteins.

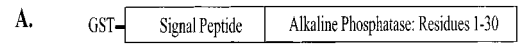
Construction of Full-Length PhoAs with Different Signal Sequences. The *Bam*HI site in the CASS3 (*35*) plasmid was removed by *Bam*HI digestion, filling in the sticky ends with DNA polymerase Klenow fragment and blunt end ligation; removal of the site was verified by subsequent resistance to *Bam*HI digestion. A *Bam*HI site was reintroduced into CASS3 just before the signal peptide using QuikChange and the primers K113 and K114 (Table 1), yielding pBCASS3. pGST/Suf, pGST/TorA, pGST/Yack, pGex/M13, and pGex/K,K were digested with *Bam*HI and *Bss*HII, and the resulting inserts were ligated into pBCASS3, yielding pBCASS3/Suf, pBCASS3/TorA, pBCASS3/Yack, pBCASS3/M13, and pBCASS3/K,K, respectively. All sequences were verified by direct DNA sequencing (*31*).

SecA−Lipid ATPase Assay. ATPase assays were conducted as described previously (*17*), and inorganic phosphate (P_i) release was determined using the malachite green colorimetric method (*36*). Liposomes were formed from *E. coli* phospholipids as described in Wang et al. (*21*). The fusion protein-stimulated SecA−lipid ATPase activity is given as the difference in the rate of ATP hydrolysis in the presence and absence of fusion protein. No aggregation was detected under the conditions employed. SecA used in the assay was overexpressed and purified as described previously (*37*).

Induction of Alkaline Phosphatase Expression. Cells were grown at 37 °C to logarithmic phase and harvested at an OD_{600} ~1.0, washed once with MOPS, no phosphate medium, and resuspended in 1 mL of the same medium supplemented with amino acids (20 mg/mL excluding methionine). Cells were incubated at 37 °C for 15 min to induce the expression of alkaline phosphatase.

Pulse−Chase Analysis. Cells were cultured, washed, and resuspended in MOPS medium as described above. Cells were radiolabeled with 40 μCi of L-[^{35}S]methionine for 40 s and chased with 4 mg/mL nonradioactive methionine for 30 s and 1 min. Alkaline phosphatase was immunoprecipitated as described previously (*38*).

Azide Treatment. Cells were incubated with sodium azide ranging in final concentration from 2 to 8 mM at 37 °C for 5 min prior to radiolabeling and chasing for 1 min as described above. Immunoprecipitated proteins were separated by electrophoresis on 7.5% SDS−PAGE[1] (*39*).



B.

Fusion Protein	Signal Peptide	Pathway
GST-AP	MKQSTI**ALALLPLLF**TPVTKA	
GST-β-lac	MSIQHFR**VALIPFFAAFCL**PVFA	*E.coli* Sec
GST-MBP	MKIKTGAR**ILALSALTTMMF**SASALA	
GST-TorA	MNNNDLFQAS*RR***RFLAQLGGLTVAGMLGPSLL**TPRRATAAQA	
GST-SufI	MSLS*RR*QFIQ**ASGIALCAGAV**PLKASA	*E.coli* Tat
GST-Yack	MQ*RR*DFLK**YSVALGVASALPLW**SRAVFA	
GST-HyaA	MNNEETFYQAMRRQGVT*RR*SF**LKYCSLAATSLGLGAGMA**PKIAWA	
GST-M13	MKKSLVLK**ASVAVATLV**PMLSFA	*E.coli* YidC

FIGURE 1: The different GST−signal peptide fusions tested. (A) Schematic representation of the GST fusion proteins. (B) Characteristics of the signal peptide segment of the fusion proteins. Core region sequences are shown in boldface, and the twin arginine motif is italicized. Definition of the core region boundaries followed the principles outlined by von Heijne (*56*). Fusions were named after the protein from which the signal peptide originated, with a GST prefix. All fusions contain the first 30 amino acids of the early mature region of alkaline phosphatase. Abbreviations: AP, alkaline phosphatase; β-lac, β-lactamase; MBP, maltose-binding protein; M13, M13 procoat protein.

Alkaline Phosphatase Activity Assay. A spectrophotometric assay was employed to provide a quantitative measure of periplasmic alkaline phosphatase as described previously (*40*).

RESULTS

To explore the substrate specificity of *E. coli* SecA and the role of SecA−signal peptide interactions in sorting among different transport pathways, we constructed a series of GST−signal peptide fusions (Figure 1). These fusions involve signal peptides from preproteins that utilize different transport pathways linked to GST. The constructs were C-terminally fused to GST to ensure that the fusion proteins would not translocate across the membrane. Following expression in *E. coli*, the incorporation of GST could be exploited for the rapid purification of the fusions from the cell lysate using glutathione−agarose affinity chromatography.

All fusion proteins also contain the first 30 amino acids of the early mature region of alkaline phosphatase in order to examine the parameters of this region, and the possible interplay with the signal peptide, which promote SecA interactions. This system was especially suitable for testing SecA−substrate specificity because of its modular design generated by the presence of unique restriction sites within the DNA coding for the constructs, thus permitting the rapid generation of parallel fusions with different signal peptides and/or segments in the early mature region. It also allows for utilizing substrates with longer polypeptides, which is not feasible using chemically synthesized peptides. A SecA−lipid ATPase assay was used to evaluate the interaction of SecA with the fusion, because it permits the examination of a large number of substrates efficiently. It serves as an earmark of a productive interaction of the signal peptide with

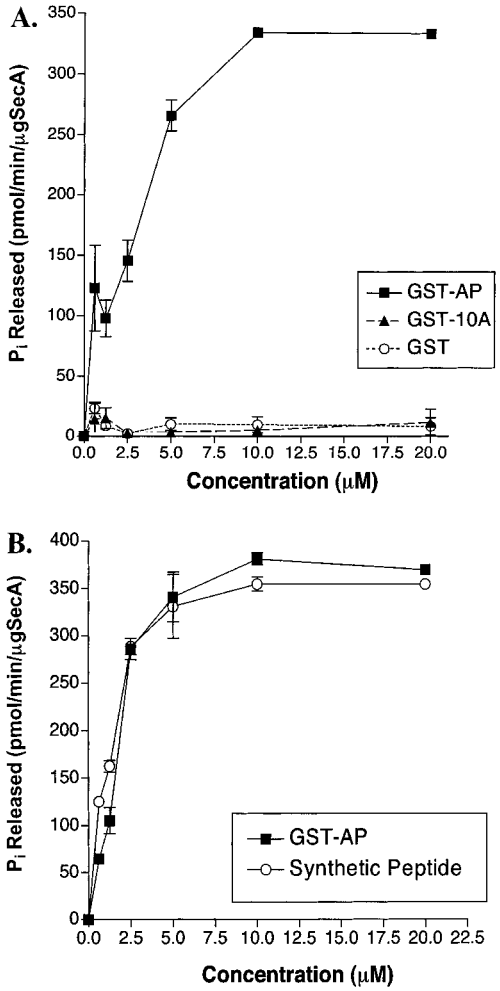


FIGURE 2: Comparison of GST, GST fusions, and a synthetic signal peptide on *E. coli* SecA−lipid ATPase activity. (A) SecA−lipid ATPase activity in the presence of GST−AP, GST−10A, and GST. The sequence of the nonfunctional signal peptide segment of the 10A fusion is MKQSTAAAAAAAAAATPVTKA. (B) Comparison of SecA−lipid ATPase activity induced by GST−AP and a synthetic peptide corresponding to the 21 residues of wild-type alkaline phosphatase signal peptide plus one C-terminal cysteine (referred to as synthetic peptide) (*17*, *21*). All assays were conducted in 50 mM HEPES−KOH (pH 7) containing 30 mM KCl, 30 mM NH_4Cl, 0.5 mM $Mg(OAc)_2$, 1 mM dithiotheritol, 4 mM ATP, BSA (0.5 mg/mL), SecA (40 μg/mL), and *E. coli* phospholipids (320 μg/mL). Concentrations of the fusions and the synthetic peptide are given in the figure. Each data point represents an average of triplicates ± SE.

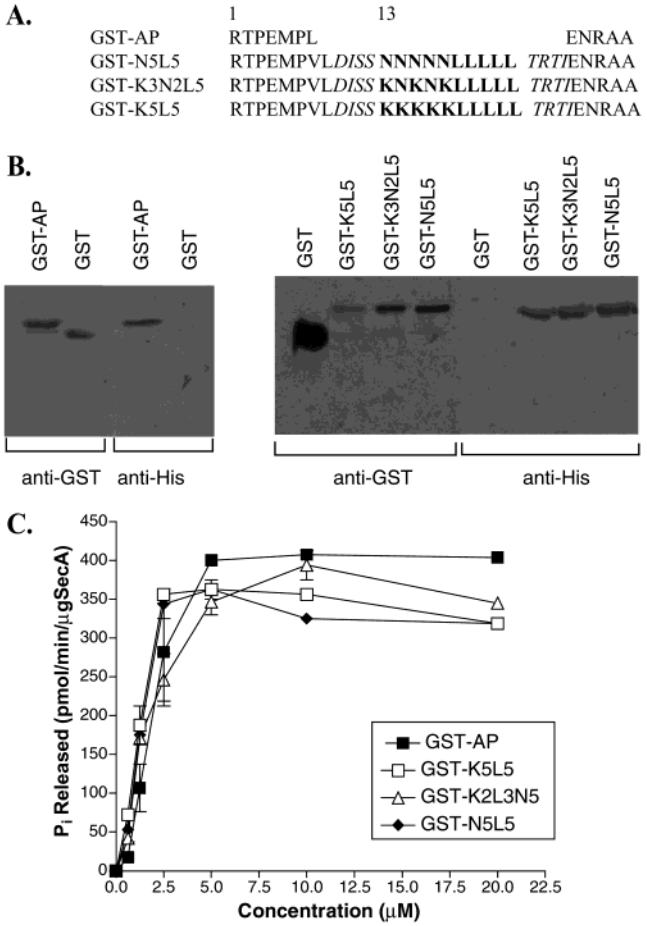
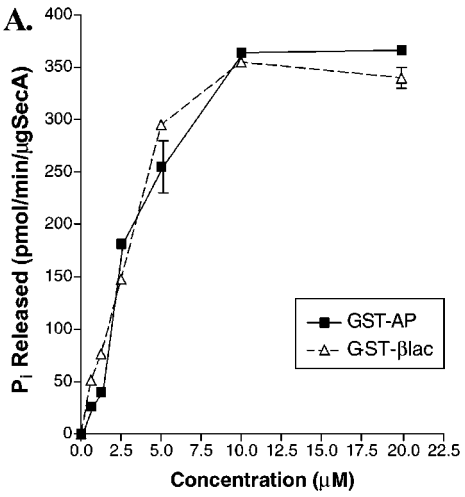


FIGURE 3: Effect of changes in the early mature region of alkaline phosphatase on stimulation of *E. coli* SecA−lipid ATPase activity. (A) Amino acid sequence of mutant GST−APs, in which additional sequences are inserted at residue 13 of the mature region. The inserted sequences are shown in boldface, and the amino acids generated during the cloning procedure are shown in italics. (B) Western blot analysis of the constructs used to verify that all C-terminal residues are intact. Constructs were probed with either anti-GST or anti-His monoclonal antibodies as indicated. (C) SecA−lipid ATPase activity generated in the presence of GST fusions with the corresponding sequences. The reaction conditions were as described in the legend of Figure 2. In addition, the reaction mixture contained 40 μM acetic acid and 80 μM urea to maintain the constructs with the additional hydrophobic residues in a soluble form as done previously (*57*). All constructs in this assay were treated identically for comparison.

SecA that indicates not only binding but also sufficient interaction to stimulate catalytic activity (*21*). Excellent correlation has been found between the stimulation of SecA−lipid ATPase activity by isolated signal peptides and the in vivo translocation activity of preproteins containing the corresponding signal sequences (*17*, *21*, *24*, *41*).

To establish the assay system and verify that GST fusions give an appropriate reading of the interaction of a particular signal peptide with SecA, we examined constructs corresponding to signal peptides that have been well studied both in vivo and in vitro. For this purpose GST fusions corresponding to the wild-type alkaline phosphatase signal peptide (GST−AP) and a nonfunctional signal peptide with a weakly hydrophobic core region (GST−10A) were generated, in addition to GST alone. When these were evaluated for stimulation of SecA−lipid ATPase activity (Figure 2A), we found that neither GST nor GST−10A had any stimulatory effect, in marked contrast to the dose-dependent stimulation seen with GST−AP. Furthermore, no significant difference in ATPase activity was observed between GST−AP and a synthetic wild-type alkaline phosphatase signal peptide (Figure 2B). These results are in good agreement with previously published work using chemically synthesized peptides corresponding to these signal peptide regions alone (*21*) and the behavior of the corresponding mutants in vivo (*41*). These results also indicate that the early mature region of alkaline phosphatase does not contribute to the observed stimulation of SecA−lipid ATPase activity.

The possible role of the early mature region was further examined using constructs with unusual sequence motifs inserted at residue 13 of the mature region of alkaline phosphatase (Figure 3A). The corresponding sequence motifs have been analyzed in vivo (*27*) and found to confer SecB



B.

Fusion Protein	Hydrophobicity		Net charge
	Kyte & Doolittle	Fauchère	
GST-AP	2.8	1.34	+2
GST-β-lac	2.6	1.25	+1
GST-10A	1.8	0.31	+2

FIGURE 4: Effect of different *E. coli* Sec signals on SecA−lipid ATPase activity parallels their hydrophobicity characteristics. (A) SecA−lipid ATPase activity in the presence of GST fusions either with the alkaline phosphatase or β-lactamase signal peptide. The reaction conditions were as described in the legend of Figure 2. (B) Hydrophobicity and net charge of the signal peptide segments of the fusions tested. GST−10A is used as an example of a fusion with a nonfunctional signal peptide. For hydrophobicity, the numbers given represent the mean hydrophobicity per residue of the core region only and are based on the scales described by Kyte and Doolittle (*58*) and Fauchère et al. (*59*).

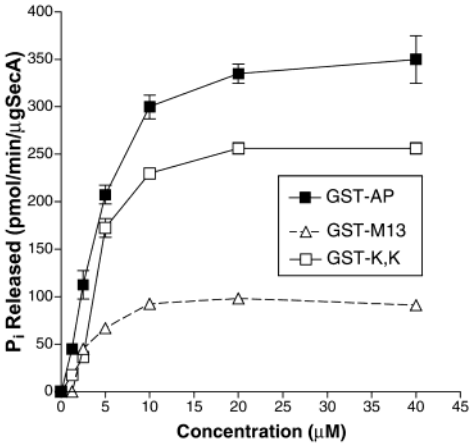
dependence to the preprotein. As an example, WT-K_5L_5 accumulated in the cytosol relative to wild-type alkaline phosphatase unless SecB was present. Thus, we considered the possibility that the motifs would influence the SecA−ATPase activity in the absence of SecB. However, we found that the ATPase activities of these constructs were comparable to that of GST−AP (data not shown). To ensure that the C-terminal end of these fusions was intact, we made a parallel set of constructs which incorporates a C-terminal hexahistidine tag. Western blot analysis verified that the full-length fusion was isolated (Figure 3B), and the ATPase activities of these constructs were similar (Figure 3C) and behaved the same as the untagged version. This further indicates that the early mature region of alkaline phosphatase has no observable impact on the signal peptide stimulated SecA−ATPase activity.

Using this experimental strategy for probing SecA−signal peptide interactions, we examined whether other *E. coli* Sec signals stimulated SecA ATPase activity to the same extent. A comparison between a fusion with the β-lactamase signal peptide (Figure 4A) and GST−AP revealed no significant difference in the extent to which these stimulated SecA−lipid ATPase activity. Furthermore, a comparable construct with maltose-binding protein gave similar results (data not shown). This is in line with the similarity of the overall signal sequence features of these peptides (Figure 4B) and the clear difference in hydrophobicity from the nonfunctional 10A. These results suggest that different *E. coli* Sec signals are recognized by, and effectively interact with, SecA in a similar manner.

M13 procoat protein serves as the prototype of proteins with typical cleavable N-terminal signal sequences that insert in the *E. coli* inner membrane via a Sec-independent mechanism (*42*−*44*). Recently, Samuelson and co-workers (*12*) showed that the membrane insertion of M13 procoat protein requires YidC. Consequently, we tested whether SecA interacts with the M13 signal peptide in vitro and if this interaction plays a role in targeting in vivo. Figure 5A shows that GST−M13 with the wild-type M13 signal peptide had low SecA−lipid ATPase activity compared to GST−AP. Inspection of the M13 signal sequence (Figure 1B) suggests that this low response may result from the effectively low hydrophobicity of the M13 core region (Figure 5B), which results from the inclusion of a lysine residue at position −16. To test this hypothesis, we constructed another fusion, GST−K,K, where we displaced this lysine to position −19 of the M13 signal sequence, thereby extending the core boundary and increasing the string of uninterrupted hydrophobic residues. This GST−K,K construct showed significantly greater ATPase simulation than GST−M13 (Figure 5A). The rank order of SecA−lipid stimulation by these fusions parallels the extent of transport in vivo (Figure 5C) of signal peptide mutants of alkaline phosphatase where the wild-type signal peptide is replaced by either M13 or the K,K signal peptide. The Sec dependence of these mutants was examined by treating the cells with sodium azide, which inhibits the translocation ATPase activity of SecA in vivo (*45*) and does not seem to inhibit the processing of full-length M13 procoat in vivo (*46*). Interestingly, precursor processing of both of these mutants was inhibited by azide (Figure 5D). In our studies the targeting elements within the mature alkaline phosphatase protein must override those of the M13 signal sequence. This is consistent with previous studies that showed the Sec dependence of procoat mutants in which the periplasmic loop of the procoat was lengthened by insertion of 60, 98 (*46*), and 174 (*44*) amino acids of the SecA-dependent protein OmpA.

The Tat pathway is used in *E. coli* to transport folded proteins to and across the inner membrane (*47*−*49*). Substrates of this pathway carry N-terminal signal peptides that retain the tripartite organization of the Sec signals, yet are characterized by a conserved twin arginine motif S/TRRX-FLK at the boundary between the N-terminal region and the hydrophobic core (*7*). It has been suggested that the low hydrophobicity of the core (*28*) and C-region basic residues (*50*) lead to avoidance of the Sec machinery. To examine whether these features preclude an interaction with SecA, we tested fusions with different *E. coli* Tat signals, namely, SufI, TorA, Yack, and HyaA, for their ability to stimulate SecA−lipid ATPase activity in vitro (Figure 6). With the exception of the moderately high response from TorA, these signals produced only a low level of ATPase activity (Figure 6A), though surprisingly more pronounced than that observed with the fusion containing a nonfunctional 10A signal peptide (Figure 2A). The response of the TorA signal was unexpected in view of the presence of Sec avoidance elements including the apparently low mean hydrophobicity of its core region.

A.



B.

Fusion Protein	Hydrophobicity		Net charge
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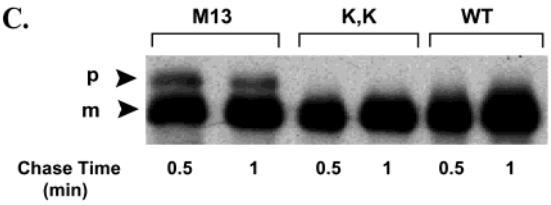
GST-M13	2.25	0.73	+3
GST-K,K	2.68	0.931	+3
GST-10 A	1.8	0.31	+2

C.



D.

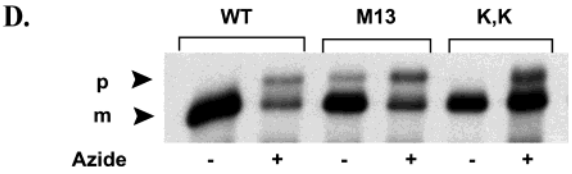


FIGURE 5: M13 signal sequence directs alkaline phosphatase to the Sec pathway. (A) SecA−lipid ATPase activity induced by fusions carrying the signal peptide of alkaline phosphatase, M13, or an M13 mutant. The sequence of the signal peptide segment of GST−K,K is MKKSK**LVLASVAVATLV**PMLSFA, with the core region sequence shown in boldface. The reaction conditions were as described in the legend of Figure 2. (B) Hydrophobicity and net charge of the signal peptide segments of the fusions. Hydrophobicity is calculated as in the legend for Figure 4B. (C) Pulse−chase analysis of in vivo transport of full-length alkaline phosphatase with mutant signal peptides. AW1043 cells were radiolabeled and chased as described under Experimental Procedures. Migration of precursor is indicated by arrowhead p; mature enzyme is indicated by m. WT = wild-type alkaline phosphatase; M13 and K,K refer to signal peptide mutants of alkaline phosphatase in which the wild-type signal peptide is replaced by the M13 and K,K signal peptides, respectively. (D) Effect of azide treatment on precursor processing of the wild-type and mutant preproteins. AW1043 cells were incubated with or without 4 mM sodium azide for 5 min prior to labeling as described under Experimental Procedures.

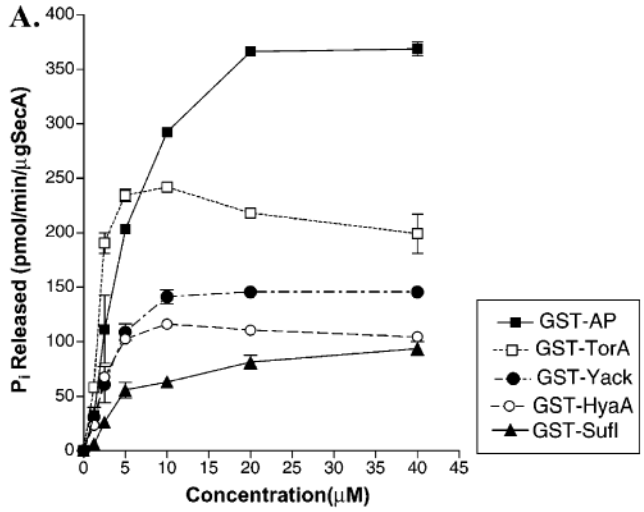
However, upon further examination, it is evident that the total hydrophobicity of its lengthy hydrophobic core (Figure 6B) is greater than that of the other Tat signals examined and nearly as high as the AP signal. Nonetheless, the transport in vivo of mutants of alkaline phosphatase for which the wild-type signal peptide is replaced by the Tat signals, SufI, TorA, and Yack, showed processing of the precursor form was inefficient (Figure 6C). Furthermore, there was no alkaline phosphatase activity detected in the periplasm (Figure 6D), suggesting that the preprotein processing that did occur reflected preproteins that were targeted to the membrane but released on the cytosolic side following processing. This possibility is consistent with observations by Sambasivarao et al. (*51*), where the dimethyl sulfoxide reductase holoenzyme was targeted to the membrane by a TorA signal peptide, processed, and then released on the cytosolic side of the membrane. We further assessed the dependence of preprotein processing of these mutants on SecA using azide sensitivity (Figure 6E) and found, in contrast to full-length alkaline phosphatase, that the mutants were unaffected by azide, indicating that these signals do not interact with SecA in vivo.

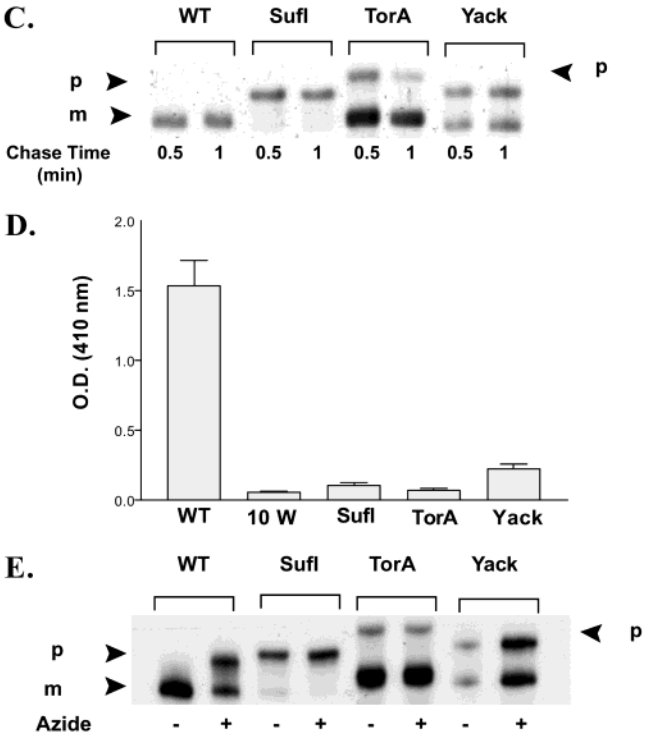
DISCUSSION

To define the role of SecA in preprotein targeting and translocation, most studies have focused on either OmpA (*15*, *52*, *53*) or OmpF-Lpp (*18*, *19*, *54*) as substrates. To begin to delineate the specificity profile of SecA, however, it is critical to test a variety of substrates, yet the purification of many different preproteins from *E. coli* makes this pursuit an enormous task. The process is made more manageable with the development of a GST fusion system which enables the examination of several different natural sequences in parallel. Through the use of this system we have demonstrated that, among the different signal peptides tested, *E. coli* Sec signals produce the most pronounced stimulation of the ATPase activity of SecA.

Since most models of preprotein translocation involve repeated cycles of ATP binding and hydrolysis by SecA coupled with the transport of successive regions of the preprotein, we designed our fusions to emulate a typical translocation loop, with the signal peptide as one arm of the loop and the early mature region as the other. Using this system we were able to address whether the presence of the early mature region of the preprotein altered the activity of SecA in the presence of the signal peptide. The first 30 amino acids of alkaline phosphatase are representative of much of the rest of the enzyme with respect to hydrophobicity, charge, and variety of amino acids and should provide an indication of the extent to which these regions could competitively bind SecA. Even the incorporation of some unusual sequence motifs in that region, which confer a marked SecB dependence in vivo, had no impact on the level of interaction with SecA in vitro. It remains possible that nonstimulatory interactions with the mature protein do occur and/or that regions distal to the signal peptide can promote ATPase activity. Nonetheless, full-length proOmpA assayed under the same conditions as utilized here stimulated SecA ATPase activity to a level comparable to that of a functional signal peptide alone (data not shown). Taken together, our results indicate that as long as an effective signal peptide is present, it will override any interaction with the mature protein; therefore, if other interactions with the preprotein do occur in vivo, the signal peptide region must become inaccessible to SecA, for example, through cleavage by leader peptidase.

In this study, we use a variety of natural peptides which differ in length, charge, and patterns of hydrophobic residues. Nonetheless, the importance of hydrophobicity in SecA





B.

Fusion Protein	Hydrophobicity		Net charge
	Kyte & Doolittle	Fauchère	
GST-AP	2.8 [28]	1.34 [13.4]	+2
GST-TorA	1.4 [27.1]	0.83 [15.9]	+4
GST-SufI	1.9 [20.6]	0.68 [7.5]	+3
GST-Yack	1.2 [19.4]	0.89 [12.4]	+3
GST-HyaA	1.2 [18.2]	0.65 [10.4]	+4
GST-10A	1.8 [18]	0.31 [3.1]	+2

FIGURE 6: *E. coli* Tat signals fail to efficiently direct the transport of alkaline phosphatase. (A) SecA−lipid ATPase activity in the presence of GST fusions with a variety of Tat signal peptides. The reaction conditions were as described in the legend of Figure 2. (B) Hydrophobicity and net charge of the signal peptide segments of the fusions. Hydrophobicity is calculated as in the legend for Figure 4B; the numbers in brackets represent the total hydrophobicity of the core region. (C) Pulse−chase analysis of in vivo transport of signal peptide mutants of alkaline phosphatase. SufI, TorA, and Yack refer to signal peptide mutants of alkaline phosphatase in which the wild-type signal peptide is replaced by Suf I, TorA, or Yack signal peptide, respectively. (D) Alkaline phosphatase activity in the periplasm of AW1043 cells expressing alkaline phosphatase with the signal peptides indicated. The assay procedure was described under Experimental Procedures. 10W is a nonfunctional mutant of alkaline phosphatase where the hydrophobic core of the wild-type signal peptide is replaced by 10 tryptophan residues. This mutant has been shown to be membrane inserted but not translocated (*40*). (E) Effect of sodium azide on precursor processing in vivo. AW1043 cells were incubated with 4 mM sodium azide for 5 min prior to labeling as described under Experimental Procedures.

interactions in vitro is noted. For example, the M13 fusion induced only a weak response from SecA while that from the K,K derivative was more marked. The latter signal peptide was identical to the former in composition, but its sequence was altered to generate a longer core. Of the signal peptides of proteins which use the Tat pathway, only the most hydrophobic, TorA, produced substantial levels of ATPase activity in vitro. Hydropathy analysis of its sequence reveals that while the mean hydrophobicity per residue is low, the total hydrophobicity over its lengthy core is comparable to that of the alkaline phosphatase signal peptide. None of the natural sequences tested are as weakly hydrophobic as the nonfunctional signal peptide, 10A, and none were as indifferent to SecA. Collectively, the data indicate that the in vitro stimulation of SecA must be substantial for a preprotein to have the possibility of utilizing SecA in vivo, but such activity does not necessarily mean that the preprotein will do so.

The TorA signal peptide interacted well with SecA in vitro but, when linked to alkaline phosphatase, was processed by an azide insensitive pathway in vivo. This suggests that the TorA signal peptide may carry a Sec avoidance signal to elements in the Sec machinery other than SecA, such as SecY or SecB, or that the signal peptide is simply not SecA accessible in vivo. Consistent with this possibility, Oresdnik et al. (*55*) showed that a protein, DmsD, interacted specifically with the signal peptide segment of TorA and DmsA preproteins, and they suggest that it escorts these proteins from the ribosome to the Tat pathway. Our in vivo studies indicate that alkaline phosphatases fused to different Tat signals are not transported via a SecA-dependent pathway due to the presence of the Tat signal. Yet it is not a consequence of direct SecA avoidance per se but rather may reflect the overriding interaction with another component, such as DmsD, which shuttles it away.

About 20% of all *E. coli* proteins are exported from the cytoplasm (*2*) via a targeting signal and enter into one of at least three different transport pathways: the Sec, YidC, or Tat pathway. Directing exported proteins into the appropriate pathway must require specific interactions at an early stage of export to maintain efficiency and avoid mistargeting. SecA provides one such point of control recognizing proteins destined for the *E. coli* Sec pathway most readily, though other points of distinction must also be involved. Future studies involving the use of the library of truncated preproteins developed here and other transport components will

provide a useful comparison and should highlight other critical interactions that influence the overall transport process.

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