An Unusual Signal Peptide Extension Inhibits the Binding of Bacterial Presecretory Proteins to the Signal Recognition Particle, Trigger Factor, and the SecYEG Complex

Considerable evidence indicates that the *Escherichia coli* signal recognition particle (SRP) selectively targets proteins that contain highly hydrophobic signal peptides to the SecYEG complex cotranslationally. Presecretory proteins that contain only moderately hydrophobic signal peptides typically interact with trigger factor (TF) and are targeted post-translationally. Here we describe a striking exception to this rule that has emerged from the analysis of an unusual 55-amino acid signal peptide associated with the *E. coli* autotransporter EspP. The EspP signal peptide consists of a C-terminal domain that resembles a classical signal peptide plus an N-terminal extension that is conserved in other autotransporter signal peptides. Although a previous study showed that proteins containing the C-terminal domain of the EspP signal peptide are targeted cotranslationally by SRP, we found that proteins containing the full-length signal peptide were targeted post-translationally via a novel TF-independent mechanism. Mutation of an invariant asparagine residue in the N-terminal extension, however, restored cotranslational targeting. Remarkably, proteins containing extremely hydrophobic derivatives of the EspP signal peptide were also targeted post-translationally. These and other results suggest that the N-terminal extension alters the accessibility of the signal peptide to SRP and TF and promotes post-translational export by reducing the efficiency of the interaction between the signal peptide and the SecYEG complex. Based on data, we propose that the N-terminal extension mediates an interaction with an unidentified cytoplasmic factor or induces the formation of an unusual signal peptide conformation prior to the onset of protein translocation.

The vast majority of bacterial presecretory proteins contain a ~20–25-amino acid cleaved signal peptide that earmarks them for translocation across the inner membrane (IM) via a highly conserved heterotrimeric protein-conducting channel called the SecYEG complex. Although signal peptides are extremely degenerate, they typically consist of a basic N region, a core of 7–12 hydrophobic amino acids (H region), and a somewhat polar C region (1). In *E. coli*, a few cleaved signal peptides are recognized by the signal recognition particle (SRP), a universally conserved ribonucleoprotein complex (reviewed in Ref. 2). *E. coli* SRP interacts predominantly with extremely hydrophobic signal peptides, although positive charges in the N region may also influence SRP recognition (3–6). SRP binds to its substrates as they emerge from translating ribosomes and then targets ribosome-nascent chain complexes to the IM cotranslationally. Most presecretory proteins, however, are bypassed by SRP and are engaged instead by a ribosome-associated chaperone called trigger factor (TF), which also binds at an early stage of translation but does not interact with the signal peptide (7–9). The binding of both SRP and TF to very short nascent chains is likely facilitated by their interaction with a ribosomal subunit (L23) that is directly adjacent to the ribosomal tunnel (9–11). TF binding promotes post-translational targeting, possibly by sterically hindering the docking of ribosomes onto the Sec complex (12–14). Recent evidence suggests that SecA, a cytoplasmic ATPase that plays a central role in the translocation reaction, may also bind to short nascent presecretory proteins (13, 15). Other chaperones such as SecB bind to presecretory proteins late during translation or after translation is complete (16). These factors are required to maintain presecretory proteins in a loosely folded conformation that is required for translocation across the IM and to keep the signal peptide exposed to gate open the Sec complex (17). The signal peptide itself may also play a role in preventing the premature folding of presecretory proteins (18, 19).

Although the superfamily of bacterial proteins known as autotransporters are translocated across the IM via the Sec pathway (20), some of them contain unusual signal peptides. Autotransporters are comprised of a large (often >100 kDa) N-terminal passenger domain that typically mediates a virulence function and a C-terminal ~30 kDa β-domain (reviewed in Ref. 21). After an autotransporter is translocated across the IM, the β-domain integrates into the outer membrane (OM) and facilitates transport of the passenger domain across the membrane by an unknown mechanism. The passenger domains of some autotransporters are then cleaved from the cell surface. About 10% of autotransporters contain signal peptides that exceed 50 amino acids in length. Although there is no obvious correlation between the presence of a long signal peptide and the effector function of the passenger domain, long signal peptides are associated with all members of one autotransporter family, the serine protease autotransporters of *E. coli* and *Shigella* (SPATEs). The C-terminal half of the long autotransporter signal peptides resembles a classical signal peptide in that it contains well defined N, H, and C regions but otherwise shows high sequence variability. The only distinguishing feature of this segment is an uncommonly high net positive charge in the N region. A recent study confirmed that the C-terminal half of the signal peptide of a SPATE produced by *E. coli* O157:H7 called EspP can act as an independent targeting signal (6). This fragment of the EspP signal peptide was previously designated ΔEspP, but is referred to here as EspP(C)_{35} (see Fig. 1).

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3 The abbreviations used are: IM, inner membrane; CTABr, cetyltrimethylammonium bromide; HA, influenza hemagglutinin epitope HA; IPTG, isopropylthiogalactoside; MBP, maltose-binding protein; OM, outer membrane; SRP, signal recognition particle; SPATE, serine protease autotransporter of *E. coli* and *Shigella*; TF, trigger factor; TPS, two-partner secretion.
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Interestingly, the N-terminal 25 amino acids of the long autotransporter signal peptides contain a unique, highly conserved sequence motif (MNK1YX(Hy).X(Hy).X(Hy).V/D(V/A)V/S/E/L/A/S R), where Hy is a large hydrophobic amino acid (22). Closely related N-terminal extensions are also found on the signal peptides of a subset of proteins that are secreted via the two partner secretion (TPS) pathway (23). Proteins secreted by the TPS pathway are transported across the OM by dedicated, coordinately expressed translocators and also tend to be very large.

The significance of the signal peptide extension is not entirely clear, although its uniqueness and high sequence conservation suggest that it has an important function. Because bacteria utilize multiple pathways to target proteins to the SecYEG complex, it is conceivable that the unusual segment dictates targeting pathway selection. Indeed it is possible that the long signal peptides route autotransporters into the SRP (Ffh) hypothesis, one study showed that the signal peptide of a SPATE called IcsA did not appear to require SRP across the IM does not require an unusual signal peptide (26). Remarkably, this study showed that the native EspP signal peptide was required primarily to prevent misfolding of the protein in the periplasm. The data suggested that the signal peptide transit slowly through the SecYEG complex and thereby prevents non-productive folding of the passenger domain by transiently tethering it to the periplasmic side of the IM.

Given the conflicting evidence on the role of the unusual autotransporter signal in specifying a targeting pathway, we decided to reevaluate the targeting of EspP and heterologous proteins that contain the EspP signal peptide under physiological conditions. Even though EspP(C)SP was clearly shown in a previous study to promote cotranslational targeting (6), we found that the full-length EspP signal peptide (EspP SP, Fig. 1) promotes post-translational targeting. Several lines of evidence indicated that the signal peptide extension routed presecretory proteins into a post-translational targeting pathway by exerting an unprecedented inhibitory effect on their interaction with SRP, TF, and the SecYEG complex. The observation that mutation of a nearly invariant residue in the N-terminal extension largely restored cotranslational targeting showed that this effect was due specifically to the presence of the conserved sequence motif. To account for the data, we propose that the N-terminal extension of EspP either recruits a novel cytoplasmic factor that mediates a post-translational targeting reaction or causes the signal peptide to adopt an unusual conformation in which it has reduced affinity for the translocation machinery.

EXPERIMENTAL PROCEDURES

Antiseras, Media, and Bacterial Strains—An antiserum generated against an N-terminal peptide of EspP has been previously described (26). Polyclonal rabbit antiseras against maltose-binding protein (MBP) and the influenza hemagglutinin epitope HA.11 (HA) were obtained from New England Biolabs and Covance, respectively. Selective media contained 100 μg/ml ampicillin or 30 μg/ml chloramphenicol as required. Unless otherwise noted, experiments were conducted at 37 °C. The E. coli K-12 strains used in this study were MC4100 (27), HD82 (MC4100 ara-), HD855 (MC4100 secB::kan; Ref. 12), HD856 (MC4100 tig::cat; Ref. 12) HD8114 (MC4100 ara-ompT::kan), HD1116 (MC4100 ara714 espB::lacZ Δlom510 clpP::cm ΔclpQY1172::tet ompT::kan), and WAM121 (MC4100 ara- pBAD33 ffh::kan tetK; Ref. 28).

Plasmid Construction—The following plasmids have been described previously: pJH28, which encodes malE, pJH28.1, which encodes the MBP*1 derivative of malE, pH36, which encodes a HA-tagged version of ompA, pJH46, which encodes an HA-tagged version of ompA containing the full-length EspP signal peptide, pJH47, which encodes malE containing the full-length EspP signal peptide, pRL56, which encodes wild-type espP and pRL51, which encodes espP with a non-cleavable passenger domain (4, 6, 12, 26). In all of these plasmids the presecretory protein is under the control of either the trc or tac promoter. An Nhel–Hind III fragment of pJH42 (112) containing the tig gene was cloned into pBAD33 (29) to create plasmid pH44. Plasmids encoding ompA with the EspP (N2A), EspP (A30P/T31P), EspP (L477T), and EspP (Hydro-) signal peptides (pJH80, pJH81, pJH82, and pJH83, respectively) were generated by site-directed mutagenesis of pJH46 using the oligonucleotides 5′-CGAGGCGCAAATATGCTCAAATTATACTCTC-3′, 5′-CCGGCAGAGTATCATCAAGACCACCTG-CTGTAAGA-3′, 5′-GCGAATTTTTTTAGGCACATTACCTGATCATAC-3′, and 5′-CCAGGCGCAATCTTTTTAGGCACATTACCTGATCATAC-3′ and 5′-CAAACCGCATACTTTTTAGGCACATTACCTGATCATAC-3′, respectively, were generated by site-directed mutagenesis of pJH46 using the oligonucleotides 5′-CGAGGCGCAAATATGCTCAAATTATACTCTC-3′, 5′-CCGGCAGAGTATCATCAAGACCACCTG-CTGTAAGA-3′, 5′-GCGAATTTTTTTAGGCACATTACCTGATCATAC-3′, and 5′-CCAGGCGCAATCTTTTTAGGCACATTACCTGATCATAC-3′ and 5′-CAAACCGCATACTTTTTAGGCACATTACCTGATCATAC-3′, respectively, were generated by site-directed mutagenesis of pJH46 using the oligonucleotides 5′-CGAGGCGCAAATATGCTCAAATTATACTCTC-3′, 5′-CCGGCAGAGTATCATCAAGACCACCTG-CTGTAAGA-3′, 5′-GCGAATTTTTTTAGGCACATTACCTGATCATAC-3′, and 5′-CCAGGCGCAATCTTTTTAGGCACATTACCTGATCATAC-3′, respectively, were generated by site-directed mutagenesis of pJH46 using the oligonucleotides 5′-CGAGGCGCAAATATGCTCAAATTATACTCTC-3′, 5′-CCGGCAGAGTATCATCAAGACCACCTG-CTGTAAGA-3′, 5′-GCGAATTTTTTTAGGCACATTACCTGATCATAC-3′, and 5′-CCAGGCGCAATCTTTTTAGGCACATTACCTGATCATAC-3′, respectively, were generated by site-directed mutagenesis of pJH46 using the oligonucleotides 5′-CGAGGCGCAAATATGCTCAAATTATACTCTC-3′, 5′-CCGGCAGAGTATCATCAAGACCACCTG-CTGTAAGA-3′, 5′-GCGAATTTTTTTAGGCACATTACCTGATCATAC-3′, and 5′-CCAGGCGCAATCTTTTTAGGCACATTACCTGATCATAC-3′, respectively, were generated by site-directed mutag...
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peptide (pJH84) was constructed by site-directed mutagenesis of pJH83 using the oligonucleotide 5′-TTATTTTATCTTTATTAATGTTTCAATCTCATATTTGCGGCCG-3′ and its complement. Plasmid pJH86, which encodes ompA with the EspP (−6) signal peptide was made by first mutagenizing pJH6 using the oligonucleotide 5′-GAGCAACTGTAAGAAAGAAAAACGCAACAGATACCTTGACTATAG-3′ and its complement to generate pJH85. Subsequently pJH85 was mutagenized using the oligonucleotide 5′-CAAGAGCAACTGTCATCTTGCAATGTTTCAATCCTCATATTCTTTTGCGGCCG-3′ and its complement. Plasmids encoding espP with the EspP (N2A) and EspP (−6) signal peptides (pJH87 and pJH88) were generated by digesting pJH80 and pJH86 with NdeI and EagI and ligating the small fragment containing the signal peptide to pRLS6 digested with the same enzymes. Plasmids encoding malE with the EspP (N2A) and EspP (Hydro) signal peptides (pJH89 and pJH90, respectively) were made by site-directed mutagenesis of pJH47 using the oligonucleotides 5′-CCAACAAGGACATAGCATATGCTAATAATACCTCTC-3′, and EspP 2L and their complements. A plasmid encoding malE with the EspP (Hydro) signal peptide (pJH91) was made by mutagenizing pJH90 using the oligonucleotide 5′-TTATTTTATCTTTATTAATGTTTCAATCCTCATATTCTTTTGCGTCAC-3′ and its complement. All mutations were made using the QuickChange mutagenesis kit (Stratagene) according to the manufacturer’s instructions.

Pulse-Chase and Cell Fractionation Experiments—For most experiments, cells were grown in M9 containing 0.2% glycerol and all the L-amino acids except methionine and cysteine. In experiments in which a secB− strain was used, cells were grown in M9 containing 0.2% glucose. Overnight cultures were washed and diluted into fresh medium at an absorbance (A550) = 0.02 (for EspP) or 0.025 (for OmpA and MBP). Unless otherwise noted, synthesis of plasmid-borne polypeptides was induced by the addition of isopropylthiogalactoside (IPTG) at a concentration of 50 μM (for OmpA and MBP) or 100 μM (for EspP) when the cultures reached A550 = 0.2. In temperature-shift experiments, cultures were shifted from 37 to 22 °C when they reached A550 = 0.2 and grown for an additional 40 min before the addition of IPTG. In TF overproduction experiments, 0.2% arabinose was added when cultures reached A550 = 0.2, and IPTG was added 30 min later. Pulse-chase labeling was conducted as previously described (30) 20 min (for OmpA and MBP) or 30 min (for EspP) after the addition of IPTG. Sodium azide (2 mM) was added 5 min (for EspP) after the addition of IPTG. Sodium azide (2 mM) was added 20 min (for OmpA and MBP) or 100 μM (for EspP) when the cultures reached A550 = 0.2. In temperature-shift experiments, cultures were shifted from 37 to 22 °C when they reached A550 = 0.2 and grown for an additional 40 min before the addition of IPTG. In TF overproduction experiments, 0.2% arabinose was added when cultures reached A550 = 0.2, and IPTG was added 30 min later. Pulse-chase labeling was conducted as previously described (30) 20 min (for OmpA and MBP) or 30 min (for EspP) after the addition of IPTG. Sodium azide (2 mM) was added 20 min (for OmpA and MBP) or 100 μM (for EspP) when the cultures reached A550 = 0.2. In temperature-shift experiments, cultures were shifted from 37 to 22 °C when they reached A550 = 0.2 and grown for an additional 40 min before the addition of IPTG. In TF overproduction experiments, 0.2% arabinose was added when cultures reached A550 = 0.2, and IPTG was added 30 min later. Pulse-chase labeling was conducted as previously described (30) 20 min (for OmpA and MBP) or 30 min (for EspP) after the addition of IPTG. Sodium azide (2 mM) was added 20 min (for OmpA and MBP) or 100 μM (for EspP) when the cultures reached A550 = 0.2, and cells were radiolabeled 30 min later. In general, radiolabeled aliquots were removed from each culture and immediately mixed with cold trichloroacetic acid to precipitate proteins. In cell fractionation experiments, radiolabeled cells were first poured over ice, and all subsequent steps were conducted at 0–4 °C. Cells were centrifuged at 2,500 × g for 10 min and converted to spheroplasts as described (30). After the addition of 10 mM MgSO4, spheroplasts were pelleted at 16,500 × g for 5 min, and the supernatant, which contained the contents of the periplasm, was discarded. Spheroplasts were then resuspended in 80 μl of 33 mM Tris-HCl (pH 8.0), 40% sucrose and lysed by the addition of 3.2 ml of H2O2. Unbroken spheroplasts were removed by centrifugation at 2,500 × g for 5 min. Subsequently the spheroplast lysate (designated “total” in these experiments) was centrifuged at 100,000 × g for 30 min to obtain cytoplasmic and membrane fractions. Proteins in each fraction were collected by trichloroacetic acid precipitation.

Trichloroacetic acid-precipitated proteins were solubilized, and immunoprecipitations were performed as described (30). In experiments that involved production of EspP or one of its derivatives, proteins were resolved by SDS-PAGE on 15 × 15 cm 4–10% gradient gels. In all other experiments, proteins were resolved on 8–16% minigels (Novex). Radiolabeled proteins were visualized using a Fuji BAS-2500 PhosphorImager, and percent precursor was calculated as previously described (31). In experiments in which the biogenesis of EspP was monitored, the data were quantitated using an analogous method. The intensity of the prepro-EspP, pro-EspP, and passenger domain bands was first normalized to take into account differences in the number of radioactive amino acids. The percent of the protein in each band was then calculated by dividing the normalized intensity of that band by the sum of the normalized intensities of all of the bands and multiplying by 100.

RESULTS

Proteins Containing EspP6 Are Targeted to the IM Post-translationally but Do Not Interact with TF—In initial experiments, we sought to determine whether EspP is translocated across the IM by the SecYEG complex. HDB114 transformed with pRLS6, a plasmid that encodes wild-type espP under the control of the trc promoter, was grown in M9 minimal medium. After the addition of IPTG to induce espP expression, cells were subjected to pulse-chase labeling in the presence or absence...
of sodium azide, a compound that blocks Sec-dependent protein translocation. Prepro-EspP, the form of EspP that contains the signal peptide, pro-EspP, the leaderless form of EspP that contains linked passenger and β-domains, and the free passenger domain were then immunoprecipitated with an anti-peptide antiserum raised against an N-terminal peptide. Consistent with previous results (26), in the absence of azide, prepro-EspP was rapidly converted to pro-EspP, and the passenger domain was then excised from pro-EspP after its translocation across the OM (Fig. 2A, lanes 1–5). In contrast, the addition of azide caused a profound defect in EspP biogenesis and led to the accumulation of prepro-EspP (Fig. 2A, lanes 6–10). These results confirmed that EspP is translocated across the IM in a Sec-dependent fashion despite the presence of an unusual signal peptide.

Interestingly, evidence that EspP is targeted to the IM post-translationally emerged from these experiments. Because signal peptides are usually removed by leader peptidase before completion of protein synthesis during cotranslational translocation, the observation that ~20–30% of the pulse-labeled protein was in the pro form in untreated cells (Fig. 2A, lane 1) was by itself diagnostic of post-translational targeting. To rule out the possibility that the appearance of the precursor was simply caused by unusually slow cleavage of the signal peptide, we performed cell fractionation experiments using pulse-labeled cells. The finding that almost all of the pro form of the protein was in the cytoplasm (Fig. 2B) provided direct evidence that at least some of the protein was completely synthesized before it was targeted to the IM. Furthermore, two additional observations suggested that the prepro-

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**FIGURE 3.** EspPSP-OmpA is targeted to the IM post-translationally in a TF-independent fashion. A. MC4100 transformed with pJH46 (Ptrc espPSP-ompA) was grown in M9, and expression of the plasmid-borne gene was induced with IPTG. Cells were subjected to pulse-chase labeling and either trichloroacetic acid-precipitated (left panel) or converted to spheroplasts and fractionated (right panel). Plasmid-derived OmpA was then immunoprecipitated with an anti-HA antiserum; p, precursor; m, mature; T, total spheroplast extract; C, cytoplasm; M, membranes. The length of the chase is indicated. B, MC4100 or HDB56 (MC4100 tig−) transformed with pJH46 or a derivative encoding espP(Hydro1)SP-OmpA or espP(Hydro2)SP-OmpA were grown as in A and shifted to 22 °C prior to pulse-chase labeling. C, the experiment described in B was repeated except that cells were pulse-labeled and subjected to a 2-min chase, and then fractionated. D, HDB20 (MC4100 ara−) was transformed with pJH44 (PBAD-tig) and either pHL36 (Ptac ompA) or pJH46. Cultures were divided in half, arabinose was added to one portion to induce overproduction of TF, and cells were subjected to pulse-chase labeling. –Ara, no arabinose added; +Ara, arabinose added.
EspP found in untreated cells was an intermediate in EspP biogenesis and not a misfolded form of the protein that was rapidly degraded in the cytoplasm. First, prepro-EspP did not appear to be a prime target for cytoplasmic proteases because it was stable in azide-treated cells for >10 min (Fig. 2A, lanes 6−10). Second, elimination of cytoplasmic proteases that have been implicated in the degradation of misfolded proteins did not increase the half-life of prepro-EspP. This conclusion was derived from pulse-chase experiments in which HDB114 and HDB116, a strain that lacks Lon and both Clp proteases, were transformed with pRLS11, a plasmid encoding a version of EspP that contains a non-cleavable passenger domain (EspP*). In both strains prepro-EspP was observed at early time points, but disappeared at later timepoints (Fig. 2C). Taken together, the data imply that at least 20−30% of the protein was targeted post-translationally. Because the synthesis and targeting of some of the protein was completed early in the pulse-labeling period; however, it is possible that a much higher fraction of the protein was targeted post-translationally.

To obtain further evidence that EspPsp promotes post-translational targeting, we next replaced the native signal peptide of OmpA with EspPsp and examined the export kinetics of the resulting chimera (EspPsp-OmpA). OmpA was chosen as a model largely because interactions between both the wild-type protein and a variety of derivatives with SRP and chaperones have been studied in detail. MC4100 transformed with a plasmid encoding HA-tagged espPsp-ompA under the control of a trc promoter (pJH46) was grown in M9. Cells were subjected to pulse-chase labeling after the addition of IPTG, and the chimeric protein was immunoprecipitated with an anti-HA antiserum. Approximately 30−50% of the pulse-labeled EspPsp-OmpA was in the precursor form but was eventually processed to mature OmpA (Fig. 3A, top panel). Cell fractionation experiments showed that the precursor was located in the cytoplasm, and therefore must have been targeted to the IM as a fully synthesized protein (Fig. 3A, right panel). When cells were grown at 22 °C, a temperature at which post-translational targeting is markedly slowed (32), virtually all of the EspPsp-OmpA in pulse-labeled cells was in the precursor form but was eventually processed to mature OmpA (Fig. 3B, left panel). Cell fractionation experiments showed that the precursor that remained after a 2-min chase was still located in the cytoplasm (Fig. 3C, lanes 1−3). In addition, when cells were converted to spheroplasts the precursor was completely resistant to digestion with proteinase K (data not shown). Thus at low temperature, most or all of the chimera was targeted post-translationally. These results were particularly striking given that only mature OmpA was observed at all time points and all temperatures when the export of OmpA containing EspP(C)sp was investigated (6).

Remarkably, we found that elevating the hydrophobicity of the EspP signal peptide did not promote cotranslational targeting of OmpA. We mutagenized the plasmid encoding HA-tagged espPsp-ompA to generate two derivatives that contain signal peptides of increasing hydrophobicity (EspP(Hydro)3sp-OmpA and EspP(Hydro)5sp-OmpA, see Fig. 1). Based on previous studies (4, 5), we expected that SRP would bind with high affinity to these unusually hydrophobic signal peptides. Indeed the export of MBP containing identical hydrophobic derivatives of EspP(C)sp is highly SRP-dependent (6). To examine the targeting of the mutant chimeras, MC4100 transformed with a mutant plasmid were grown at 22 °C and subjected to pulse-chase labeling as described above. Like EspPsp-OmpA, both EspP(Hydro)3sp-OmpA and EspP(Hydro)5sp-OmpA appeared to be exported in a post-translational fashion (Fig. 3B, middle and bottom panels). These results strongly suggest that EspP is targeted post-translationally because the presence of the N-terminal extension prevents SRP from binding to the signal peptide.

Perhaps equally surprisingly, we found that the post-translational targeting of EspPsp-OmpA was not caused by an interaction of the protein with TF. Consistent with the view that the binding of TF to presecretory proteins prevents docking of ribosomes onto the SecYEG complex and thereby promotes post-translational targeting, previous work has shown that elimination of TF accelerates the export of OmpA and other proteins in vivo whereas overproduction of TF delays protein export (12). Moreover, cross-linking experiments have yielded considerable evidence that TF interacts with OmpA directly (8, 13, 33). To test the effect of TF binding on the export of EspPsp-OmpA, we initially compared the rate of export of the protein at 22 °C in MC4100 and HDB56, a strain that lacks TF. Although wild-type OmpA is exported much faster in HDB56 than MC4100 at this temperature (12), elimination of TF had no effect on the rate of EspPsp-OmpA export (Fig. 3B, top panel, lanes 5–8). As in MC4100, essentially all of the EspPsp-OmpA precursor was found in the cytoplasm (Fig. 3C, lanes 4–6). Subsequently we transformed MC4100 with either pH36 (ompA- HA+) or pH46 (espPsp-ompA-HA-) and pH44, a plasmid that encodes TF under the control of an arsRAD promoter. Cells were radiolabeled after the addition of arabinose to induce expression of the plasmid-borne tig gene. Overproduction of TF strongly retarded the export of wild-type OmpA, but did not significantly affect the export of EspPsp-OmpA (Fig. 3D).

These results strongly suggest that the exclusion of proteins containing EspPsp from the SRP pathway results from the inaccessibility of the hydrophobic core rather than from the binding of TF. In the absence of TF binding, either inefficient interaction of the signal peptide with the SecYEG complex or the binding of another cytoplasmic factor would presumably hinder cotranslational translocation.

To rule out the possibility that the unexpected results we obtained were simply a consequence of producing EspPsp-OmpA at a moderately high level, we grew MC4100 containing pH46 as described above but did not add IPTG prior to radiolabeling. Under these conditions the expression of the plasmid-borne gene was reduced nearly 20-fold to a level that was ~10-fold lower than that of the endogenous ompA gene (data not shown). The large reduction in the synthesis of EspPsp-OmpA, however, did not significantly alter the kinetics of export at either 37 °C or 22 °C (Fig. 4A). The results imply that the post-translational export of EspPsp-OmpA described above is not due to the saturation of a target-
ing factor that has a limited capacity or to a concentration-dependent self-association.

Efficient Export of Presecretory Proteins Containing EspP<sub>SP</sub> Is Dependent on SecB but Not SRP—To obtain further evidence that proteins containing EspP<sub>SP</sub> are targeted post-translationally, we next examined the fate of EspP and a chimeric version of MBP containing EspP<sub>SP</sub> (EspP<sub>SP</sub>-MBP) in cells that lack SecB. MBP was chosen as a reporter because export of the wild-type protein is particularly SecB-dependent (34) and because the targeting of derivatives containing a variety of signal peptides has been studied in detail. In initial experiments MC4100 and HDB55, a strain that contains a secB-null allele, were transformed with pRLS6 (espP/H11001). Cells were subjected to pulse-chase labeling after the addition of IPTG, and immunoprecipitations were performed using the N-terminal EspP antiserum (A) or anti-MBP (B). The length of the chase is indicated. p, precursor; m, mature.

Consistent with the observation that EspP(Hydro<sup>+</sup>)<sub>SP</sub>-OmpA and EspP(Hydro<sup>+</sup>)<sub>SP</sub>-OmpA are targeted to the SecYEG complex post-translationally, we found that increasing the hydrophobicity of the signal peptide did not reduce the SecB-dependence of EspP<sub>SP</sub>-MBP export. MC4100 and HDB55 were transformed with plasmids that produce EspP(Hydro<sup>+</sup>)<sub>SP</sub>-MBP or EspP(Hydro<sup>+</sup>)<sub>SP</sub>-MBP (pJH90 and pJH91) and the export of the chimeric proteins was examined in pulse-chase experiments as described above. Elimination of SecB produced a modest export defect very similar in magnitude to that observed when the biogenesis of EspP<sub>SP</sub>-MBP was analyzed (Fig. 5B, lanes 13–24). In contrast, the targeting of MBP containing highly hydrophobic signal peptides (including hydrophobic derivatives of EspP(C)<sub>SP</sub>) has previously been shown to be completely SecB-independent (4, 6). In addition, a signifi-
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FIGURE 6. Export of proteins containing EspPSP does not require SRP. WAM121 (MC4100 fffh) was transformed with plasmids that produce MBP*1, EspPSP-MBP, EspPHydro*1SP-MBP or EspPHydro*1SP-MBP. Cells were grown in the presence of arabinose (+ Ara) or glucose (+ Dex) and subjected to pulse-chase labeling after the addition of IPTG. Immunoprecipitations were performed using anti-MBP. The length of the chase is indicated: p, precursor; m, mature.

cant amount of the precursor form of the protein (which is diagnostic of post-translational targeting) was observed in pulse-labeled MC4100 (Fig. 5B, lanes 13 and 19). Taken together, these data provide additional evidence that the binding of SRP to the full-length EspP signal peptide is hindered.

More direct evidence that SRP fails to recognize EspPSP emerged from SRP depletion experiments. We first transformed WAM121, a strain in which the gene encoding the protein subunit of SRP (ffh) is under the control of the araBAD promoter, with pJH47, pJH90, or pJH91. As a positive control, WAM121 was also transformed with pJH28.1, a plasmid that encodes a derivative of MBP containing a highly hydrophobic signal peptide (MBP*1; see Ref. 4). The cells were then grown in M9 containing either arabinose (to maintain Ffh synthesis) or glucose (to deplete Ffh) and radiolabeled after the addition of IPTG. Consistent with previous results, we found that the export of MBP*1 was strongly impaired in cells that lacked Ffh (Fig. 6, lanes 1–6). A small amount of the MBP*1 precursor was observed in pulse-labeled WAM121 grown in the presence of arabinose (Fig. 6, lane 1) but not in MC4100 (data not shown) and probably reflects a slight defect in the SRP pathway caused by placing fffh under an inducible promoter. By contrast, Ffh depletion did not significantly affect the export of EspPSP-MBP, EspPHydro*1SP-MBP or EspPHydro*1SP-MBP (Fig. 6, lanes 7–24). These results are remarkable because (as mentioned above) the export of MBP containing identical hydrophobic derivatives of EspP(SP) is highly SRP-dependent (6). Curiously, we found that Ffh depletion slowly delayed the export of native EspP (data not shown). This observation is consistent with results obtained in a study on the targeting of Hbp (20). Given that all of our other data indicate that EspPSP evades recognition by SRP and that perturbation of the SRP pathway has been shown to indirectly inhibit the export of at least one presecretory protein that is not a bona fide substrate (35); however, it is likely that the defect in EspP biogenesis is a secondary effect of SRP depletion.

Mutation of a Conserved Residue in the N-terminal Extension of EspPSP Accelerates Protein Export—We suspected that the N-terminal extension of EspPSP exerts a unique effect on protein targeting through its highly conserved amino acid sequence. To test this hypothesis, we created a mutant version of EspPSP in which the nearly invariant asparagine residue that follows the initiator methionine was changed to alanine (EspP(N2A)SP; see Fig. 1). HDB114 transformed with a plasmid producing EspP(N2A)SP-EspP, and MC4100 and HDB55 transformed with a plasmid producing EspP(N2A)SP-OmpA or EspP(N2A)SP-MBP were subjected to pulse-chase labeling after the addition of IPTG as described above. In contrast to the experiments performed with wild-type EspP, significant amounts of the prepro form of EspP(N2A)SP-EspP were not observed at any time point (Fig. 7A). Likewise, almost none of the precursor form of EspP(N2A)SP-OmpA was seen in pulse-labeled cells at 37 °C (Fig. 7B, left panel). At 22 °C, a small fraction of the protein was found in the precursor form, but only at early time points (Fig. 7B, right panel). Finally, only 3 and 10% of the EspP(N2A)SP-MBP was in the precursor form in pulse-labeled MC4100 and HDB55, respectively (compared with 18 and 46% for EspPSP-MBP) (Fig. 7C, lanes 1 and 4). All of these results indicate that mutation of a key residue in the N-terminal extension of the EspP signal peptide almost completely restores cotranslational targeting, presumably by suppressing the inhibitory effect that the conserved segment exerts on SRP binding. Moreover, these results imply that the presence of a specific amino acid sequence upstream of the hydrophobic core of EspPSP is (rather than the mere presence of 30 extra amino acids) promotes post-translational targeting.

Mutations in the C-terminal Half of EspPSP Significantly Delay Export—The analysis of an EspP derivative that contains a mutation in the C-terminal half of its signal peptide strongly reinforced our conclusion that the protein can be targeted to the IM effectively via a post-translational mechanism despite its large size. In this derivative we changed all of the charged amino acids upstream of the hydrophobic core of EspPSP to glutamine to create EspP(-6)SP (rather than the mere presence of 30 extra amino acids) promotes post-translational targeting.

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Function of an Unusual Signal Peptide Extension

In this report we show that a ~25 amino acid N-terminal extension associated with a long autotransporter signal peptide dramatically alters its targeting function. We found that both EspP and heterologous proteins containing EspPSP are targeted to the SecYEG complex post-translationally even though EspPSP, which resembles a classical signal peptide, has been shown to promote cotranslational targeting. Like other proteins that are targeted post-translationally, EspPSP-containing proteins required SecB for optimal export but did not require SRP. The post-translational targeting reaction mediated by EspPSP, however, was completely novel in two respects. First, proteins containing EspPSP could not be rerouted into the SRP pathway by increasing the hydrophobicity of the signal peptide. The data are particularly striking because there are no known instances in which the sequence upstream of a targeting signal blocks SRP binding. Indeed SRP recognizes the first half of EspPSP also retarded protein export. To reduce the hydrophobicity of EspPSP, a leucine in the middle of the H region was changed to threonine to create EspP(L47T)SP (see Fig. 1). MC4100 transformed with a plasmid encoding espPL47TSPompA was subjected to pulse-chase labeling as described above. Because the H region of EspPSP is more hydrophobic than that of most other signal peptides, we did not expect this mutation to affect the targeting of the protein. Indeed previous work has shown that introduction of the equivalent mutation into EspP(C)SP has no discernable effect on the rate of protein export (6). Surprisingly, EspP(L47T)SP-OmpA was targeted to the IM much more slowly than EspPSP-OmpA (compare Fig. 8D, middle panel to Fig. 3A, left panel). A possible explanation of the data is that the H region of the signal peptide is partially masked in such a way that a slight reduction in hydrophobicity hinders binding to the SecYEG complex. This interpretation also provides an explanation for the finding that presecretory proteins containing exceptionally hydrophobic derivatives of EspPSP (e.g. EspP(Hydro)e) can be targeted to the IM post-translationally without aggregating. In addition, a double point mutation in a highly variable region of EspPSP that resides between the N-terminal extension and the basic N region (A30P/T31P) also retarded protein export, although more modestly than L47T (Fig. 8D, bottom panel). These data are consistent with a scenario in which EspPSP-OmpA and EspP(L47T)SP-OmpA all prolong the targeting delay associated with the wild-type signal peptide. The mutations might inhibit dissociation of an external factor that normally binds to EspPSP and stabilize a unique conformation that normally hinders binding of EspPSP to the SecYEG complex. Given the distinct character of the mutations, however, it is unlikely that they all fortuitously promote an aberrant reaction between the signal peptide and a non-physiological receptor.

DISCUSSION

The time course whereas the cleaved passenger domain continued to accumulate (Fig. 8B). The simplest explanation of the data is that the pro form was continuously generated over a period of several minutes from the prepro form and cleaved into discrete passenger and β-domains at about the same rate. Interestingly, about 80% of the protein was eventually translocated across the IM. The results indicate that the signal peptide mutation dramatically delays but does not prevent translocation, and imply that prepro-EspP can remain translocation-competent in the cytoplasm for a prolonged period.

To confirm that the slow export of EspP(-6)SP-EspP was caused by the presence of the mutant signal peptide, we examined the export of EspP(-6)SP-OmpA in MC4100 in pulse-chase experiments. As expected, the EspP(-6)SP-OmpA precursor was converted to the mature form much more slowly than the EspPSP-OmpA precursor (compare Fig. 8D, top panel to Fig. 3A, left panel). Despite the slow conversion, all of the precursor was found in the cytoplasm and therefore must have been targeted to the IM as a fully synthesized protein (Fig. 8E). These results are striking because previous work showed that introduction of the same mutation into EspP(C)SP, had no discernable effect on the rate of protein export (6). To rule out the possibility that the signal peptide mutation delayed targeting by promoting an artificial association between EspP(-6)SP-OmpA molecules when the protein was synthesized at a moderately high level, we repeated the pulse-chase experiments described above but did not add IPTG prior to radiolabeling. The sharp drop in the production of EspP(-6)SP-OmpA, however, did not significantly affect the rate of export (Fig. 4B). Taken together, the data suggest that the charge mutation either exacerbates the inefficiency in protein targeting associated with the wild-type signal peptide or promotes the binding of the signal peptide to a factor that acts as an adventitious receptor.

Interestingly, two very different types of mutations in the C-terminal half of EspPSP also retarded protein export. To reduce the hydrophobicity of EspPSP, a leucine in the middle of the H region was changed to threonine to create EspP(L47T)SP (see Fig. 1). MC4100 transformed with a plasmid encoding espPL47TSPompA was subjected to pulse-chase labeling as described above. Because the H region of EspPSP is more hydrophobic than that of most other signal peptides, we did not expect this mutation to affect the targeting of the protein. Indeed previous work has shown that introduction of the equivalent mutation into EspP(C)SP has no discernable effect on the rate of protein export (6). Surprisingly, EspP(L47T)SP-OmpA was targeted to the IM much more slowly than EspPSP-OmpA (compare Fig. 8D, middle panel to Fig. 3A, left panel). A possible explanation of the data is that the H region of the signal peptide is partially masked in such a way that a slight reduction in hydrophobicity hinders binding to the SecYEG complex. This interpretation also provides an explanation for the finding that pressecretory proteins containing exceptionally hydrophobic derivatives of EspPSP (e.g. EspP(Hydro)e) can be targeted to the IM post-translationally without aggregating. In addition, a double point mutation in a highly variable region of EspPSP that resides between the N-terminal extension and the basic N region (A30P/T31P) also retarded protein export, although more modestly than L47T (Fig. 8D, bottom panel). These data are consistent with a scenario in which EspPSP-OmpA and EspP(L47T)SP-OmpA all prolong the targeting delay associated with the wild-type signal peptide. The mutations might inhibit dissociation of an external factor that normally binds to EspPSP and stabilize a unique conformation that normally hinders binding of EspPSP to the SecYEG complex. Given the distinct character of the mutations, however, it is unlikely that they all fortuitously promote an aberrant reaction between the signal peptide and a non-physiological receptor.
the properties of the signal peptide. Consistent with this notion, mutation of the most highly conserved residue in the motif appeared to diminish the effect of the N-terminal extension on protein targeting and largely restored cotranslational export.

Our results raise the possibility that the N-terminal extension promotes the interaction of an unidentified cytoplasmic factor with EspPSP at an early stage of translation. This interaction might block the binding of SRP and TF to the nascent chain and promote post-translational translocation by inhibiting the association of the signal peptide with the SecYEG complex. The interaction of a cytoplasmic factor with EspPSP might account for the surprisingly effective post-translational export of proteins containing EspP(Hydro/His)SP and EspP(Hydro/His)SP. The results strongly suggest that the H region of these signal peptides is at least partially sequestered because a high degree of hydrophobicity tends to promote rapid aggregation and generally imposes a requirement for cotranslational targeting (6, 12). Attempts to identify a novel factor that binds to EspPSP by cross-linking (both in vivo and in vitro) or to show a novel interaction between polypeptides containing EspPSP and the ribosome by copurification, however, have been unsuccessful (Refs. 20 and 25).4 Curiously, we and others have observed cross-linking between SPATE signal peptides and SRP in vitro (Ref. 20).4 The incompatibility between the results obtained in vivo and in vitro suggests that any interaction between a novel factor and EspPSP is unstable in cell extracts. Biochemical studies have suggested that SecA can interact with signal peptides that have a highly basic N region at an early stage of translation (15, 38), but because EspP(-6)SP (which contains an uncharged N region) promotes post-translational targeting, it does not appear likely that SecA recognizes EspPSP.

Alternatively, it is possible that EspPSP-containing proteins are targeted post-translationally because the N-terminal extension drives the signal peptide into a conformation in which it binds to the SecYEG complex relatively slowly (Fig. 9). Presumably the H region of the signal peptide would be shielded from aggregation in this conformational state. In light of recent evidence that nascent polypeptides can acquire secondary structure in the ribosome tunnel (39), it is conceivable that EspPSP folds partially before it encounters SRP and TF and thereby prevents an effective interaction between the nascent chain and the ribosome-associated factors. It is also possible, however, that a conformational change occurs at a later stage of translation. In this scenario the N-terminal extension might bypass SRP and TF by transmitting a signal from the ribosome tunnel to the exterior of the ribosome that would force their dissociation from L23 or by directing nascent presecretory proteins to one of the alternate ribosome tunnels recently identified by cryo-electron microscopy (40). In any case, the finding that SRP can be cross-linked to SPATE signal peptides strongly suggests that any distinctive conformational state is difficult to reproduce or maintain in vitro.

Although our data are compatible with both of the above models, the conformational model has the distinct advantage of providing a possible structural basis for the remarkable ability of the long signal peptide to facilitate late stages of EspP biogenesis (26). The observation that overproduction of a protein containing EspPSP (but not EspP(C)SP) delayed

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the export of other proteins in trans led to the proposal that EspP
transits slowly through the SecYEG complex and thereby acts as a tran-
sient membrane anchor that prevents misfolding of the passenger
domain after its transport into the periplasm. In light of these results, it
seems plausible that autotransporter signal peptides acquired the ability
to adopt a unique conformation not to promote post-translational tar-
getting (which is simply an epiphenomenon) but rather to facilitate slow
dissociation from the SecYEG complex or to slow cleavage by leader
peptidase (Fig. 9). This is an attractive hypothesis because it explains
why a signal peptide extension that has the curious property of delaying
the onset of translocation would have ever evolved. In addition, the
observation that EspP(N2A)SP restores cotranslational targeting (Fig. 7)
but does not cause the defect in EspP biosynthesis that is associated
with deletion of the N-terminal extension (26) is more readily explained
by the conformational model. The data suggest that the mutation delays,
but does not abolish, the structural transition that is required for the
signal peptide to promote effective EspP biosynthesis. Consistent with
this interpretation, we found that a derivative of EspPSP-OmpA containing
both the N2A and L47T mutations is exported extremely slowly.4 If
the N2A mutation completely abolished the effect of the N-terminal
extension on signal peptide function, then the double mutant should
be exported as rapidly as the N2A mutant. The data suggest that despite
the presence of the N2A mutation, a conformational change that accounts
for the slow interaction of EspP(L47T)SP-OmpA with the SecYEG com-
plex (Fig. 8D) ultimately still occurs.

Finally, our results provide strong evidence against the hypothesis
that autotransporters must be targeted to the SecYEG complex cotran-
slationally to avoid folding prematurely in the cytoplasm. On the con-
tary, the data show that EspP can be maintained in a translocation-
competent state for a surprisingly long period of time and imply that
protein size does not accurately predict the targeting requirements of a
bacterial protein. In this regard it is interesting to note that secondary
structure predictions as well as crystallographic analysis have indicated
that both autotransporters and proteins secreted via the TPS pathway
are largely comprised of β-strands that form β-helical and β-barrel
structures (41–44). It has previously been shown that OM proteins such
as OmpA and LamB that fold into a β-barrel (but not periplasmic pro-
teins that contain a mixture of α-helices and β-strands) can be main-
tained in a prolonged translocation-competent conformation in the
cytoplasm by DnaK (45). In light of these considerations and the results
that we describe here, it is tempting to speculate that the effectiveness
of post-translational targeting may be more closely related to the compo-
sition of a protein than to its size.

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