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Different *sec*-requirements for signal peptide cleavage and protein translocation in a model *E. coli* protein

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We describe a secretory *E. coli* protein with a novel phenotype: signal peptide cleavage is largely unaffected whereas chain translocation is efficiently blocked under conditions where SecA, a central component of the secretory machinery, is rendered non-functional, and we have traced this phenotype to the presence of a mildly hydrophobic segment located ~30 residues downstream of the signal peptide. When this segment is deleted, normal SecA-dependent signal peptide cleavage and chain translocation is observed; when its hydrophobicity is increased, it becomes a permanent membrane anchor with cleavage of the signal peptide and membrane insertion both being SecA-independent. These findings suggest that the initial insertion of the signal peptide across the membrane can be uncoupled from the translocation process proper.

Protein secretion; Protein export; Signal peptide

1. INTRODUCTION

In *E. coli*, protein export across the inner membrane involves at least five distinct proteins: the peripheral inner membrane protein SecA, and the integral inner membrane proteins SecY, SecE, SecD, and SecF [1–3]. In addition, to be efficiently exported, many but not all exported proteins must be prevented from premature folding or aggregation by cytoplasmic chaperones such as SecB, DnaK, or GroEL [4–6].

On the other hand, certain inner membrane proteins can be inserted into the membrane under conditions where the normal functioning of SecA or SecY is perturbed [7,8]. This kind of '*sec*-independent' insertion is possible only when the translocated segment of the protein is less than 50–60 residues long and does not contain too many positively charged residues [9–12].

In general, when the *sec*-machinery is inactivated in vivo, both cleavage of the signal peptide and translocation of the nascent chain is prevented. We now report on a novel phenotype, not hitherto observed in *E. coli*, where translocation depends on an intact *sec*-machinery while signal peptide cleavage does not. This is observed for a molecule where the signal peptide is followed by a mildly hydrophobic segment: when this segment is deleted, a normal phenotype with *sec*-dependent signal peptide cleavage and translocation is observed; when, on the other hand, the hydrophobicity of this segment is increased, the protein becomes permanently anchored to the inner membrane. These findings suggest that the initial insertion of the signal peptide across the membrane can be uncoupled from the translocation process proper.

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2. MATERIALS AND METHODS

2.1. *Enzymes and chemicals*

Trypsin, soybean trypsin inhibitor, chicken egg white lysozyme, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma. All other enzymes were from Promega and Pharmacia (T7 DNA polymerase).

2.2. *Strains and plasmids*

Leader peptidase mutants were expressed from the pING1 plasmid [13] in *E. coli* strains MC1061 (*ΔlacX74 araD139 Δ(ara, leu)7697, galU, galK, hsr, hsm, strA*) [14] and MC4100 (F⁻ *ΔlacU169 araD139 relA rpsL thi*) [15].

2.3. *DNA techniques*

Site-specific mutagenesis was performed according to the method of Kunkel [16], as modified by Geisselsoder et al. [17]. All mutants were confirmed by DNA sequencing of single-stranded M13 DNA using T7 DNA polymerase. Cloning into the pING1 plasmid was performed as described [18].

2.4. *Assay of membrane topology*

E. coli strains MC1061 and MC4100 transformed with the pING1 vector carrying mutant leader peptidase (*lep*) genes under control of the arabinose promoter were grown at 37°C in M9 minimal medium supplemented with 100 μg/ml ampicillin, 0.5% fructose, and all amino acids (50 μg/ml each) except methionine. Overnight cultures were diluted 1:25 in 1 ml fresh medium, shaken for 3.5 h at 37°C, induced with arabinose (0.2%) for 5 min, and labeled with [^{35}S]methionine (150 μCi/ml). After 2 min, nonradioactive methionine was added (500 μg/ml) and, after an additional 1 min chase, incubation was stopped by chilling on ice. To test for SecA dependence, constructs were induced with arabinose as above, and sodium azide was added (2 mM final concentration) 4 min after induction. One minute later, cells were labeled with [^{35}S]Met for 2 min, chased in the presence of excess nonradioactive methionine for 1 min, and put on ice. Cells were spun at 15,000 rpm for 2 min, resuspended in ice-cold buffer (40% w/v

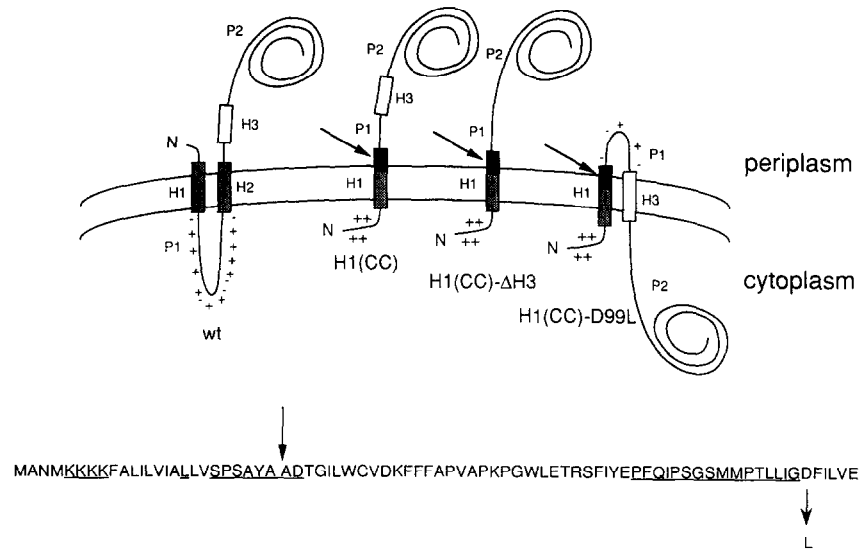

Fig. 1. Mutants discussed in the text and their relation to wild-type Lep. To make H1-CC, four lysines were added between residues 4 and 5 to the N-terminus of the H1 transmembrane region (residues 4–22), Thr^{13} in H1 was changed to Leu, a 'cleavage cassette' (CC, black) was added to H1 between residues 15 and 16, and residues 30–52 in P1 were deleted together with H2 (residues 62–76), converting H1 into a cleavable signal sequence [19,22]. To make H1(CC)-ΔH3, residues 83–98 were also deleted, and to make H1(CC)-D99L, Asp^{99} at the end of H3 was changed to Leu. The sequence of H1(CC) is also shown, with the N-terminal lysines, Leu^{13}, and the 'cleavage cassette' not present in wild-type Lep underlined, the Lep cleavage-site in the cleavage cassette indicated by an arrow, H3 underlined, and the Asp^{99}→Leu mutation marked.

sucrose, 33 mM Tris pH 8.0), and incubated with lysozyme (5 μg/ml) and 1 mM EDTA for 15 min on ice. Aliquots of the cell suspension were incubated 1 h on ice, either with 0.75 mg/ml TPCK-treated trypsin or with (0.75 mg/ml TPCK-trypsin + 1.0 mg/ml trypsin inhibitor + 0.33 mg/ml PMSF). After addition of trypsin inhibitor and PMSF, samples were acid-precipitated (trichloroacetic acid, 10% final conc.), resuspended in 10 mM Tris/2% SDS, immunoprecipitated with antisera to Lep, OmpA (an outer membrane control, not shown), and AraB (a cytoplasmic control, not shown), washed, and analyzed by SDS-PAGE and fluorography.

3. RESULTS

We have previously described mutants of the inner membrane protein leader peptidase (Lep) from *E. coli* with both one and two transmembrane segments and with different membrane orientations [8,12,19–21]. Here, we focus on constructs where the second transmembrane segment in the wild-type protein has been deleted, and where the first transmembrane segment, which is normally oriented with its N-terminus facing the periplasm, has been converted to a cleavable signal sequence that promotes the translocation of a large C-terminal domain into the periplasm, Fig. 1. One such construct has already been described [22]. This mutant, called H1(CC), differs from wild-type Lep in three regions: it has four extra lysines inserted between codons 4 and 5, it has Thr^{13} replaced with Leu, a 'cleavage cassette' Ser-Pro-Ser-Ala-Tyr-Ala^{-1}↓Ala^{+1}-Asp inserted between codons 15 and 16, and residues 30–52 in the P1-region have been deleted, together with the second transmembrane domain H2 (residues 62–76), Fig. 1. In H1(CC), the H1-region acts as a signal peptide: it is cleaved by the chromosomally encoded leader peptidase present in wild-type cells at the intended site between Ala^{-1} and Ala^{+1} in the cleavage cassette (arrow), and the P1–P2 region is translocated to the periplasm [22].

Since the combined P1–P2 region is 271 residues long in H1(CC), and since H1 seems to act as a normal signal peptide in this context, we expected that H1(CC) would show the same dependence on the *sec*-machinery as most other secretory proteins [12]; i.e. that both signal peptide cleavage and translocation of the chain would be blocked under conditions where the SecA protein was rendered non-functional. However, when SecA function was blocked by treatment of the cells with 2 mM sodium azide prior to [^{35}S]Met labeling of the mutant protein [23] only a mild effect on signal peptide cleavage was observed, whereas translocation of the P1–P2 region was fully inhibited as assayed by its protease-resistance in spheroplasts, Fig. 2. During a prolonged chase (up to 5 min) in azide-treated cells, the fraction of cleaved, non-translocated molecules was constant even though the SecA-dependent outer membrane protein OmpA was completely translocated (data not shown), suggesting that the cleaved form of the H1(CC) mutant cannot be posttranslationally translocated. Further, both the cleaved and non-cleaved forms that accumulate in azide-treated cells fractionated with the supernatant after sonication (data not shown); thus, the cleaved form does not remain bound to the membrane but rather slips back into the cytoplasm, even though the 'cleavage cassette' must at one point have been exposed to the periplasmically located active site of the chromosomally encoded leader peptidase [24]. It

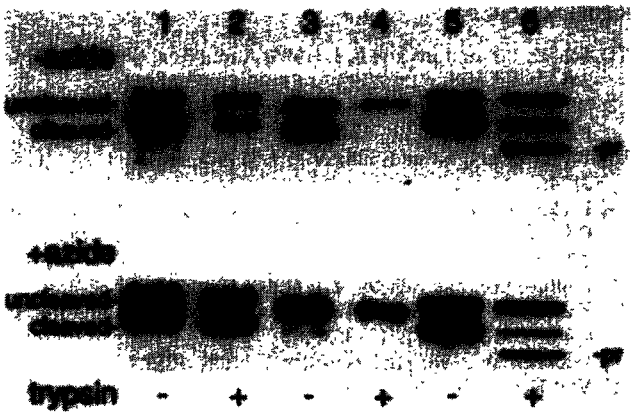

Fig. 2. Topology and SecA-dependence of the mutants discussed in the text. Lanes 1 and 2: H1(CC). Lanes 3 and 4: H1(CC)-ΔH3. Lanes 5 and 6: H1(CC)-D99L. Expression of mutant Lep proteins was induced by arabinose, cells were labeled with [^{35}S]Met for 2 min and chased with non-radioactive Met for an additional 1 min, converted into spheroplasts, treated with trypsin (even-numbered lanes) or trypsin + trypsin inhibitor (odd-numbered lanes), TCA-precipitated, resuspended, immunoprecipitated with Lep antiserum, and analyzed by SDS-PAGE. To test the SecA-dependence, cells were incubated with 2 mM sodium azide for 1 min prior to pulse-labeling (bottom panel). Mutants H1(CC) and H1(CC)-ΔH3 were expressed in strain MC1061, whereas H1(CC)-D99L, which was very poorly expressed in this strain, was analyzed in MC4100. The protease-resistant fragment (*pr*) resulting from trypsin-cleavage in the P1 loop in mutant H1(CC)-D99L is indicated. Note that some signal peptidase-cleaved but trypsin-resistant material remains in the H1(CC) and H1(CC)-D99L mutants even in the '-azide' lanes; this may represent molecules that have slipped back into the cytoplasm after cleavage of the signal peptide.

thus appears that the initial insertion of the signal peptide across the membrane and its removal by leader peptidase is effectively uncoupled from the *sec*-dependent translocation of the large periplasmic P1–P2 region in this mutant.

We noted that, in the H1(CC) mutant, the signal peptide is closely followed by a mildly apolar region H3 (residues 83–98) that is part of the periplasmic region in wild-type Lep [25], Fig. 1. Since we have previously found that the presence of the more hydrophobic H2 region in approximately the same position where H3 is found in H1(CC) allows the H1–P1–H2 region to insert into the inner membrane as a 'helical hairpin' independently of SecA [26], we tested whether H3 could in any way be responsible for the peculiar *sec*-dependence of H1(CC). To this end, two new mutants were made: H1(CC)-ΔH3 and H1(CC)-D99L, Fig. 1. In the former, H3 (residues 83–98) was deleted; in the latter, the length and hydrophobicity of H3 were increased by the replacement of Asp^{99} (as numbered in the wild-type sequence) with Leu.

As shown in Fig. 2, H1(CC)-ΔH3 behaves as a normal secretory protein, with both signal peptide cleavage and translocation being dependent on SecA. On the other hand, a large fraction of the H1(CC)-D99L molecules insert into the inner membrane independently of SecA (as demonstrated by signal peptide cleavage), and are left spanning the membrane with a small periplasmic segment that can be cleaved by protease added to intact spheroplasts, Fig. 2. H1(CC)-D99L thus behaves in the same way as previously described Lep-constructs where the H1 and H2 regions together bring about the *sec*-independent translocation of a short intervening periplasmic loop [8,12,26]. From these observations, we conclude that the H3 region is indeed responsible for the different *sec*-requirements of signal peptide cleavage and chain translocation of the H1(CC) mutant.

4. DISCUSSION

In this paper, we have described a secretory *E. coli* protein with a novel phenotype: signal peptide cleavage is largely unaffected under conditions where SecA is rendered non-functional whereas chain translocation is fully *sec*-dependent, and we have traced this phenotype to the presence of a mildly hydrophobic segment located ~30 residues downstream of the signal peptide. When this segment is deleted, normal SecA-dependent signal peptide cleavage and chain translocation is observed; when its hydrophobicity is increased, it becomes a permanent membrane anchor and cleavage of the signal peptide and membrane insertion are both SecA-independent.

Secretion intermediates with cleaved signal peptides have been described before. C-terminal deletions in β-lactamase result in cleaved intermediates that are transiently bound to the periplasmic face of the inner membrane [27], and similar intermediates are observed when cells are grown at low temperature [28] or when the membrane electrochemical potential is reduced [29]. In all these cases, both signal peptide cleavage and translocation appear to be normal, and the effects are seen at later stages of release of the protein from the periplasmic surface of the membrane. Two reports of cytoplasmically located proteins with cleaved signal peptides have appeared. In one, a suppressor mutation in SecY (PrlC8) was shown to promote signal peptide cleavage but did not allow translocation out of the cytoplasm [30]; in the other, a cleaved form of maltose-binding protein that was still not exposed to the periplasmic space was found in cells harboring temperature-sensitive *secA* or *secY* mutants when they were grown at the permissive temperature [31]. Finally, in an in vitro translocation system based on inverted *E. coli* inner membrane vesicles, an early OmpA-intermediate where the signal peptide has been cleaved but where little or no translocation has taken place accumulates when a non-hydrolyzable ATP analog is used to block the SecA function [32]. Similarly, in the dog pancreas microsome system, cleaved but non-translocated forms of the hepatitis B virus precore protein [33] and a fragment of the bactericidal/permeability-increasing protein [34] have been observed.

The properties of the H1(CC)-mutants provide some further insights into the interactions between a nascent, secretory protein and the *sec*-machinery. It seems that our observations can be explained in the context of an extension of the Wickner model for protein translocation [2] that we recently put forward to account for our findings relative to the correlation between *sec*-dependence and the length of the translocated chain [12]. It is thought that SecA binds both to the signal peptide and to some more C-terminal region of the nascent chain, that this binding activates an ATP-binding site on SecA, that binding of ATP releases the signal peptide and allows its insertion into the membrane, that the subsequent hydrolysis of the bound ATP releases the nascent chain and allows a portion of the chain to translocate, that the nascent chain rebinds to SecA, and that this process is repeated until the whole chain has been transferred across the membrane [32,35,36]. If, on the other hand, the signal peptide is closely followed by a second hydrophobic region, the initial release of the signal peptide from SecA may allow the simultaneous insertion into the membrane of the signal peptide and the downstream hydrophobic region as a 'helical hairpin' [37]; this would be scored as a '*sec*-independent' insertion, since no ATP hydrolysis on SecA would be required [12].

This is how we picture the membrane insertion of the H1(CC)-D99L mutant; in the case of the H1(CC) mutant, we would suggest that H3 is sufficiently hydrophobic to allow the initial helical hairpin insertion and cleavage of the signal peptide, but that it is not sufficiently hydrophobic to permanently anchor the protein in the membrane. Rather, the *sec*-machinery will be able to overcome the hydrophobicity of H3 and extrude it to the periplasmic side of the membrane, unless *sec*-function is blocked. Under such conditions no translocation would take place, and the molecule would simply slip back into the cytoplasm. The H1(CC)-ΔH3 mutant lacks H3 altogether, and hence would behave as a normal secretory protein.

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