The EMBO Journal vol.15 no.19 pp.5209–5217, 1996

Targeting of signal sequenceless proteins for export in *Escherichia coli* with altered protein translocase

William A.Prinz, Christoph Spiess[1], Michael Ehrmann[1], Clark Schierle and Jon Beckwith[2]

Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115, USA and [1]Universität Konstanz, Fakultät für Biologie, Postfach 5560, D606, D-78434 Konstanz, Germany

[2]Corresponding author

Most extracytoplasmic proteins are synthesized with an N-terminal signal sequence that targets them to the export apparatus. *Escherichia coli prlA* mutants (altered in the *secY* gene) are able to export cell envelope proteins lacking any signal sequence. In order to understand how such proteins are targeted for export, we isolated mutations in a signal sequenceless version of alkaline phosphatase that block its export in a *prlA* mutant. The mutations introduce basic amino acyl residues near the N-terminus of alkaline phosphatase. These changes do not disrupt an N-terminal export signal in this protein since the first 25 amino acids can be removed without affecting its export competence. These findings suggest that signal sequenceless alkaline phosphatase does not contain a discrete domain that targets it for export and may be targeted simply because it remains unfolded in the cytoplasm. We propose that basic amino acids near the N-terminus of a signal sequenceless protein affect its insertion into the translocation apparatus after it has been targeted for export. These findings allow the formulation of a model for the entry of proteins into the membrane-embedded export machinery.

Keywords: *Escherichia coli*/*prlA* mutations/protein export/protein targeting/signal sequenceless proteins

Introduction

Most extracytoplasmic proteins are synthesized with an N-terminal signal sequence that targets them for export. The function and structure of these sequences are conserved from bacteria to mammals and signal sequences from different organisms are often interchangeable (von Heijne, 1985; Gierasch, 1989). Signal sequences are generally about 25 amino acids long and share three physical characteristics: a positively charged N-terminal, a core of at least six hydrophobic amino acids, and a more polar C-terminal domain lacking charged amino acids where cleavage by signal peptidase takes place (see Izard and Kendall, 1994, for a recent review). Mutations in the first two domains can affect the rate at which a protein is translocated both *in vivo* and *in vitro* (Gennity *et al.*, 1989).

While the function and physical properties of signal sequences are preserved across species, a variety of ways in which signal sequences target proteins for export have been identified. In mammalian cells, proteins with signal sequences are bound by a 16S ribonucleoprotein particle, signal recognition particle (SRP), as they emerge from the ribosome (Walter and Johnson, 1994). The SRP targets the translating ribosome to the endoplasmic reticulum (ER) membrane where a second signal sequence recognition event is performed by a component of the protein translocation apparatus (Jungnickel and Rapoport, 1995; Belin *et al.*, 1996). In addition, nascent-polypeptide-associated complex (NAC) may play a role in protein targeting by preventing SRP from binding proteins without signal sequences (Lauring *et al.*, 1995). In contrast, many proteins in *Saccharomyces cerevisiae* do not require SRP for export (Walter and Johnson, 1994; High, 1995). It is not known how signal sequences are recognized for SRP-independent transport, but it is likely that a heterotetrameric complex of membrane proteins, the Sec62/63p complex, plays a role in this process (Panzner *et al.*, 1995).

Less is known about how signal sequences target proteins for export in *Escherichia coli*. Signal sequences can interact with the mature portion of a secreted protein to help maintain it in an unfolded translocation-competent state (Park *et al.*, 1988; Laminet and Plückthun, 1989). The export-specific cytoplasmic chaperone SecB interacts with a subset of exported proteins and prevents them from folding. While it has been suggested that SecB recognizes signal sequences (Altman *et al.*, 1990; Watanabe and Blobel, 1989, 1995), the preponderance of evidence indicates that SecB binds to the mature domains of secreted proteins (Randall *et al.*, 1990; Derman *et al.*, 1993a). SecB is also thought to target its substrate proteins to the translocation machinery in the membrane (Kumamoto, 1990). While *E.coli* has an SRP homolog which can bind proteins with signal sequences (Poritz *et al.*, 1990; Ribes *et al.*, 1990; Luirink *et al.*, 1992), SRP does not appear to be required for the export of many proteins (Phillips and Silhavy, 1992) and the role of SRP in protein translocation is not clear. It has also been suggested that SecA recognizes signal sequences (Lill *et al.*, 1990). Finally, studies on the interaction of signal sequences with lipid bilayers have been used to argue that the insertion of signal sequences into the cytoplasmic membrane facilitates protein export (Jones *et al.*, 1990).

One method that has been used to try to identify *E.coli* proteins which interact with signal sequences and recognize them as export signals *in vivo* is to isolate mutants that are able to export proteins with defective signal sequences (Emr *et al.*, 1981). These mutants might have compensatory mutations that allow the recognition of a defective signal sequence. This class of mutation, the *prl* mutations, has been detected in many of the genes encoding components of the secretion apparatus including *secY*, *secA*, *secE* and *secG* (Bieker *et al.*, 1990; Schatz

and Beckwith, 1990; D.Belin, personal communication). *prl* mutants are not allele-specific; they can export proteins with a wide variety of signal sequence defects (Randall *et al.*, 1987). One explanation for *prl* mutations is that they disrupt a proof-reading system of the secretory apparatus that ordinarily prevents proteins with defective signal sequences from entering the export pathway (Osborne and Silhavy, 1993).

The strongest *prl* mutations (those best able to promote the export of proteins with defective signal sequences) are found in *secY* and are named *prlA* mutations. *prlA* mutations even allow the export of secreted proteins for which the signal sequence has been totally deleted (Derman *et al.*, 1993a). To date, this latter finding has held for all proteins tested, including alkaline phosphatase (AP), MalE, β-lactamase, LamB and MalS (Bowden *et al.*, 1992; Derman *et al.*, 1993a; Flower *et al.*, 1994; M.Ehrmann, unpublished results). In the case of AP without its signal sequence, ~30% of the AP is exported in a *prlA4* strain (a strong *prlA* mutation). If cell envelope proteins without their signal sequences can be efficiently translocated, one might expect that cytoplasmic proteins would also be exported in *prlA* mutants. However, cytoplasmic proteins are not exported in *prlA* strains to any significant extent. Fractionation of wild-type cells and *prlA* mutants (including *prlA421* cells; see Results) followed by two-dimensional SDS–PAGE revealed no significant difference in the distribution of proteins between the periplasm and cytoplasm of these strains (D.Boyd and S.Pedersen, personal communication).

Why do *prlA* mutants export cell envelope proteins missing their signal sequences, but not cytoplasmic proteins? One possibility is that all exported proteins contain sequences, in addition to the N-terminal signal sequence, that reside in their mature regions and contribute to their targeting for export. Alternatively, it is possible that all exported proteins have evolved so as to fold slowly or incompletely when expressed in the cytoplasm and any unfolded protein is targeted for export in *prlA* mutants (Derman *et al.*, 1993a). In order to distinguish between these possibilities, and to learn more about how proteins are targeted for export, we isolated mutations in signal sequenceless AP and MalS that prevent these proteins from being exported in *prlA4* cells.

Results

Mutations in APΔ2-22 that block its export in a prlA4 strain

The plasmid pAID135 encodes a version of alkaline phosphatase, called APΔ2-22, which lacks its entire signal sequence and the first amino acid of the mature protein (Derman *et al.*, 1993a). When expressed in *prlA*$^+$ cells, APΔ2-22 is not exported to the periplasm so that colonies of this strain are white on plates with 5-bromo-3-chloro-3-indolyl-phosphate (XP, a chromogenic substrate for AP). By contrast, *prlA4* cells export APΔ2-22 to the periplasm and form blue colonies on plates with XP. Thus, it is possible to screen for mutations in APΔ2-22 that prevent it from being exported in a *prlA4* strain by screening for colonies that are white or light blue on media containing XP. However, this approach would also yield mutations that reduce the amount of APΔ2-22 or its specific activity. In order to avoid having to screen out these mutations—which would be quite frequent—the screen was performed under conditions which require that the mutant proteins still be capable of exhibiting AP activity. This can be done by including a genetic selection that requires an active AP in the cytoplasm.

In wild-type cells, APΔ2-22 cannot fold into an active conformation in the cytoplasm because its essential disulfide bonds do not form (Derman *et al.*, 1993b). However, in the cytoplasm of cells lacking thioredoxin reductase (encoded by *trxB*), the reducing environment is altered so that disulfide bonds can form in APΔ2-22 and the protein folds into an enzymatically active conformation. In a *trxB* strain, active APΔ2-22 can substitute for a number of cytoplasmic phosphatases including phosphoserine phosphatase (the product of *serB*; Derman *et al.*, 1993b). A *serB* strain is not able to grow on media lacking serine. By contrast, a *trxB serB* strain expressing APΔ2-22 is able to grow on media without serine because APΔ2-22 is able to substitute for phosphoserine phosphatase. Reducing the amount of APΔ2-22 in this strain by as little as 10-fold prevents it from growing without serine supplementation (data not shown). Thus, these properties allow us to select for the continued presence of active alkaline phosphatase in the cytoplasm. Mutations which dramatically affect the amount or specific activity of APΔ2-22 will prevent a *trxB serB* strain from growing without serine. A *trxB serB prlA4* strain producing APΔ2-22 is also able to grow on media without serine and forms blue colonies on plates containing XP. An isogenic *prlA*$^+$ strain does not form blue colonies (presumably because XP is not able to enter the cytoplasm). Therefore, a mutation in APΔ2-22 that still allows growth without serine, but causes a *trxB serB prlA4* strain to be white or light blue on media containing XP will probably be a mutation that prevents the export of APΔ2-22 while not affecting the ability of APΔ2-22 to substitute for phosphoserine phosphatase in the cytoplasm.

The genetic screen and selection was performed by transforming WP610 with hydroxylamine-mutagenized pAID135 and plating on minimal media containing XP and lacking serine. Sixteen independent light blue colonies were characterized. In order to determine whether the APΔ2-22 encoded by the mutant plasmids was defective for export in a *prlA4* strain, but could still fold into an active conformation in the cytoplasm of a *trxB* strain, the plasmid from each mutant was isolated and used to transform AD494 (*trxB prlA*$^+$) and WP637 (*trxB*$^+$ *prlA4*) and the AP activity of the resulting strains assessed. The plasmids from 11 of the 16 mutants encoded APΔ2-22 that was exported poorly in WP637, but was still active in the cytoplasm of AD494. The plasmids from the remaining five mutants encoded APΔ2-22 that had low activity in both strains and were not further characterized. The *phoAΔ2-22* allele on these 11 plasmids was sequenced; in nine the fourth codon of *phoAΔ2-22* was changed from GAA to AAA while in the remaining two the ninth codon of *phoAΔ2-22* was changed from GAA to AAA. These mutations convert the glutamate residues at positions four and nine of APΔ2-22 to lysines (Figure 1). Table II shows the AP activity of these mutants in various strains.

Since both mutations introduce basic amino acids near the N-terminus of APΔ2-22, we assessed whether another

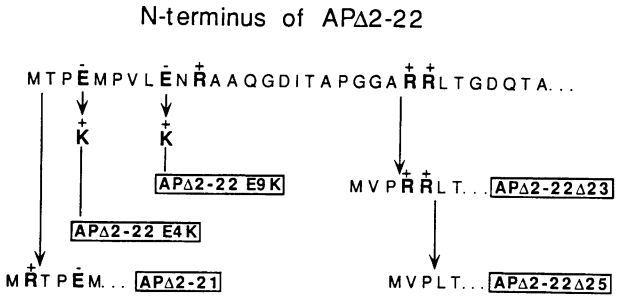


Fig. 1. Sequence of the N-termini of APΔ2-22 and various derivatives.

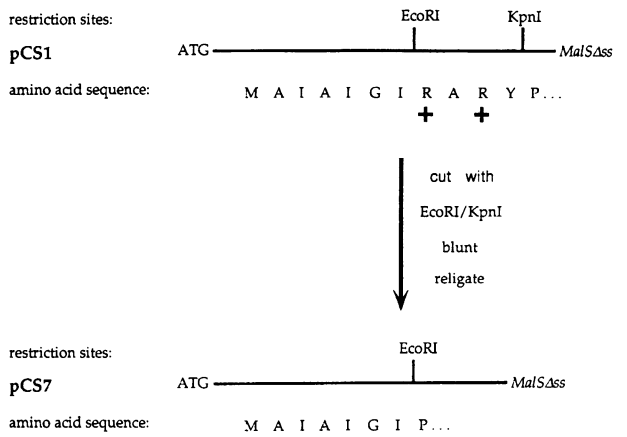


Fig. 2. Sequence of the N-termini of ΔssMalS and ΔssΔR'sMalS and an outline of how pCS7 was derived from pCS1.

construct in which a basic amino acyl residue is introduced near the N-terminus of APΔ2-22 would have the same effect. The plasmid pAID134 encodes APΔ2-21 (Derman, 1994), which is identical to APΔ2-22 except that it contains an additional arginine following the N-terminal methionine of the protein (Figure 1). In order to determine whether this addition affects the ability of APΔ2-21 to be exported in a *prlA4* strain, pAID134 was introduced into WP637 and AD494 and the AP activity of the resulting strains was determined (Table II). Relative to the amount of APΔ2-22 made from pAID135, cells containing pAID134 make ~10-fold less APΔ2-21 (Derman, 1994). Accordingly, AD494 expressing APΔ2-21 has ~10-fold less AP activity than the same strain expressing APΔ2-22 (Table II). Since APΔ2-21 is exported more than 170-fold less efficiently in a *prlA4* strain than is APΔ2-22 (Table II), the additional arginyl group near the N-terminus of APΔ2-21 greatly reduces the ability of APΔ2-21 to be exported in a *prlA4* mutant. Therefore, introducing a positively charged residue at a number of positions near the N-terminus of APΔ2-22 blocks its export in a *prlA4* mutant.

Positively charged amino acids near the N-terminus of ΔssMalS affect its ability to be exported in a prlA4 strain

MalS is a periplasmic *E.coli* amylase (Schneider *et al.*, 1992). A plasmid, pCS1, was constructed which encodes MalS without its signal sequence under the control of the P_{BAD} promoter of the arabinose operon (Figure 2). This plasmid encodes a protein (ΔssMalS) in which the signal sequence of MalS has been replaced by ten amino acids encoded by pBAD18s. These ten amino acids include two arginyl residues (Figure 2). MalS is enzymatically active when it is exported to the periplasm, but remains inactive in the cytoplasm (unpublished results). Therefore, the amount of amylase activity in strains expressing different versions of MalS reflects how much of the protein is exported. When pCS1 was introduced into CS12 (*prlA*$^+$) and CS18 (*prlA4*), MalS was not efficiently exported in either strain (Table III). A second plasmid was constructed from pCS1 that encodes a version of ΔssMalS (ΔssΔR'sMalS) that does not include the two arginyl residues near the N-terminus of this protein (Figure 2). Unlike the product of the initial construct, the amylase without these two arginyl residues is exported in a *prlA4* strain (Table III). Eliminating these residues does not change the level of expression or stability of ΔssMalS (data not shown). Therefore, as with APΔ2-22, positively charged amino acyl residues near the N-terminus of ΔssMalS block its export in a *prlA4* mutant.

The N-terminus of APΔ2-22 is not essential for export

One explanation for our results is that AP contains export targeting information within the N-terminal portion of the mature protein, and the introduction of positively charged residues disrupts this signal in APΔ2-22. If this were true, the deletion of the N-terminus of APΔ2-22 would prevent it from being exported in *prlA* mutants. Two plasmids were constructed that encode versions of APΔ2-22 with N-terminal truncations. APΔ2-22Δ23, encoded by pWP851, lacks the first 23 amino acids of the mature sequence of APΔ2-22, while APΔ2-22Δ25, encoded by pWP852, is missing the first 25 amino acids of APΔ2-22 (Figure 1). The first three amino acids of both proteins are MVP. APΔ2-22Δ23 contains two arginyl groups near its N-terminus which are absent in APΔ2-22Δ25.

The plasmids encoding these proteins were introduced into DHB4 (*prlA*$^+$) and WP827 (*prlA421*, a *prlA* allele that will be described below). Since AP is oxidized to generate disulfide bonds when it is exported to the periplasm, the amount of oxidation of APΔ2-22Δ23 and APΔ2-22Δ25 indicates the degree to which they are exported. Pulse–chase labeling of these strains followed by non-reducing SDS–PAGE was used to determine the oxidation state of these proteins when they are expressed in DHB4 and WP827. The strains were labeled for 1 min with [^{35}S]methionine and chased with cold methionine for 10 min. Samples were taken at the times indicated in Figure 3. A protein band running at a position corresponding to oxidized AP is seen only when APΔ2-22Δ25 is expressed in WP827 (Figure 3). That this is indeed oxidized AP is indicated by the finding that when the samples in Figure 3 were run on SDS–PAGE with 50 mM β-mercaptoethanol, only reduced APΔ2-22Δ25 was visible (data not shown). These results show that the N-terminus of APΔ2-22 is not essential for its export in *prlA* mutants. In addition, the basic amino acyl residues near the N-terminus of APΔ2-22Δ23 block its export. Thus, basic amino acyl residues have the same affect on the export of APΔ2-22 with an N-terminal deletion of the mature sequence as they do on APΔ2-22 itself.

Screen for suppressors that are able to export APΔ2-22 E4K

One way to learn more about how positively charged residues block the export of APΔ2-22 and ΔssMalS is to

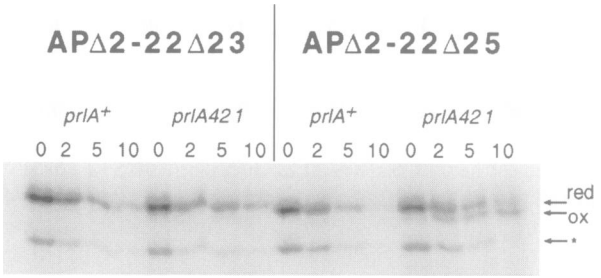


Fig. 3. APΔ2-22Δ25, but not APΔ2-22Δ23, is exported in a *prlA421* mutant. Strains WP851, WP852, WP857 and WP858 were labeled with [^{35}S]methionine for 1 min and chased with cold methionine for 10 min. Samples were taken 0, 2, 5 and 10 min after the start of the chase (times indicated by numbers above lanes). Reduced and oxidized APΔ2-22Δ25 are indicated by 'red' and 'ox' respectively. The material marked '*' is probably a break-down product of AP.

obtain suppressor mutations that allow APΔ2-22 E4K to be exported in a *prlA4* strain. These mutations might directly affect the process that is disrupted by the positive charges. Suppressors were isolated by mutagenizing WP739 (which expresses APΔ2-22 E4K and carries the *prlA4* mutation) with nitrosoguanidine and screening for mutants that form blue colonies on media containing XP. After screening 5×10^6 colonies, two mutants were isolated that have elevated AP activity. One contained a direct reversion of the original mutation that caused the E4K change in APΔ2-22 (data not shown). The second, CFS21, maps to the *secY* region by P1 transduction (data not shown).

This second mutation was further characterized by determining how well it was able to allow export of different versions of AP with defective or missing signal sequences. The *secY* region of CFS21 was transduced into a number of strains encoding AP with various signal sequence defects. In all cases, the resulting strains were able to export AP more efficiently than isogenic *prlA4* or *prlA*$^+$ strains (Table IV). Thus, this mutation does not appear specifically to counteract the effects of basic residues on the export of AP. In addition, as with *prlA4*-mediated export of APΔ2-22 (Bieker *et al.*, 1990), the export of APΔ2-22 E4K in CFS21 is dependent on SecB (data not shown). Finally, all strains containing the *prlA* allele from CFS21 grow more slowly than otherwise isogenic *prlA*$^+$ strains (data not shown). We have named the *prlA* allele in CFS21, *prlA421*.

Discussion

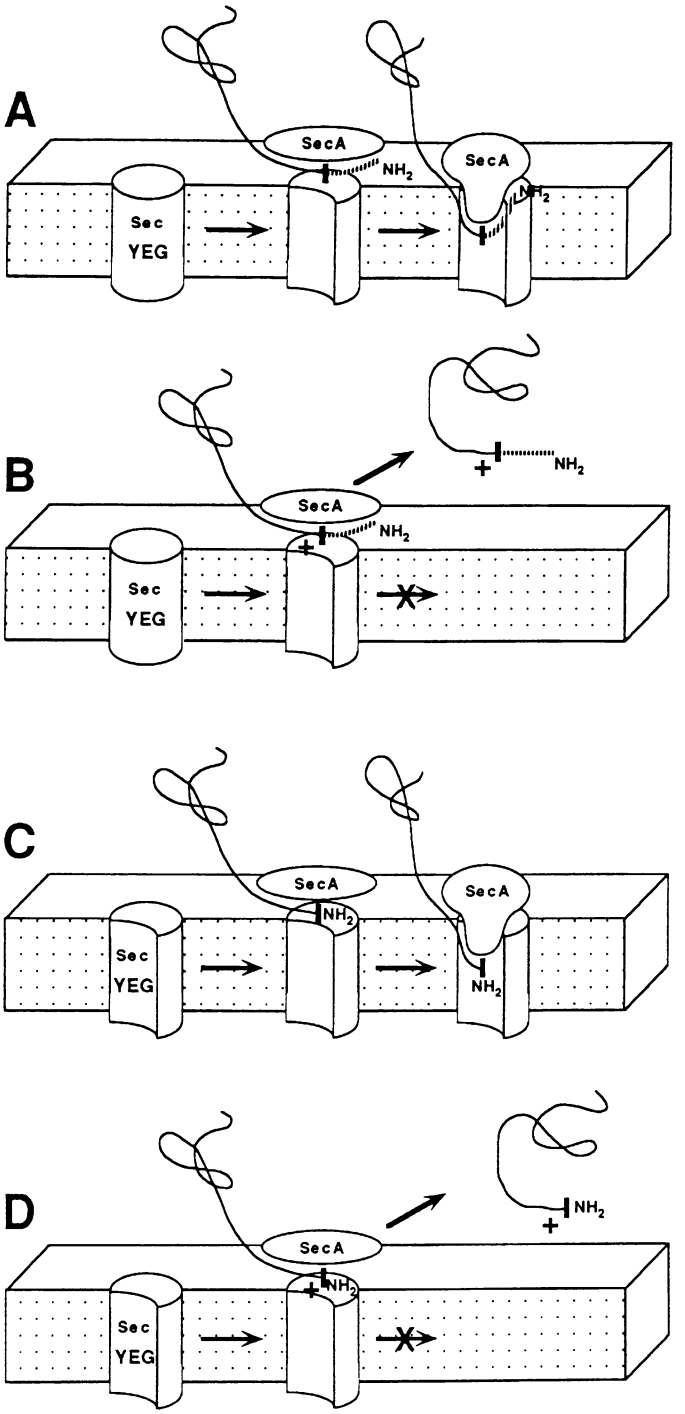
Targeting of signal sequenceless proteins in prlA mutants

Signal sequenceless extracytoplasmic proteins, such as APΔ2-22, are exported in *prlA* mutants while cytoplasmic proteins are not exported. We have considered two explanations for this difference between exported proteins and cytoplasmic proteins. According to the first explanation, the mature portion of all secreted proteins contains export signals that are missing in cytoplasmic proteins. For example, the N-termini of the mature domains of exported proteins may function as cryptic signal sequences. Alternatively, since APΔ2-22 is dependent on SecB for export (Derman *et al.*, 1993a), the signal may simply be a SecB-binding site. However, SecB can bind to a wide variety of polypeptides and does not appear to bind to a specific sequence (Hardy and Randall, 1993). According to the second explanation, all exported proteins, in contrast to cytoplasmic proteins, have evolved to remain unfolded or to fold slowly in the cytoplasm, and unfolded proteins are targeted for export in *prlA* mutants. If the first explanation were correct, then it might be possible to identify these cryptic targeting sequences by obtaining mutations which disrupt them. We sought such mutations in APΔ2-22 and found that altering either Glu4 or Glu9 to Lys blocks the export of APΔ2-22 in a *prlA4* mutant. In addition, a version of APΔ2-22 with an additional arginyl residue in the N-terminus (APΔ2-21; Figure 1) is not exported efficiently in a *prlA4* mutant. Thus, mutations which introduce positively charged amino acyl residues near the N-terminus of APΔ2-22 block its export in a *prlA4* mutant. Similar results were obtained with a signal sequenceless version of MalS (Table III).

Since all the mutations which block the export of APΔ2-22 in a *prlA4* mutant cluster near the N-terminus of APΔ2-22, we considered whether the N-terminus of this protein functions as a signal sequence in *prlA* mutants. Functional signal sequences always contain a hydrophobic core and a positively charged N-terminal domain. The mutations which block the export of APΔ2-22 do not disrupt any obvious stretch of hydrophobic amino acids. In addition, since all of the mutations introduce positively charged residues near the N-terminus of APΔ2-22, one might expect that these changes would make the N-terminus of APΔ2-22 more rather than less like a functional signal sequence. Finally, if APΔ2-22 contains a cryptic signal sequence, then deleting the N-terminal 25 residues of APΔ2-22 should prevent its export in a *prlA* mutant. However, this is not the case (Figure 3). Therefore, the N-terminus of APΔ2-22 does not function as a cryptic signal sequence in *prlA* mutants; the mutations which block the export of APΔ2-22 do not disrupt an N-terminal targeting signal but must affect the export competence of APΔ2-22 in some other way.

Our findings make less likely the explanation for *prlA*-based translocation that posits an export signal in the mature portion of cell envelope proteins. We expected mutations which disrupt such a signal in our genetic screen; but instead, among 11 independent mutants, we found only the Glu→Lys changes early in APΔ2-22. While our failure to find mutations in a putative targeting site suggests that the site does not exist, such negative results should be interpreted with caution.

Nevertheless, we believe that our results indicate that a more likely explanation for *prlA*-based translocation is that slowly folding or partially unfolded proteins are targeted for export in *prlA* mutants (Derman *et al.*, 1993a). AP and other proteins that require disulfide bonds remain unfolded in the cytoplasm (Derman and Beckwith, 1991; Derman *et al.*, 1993b). Furthermore, AP without its signal sequence, MalE, and other proteins are maintained in an export-competent, unfolded state by SecB (Randall *et al.*, 1990; Derman *et al.*, 1993a). In addition, the fact that many exported proteins are unstable when they are expressed in the cytoplasm (Emr and Bassford, 1982; Michaelis *et al.*, 1986) suggests that they fold slowly in the cytoplasm. One way to test whether slowly folding proteins are targeted for export in *prlA* mutants would be to find



Signal sequence	N-terminal + charge	*prlA* allele
wild-type	none	*prlA*+
wild-type	+	*prlA*+
none	none	*prlA4*
none	+	*prlA4*

Fig. 4. Model of the initiation of protein translocation. In the protein to be exported, the hatched line represents the signal sequence and the filled bar, the N-terminus of the mature protein. (**A**) The signal sequence (striped line) of a protein to be translocated is positioned at a recognition site in SecA and causes a conformational change in the SecYEG complex (indicated by a change from a cylinder to a cylinder with a concave surface). Next, the C-terminus of the signal sequence and the N-terminus of the mature portion of the translocating protein are inserted as a loop into the secretion machinery. (**B**) When a positively charged amino acyl residue is introduced immediately downstream of the signal sequence cleavage site (black bar), the second step of the initiation process is blocked. (**C**) When a signal sequenceless protein is targeted for export, it is bound by SecA, but is unable to trigger the conformational change in the SecYEG complex. However, in *prl* mutants the SecYEG complex is locked into the activated conformation and the N-termini of signal sequenceless proteins are inserted into the secretion machinery. (**D**) Positively charged amino acids near the N-terminus of a signal sequenceless protein block the second step in the initiation process.

mutations that alter the folding of a cytoplasmic protein so that it is exported in a *prlA* mutant. We looked for mutations in MalZ, a cytoplasmic maltodextrin glucosidase (Tapio *et al.*, 1991), that allow it to be exported in a *prlA* mutant since MalZ is highly homologous to MalS, an exported protein. So far, we have failed to find such mutations (unpublished results).

Several lines of evidence suggest that the secretory apparatus recognizes the mature domains of exported proteins in wild-type cells as well as *prlA* mutants. The

Table I. Strains and plasmids used in this paper

Strain or plasmid	Description or relevant genotype	Source
Plasmids		
pBAD18s		Guzman *et al.* (1995)
pBAD22		Guzman *et al.* (1995)
pAID135	expresses APΔ2-22 under tac promoter	Derman *et al.* (1993a)
pAID134	expresses APΔ2-21 under tac promoter	Derman (1994)
pWP620	expresses APΔ2-22 E4K under tac promoter	this study
pWP643	expresses APΔ2-22 E9K under tac promoter	this study
pWP851	expresses APΔ2-22Δ23 under *araBAD* promoter	this study
pWP852	expresses APΔ2-22Δ25 under *araBAD* promoter	this study
pUMa103	*malS* in pBR322	Schneider *et al.* (1992)
pCS1	expresses ΔssMalS under *araBAD* promoter	this study
pCS7	expresses ΔssΔR′sMalS under *araBAD* promoter	this study
Strains		
DHB4	F′ *lac-pro lacI*^{Q}/Δ(*ara-leu*)7697 *araD*139 Δ*lac*X74 *galE galK rpsL phoR* Δ(*phoA*)PvuII Δ*malF*3 *thi*	lab collection
Mph42	F^{-} Δ(*ara-leu*)7697 *araD*139 Δ*lac*X74 *galU galK phoR rpsL*	lab collection
AD494	DHB4 *trxB*::Kan^{r}	Derman *et al.* (1993b)
WP637	DHB4 *prlA4* Tn*10*	this study
WP827	DHB4 *prlA421* Tn*10*	this study
WP739	WP637 + pWP620	this study
WP610	WP637 *trxB*::Kan^{r} Δ(*deoD-serB*)252	this study
CFS21	WP739 but *prlA421*	this study
Mph53	Mph42 but *phoA82*	lab collection
Mph54	Mph42 but *phoA21*	lab collection
Mph56	Mph42 but *phoA73*	lab collection
WP880	Mph54 *prlA4* Tn*10*	this study
WP881	Mph53 *prlA4* Tn*10*	this study
WP882	Mph56 *prlA4* Tn*10*	this study
WP883	Mph42 *prlA4* Tn*10*	this study
WP884	Mph54 *prlA421* Tn*10*	this study
WP885	Mph53 *prlA421* Tn*10*	this study
WP886	Mph56 *prlA421* Tn*10*	this study
WP887	Mph42 *prlA421* Tn*10*	this study
WP551	DHB4 + pAID135	this study
WP552	WP551 *trxB*::Kan^{r}	this study
WP636	WP551 *prlA4* Tn*10*	this study
WP888	DHB4 + pWP620	this study
WP890	WP888 *trxB*::Kan^{r}	this study
WP739	WP888 *prlA4* Tn*10*	this study
WP889	DHB4 + pWP643	this study
WP891	WP889 *trxB*::Kan^{r}	this study
WP892	WP889 *prlA4* Tn*10*	this study
WP760	DHB4 + pAID134	this study
WP893	WP760 *trxB*::Kan^{r}	this study
WP761	WP760 *prlA4* Tn*10*	this study
WP828	WP551 *prlA421* Tn*10*	this study
WP829	WP888 *prlA421* Tn*10*	this study
WP851	DHB4 + pWP851	this study
WP852	DHB4 + pWP852	this study
WP857	WP851 *prlA421*	this study
WP858	WP852 *prlA421*	this study
MC4100	F^{-} *araD139* Δ*lac*U169 *rpsL thi rbsR*	lab collection
CS10	MC4100 *malTc* Δ*malQ* ϕ(*malP-lacZ*) *malS malZ*	this study
CS12	CS10 Δ*ara*714 .... *leu*::Tn*10*	this study
CS18	CS12 *prlA4* Tn*10*	this study

secretion machinery in *prlA* mutants is able to distinguish signal sequenceless cell envelope proteins from cytoplasmic proteins. This ability must reflect differences in the properties of the mature domains of exported proteins and cytoplasmic proteins. The existence of these differences suggests that they must be required for the efficient targeting and export of cell envelope proteins in wild-type cells and not just *prlA* mutants. This idea is supported by the findings that both SecA and SecB recognize the mature domains of exported proteins *in vitro* (Cunningham and Wickner, 1989; Lill *et al.*, 1990; Randall *et al.*, 1991). In addition, studies on MalE suggest that, in wild-type cells, SecB recognizes the mature domains of cell envelope proteins *in vivo* (Bankaitis and Bassford, 1984; Collier *et al.*, 1988).

The effect of basic amino acids on export competence

Introducing basic residues near the N-terminus of a signal sequenceless protein affects its export competence, not by disrupting its ability to be targeted for export, but by some other mechanism. This finding is reminiscent of results seen when basic amino acids are introduced into proteins with signal sequences. Basic residues early in the mature

portion of wild-type AP, immediately after the signal sequence, cause a substantial reduction in export (Li *et al.*, 1988). Similar findings have been reported for other exported proteins (Gennity *et al.*, 1989). To explain these observations, it has been proposed that the basic amino acids block translocation by preventing the formation in the membrane of a loop structure that includes the signal sequence (Li *et al.*, 1988). Our results show that inserting a basic amino acyl residue early in the mature portion of AP affects the export competence of AP whether it is expressed with a signal sequence or not. We suspect that the same mechanism accounts for the export block in both cases. If this is so, basic amino acids must affect a step in the process that does not require a signal sequence or, by extension, the formation of a loop structure by the signal sequence.

A model for protein export

We have developed a model for the initiation of protein translocation that incorporates our current findings, explains the properties of *prlA* mutants and suggests how basic amino acids block translocation. This model is based, in part, on the assumption that basic amino acids at the N-terminus of the mature protein cause a block in translocation by the same mechanism whether the protein has its signal sequence or not. This assumption would lead to the further assumption that, at an early stage of the process, the N-terminus of the mature protein is positioned at the same location in the secretion machinery irrespective of whether it carries a signal sequence. In our model, we make no assumptions about whether a secreted protein enters directly into a channel formed by the membrane-embedded components or is translocated through some interface between those components and SecA or the lipid bilayer itself. For simplicity's sake, we will refer to this process as entry into the membrane, although the model, as depicted in Figure 4, shows entry into a pore that is partially exposed to the lipid bilayer.

We propose that basic amino acids at the N-terminus of a mature protein directly block entry of a protein into the membrane. In the case of the wild-type secretion machinery interacting with a normal signal sequence, these basic amino acids block the process of initiation of insertion of the signal sequence into a loop structure in the membrane (Figure 4A and B). If this is correct, then the initiation of formation of the loop structure occurs not by insertion of the hydrophobic signal sequence into the membrane, but rather by the insertion of the region of exported proteins that includes the C-terminus of the signal sequence and the N-terminus of the mature protein (Figure 4A). According to this picture, the loop structure is a result of the initiation of translocation, not a cause.

It seems simplest to us to imagine the following steps in the export process for proteins carrying signal sequences: (i) An unfolded protein interacts with the secretion machinery. It may be that this machinery will bind any unfolded protein. Some evidence exists for a function of SecA as a chaperone protein (Cunningham and Wickner, 1989; Lill *et al.*, 1990; Kumamoto, 1991), in addition to its direct role in the translocation process. Alternatively, SecB or other chaperones (e.g. SRP) may specifically target any unfolded protein to the membrane apparatus. (ii) After interaction with SecA, proteins to be translocated are aligned in the machinery so that the signal sequence is positioned at a recognition site involving perhaps both the hydrophobic membrane components (SecY, E and G) and SecA (Figure 4). At this point, the machinery utilizes a proof-reading mechanism to reject proteins with either defective or missing signal sequences (Osborne and Silhavy, 1993). After successful proof-reading, the machinery undergoes a conformational change that allows the translocation process to begin. The alignment of a functional signal sequence in the machinery will position the C-terminus of the signal sequence and N-terminus of the mature protein so that they can enter the membrane as a loop. (iii) The translocating protein is

Table II. AP activity of strains expressing wild-type and mutant forms of APΔ2-22

AP synthesized	Relevant genotype			Number isolated
	prlA$^+$ *trxB*$^+$	*prlA4 trxB*$^+$	*prlA*$^+$ *trxB*	
APΔ2-22	43	1200	910	n.r.
APΔ2-22 E4K	7.1	32	810	9
APΔ2-22 E9K	4.2	37	670	2
APΔ2-21	1.0	6.9	82	n.r.

n.r., not relevant.

Table IV. AP activity of strains with different *phoA* alleles

phoA allele	Relevant genotype		
	prlA$^+$	*prlA4*	*prlA421*
phoAΔ2-22	40	1200	1800
phoA21	28	200	340
phoA82	130	480	540
phoA73	300	480	510
phoA$^+$	640	660	540
phoAΔ2-22 E4K	7.1	32	260

Table III. Amylase activity of strains expressing various MalS derivatives

Strain	MalS synthesized	*prlA* allele	MalS activity	Relative increase
CS12+pCS1	ΔssMalS	*prlA*$^+$	0.005[a]	1[b]
CS18+pCS1	ΔssMalS	*prlA4*	0.006	1
CS12+pCS7	ΔssΔR′sMalS	*prlA*$^+$	0.036	8
CS18+pCS7	ΔssΔR′sMalS	*prlA4*	0.590	131
CS12+pUMa103	MalS	*prlA*$^+$	1.950	n.r.

[a]One unit corresponds to 1 nmol *p*-nitrophenyl-hexoside hydrolyzed/min/mg cellular protein at room temperature.
[b]Ratio with respect to CS12+pCS1.
n.r., not relevant.

then inserted into the membrane. Recent evidence suggests that this process requires the insertion of SecA, along with the protein to be exported, into the membrane (Economou and Wickner, 1994; Kim *et al.*, 1994; Economou *et al.*, 1995). A protein that carries a net positive charge at the N-terminus of the mature protein will not be able to enter the membrane and will be rejected by the machinery (Figure 4B and D). (iv) Many of the details of translocation itself, including the energetics of the process, are proposed elsewhere (Schiebel *et al.*, 1991; Wickner *et al.*, 1991).

prlA mutants and basic amino acids

How do *prlA* mutants alter the translocation machinery so that signal sequenceless proteins can be exported? Osborne and Silhavy (1993) have suggested that *prlA* mutants eliminate or weaken the proof-reading activity of the machinery. A similar post-targeting signal sequence recognition step has recently been discovered in mammalian systems (Jungnickel and Rapoport, 1995; Belin *et al.*, 1996). Thus, the secretion machinery in a *prlA* mutant will accept an unfolded protein without a signal sequence. We propose that when such a protein enters the secretion apparatus, the N-terminus does not position itself along the signal sequence-binding–proof-reading site, but remains at the site where the mature portion of proteins with signal sequences bind. The N-terminus of the protein then enters the translocation machinery facilitated by SecA (Figure 4C). If there are basic amino acids at the N-terminus of the protein, entry is blocked in the same way as with a protein with a normal signal sequence followed by basic amino acids (Figure 4D).

We have found that *prlA421* cells are able to export signal sequenceless AP even when it is expressed with a basic residue near its N-terminus. It is unlikely that this mutation affects the ability of positively charged residues to be inserted into the membrane [step (iii), above] since it does not specifically counteract the effects of basic residues on the export of AP. Rather, *prlA421* cells probably allow a greater fraction of signal sequenceless AP to proceed to the membrane insertion step of protein translocation than *prlA4* cells. This, in turn, allows a small fraction of signal sequenceless AP with a basic residue near its N-terminus to be exported.

Materials and methods

Materials

Media and chemical reagents were prepared or purchased as previously described (Derman *et al.*, 1993a). Oligonucleotides were purchased from Genosys Biotechnologies Inc.

Strains and plasmids

The strains and plasmids used in this study are listed in Table I. Strains were constructed using standard techniques (Miller, 1992). Plasmids pWP620 and pWP632 encode APΔ2-22 E4K and APΔ2-22 E9K respectively (Figure 1) and were isolated in a screen described below. The sequence of the *phoA* alleles in these plasmids was confirmed by sequencing. The plasmid pWP851, which encodes APΔ2-22Δ23 (Figure 1), was constructed as follows. Using *phoA* as a template, the polymerase chain reaction (PCR) was performed with primers phoA-7 (5′-GGGGGGTACCTCGCCGTTTAACGGGTGATCAG-3′) and phoA-9 (5′-GGGGTCTAGATTATTTCAGCCCCAGAGCGGC-3′). The resulting product was cut with *Eco*RI and *Xba*I and ligated into pBAD22 (Guzman *et al.*, 1995). The same procedure was used to make pWP852, which encodes APΔ2-22Δ25, except that the primer phoA-8 (5′-GGGGGGTACCTTTAACGGGTGATCAGACTGCC-3′) was used in place of phoA-7. The plasmid pCS1 was constructed by cutting pUMa103 with *Pvu*I and *Hin*dIII, and ligating the resulting fragment containing *malS* into pBAD18s (Guzman *et al.*, 1995) cut with *Sma*I and *Hin*dIII. The plasmid pCS7 was derived from pCS1 by cutting pCS1 with *Eco*RI and *Kpn*I, blunting the ends, and re-ligating (Figure 2).

Isolation of mutations in APΔ2-22 that block its export

The plasmid pAID135 was mutagenized with hydroxylamine as described by Mendenhall *et al.* (1988) except that it was kept at 70°C for 2 h. This mutagenized plasmid was used to transform WP610 and the cells were plated on M63 medium containing 0.2% glucose, 100 μg/ml each of isoleucine and leucine, 200 μg/ml ampicillin and 40 μg/ml XP. Light blue or white colonies were picked after incubation for 2 days at 37°C.

Isolation of mutants that can export APΔ2-22 E4K

WP739 was mutagenized with nitrosoguanidine as described in Miller (1992) and plated on NZ–amine-A medium containing 200 μg/ml ampicillin and 40 μg/ml XP. Dark blue colonies were picked after incubation for 1 day at 37°C.

Alkaline phosphatase and amylase assays

For alkaline phosphatase assays, cells were grown in NZ–amine-A plus 200 μg/ml ampicillin and 5 mM isopropyl-thio-β-D-thiogalactopyranoside (IPTG) to a final OD_{600} of ~0.4. They were then incubated on ice for 20 min in the presence of 100 mM iodoacetamide. The remainder of the assay was performed as described in Derman *et al.* (1993) except that 100 mM iodoacetamide was used instead of 1 mM iodoacetamide in the wash buffer. The assays were performed in duplicate and results varied by <5%.

Amylase activity was determined in whole cells using *p*-nitrophenyl-hexoside (PNP6) as a substrate. The release of *p*-nitrophenol from PNP6 by MalS was measured at room temperature. Overnight cultures grown in minimal medium 9 (M9) and 0.4% glycerol were harvested, washed in M9 and resuspended in M9 to an OD_{578} of 2.0. Five hundred microlitres of cells were permeabilized with 25 μl of 0.1% SDS and 25 μl $CHCl_3$. After incubation for 10 min at room temperature, PNP6 was added to a final concentration of 2 mM. After the appearance of a pale yellow color, the reaction was stopped by adding 170 μl of 20% trichloroacetic acid. Samples were kept on ice for 15 min. After centrifugation, 400 μl of supernatant was transferred to a tube containing 600 μl 1 M Na_2CO_3. *p*-Nitrophenol was assayed by determining absorbance at 405 nm. The extinction coefficient of *p*-nitrophenol is 0.014 μM^{-1} cm^{-1} (Walsh, 1979). One unit of MalS activity corresponds to 1 nmol of PNP6 hydrolyzed per minute; activity is expressed as units per mg cellular protein.

Radiolabeling and immunoprecipitation

Cells were grown to mid-log phase in M63 medium containing 0.2% glycerol, 50 mg/ml of all amino acids except cysteine and methionine, and 100 mg/ml ampicillin and then induced with 0.2% arabinose for 10 min before labeling. Labeling, immunoprecipitation, non-reducing SDS–PAGE and autoradiography were performed as previously described (Derman *et al.*, 1993a) except that the cells were treated with 100 mM iodoacetamide for 20 min on ice immediately after labeling.

Acknowledgements

This work was supported by a predoctoral fellowship from the National Eye Institute to W.P. and by a grant from the National Institute of General Medical Sciences and an American Cancer Society Research Professorship to J.B. M.E. was supported by Deutsche Forschungsgemeinschaft.

References

Altman,E., Emr,S.D. and Kumamoto,C.A. (1990) The presence of both the signal sequence and a region of mature LamB protein is required for the interaction of LamB with the export factor SecB. *J. Biol. Chem.*, **265**, 18154–18160.

Bankaitis,V.A. and Bassford,P.J.,Jr (1984) The synthesis of export-defective proteins can interfere with normal protein export in *Escherichia coli*. *J. Biol. Chem.*, **259**, 12 193.

Belin,D., Bost,S., Vassalli,J.D. and Strub,K. (1996) A two step recognition of signal sequences determines the translocation efficiency of proteins. *EMBO J.*, **15**, 468–478.

Bieker,K.L., Phillips,G.J. and Silhavy,T.J. (1990) The *sec* and *prl* genes of *Escherichia coli*. *J. Bioenerg. Biomembr.*, **22**, 291–310.

Bowden,G.A., Baneyx,F. and Georgiou,G. (1992) Abnormal fractionation of β-lactamase in *Escherichia coli*: evidence for an interaction with the inner membrane in the absence of a leader peptide. *J. Bacteriol.*, **174**, 3407–3410.

Collier,D.N., Bankaitis,V.A., Weiss,J.B. and Bassford,P.J.,Jr (1987) The antifolding activityy of SecB promotes the export of the *E.coli* maltose binding protein. *Cell*, **53**, 272–283.

Cunningham,K. and Wickner,W. (1989) Specific recognition of the leader region of precursor proteins is required for the activation of the translocation ATPase of *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **86**, 8630–8634.

Derman,A.I. (1994) Alkaline phosphatase lacking its signal sequence: folding in the cytoplasm and export to the periplasm. PhD Thesis, Harvard University, Cambridge, MA.

Derman,A.I. and Beckwith,J. (1991) *Escherichia coli* alkaline phosphatase fails to acquire disulfide bonds when retained in the cytoplasm. *J. Bacteriol.*, **173**, 7719–7722.

Derman,A.I., Puziss,J.W., Bassford,P.J.,Jr and Beckwith,J. (1993a) A signal sequence is not required for protein export in *prlA* mutants of *Escherichia coli*. *EMBO J.*, **12**, 879–888.

Derman,A.I., Prinz,W.A., Belin,D. and Beckwith,J. (1993b) Mutations that allow disulfide bond formation in the cytoplasm of *Escherichia coli*. *Science*, **262**, 1744–1747.

Economou,A. and Wickner,W. (1994) SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. *Cell*, **78**, 835–843.

Economou,A., Pogliano,J.A., Beckwith,J., Oliver,D.B. and Wickner,W. (1995) SecA membrane cycling at SecYEG is driven by distinct ATP binding and hydrolysis events and is regulated by SecD and SecF. *Cell*, **83**, 1171–1181.

Emr,S.D. and Bassford,P.J.,Jr (1982) Localization and processing of outer membrane and periplasmic proteins in *Escherichia coli* strains harboring export-specific suppressor mutations. *J. Biol. Chem.*, **257**, 5852–5860.

Emr,S.D., Hanley-Way,S. and Silhavy,T.J. (1981) Supressor mutations that restore export of a protein with a defective signal sequence. *Cell*, **23**, 79–88.

Flower,A.M., Doebele,R.C. and Silhavy,T.J. (1994) PrlA and PrlG suppressors reduce the requirement for signal sequence recognition. *J. Bacteriol.*, **176**, 5607–5614.

Gennity,J., Goldstein,J. and Inouye,M. (1989) Signal peptide mutants of *Escherichia coli*. *J. Bioenerg. Biomembr.*, **22**, 233–269.

Gierasch,L.M. (1989) Signal sequences. *Biochemistry*, **28**, 923–930.

Guzman,L.M., Belin,D., Carson,M.J. and Beckwith,J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.*, **177**, 4121–4130.

Hardy,S.J. and Randall,L.L. (1993) Recognition of ligands by SecB, a molecular chaperone involved in bacterial protein export. *Philos. Trans. R. Soc. Lond. [Biol.]*, **339**, 343–352.

High,S. (1995) Protein translocation at the membrane of the endoplasmic reticulum. *Prog. Biophys. Mol. Biol.*, **63**, 233–250.

Izard,J.W. and Kendall,D.A. (1994) Signal peptides: exquisitely designed transport promoters. *Mol. Microbiol.*, **13**, 765–773.

Jones,J.D., McKnight,C.J. and Gierasch,L.M. (1990) Biophysical studies of signal peptides: implications for signal sequence functions and the involvement of lipid in protein export. *J. Bioenerg. Biomembr.*, **22**, 213–232.

Jungnickel,B. and Rapoport,T.A. (1995) A posttargeting signal sequence recognition event in the endoplasmic reticulum membrane. *Cell*, **82**, 261–270.

Kim,Y.J., Rajapandi,T. and Oliver,D. (1994) SecA protein is exposed to the periplasmic surface of the *E. coli* inner membrane in its active state. *Cell*, **78**, 845–853.

Kumamoto,C.A. (1990) SecB protein: a cytosolic export factor that associates with nascent exported proteins. *J. Bioenerg. Biomembr.*, **22**, 337–351.

Kumamoto,C.A. (1991) Molecular chaperones and protein translocation across the *Escherichia coli* inner membrane. *Mol. Microbiol.*, **5**, 19–22.

Laminet,A.A. and Plückthun,A. (1989) The precursor of β-lactamase: purification, properties and folding kinetics. *EMBO J.*, **8**, 1469–1477.

Lauring,B., Kreibich,G. and Weidmann,M. (1995) The intrinsic ability of ribosomes to bind to endoplasmic reticulum membranes is regulated by signal recognition particle and nascent-polypeptide-associated complex. *Proc. Natl Acad. Sci. USA*, **92**, 9435–9439.

Li,P., Beckwith,J. and Inouye,H. (1988) Alteration of the amino terminus of the mature sequence of a periplasmic protein can severely affect protein export in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **85**, 7685–7689.

Lill,R., Dowhan,W. and Wickner,W. (1990) The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature regions of precursor proteins. *Cell*, **60**, 271–280.

Luirink,J., High,S., Wood,H., Giner,A., Tollervey,D. and Dobberstein,B. (1992) Signal-sequence recognition by an *Escherichia coli* ribonucleoprotein complex. *Nature*, **359**, 741–743.

Mendenhall,M.D., Richardson,H.E. and Reed,S.I. (1988) Dominant negative protein kinase mutations that confer a G1 arrest phenotype. *Proc. Natl Acad. Sci. USA*, **85**, 4426–4430.

Michaelis,S., Hunt,J.F. and Beckwith,J. (1986) Effects of signal sequence mutations on the kinetics of alkaline phosphatase export to the periplasm in *Escherichia coli*. *J. Bacteriol.*, **167**, 160–167.

Miller,J.H. (1992) *A Short Course in Bacterial Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Osborne,R.S. and Silhavy,T.J. (1993) PrlA suppressor mutations cluster in regions corresponding to three distinct topological domains. *EMBO J.*, **12**, 3391–3398.

Panzner,S., Dreier,L., Hartmann,E., Kostka,S. and Rapoport,T.A. (1995) Posttranslational protein transport in yeast reconstituted with a purified complex of Sec proteins and Kar2p. *Cell*, **81**, 561–570.

Park,S., Liu,G., Topping,T.B., Cover,W.H. and Randall,L.L. (1988) Modulation of folding pathways of exported proteins by leader sequence. *Science*, **239**, 1033–1035.

Phillips,G.J. and Silhavy,T.J. (1992) The *E.coli ffh* gene is necessary for viability and efficient protein export. *Nature*, **359**, 744–746.

Poritz,M.A., Berstein,H.D., Strub,K., Zopf,D., Wilhelm,H. and Walter,P. (1990) An *E. coli* ribonucleoprotein containing 4.5S RNA resembles mammalian signal recognition particle. *Science*, **250**, 1111–1117.

Randall,L.L., Hardy,S.J. and Thom,J.R. (1987) Export of protein: a biochemical view. *Annu. Rev. Microbiol.*, **41**, 507–541.

Randall,L.L., Topping,T.B. and Hardy,S.J. (1990) No specific recognition of leader peptide by SecB, a chaperone involved in protein export. *Science*, **248**, 860–863.

Ribes,V., Romisch,K., Giner,A., Dobberstein,B. and Tollervey,D. (1990) *E. coli* 4.5S RNA is part of a ribonucleoprotein particle that has properties related to signal recognition particle. *Cell*, **63**, 591–600.

Schatz,P.J. and Beckwith,J. (1990) Genetic analysis of protein export in *Escherichia coli*. *Annu. Rev. Genet.*, **24**, 215–248.

Schiebel,E., Driessen,A.J., Hartl,F.U. and Wickner,W. (1991) $\Delta\mu$ H^+ and ATP function at different steps of the catalytic cycle of preprotein translocase. *Cell*, **64**, 927–939.

Schneider,E., Freundlieb,S., Tapio,S. and Boos,W. (1992) Molecular characterization of the MalT-dependent periplasmic alpha-amylase of *Escherichia coli* encoded by malS. *J. Biol. Chem.*, **267**, 5148–5154.

Tapio,S., Yeh,F., Shuman,H.A. and Boos,W. (1991) The *malZ* gene of *Escherichia coli*, a member of the maltose regulon, encodes a maltodextrin glucosidase. *J. Biol. Chem.*, **266**, 19450–19458.

von Heijne,G. (1985) Signal sequences. The limits of variation. *J. Mol. Biol.*, **184**, 99–105.

Walsh,C. (1979) *Enzymatic Reaction Mechanisms*. W.H.Freeman and Co. Press, San Francisco, CA, p. 68.

Walter,P. and Johnson,A.E. (1994) Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. *Annu. Rev. Cell Biol.*, **10**, 87–119.

Watanabe,M. and Blobel,G. (1989) SecB functions as a cytosolic signal recognition factor for protein export in *E. coli*. *Cell*, **58**, 695–705.

Watanabe,M. and Blobel,G. (1995) High affinity binding of *Escherichia coli* SecB to the signal sequence region of a presecretory protein. *Proc. Natl Acad. Sci. USA*, **92**, 10133–10136.

Wickner,W., Driessen,A.J. and Hartl,F.U. (1991) The enzymology of protein translocation across the *Escherichia coli* plasma membrane. *Annu. Rev. Biochem.*, **60**, 101–124.

Received on May 21, 1996; revised on June 18, 1996