Precursor-specific Requirements for SecA, SecB, and ΔpH during Protein Export of Escherichia coli

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We compare translocation into inside-out plasma membrane vesicles (INV) of the in vitro synthesized outer membrane proteins LamB and OmpA and the periplasmic protein Skp of Escherichia coli and demonstrate a precursor-specific dependence on the export factors SecA, SecB, and the proton-motive force (ΔpH). A partial reduction in soluble SecA caused a 50% decrease in translocation of preOmpA. In contrast, removal of INV-bound SecA by urea extraction was required to see a decrease in translocation of preOmpA and preSkp, with 8% of preSkp still being translocated into urea-treated INV. Translocation of the three precursors into INV showed a corresponding differential sensitivity toward dissipation of ΔpH, following removal of the F, ATPase from the INV. While depletion of both F, and SecA or simply lowering of the reaction temperature resulted in an inhibition of complete transmembrane translocation, it interfered less severely with signal sequence cleavage, indicating the formation of translocation intermediates under these conditions. The relative amounts of intermediate obtained were also different for the three preproteins correlating a low requirement for SecA and ΔpH with a facilitated initiation of translocation. Whereas preSkp was translocated independently of SecA, preLamB was not even targeted to the INV in its absence. Functional targeting of preOmpA required the presence of SecB during incubation of the precursor with INV and not during its synthesis. SecB, exogenously added during the period of synthesis, did not prevent the formation of translocation-incompetent preLamB. The latter results are consistent with an important targeting function of SecB, which so far has mostly been described as a molecular chaperone. The findings are discussed with respect to current models of bacterial protein export usually derived from the analysis of a single precursor.

The export of periplasmic and outer membrane proteins across the plasma membrane of the Gram-negative bacterium Escherichia coli involves a distinct set of Sec proteins. The Sec proteins were originally identified via conditionally lethal mutations which are phenotypically characterized by a general export defect (1, 2). The essential role most of these Sec proteins play in protein export in E. coli was biochemically demonstrated by the reconstitution of the transmembrane translocation of preOmpA from purified precursor, SecA, SecYSecE-containing liposomes, and ATP (3, 4). According to a current model (5), precursor proteins, which are synthesized on free ribosomes, interact with cytosolic chaperones such as SecB. The SecA-SecB complex binds to the so-called preprotein translocase consisting of the membrane-attached SecA and the integral membrane proteins SecY and SecE. SecA and SecB are therefore involved in targeting the precursor to the plasma membrane (6, 7). SecY and SecE, which presumably function in concert with each other (3, 8) and with other membrane components (9), most likely participate in the formation of an aqueous channel for the transmembrane passage of exported proteins (10, 11). Export of proteins is driven by two sources of energy, i.e. ATP and the proton-motive force (ΔpH).1 Functional binding of precursors to the plasma membrane occurs in the absence of ATP (6). Formation of a translocation intermediate, that has already undergone proteolytic cleavage of its signal sequence but remains for the most part of its sequence untranslocated, proceeds by the aid of ATP (6, 12). The latter process can be driven by binding of nonhydrolyzable ATP to SecA (13). Complete translocation of the polyprotein chain depends on ATP hydrolysis and/or the ΔpH (13, 14). Most of these findings were derived from studies employing in vitro synthesized precursor proteins and inside-out plasma membrane vesicles (INV). Studies were performed either with crude cytosolic extracts containing the newly synthesized precursors (15, 16) or with a purified precursor kept unfolded by 6 M urea (17). However, results obtained with these cell-free systems are not always in agreement. Thus, SecB is not essential for translocation of preOmpA into INV (18) in contrast to that of preLamB (6). In the absence of SecA, preOmpA binds to INV (19), albeit nonproductively, whereas membrane-targeting of preLamB was not observed under these conditions (6). Removal of INV-associated SecA by urea treatment of the vesicles prevents translocation of preOmpA (19) but not that of a truncated form of preMBP (maltose-binding protein) (20). This latter precursor differs from many others also in that it was reported to be translocated in the absence of SecY (21). The extent to which post-translational translocation into INV occurs also varies with the precursor under investigation (for references see "Discussion"), and similar differences apply to the dependence of the export on the ΔpH (22).

We therefore undertook a comparative analysis of the conditions under which transport across the plasma membrane occurs in vitro for the two outer membrane proteins LamB and OmpA and the periplasmic protein Skp (HlpA, OmpH). Skp is a small periplasmic protein (23) whose precise function in vitro is unknown. The abbreviations used are: ΔpH, proton-motive force; INV, inside-out plasma membrane vesicles; S-135, high-speed supernatant obtained by centrifugation of an E. coli cell homogenate at 135,000 g; MBP, maltose-binding protein.

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has so far remained unknown (24). We demonstrate here that the export of the three precursors differs in its requirements for SecA, SecB, and ΔmH+ and that translocation of preLamB proceeds in the least canonical manner with respect to current models of protein export in E. coli.

EXPERIMENTAL PROCEDURES

Cell-free synthesis of LamB, OmpA, and Skp by transcription/translation from plasmids pH7012 (25), pRD87 (26), and pGAH317 (23), respectively, was performed as described (27) using cell extract cells (15) from E. coli K12 sec wild type strains MC4100 (28) and MZ9 (6), the secA amber mutant MM66 (28), and the secB amber mutant CK1953 (29). If not stated otherwise, sucrose gradient-purified INV (15) were present during synthesis. Extraction of INV with 6-urea (19) and low salt (25) was carried out as previously described. Pulse-labeling of E. coli cells (Fig. 5) and immunoprecipitation of OmpA and Skp have been described (23). Experimental details of the analysis shown in Fig. 4 and Table I are given in Ref. 6.

RESULTS

Due to its partitioning between the cytoplasm and the plasma membrane in E. coli, SecA is present in both the soluble fraction (S-135) and attached to INV of the in vitro synthesis/export system used in the present studies. To examine the sensitivity of various E. coli precursor proteins toward different degrees of SecA depletion, SecA was separately removed from the S-135 and the INV. This was achieved by preparing the S-135 from a secA amber mutant grown at the restrictive temperature of 42°C, at which the nonsense mutation is not suppressed (28), and by urea extraction of the INV (19). Fig. 1 compares processing (odd-numbered lanes) by, and translocation (even-numbered lanes) into, these vesicles of in vitro synthesized LamB, OmpA, and Skp. Whereas a mere lack of soluble SecA, i.e., usage of a secA-mutant S-135 in combination with SecA-containing membranes, led to a 50% reduction in processing and translocation of preLamB (LamB, lanes 1–4), it influenced the other two precursors only marginally (OmpA, lanes 1–4) or not at all (Skp, lanes 1–4). To see a dramatic decrease in the export of OmpA, the INV-associated SecA had to be removed in addition to the soluble fraction (OmpA, lanes 5 and 6). For Skp, the same conditions still allowed for processing of 26% of the precursor but diminished translocation to 8% (Skp, lanes 5 and 6). Thus, while LamB reacted most sensitively to a decreased level of SecA, the export of Skp seems to require very little of this export factor.

As can be seen in Fig. 1, the ratio of the percentages of processing and translocation for a given experimental situation varied among the three precursors. Thus, whereas the amounts of processed and translocated, i.e., protease-resistant, mature LamB were usually about equal (open arrowheads), there was always somewhat less protease-resistant mature OmpA and Skp than processed material. This discrepancy was most striking when Skp was synthesized in the presence of urea-extracted INV (Skp, lanes 5 and 6). Obviously, most of the Skp which was processed by urea-extracted INV was not sequestered within the vesicles but only partially translocated across the lipid bilayer. In this manner, cleavage of the signal sequence could occur without rendering the whole molecule protected against protease K.

By subfractionating the cell-free translation products, we have recently demonstrated that the decreased translocation of in vitro synthesized LamB observed upon depletion of SecA was the result of a diminished membrane targeting of preLamB (6). In contrast, preOmpA had been reported by others to bind to INV in the complete absence of SecA (19). Fig. 2 shows that preOmpA also remained largely soluble (compare lane 1 with 4) when the content of SecA in the in vitro system was decreased to a level at which an impairment of processing and translocation of OmpA became apparent (cf. Fig. 1, OmpA, lanes 3–6). Since a similar behavior was found for Skp (not shown), membrane targeting in general requires the presence of SecA.

The in vitro export of the three precursors studied differed also in its sensitivity toward dissolution of ΔmH+. The data plotted in Fig. 3 were obtained by comparing the efficiencies of processing and translocation between native and low salt-washed INV. The latter are de-energized because of a loss of the F1 part of the H+-translocating ATPase. This loss renders them largely inactive for protein translocation due to a collapse of ΔmNp (25). Again, LamB was the protein most affected, displaying a 90% loss of translocation after F1 removal (cross-hatched bar). Translocation of OmpA was less severely diminished and that of preSkp by only 60%. A similar correlation was found also for the three precursors and their efficiencies of processing (black bars) with Skp being almost as efficiently processed whether or not INV had been depleted of F1. However, with all three proteins analyzed, transmembrane translocation was affected considerably more by the F1 removal than the processing activity. This finding indicates that initiation of the transmembrane transport leading to an exposure of the signal sequence

![Fig. 1. The amount of SecA required for translocation into plasma membrane vesicles varies with the precursor studied.](image-url)
Although it had been demonstrated in vitro that SecB, much of, the variation in the dependence on SecA observed for the three precursor proteins tested paralleled that on ΔH₂₅. A differing response of E. coli precursors toward SecB has been reported (30). We therefore wished to compare the dependence on SecB of the in vitro export of LamB, OmpA, and Skp. Although it had been demonstrated in vitro that SecB, much like SecA, functioned as a targeting factor for LamB (6), preOmpA was found associated with membrane vesicles in the complete absence of SecB (7). In order to examine the action of SecB on the functional binding of precursor proteins leading to subsequent transmembrane translocation, as opposed to sole membrane association, a previously developed assay was employed (6, 27). This assay is based on the fact that a portion of completely synthesized precursor molecules binds to INV at low temperature (13 °C) and is translocated from the bound state across the lipid bilayer upon raising the reaction temperature. Fig. 4 illustrates this method of analysis for LamB, OmpA, and Skp. Lane 1 shows the amount of precursor that did not bind to INV at 13 °C, lanes 2 and 3 reflect INV-bound material and the protease-resistant fraction thereof, and lanes 4 and 5 depict the extent of processing and translocation following incubation of the INV-bound material at 37 °C. A detectable amount of all three precursors was processed but not translocated to a significant extent during the low temperature binding step (lanes 2 and 3). This is yet another situation in which partial translocation occurred leading to signal sequence cleavage but not protease resistance. Both processing and complete transmembrane transport of the membrane-associated preproteins took place when the reaction temperature was raised (lanes 4 and 5). The right half of Fig. 4 shows identical experimental conditions with the exception that synthesis of the precursors was performed in the absence of SecB by use of an S-135 prepared from a secBmutant. There was almost no binding of preLamB to INV in the absence of SecB confirming our previous results obtained with a different experimental approach (6). In contrast, preOmpA and preSkp did bind to the membrane vesicles after synthesis in the absence of SecB with an efficiency equal to (OmpA) or greater than (Skp) as in its presence. However, whereas the preOmpA which had bound to INV at 13 °C in the absence of SecB was very poorly translocated upon raising the temperature, translocation of preSkp clearly proceeded very efficiently under these conditions. Thus, preOmpA binds to INV in the absence of SecB but in a non-productive manner because subsequent translocation does not result. This is in agreement with previous findings (7). Under identical conditions, Skp was translocated virtually independently of SecB.

The slight differences in the efficiencies of processing and translocation of Skp synthesized in the presence or absence of SecB (Fig. 4, Skp, compare lanes 4 and 5 to 9 and 10) are not significant enough to indicate SecB dependence for this export protein. This was confirmed by in vivo pulse-labeling with [35S]methionine of whole cells followed by immunoprecipitation as shown in Fig. 5. Whereas preOmpA accumulated during a

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**Fig. 2.** The precursor of OmpA when synthesized in a cytosolic environment does not bind to membrane vesicles in the absence of SecA. PreOmpA was synthesized by a secAmutant S-135 in the presence of SecA-containing (secA⁺) and urea-extracted, i.e. SecA-free (secA⁻) INV. Prior to SDS-PAGE and autoradiography, translation products were separated on mini-sucrose gradients into soluble (sol), membrane-associated (mbs), and aggregated (pel) material. The relative amount of both precursor and mature forms of OmpA recovered from each fraction was calculated with respect to the sum of all three fractions. The majority of preOmpA remains soluble when the SecA content is sufficiently low.

**Fig. 3.** Differential sensitivity of the export of three precursor proteins toward ΔH₂₅. The three export proteins were synthesized in vitro in the presence of native and F₁,ATPase-depleted INV. Processing and translocation were quantitatively determined as described, and the efficiencies remaining with F₁-depleted vesicles were plotted relative to the values obtained for F₁-containing INV. Translocation in general is more severely affected by the dissipation of ΔH₂₅ than signal sequence cleavage. Solid boxes, processing; hatched boxes, translocation.
FIG. 4. The three precursor proteins differ in their dependence on SecB for functional membrane targeting. The precursors indicated were synthesized in 5-fold scaled-up reactions (125 nl) lacking INV either by an S-135 containing wild type levels of SecB (lanes 1–5) or an S-135 derived from a secBmutant (lanes 6–10). The soluble precursor of each 125-ml reaction was subsequently freed from ribosomes and aggregated material by centrifugation in a Beckman Airfuge and incubated at 13 °C for 5 min with 5 eq of INV. One eq of INV is the amount usually added to a single cell-free synthesis reaction of 25 nl. After incubation, membrane-bound precursor was recovered from the reaction by pelleting the INV in the Airfuge. A fraction of resuspended membranes containing 1 eq of INV was precipitated with 1 volume of 10% trichloroacetic acid (lanes 3 and 8), and another was incubated with 1 volume of 1 mg/ml proteinase K (prot K) at 25 °C for 20 min, after which the digestion was terminated by the addition of 2 ml phenylmethylsulfonyl fluoride and 1 volume of 10% trichloroacetic acid (lanes 5 and 10) and proteinase K-resistant (lanes 5 and 10) precursor (closed arrowheads) and mature species (open arrowheads). Lanes 1 and 6 each show the precursor (representing 0.7 of a single reaction) not associating with INV at 13 °C. Post-targeting transport of LamB and OmpA, in contrast to that of Skp, were severely affected when synthesis proceeded in the absence of SecB. There was always some mature OmpA and Skp present in the fraction of precursor which had not bound to INV (lanes 1 and 6). This material must stem from the incubation with the membranes at 13 °C due to the removal of the signal sequence. However, it has not remained with the INV but slipped back probably because of reverse translocation (13).

20-s pulse to roughly 50% in the secBmutant (lane 2), no precursor of Skp could be detected under identical experimental conditions (lane 4). Thus, Skp appears to be a protein whose export is truly independent of SecB.

The fact that preOmpA binds to membranes in the absence of SecB but requires SecB for the post-targeting translocation raised the question as to when during the export process SecB had to be present in order to achieve efficient transport of preOmpA into INV. The data are summarized in Table I. Listed are the efficiencies of binding, processing, and translocation of preOmpA during incubation with INV at 13 °C and subsequently at 37 °C using the experimental strategy illustrated in Fig. 5. Export of Skp in vivo proceeds independently of SecB. Cells of wild type E. coli strain MC4100 (lanes 1 and 3) and the secBmutant, mutant strain CK1953 (lanes 2 and 4) were pulse-labeled for 20 s with [35S]methionine, and OmpA and Skp species were immunoprecipitated. Only the precursor of OmpA (closed arrowhead) accumulated under SecB-free conditions.

Table I

| Synthesis | Binding of pOmpA to INV (13 °C) | Translocation of bound OmpA into INV (37 °C) |
|-----------|--------------------------------|----------------------------------|
| SecB+     | Binding 3                      | Translocation 3                   |
| SecB−     | Binding 7                      | Translocation 4                   |
| +SecB     | Binding 4                      | Translocation 3                   |

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Differences in the Export of Three E. coli Precursors

Fig. 6. An excess of SecB does not prevent loss of translocation competence of preLamB. PreLamB was synthesized in vitro by an S-135 containing wild type levels of SecC (C) or with purified SecB added (3.5 μg/single reaction) (B). INV were added at the times indicated on the abscissa, and reactions were further incubated for 20 min. The extent of processing (—) and translocation (—) of preLamB was then determined as described in the legend to Fig. 1.

location, indicating that under normal in vitro conditions SecB was a limiting factor for the export of preLamB. However, additional SecB did not prevent the loss in translocation competence observed when INV were added at various times after the onset of synthesis of preLamB (the delayed decrease in translocation between 10 and 20 min (Fig. 6) is due to an experimental error because it is not reflected by the degree of processing and was not observed in parallel experiments (not shown)). Thus, these results also suggest an important function of SecB as a targeting factor in addition to being an export-specific chaperone.

DISCUSSION

Using an in vitro approach, we have compared translocation across the plasma membrane of three cell envelope proteins of E. coli with respect to their sensitivity toward SecA and ΔμH+. A current idea states (31) that prokaryotic precursors, once synthesized, partition between entering the export pathway and folding into an export-incompetent structure. In this scenario, any impairment of the export process, such as a decrease in the level of available SecA or a de-energetization of the INV, is likely to shift the equilibrium in favor of the folding pathway. The accumulation of translocation-incompetent precursor as a reflection of an irreversible aggregation/denaturation occurring under these conditions was considerably more pronounced with preLamB than with the two other precursors. The difference could be explained by some inherent properties of preOmpA and preSkp, such as slow folding kinetics or, alternatively, slower rates of synthesis in our in vitro system. Although studies in vivo have in fact shown that slowing down translation by antibiotics can compensate for a partial loss of SecA (32), and suppressor mutations of secA frequently map to genes encoding components of the translation machinery (reviewed in Ref. 1), during the study of many different E. coli precursors in this in vitro system we have never obtained clearcut experimental evidence for grossly different rates of synthesis.

The most likely explanation for the different sensitivities of the three precursors toward a lack of SecA and dissipation of ΔμH+ would then be that preLamB tightly folds or aggregates faster than preOmpA and this in turn faster than preSkp. An indication for this in fact being the case are the different efficiencies of post-translational translocation of these proteins. Whereas preLamB is very poorly translocated into INV after completion of synthesis (6), preOmpA crosses the plasma membrane very efficiently in a post-translational manner (e.g. Ref. 33), and the extent of post-translational translocation of preSkp into INV sometimes even exceeds that of a co-translational assay (23). This behavior can actually be seen in Fig. 4 showing post-translational translocation into INV of the three precursors after membrane binding at low temperature. Since the tendency of the three precursors to acquire tight tertiary structures, as reflected by the degree of translocation incompetence, is obviously paralleled by the length of each polypeptide chain, the export of shorter precursors seems to be more favorable compared to that of the longer ones. On the other hand, NH₂-terminal fragments of preOmpA were recently reported to translocate in vitro with the same Vmax as the full-length precursor (34) suggesting that some as yet poorly defined, inherent property of the precursor, such as its affinity for translocation factors, might determine the rate of translocation.

We have shown here that for translocation of preOmpA into INV to occur, SecB need not be present during synthesis but only post-translationally, merely during the membrane-targeting step. Since in the experimental setup used SecB was not present during the 30-min period of synthesis at 37 °C, but only during a 5-min co-incubation of the precursor with INV at 13 °C, it is hard to believe that its translocation-supporting effect was due to an interference with the folding of the precursor. Accordingly, SecB when added in excess increased the efficiency of translocation without prolonging the translocation-competent state of preLamB.

These results disagree with the idea of SecB functioning solely as a molecular chaperone. Such a function is suggested by (i) the accumulation of the tightly folded precursor of maltose-binding protein (MBP) in vitro in the absence of SecB (35), (ii) the prevention of preMBP from forming a protease-resistant structure by pure SecB (36, 37), (iii) the blockage of refolding of denatured preMBP in the presence of SecB (38), and (iv) the isolation of extragenic suppressors of a secB-null mutation which are regulated by the heat shock transcription factor σ² (39). Furthermore, SecB was shown to retard the loss of translocation competence of prePhoE (40) which, however, is in contrast to the results presented herein for preLamB. One obvious difference between the two approaches is that Kusters et al. (40) did not consider comparing the 30-min period of synthesis at 37 °C, but only during a 5-min co-incubation of the precursor with INV at 13 °C, it is hard to believe that its translocation-supporting effect was due to an interference with the folding of the precursor. Accordingly, SecB when added in excess increased the efficiency of translocation without prolonging the translocation-competent state of preLamB.

This study gives several examples for a dissociation of processing and translocation of a precursor molecule leading to a partially translocated protein whose signal sequence has been cleaved off. Such a situation occurs for all three proteins when interacting with the plasma membrane at low temperature. Since a decrease in temperature affects membrane fluidity and thereby the activity of membrane proteins, it appears that initial translocation leading to insertion of the signal peptide might not require the same assembly of the membrane pore as the remainder of the molecule. Partial translocation was also much less affected when INV were depleted of their F₁-ATPase in agreement with previous reports that the initial translocation of a bacterial precursor does not require ΔμH+ (12-14). On the other hand, processing of preOmpA and most extensively of preLamB was impaired by the loss of ΔμH+, indicating that ΔμH+ does influence even the initial steps of export of distinct
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precursor proteins. In all situations tested, incomplete translocation occurred more markedly with OmpA and Skp than with LamB, and it was particularly pronounced for Skp when urea-extracted vesicles had been used, i.e., when SecA was limiting. These findings point to a simplified initial translocation of OmpA and Skp compared to that of LamB.

The results presented here reveal preLamB as the precursor whose mode of translocation across the plasma membrane is the least canonical one. Low levels of SecA and ΔψH+, that still allow for translocation of preOmpA and preSkp, severely inhibit that of preLamB. The initiation of translocation of preLamB appears to be rendered more difficult than that of preOmpA and preSkp. Compared with other precursors examined here and elsewhere (43, 44), preLamB exhibits the least capability of translocating post-translationally into INV. Thus, preLamB appears to have a particularly pronounced tendency to fold into an export-incompetent structure. This property is at first sight inconsistent with the possibility that preLamB molecules have associated with chaperoning proteins such as SecB. It is therefore possible that a portion of preLamB molecules does not, or not stably, interact with SecB and/or other chaperones when synthesized in our in vitro system and as a consequence quickly aggregates/denatures. A matter of fact, SecB was found to be limiting for the translocation of preLamB into INV. However, even an excess of SecB did not prevent the acquisition of a translocation-incompetent conformation of the precursor. Presumably, the putative complex of preLamB and chaperone(s), for unknown reasons, has lost its capability to be recognized and consequently targeted to the plasma membrane. In complete agreement, a soluble ternary complex consisting of preLamB, SecA, and SecB which is poorly translocated into INV has recently been identified (45). Conceivable explanations for the behavior of preLamB would be that it requires targeting functions in addition to SecA and SecB or that targeting of preLamB by SecA and SecB must occur co-translationally in order to prevent tight folding of the precursor. Translocation of preLamB thus does not seem to be sufficiently explained by current models of bacterial protein export based on in vitro systems which were reconstituted from a minimal set of export components.

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