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Communication

The Signal Recognition Particle-targeting Pathway Does Not Necessarily Deliver Proteins to the Sec-translocase in *Escherichia coli**

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ProW is an *Escherichia coli* inner membrane protein that consists of a 100-residue-long periplasmic N-terminal tail (N-tail) followed by seven closely spaced transmembrane segments. N-tail translocation presumably proceeds in a C-to-N-terminal direction and represents a poorly understood aspect of membrane protein biogenesis. Here, using an *in vivo* depletion approach, we show that N-tail translocation in a ProW derivative comprising the N-tail and the first transmembrane segment fused to the globular P2 domain of leader peptidase depends both on the bacterial signal recognition particle (SRP) and the Sec-translocase. Surprisingly, however, a deletion construct with only one transmembrane segment downstream of the N-tail can assemble properly even under severe depletion of SecE, a central component of the Sec-translocase, but not under SRP-depletion conditions. To our knowledge, this is the first demonstration that the SRP-targeting pathway does not necessarily deliver SRP-dependent inner membrane proteins to the Sec-translocase. The data further suggest that N-tail translocation can proceed in the absence of a functional Sec-translocase.

N-tail[1] membrane proteins have a translocated N-terminal segment upstream from the first transmembrane domain and lack an N-terminal signal peptide. N-tail proteins are quite common both in prokaryotic and eukaryotic organisms (1). In *Escherichia coli*, most inner membrane proteins are targeted to the inner membrane via the SRP/SecAYEG-translocase pathway (2–5), but the role of SRP and the Sec-translocase during N-tail translocation is not well understood.

The *E. coli* N-tail inner membrane protein ProW, a constituent of the ProU osmoregulatory system (6, 7), has seven transmembrane segments and an unusually long N-tail of 100 residues (7), Fig. 1, and has been established as a convenient model protein for studying N-tail translocation. Previous work has shown that translocation of the ProW N-tail is proton motive force-dependent, that it can be blocked by the introduction of positively but not negatively charged amino acids, and that it is not affected in conditional *secA* and *secY* mutant strains or by treatment with sodium azide (which inhibits the ATPase activity of the preprotein translocase subunit SecA) (8, 9). It should be noted, however, that the use of conditional mutant strains or sodium azide to study Sec-translocase dependence has been found not to be sufficient to prove Sec-translocase independence (3, 4).

Here, we have studied the translocation of the ProW N-tail across the inner membrane in more detail using tight SRP-4.5S RNA and SecE-depletion strains rather than conditional mutant strains. We show that the ProW N-tail cannot be translocated across the inner membrane by itself but requires a C-terminal signal-anchor sequence. Surprisingly, we find that an operational SRP-targeting pathway is always required for translocation of the ProW N-tail, whereas the Sec-translocase appears not to be needed for the proper membrane insertion of a truncated ProW molecule consisting only of the N-tail and the first transmembrane segment. This suggests that the SRP-targeting pathway does not necessarily deliver SRP-dependent proteins to the Sec-translocase, as has hitherto been assumed to be the case, and that N-tail translocation can be Sec-independent.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions—Strain MC4100 (11) was cultured in M9 minimal medium supplemented with 0.2% glucose. The 4.5 S RNA (SRP) conditional strain FF283 was cultured as described previously (12). To deplete cells for 4.5 S RNA, cells were grown to mid-log phase in the absence of IPTG. The SecE-depletion strain CM124 (13) was cultured in M9 minimal medium supplemented with 0.4% glucose and 0.2% L-arabinose. Over night cultures were washed once with M9 medium and backdiluted 1:20. To deplete cells for SecE, cells were, if not stated differently, grown to mid-log phase in the absence of L-arabinose. Depletion of SecE was checked by monitoring the accumulation of pro-OmpA during a short pulse labeling with [^{35}S]methionine. Where appropriate, ampicillin (final concentration 100 μg/ml) and kanamycin (final concentration 50 μg/ml) were added to the medium.

ProW Nt (the first 92 amino acids of the ProW N-tail), ProW Nt/TM1/3K (9), and ProW Nt/TM1/P2 (8) constructs were constructed using a polymerase chain reaction-based approach. ProW N-tail constructs were expressed by L-arabinose induction from the pING1 vector (14) or from the pBAD24 vector (15) in strains MC4100 and FF283 and by IPTG induction from the pDHB5700 vector in strain CM124 (3).

Assay for Membrane Targeting and Assembly—For all experiments cells were grown to mid-log phase. Expression of the ProW N-tail constructs was induced for 5 min with either IPTG (final concentration 1 mM) or L-arabinose (final concentration 0.2%). Cells were labeled with [^{35}S]methionine (150 μCi/ml, Ci = 37 GBq) for 15 s whereupon nonradioactive methionine was added (final concentration 500 μg/ml). After labeling, cells were converted to spheroplasts. For spheroplasting, cells were collected at 14,000 rpm for 2 min in a microcentrifuge, resuspended in ice-cold buffer (40% w/v sucrose, 33 mM Tris pH 8.0), and incubated with lysozyme (final concentration 5 μg/ml) and 1 mM EDTA for 15 min on ice. Aliquots of the spheroplast suspension were incubated

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[1] The abbreviations used are: N-tail, N-terminal tail; SRP, signal recognition particle; IPTG, isopropyl-1-thio-β-D-galactopyranoside; OmpA, outer membrane protein A.

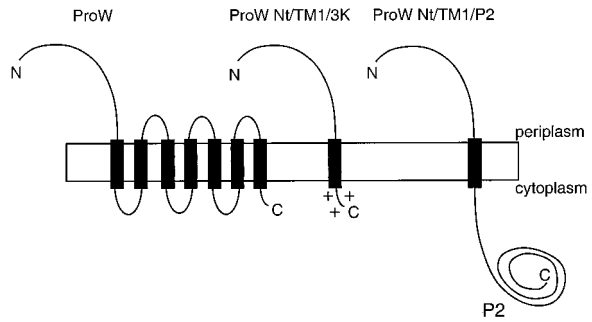

FIG. 1. **Topology of ProW and its derivatives ProW Nt/TM1/3K and ProW Nt/TM1/P2.** In spheroplasts, if the ProW N-tail is translocated across the inner membrane, proteinase K degrades the ProW N-tail. No immunoprecipitable material remains when the ProW N-tail has been digested.

on ice for 1 h either in the presence or absence of proteinase K (final concentration 0.3 mg/ml). Subsequently, phenylmethylsulfonyl fluoride was added to the spheroplast suspension (final concentration 0.33 mg/ml). After addition of phenylmethylsulfonyl fluoride, samples were precipitated with trichloroacetic acid (final concentration 10%), resuspended in 10 mM Tris, 2% SDS, immunoprecipitated with antisera to the ProW N-tail, OmpA (a periplasmic control (16) and AraB/bandX (a cytoplasmic control (16)), washed, and analyzed by standard SDS-polyacrylamide gel electrophoresis (17). Gels were scanned in a Fuji BAS1000 phosphoimager and quantitated using the MacBAS software (version 2.31).

RESULTS

The ProW N-tail Requires a C-terminal Signal-Anchor Sequence for Translocation across the Inner Membrane—To rule out the presence of cryptic targeting signals in the ProW N-tail itself, translocation of a deletion mutant, ProW Nt, comprising only the N-tail without any transmembrane segments was monitored by protease treatment of spheroplasts, Fig. 2. No translocation was observed, indicating that the ProW N-tail cannot be translocated by itself but requires additional information for translocation across the inner membrane. As already shown (9), the addition of a C-terminal signal-anchor sequence (the first ProW transmembrane segment followed by three C-terminal lysines: ProW Nt/TM1/3K (Fig. 1)) is sufficient to bring about efficient translocation of the N-tail across the inner membrane, Fig. 3. Efficient N-tail translocation was also seen when the Nt/TM1/3K construct was lengthened at the C terminus with the globular P2 domain of leader peptidase (Ref. 8 and Figs. 1 and 4). We conclude that a single transmembrane domain is sufficient to initiate translocation of the ProW N-tail across the inner membrane.

Translocation of the ProW N-tail Requires SRP but Not Necessarily the Sec-translocase—Translocation of the ProW N-tail was previously shown not to be affected by sodium azide or by inactivation of SecA and SecY in conditional mutant strains (8, 9). However, it was found recently that for some proteins Sec dependence may only be uncovered by the use of tight depletion strains (10, 13). Therefore, we decided to study translocation of the ProW N-tail in SRP (4.5 S RNA) and Sec-translocase (SecE) depletion strains. SecY is rapidly degraded in the absence of SecE (18, 19), and the SecE depletion strain CM124 has the strongest *sec* phenotype known to date (13).

The translocation of the ProW N-tail was both in Nt/TM1/3K and Nt/TM1/P2 strongly inhibited under SRP-depletion conditions, Figs. 3*A* and 4*A*, suggesting that SRP is required for targeting ProW to the inner membrane.

To our surprise, N-tail translocation in Nt/TM1/3K was not affected even after prolonged depletion of SecE, while translocation of the Sec-dependent protein OmpA was completely blocked under these conditions, Fig. 3*B*. In contrast, when the Nt/TM1/3K construct was extended at the C terminus with the

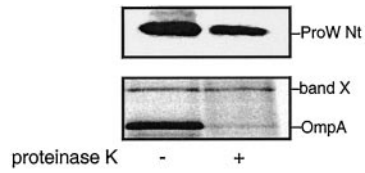

FIG. 2. **The ProW N-tail cannot be translocated by itself.** Proteinase K accessibility of ProW Nt (*top*) and OmpA/bandX (*bottom*) in MC4100 spheroplasts. The ProW Nt construct was expressed in strain MC4100. Cells were pulse-labeled and processed as described under "Materials and Methods." OmpA is only degraded by proteinase K in cells with a disrupted outer membrane. Band X is a cytoplasmic control and not degraded by proteinase K in spheroplasts.

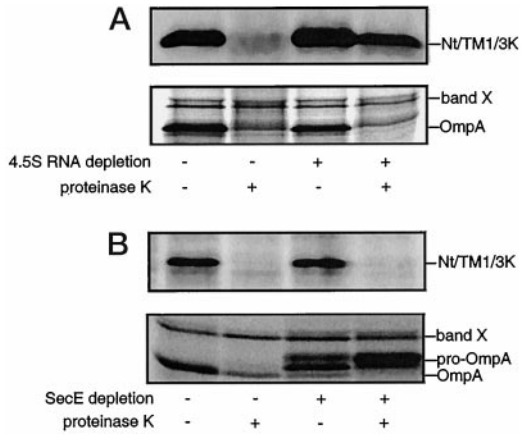

FIG. 3. **N-tail translocation in ProW Nt/TM1/3K depends on SRP but not on SecE.** *A*, proteinase K accessibility of ProW Nt/TM1/3K (*top*) and OmpA/bandX (*bottom*) in FF283 spheroplasts not depleted (−) and depleted (+) for 4.5 S RNA. Nt/TM1/3K was expressed in strain FF283. Cells were pulse-labeled and processed as described under "Materials and Methods." OmpA is only degraded by proteinase K in cells with a disrupted outer membrane. OmpA secretion/processing is not affected upon SRP depletion (16). Band X is a cytoplasmic control and not degraded by proteinase K in spheroplasts. *B*, proteinase K accessibility of ProW Nt/TM1/3K (*top*) and OmpA/bandX (*bottom*) in CM124 spheroplasts not depleted and depleted for SecE. In contrast to the other experiments where mid-log phase cells were used, in this experiment stationary phase cells were used in order to study the assembly of the Nt/TM1/3K construct in cells that had been depleted for SecE as much as possible. Cells were pulse-labeled and processed as described under "Materials and Methods." Depletion of SecE was checked by monitoring the accumulation of pro-OmpA. Pro-OmpA runs as a doublet upon depletion of SecE, as has been observerved before after compromising the Sec machinery (10). Band X is a cytoplasmic control and not degraded by proteinase K in spheroplasts.

P2-domain of leader peptidase, N-tail translocation was inhibited even upon mild depletion of SecE, Fig. 4*B*.

An alternative protein translocation pathway has recently been identified: the so-called twin arginine translocation pathway found both in thylakoids and bacteria (20, 21). To rule out the (unlikely) involvement of the twin arginine translocation pathway in translocation of the ProW N-tail, assembly of constructs Nt/TM1/3K and Nt/TM1/P2 was tested in a *tatC* mutant strain (22). As expected, no effect on translocation was seen in this strain (data not shown).

DISCUSSION

In this communication we show that the ProW N-tail requires a C-terminal signal anchor sequence and an operational SRP-targeting pathway for translocation across the inner membrane. In addition, the Nt/TM1/P2 construct depends on the Sec-translocase for membrane insertion. Interestingly, however, the short Nt/TM1/3K construct inserts efficiently into the inner membrane even after severe depletion of SecE, suggesting that SRP-dependent proteins may not necessarily be targeted to the Sec pathway. Since a C-terminal lengthening of

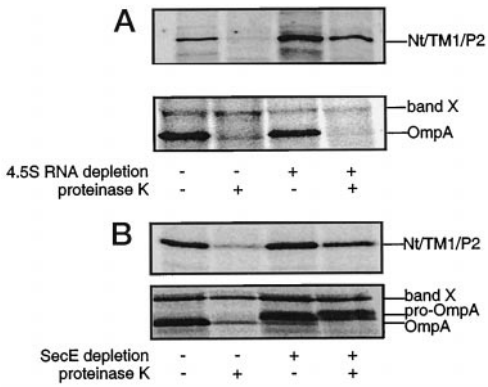

FIG. 4. **N-tail translocation in ProW Nt/TM1/P2 depends on both SRP and SecE.** *A*, proteinase K accesibility of ProW Nt/TM1/P2 (*top*) and OmpA/bandX (*bottom*) in FF283 spheroplasts not depleted and depleted for 4.5 S RNA. Cells were pulse-labeled and processed as described under "Materials and Methods." OmpA is only degraded by proteinase K in cells with a disrupted outer membrane. OmpA secretion/processing is not affected upon SRP depletion (16). Band X is a cytoplasmic control and not degraded by proteinase K in spheroplasts. *B*, proteinase K accesibility of ProW Nt/TM1/P2 (*top*) and OmpA/bandX (*bottom*) in CM124 spheroplasts not depleted and depleted for SecE. Cells were pulse-labeled and processed as described under "Materials and Methods." Depletion of SecE was checked by monitoring the accumulation of pro-OmpA. Band X is a cytoplasmic control and not degraded by proteinase K in spheroplasts.

the SecE-independent Nt/TM1/3K construct renders it SecE-dependent, one possibility is that a co-translational interaction between the substrate protein and SRP is necessary (and sufficient?) for targeting to the Sec-translocase.

Recently, it has been reported that translocation of large protein domains (β-lactamase, PhoA) across the inner membrane can be initiated by a downstream hydrophobic targeting signal and that translocation is both SecA- and SecB-dependent (23, 24). This is consistent with the SecE dependence noted above for the ProW Nt/TM1/P2 construct and further underscores the surprising finding that SecE depletion does not affect the translocation of the short Nt/TM1/3K construct. On the other hand, the addition of just a few positively charged residues to the ProW N-tail prevents its translocation (8, 9), while both β-lactamase and PhoA contain many basic residues, suggesting that there may be important differences in their mode of translocation yet to be identified.

In yeast mitochondria an inner membrane protein, Oxa1p, has been shown to be involved in the assembly in N-tail inner membrane proteins (25, 26). In *E. coli* there is a homologue of this protein, $Oxa1^{Ec}$ (27). However, no function has yet been assigned to $Oxa1^{Ec}$, and it remains to be seen if it plays a role in the assembly of N-tail inner membrane proteins.

In conclusion, we have shown that the SRP-targeting pathway does not necessarily deliver proteins to the Sec-translocase and that N-tail translocation can be Sec-independent.

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