The assembly of bacterial membrane proteins with large periplasmic loops is an intrinsically complex process because the SecY translocon has to coordinate the signal recognition particle-dependent targeting and integration of transmembrane domains with the SecA-dependent translocation of the periplasmic loop. The current model suggests that the ATP hydrolysis by SecA is required only if periplasmic loops larger than 30 amino acids have to be translocated. In agreement with this model, our data demonstrate that the signal recognition particle- and SecA-dependent multiple spanning membrane protein YidC becomes SecA-independent if the large periplasmic loop connecting transmembrane domains 1 and 2 is reduced to less than 30 amino acids. Strikingly, however, we were unable to render single spanning membrane proteins SecA-independent by reducing the length of their periplasmic loops. For these proteins, the complete assembly was always SecA-dependent even if the periplasmic loop was reduced to 13 amino acids. If, however, the 13-amino acid-long periplasmic loop was fused to a downstream transmembrane domain, SecA was no longer required for complete translocation. Although these data support the current model on the SecA dependence of multiple spanning membrane proteins, they indicate a novel function of SecA for the assembly of single spanning membrane proteins. This could suggest that single and multiple spanning membrane proteins are processed differently by the bacterial SecY translocon.

Membrane protein assembly in both eukaryotes and prokaryotes is initiated by the cotranslational targeting of ribosome-associated nascent chains (RNCs) to the Sec translocons in the endoplasmic reticulum or the bacterial cytoplasmic membrane. This requires binding of the signal recognition particle (SRP) to the signal anchor sequence of a membrane protein when it emerges from the ribosomal exit tunnel and the subsequent interaction of the SRP-RNC complex with the membrane-attached SRP receptor (SR) (1, 2). The subsequent transfer of the RNC to the Sec translocon is probably favored by the proposed close vicinity of the SR to the Sec translocon (3). A direct interaction between FtsY, the bacterial SR, and SecY has recently been demonstrated in Escherichia coli (4). In contrast to the eukaryotic SRP, which targets both membrane and secretory proteins, the bacterial SRP is predominantly engaged in membrane protein targeting. The vast majority of bacterial secretory proteins, i.e. proteins that are destined to reach the periplasmic space or the outer membrane, are post-translationally targeted by the bacteria-specific SecA/SecB pathway (5). In this pathway, SecB functions as a secretion-specific chaperone for most secretory proteins, whereas SecA is proposed to translocate the preprotein in a stepwise translocation of stretches of about 30 amino acids (6–8).

The translocation of large luminal domains in eukaryotic membrane proteins does not depend on cytosolic proteins other than SRP (3). This is different for bacterial membrane proteins with large periplasmic domains, which are cotranslationally targeted to the membrane by SRP/SR but are fully assembled only in the presence of SecA (9–11). For these proteins, the ATP hydrolysis by SecA is thought to provide the energy for the translocation of the large periplasmic loops (12). Additionally the proton motive force appears to be required for complete translocation (13). In contrast, neither SecA nor the proton motive force is required for the complete assembly of bacterial membrane proteins without extended periplasmic domains. This has been shown for the multiple spanning membrane proteins mannitol permease and SecY (14, 15).

In this study we analyzed the SRP- and SecA-dependent steps during the assembly of two SRP- and SecA-dependent membrane proteins: the single spanning type II model protein Momp2 (9) and the multiple spanning membrane protein YidC (16, 17). In agreement with the proposed model we demonstrate that the assembly of YidC requires SecA only if periplasmic loops larger than 30 amino acids have to be translocated. Unexpectedly, however, the single spanning Momp2 was, like single spanning YidC derivatives, always SecA-dependent irrespective of the length of the periplasmic loop. If, however, small periplasmic loops were fused to a downstream transmembrane (TM) domain, their translocation became SecA-independent. These data indicate that the SecA dependence of a bacterial membrane protein is not solely determined by the length of the periplasmic loop but also by the presence of a downstream TM domain.

**EXPERIMENTAL PROCEDURES**

*Strains*—The following *E. coli* strains were used: BL21 (DE3) pLysS (Novagen, Bad Soden, Germany), MRE600 (18), MC4100 (19), TY1 (ompT::kan, secY205) (20), CM124 (secED19-111, pCM22) (21), KN553 (ΔsecB::Cm:Trn10 ΔsecC::kan) (22), and EK414 (MC4100ara ΔsecG::kan) (22).

*Plasmids and Plasmid Construction*—For *in vitro* protein synthesis, the following plasmids were used: pMomp2 (22), p717-MtIA (mannitol permease) (9), and pDMB (pOMP2A) (23). For *in vitro* expression of YidC we used pKSM717-YidC, which was constructed by ligating the Ncol/EcoRI fragment of plasmid pROEX-HTB-yidC (24) into pKSM717 (25). YidC deletion plasmids were constructed by PCR introducing a first BglII site at codon 336 of YidC. A second BglII site was created by...
then introduced at either codons 30, 51, 61, 71, or 173, respectively. Digestion with BgIII and religation yielded the plasmids pKSM717-YidC-D307, pKSM717-YidC-D286, pKSM717-YidC-D276, pKSM717-YidC-D266, and pKSM717-YidC-D164, respectively. To obtain YidC deletion mutants containing only one or two TM domains, TGA stop

YidC

(YidC)(4/H9262/H9262/mM creatine phosphate, and 40
cultures were grown at 37 °C up to an

tion gradient analyses, the reaction mixture was adjusted to 1.6M

Beckman TLA-100.2 rotor at 70,000 rpm at 4 °C for 30 min. For flota-
bated further for 20 min at 37 °C in the presence of 0.8 mM puromycin
gradient, representing the vesicle fractions, were withdrawn and incu-
gradient and trichloroacetic acid-precipitated (5% final concentration).

In Vitro Synthesis—In vitro protein synthesis and the composition of the reconstituted transcription/translation system of

E. coli

purification of its components, and protease protection assay used in this study have been described previously (14, 9). Synthesis of RNCs was achieved as described in Beck et al. (26) by the addition of the following oligode-

oxynucleotides: Momp2-60, 5’-GTGTGATGAAACCATATCAG-3’

(4 µg/25 µl); Momp2-65, 5’-TCGGGCCCATTGTGTGATG-3’

(4 µg/25 µl); Momp2-70, 5’-CCAGTTGTTTTCTAGGGGTCG-3’

(2.5 µg/25 µl); Momp2-86, 5’-CAAGGCAACATACGGGTTAAC-3’

(3 µg/25 µl); Momp2-146, 5’-TAAACGTGATTAGTGTCG-3’

(3 µg/25 µl); Momp2-201, 5’-GGAGATCAGGTAATATACACAC-3’

(4 µg/25 µl); Momp2-329, 5’-CTGTTTCACTGGTTCACAGGTTG-3’

(4 µg/25 µl); YidC-330, 5’-AGAGATGAAACCAACACCAAC-3’

(3 µg/25 µl); YidC-422, 5’-GATCACGAGCCGGGAAAGCCGACCC-3’

(3 µg/25 µl); and YidC-447, 5’-CACAGTGCAACACCGGTCCTG-3’

(3 µg/25 µl). RNase H (1 unit/25 µl) and 100 µg RNA antisense oligode-

oxynucleotide (5’-TTAACGCTGAATACGCTTGTTCCGTT-3’, 0.25 µg/25 µl) were added routinely. To release the RNCS from the ribosome, puromycin was added to the reaction mixture at a final concentration of 0.8 mM with a further incubation for 15 min at 37 °C. For carbonate extraction of membrane-bound RNCSs, samples were treated with freshly prepared 0.2 M Na2CO3, pH 11.3 (27) for 30 min on ice. Membranes were then recovered by centrifugation in a Beckman TLA-100.2 rotor at 70,000 rpm at 4 °C for 30 min. For flota-
gation gradient analyses, the reaction mixture was adjusted to 1.6 M

sucrose (final volume, 100

µl) and overlaid with 200

tl. These concentrations, like those of Ffh (150 ng/25 µl); Momp2-86, 5

µl); Momp2-146, 5

µl); Momp2-201, 5

µl); YidC-330, 5

µl); YidC-422, 5

µl); YidC-447, 5

µl) and

10s RNA antisense oligode-

oxynucleotide (5’-TTAACGCTGAATACGCTTGTTCCGTT-3’, 0.25 µg/25 µl) were added routinely. To release the RNCSs from the ribosome, puromycin was added to the reaction mixture at a final concentration of 0.8 mM with a further incubation for 15 min at 37 °C. For carbonate extraction of membrane-bound RNCSs, samples were treated with freshly prepared 0.2 M Na2CO3, pH 11.3 (27) for 30 min on ice. Membranes were then recovered by centrifugation in a Beckman TLA-100.2 rotor at 70,000 rpm at 4 °C for 30 min. For flota-
gation gradient analyses, the reaction mixture was adjusted to 1.6 M

sucrose (final volume, 100 µl) and overlaid with 200 µl of 1.25 M sucrose and 100 µl of 0.25 M sucrose, each prepared in 40 mM triethanolamine acetate, 5 mM magnesium acetate, and 70 mM potassium acetate. After centrifugation for 90 min at 100,000 rpm in a Beckman TLA-100.2 rotor, four fractions of 100 µl each were withdrawn from the top of the gradient and trichloroacetic acid-precipitated (5% final concentration). The pellet was directly dissolved in SDS loading buffer. For subsequent translocation assays of membrane-bound RNCSs, fractions 2 and 3 of the gradient, representing the vesicle fractions, were withdrawn and incubated further for 20 min at 37 °C in the presence of 0.8 mM puromycin and an ATP-regenerating system (2.5 mM ATP, 2 mM dithiothreitol, 8 mM creatine phosphate, and 40 µg/ml creatine phosphokinase). SecA36 (20) was present at concentrations of 300 ng/25 µl during this incubation where indicated; wild type SecA was added at a concentration of 900 ng/25 µl. These concentrations, like those of Ffh (150 ng/25 µl) and

FtsY (500 ng/25 µl), were shown to efficiently stimulate protein transpor-
to U-INVVs (14), which are devoid of these proteins. The purifica-
tions of SecA, SecA36, Ffh, and FtsY have been described previously
(14, 28).

Sample Analysis and Quantification—SDS-PAGE (15 and 17%) was carried out according to Laemmli (29). For Momp2-60, Momp2-65, and Momp2-70, a Tris-Tricine (6–16.5%) SDS-PAGE system was performed as described previously (30). YidC Δ307 and its derivatives were separated by 22% urea-SDS-PAGE. Radiolabeled proteins were visual-
ized by phosphorimaging using an Amersham Biosciences Phosphor-
Imager and quantified using ImageQuant software from Amersham Biosciences.

Reagents—Growth media components and chemicals were obtained from Roth (Karlsruhe, Germany), Sigma, and Promega (Mannheim, Germany). Oligodeoxynucleotides were from MWG Biotech (Ebersberg, Germany). The [35S]Met/Cys labeling mixture was from Amersham Biosciences.

RESULTS

SRP and SecA Are Involved in Distinct Stages during the Transport of Bacterial Membrane Proteins—For analyzing the SRP- and SecA-dep-

endent stages of membrane protein assembly individually we used the

secY205 mutant in which the SecA-SecY interaction is impaired (21). SecA-dependent proteins are not translocated into INVVs derived form the

secY205 mutant as shown in Fig. 1A for the SecA-dependent secretory protein OmpA. In the presence of wild type INVVs, almost 50% of the in vitro synthesized material was translocated into the lumen of these vesicles as deduced from its proteinase K resistance. In the presence of secY205 INVVs, however, translocation was reduced to 3%, which is con-
sistent with the SecA-dependent translocation of OmpA. SecA36, a highly active SecA derivative, has been shown to specifically suppress the

secY205 defect (21, 28). In agreement with this, the translocation of OmpA into secY205 INVVs was completely restored by the addition of purified SecA36 (Fig. 1A) but not by the addition of wild type SecA.

The SecA dependence of membrane proteins like Momp2 and YidC also can be disclosed by use of the secY205 mutant. In the presence

of wild type INVVs (Fig. 1A), a membrane-protected fragment of Momp2 (Momp2-MPF) was observed that corresponds to the TM domain and the 320-amino acid-long periplasmic domain translocated into the lumen of the INVVs. The reduction in size is due to proteinase K cleavage of the major part of the N-terminal amphiphilic helix (9). The occurrence of the Momp2-MPF was significantly reduced in the presence of secY205 INVVs unless SecA36 was added. Similar results were also observed for YidC in which TM domains 1 and 2 are connected by a 42-kDa protease-protected fragment of YidC, observed in the presence of wild type INVVs (Fig. 1A), but only barely detectable in the presence of secY205 INVVs. This frag-

ment corresponds to the first two TM domains and the connecting periplasmic loop (16, 31). Only by adding SecA36 to secY205 INVVs was a significant translocation of the periplasmic loop observed (Fig. 1A). In contrast to Momp2 and YidC, the SecA-independent membrane protein mannitol permease (MfIA) (14), which lacks extended periplasmic loops, was not affected by the secY205 mutation, and its integration was not stimulated by the addition of SecA36 (Fig. 1A).

SecA-dependent membrane proteins are, like SecA-independent membrane proteins, cotranslationally targeted to the SecY translocon by SRP. We therefore analyzed whether the secY205 mutation would affect the membrane targeting of Momp2 and YidC. As shown previ-
ously (9), the cotranslational binding of RNCSs to the membrane can be analyzed by flotation gradient centrifugation. If Momp2-329, a nascent
A chain of 329 amino acids, was subjected to flotation gradient centrifugation in the absence of INVs (Fig. 1B), about 90% of the nascent chains were recovered from the bottom fractions (fractions 4 and 5) of the gradient. In contrast, in the presence of wild type INVs more than 80% of the material was found in the membrane-containing fractions 2 and 3 of the gradient. The same partitioning into the membrane fractions of the gradient was observed when Momp2-RNCs were synthesized in the presence of secY205. This reflects a SecA-independent targeting of Momp2-RNCs to the SecY205 translocon.

To directly demonstrate that the binding of Momp2-RNCs to the secY205 INVs was due to cotranslational targeting mediated by SRP, the secY205 INVs were extracted with 4M urea. This treatment has been shown to remove the membrane-bound SRP, SR, and SecA (14). Membrane binding of Momp2-RNCs to urea-treated secY205 INVs was drastically reduced but could be completely restored if purified Ffh, the protein component of the bacterial SRP, and purified FtsY, the bacterial SR, were added (Fig. 1B). The addition of 4.5 S RNA, which together with Ffh forms the bacterial SRP, was not necessary in these experiments due to sufficient amounts of 4.5 S RNA in the in vitro system used (14). The same SRP- and FtsY-dependent cotranslational targeting to secY205 INVs was observed for YidC-447-RNCs, a nascent chain of 447 amino acids (Fig. 1B). Thus, although the impaired SecA-SecY interaction in the secY205 INVs blocks the complete assembly of SecA-dependent membrane proteins, it does not reduce their SRP-dependent membrane targeting.

To examine whether the SRP-dependent targeting of SecA-dependent membrane proteins to secY205 INVs resulted in lipid anchorage of the TM domain, we used alkaline extraction as a suitable method to differentiate between lipid-inserted proteins and peripherally bound E. coli proteins (10). Momp2-329-RNCs were synthesized in the

**FIGURE 1.** Discrimination between SecA- and SRP-dependent steps during assembly of the type II membrane protein Momp2. A, OmpA, Momp2, YidC, and MtlA were synthesized in vitro in the absence or presence of E. coli inside-out INVs. SecA36 (300 ng/25-μl reaction mixture) or wild type SecA (WT) (900 ng/25-μl reaction mixture) were added when indicated. Samples were either precipitated directly with trichloroacetic acid or after incubation with 0.5 mg/ml protease K (Prot. K). Indicated are the positions of the precursor (pOmpA) and the mature form of OmpA and of full-size Momp2, YidC, and MtlA and their protease K-resistant MPFs. The percentage of translocation or integration, respectively, was calculated after quantification of the radioactivity of the individual protein bands using an Amersham Biosciences PhosphorImager and ImageQuant software and calculating the ratio between the amounts present in the protease K-treated sample and the trichloroacetic acid-precipitated sample. A representative example of several independent experiments is shown. The Momp2, YidC, and MtlA values obtained were corrected for the loss of Met and Cys residues occurring during cleavage. On the right, the topologies of the substrates and the sizes of their periplasmic loops are shown. Indicated are the signal peptidase or proteinase K cleavage sites. B, in vitro synthesis of Momp2-329- and YidC-447-RNCs was performed in the absence or presence of wild type (WT), secY205, or urea-treated secY205 (U-secY205) INVs. When indicated, Ffh (150 ng/25-μl reaction mixture) and FtsY (500 ng/25-μl reaction mixture) were present during synthesis. The reaction mixture was separated by flotation gradient centrifugation as described under “Experimental Procedures.” For calculating the percentage of membrane binding, the signal present in all five fractions was summed and set as 100%. Cytopl., cytoplasm; Peripl., periplasm.
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FIGURE 2. Lipid insertion of the signal anchor sequence requires SecY and precedes ribosome release. Momp2-329-RNCs were synthesized in vitro in the absence or presence of INVs derived from the wild type strain (WT), the secY205 mutant, and strain CM124 induced (SecYE') or not induced (SecYE-) for SecE expression. After synthesis, the reaction mixtures were incubated with 0.2 M sodium carbonate for 30 min on ice. Carbonate-resistant and -soluble material was separated by ultracentrifugation. The supernatant (S) was precipitated with trichloroacetic acid, and the pellet (P) was directly dissolved in loading buffer and subjected to SDS-PAGE. The inset shows immune detection of SecY in wild type cells and CM124 cells induced (SecYE') or not (SecYE-) for SecE expression.

Collectively our data demonstrate that the SRP-dependent cotranslational targeting of bacterial membrane proteins with extended periplasmic loops leads to a stable binding of the RNCs to the membrane. Importantly during this step the signal anchor sequence gains access to the lipid phase in the absence of a functional SecA-SecY interaction. The subsequent translocation of the periplasmic loop then requires a functional interaction between the Sec translocon and SecA.

Ribosome Release Is Required for Complete Translocation of the Periplasmic Loop in Single But Not in Multiple Spanning Membrane Proteins—The release of the ribosome was not required for lipid insertion of the TM domain of Momp2 (Fig. 2). To address the question whether also the SecA-dependent translocation of the periplasmic loop would occur while the ribosome was still attached, we analyzed protease protection of Momp2-RNCs prior to or after ribosome release by puromycin. In the presence of wild type INVs about 50% of Momp2-329-RNCs were protease-protected after puromycin treatment, but only 17% were protease-protected in the absence of puromycin (Fig. 3B, upper panel, lanes 5 and 6). These remaining 17% most likely result from a puromycin-independent detachment of the ribosome during handling of the RNCs. Consistent with the SecA-dependent translocation of the periplasmic loop, significant protease protection of Momp2-329 in the presence of secY205 INVs was only observed if puromycin and SecA36 were added (Fig. 3B, upper panel, lanes 11 and 12). These results indicate that the release of the ribosome is a prerequisite for the SecA-dependent translocation of the periplasmic loop in Momp2 presumably because SecA does not have sufficient access to even long hydrophilic loops that are still bound to the ribosome.

Surprisingly, however, if YidC-447-RNCs, consisting of three TM domains (Fig. 3A), were analyzed in the same experimental setup, protease protection of the 320-amino acid-long periplasmic loop was independent of the addition of puromycin (Fig. 3B, middle panel, lanes 5 and 6). To confirm that the translocation of YidC-447-RNCs in the presence of the ribosome was still SecA-dependent, protease protection in secY205 INVs was analyzed in the presence or absence of SecA36. The periplasmic loop of YidC-447 was translocated into secY205 INVs only when SecA36 was present, but the release of the ribosome was not required to obtain a stable translocation product (Fig. 3B, middle panel, lanes 11 and 12). We next tested the effect of puromycin on the translocation of YidC-330-RNCs, which like the Momp2-RNCs consist of a single TM domain, connected to a 307-amino acid-long periplasmic loop (Fig. 3A). As for Momp2-329-RNCs, efficient protease protection of YidC-330-RNCs was observed only after the ribosome had been released by the addition of puromycin (Fig. 3B, lower panel, lanes 5 and 6) and was clearly SecA-dependent (Fig. 3B, lower panel, lanes 9 and 12).

In summary, for single spanning membrane proteins like Momp2-329 or YidC-330, complete SecA-dependent translocation is observed only after the release of the ribosome, i.e. after protein synthesis is terminated. In contrast, if the periplasmic domain is followed by a downstream TM domain, its SecA-dependent translocation occurs before protein synthesis is terminated, i.e. in the presence of the ribosome.

The SecA Dependence of the Single Spanning Momp2 Is Not Determined by the Length of the Periplasmic Loop—A proposed model of the SecA function suggests that SecA catalyzes the stepwise translocation of 30 amino acids by inserting together with its substrate into the translocon. Multiple ATP-dependent cycles of SecA insertion and deinsertion would then completely translocate the cargo (6, 8). This idea is supported by data indicating that only periplasmic domains of multiple spanning membrane proteins larger than 30 amino acids require SecA for translocation (33). Thus, if the role of SecA were limited to provide the driving force for translocation of only those periplasmic domains

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4 S. Deitermann, G. S. Sprie, and H.-G. Koch, unpublished data.
significantly larger than 30 amino acids, one would expect to see a reduction in the SecA dependence of Momp2-RNCs by reducing the length of their periplasmic domains. Surprisingly we did not observe that short nascent chains of Momp2 were less SecA-dependent than long nascent chains (Fig. 4A). Even the translocation of Momp2-60, harboring a periplasmic loop of just 13 amino acids, appeared to be as sensitive toward the impaired SecA-SecY interaction in secY205 INVs as Momp2-329 harboring a periplasmic loop of 287 amino acids. These data suggest that, independently of the length of the periplasmic domain, complete assembly of the single spanning Momp2 is not possible without a functional SecA-SecY interaction. Neither Momp2-329 nor Momp2-60-RNCs did acquire protease resistance in INVs derived from the SecG deletion strain KN553 (22) (data not shown). Because SecG, which is the third component of the heterotrimeric SecY translocon, is suggested to support the catalytic cycle of SecA, these data confirm the SecA-dependent translocation of even small periplasmic loops in single spanning membrane proteins.

To directly demonstrate that even for these very short Momp2-RNCs, the role of SecA is restricted to sustain the translocation of the periplasmic domain, we experimentally separated the targeting reaction from the translocation reaction. In a first step, Momp2-60-RNCs that were cotranslationally targeted to the SecY translocon were isolated by flotation gradient centrifugation. Fig. 4B (compare lanes 1 and 3) shows that Momp2-60-RNCs were as efficiently targeted to wild type INVs as to secY205 INVs because about equal amounts of Momp2-60 were present in the respective INV-containing fractions. In a second step, the isolated membrane-bound RNCs were treated with puromycin to release the ribosome and then treated with proteinase K. Protease resistance of INV-associated Momp2-329, indicative of translocation of the periplasmic loop into the vesicle lumen, was sufficiently obtained only with wild type but not with secY205 INVs (Fig. 4B, lanes 2 and 4). If, however, Momp2-60-RNCs that had been targeted to secY205 INVs were incubated with SecA36 (Fig. 4B, lane 5), a significant stimulation of the protease protection was observed (Fig. 4B, lane 6). As a control, we analyzed Momp-329-RNCs, which like Momp-60 were efficiently targeted to secY205 INVs but not translocated unless SecA36 had been added (Fig. 4B). In summary, these data suggest that a periplasmic loop as short as 13 amino acids does not reach the periplasmic side of the membrane in the absence of SecA and SecG.

The SecA Dependence of a Small Periplasmic Loop Is Abolished by the Presence of a Downstream TM Domain—The above described observations were unexpected because previous studies had demonstrated that multiple spanning membrane proteins like MtlA, which contains only small periplasmic loops varying between 6 and 22 amino acids (Fig. 1A), are integrated independently of SecA and SecG (14). SecA- and SecG-independent integration has also been shown for SecY, which like MtlA contains only small periplasmic loops (15). This would suggest that small periplasmic loops can be translocated independently of SecA if they are followed by a TM domain as in MtlA or SecY. To test this hypothesis, we created different YidC constructs in which the size of the periplasmic loop connecting TM1 and TM2 domains was gradually reduced from 320 to 13 amino acids (Fig. 5A). The SecA dependence of these constructs was analyzed by testing their SecA36-dependent integration into secY205 INVs. For the constructs YidCΔ164, YidCΔ266,
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Strikingly if the loop size was reduced to 13 amino acids as in YidCΔ307, we no longer observed a significant difference between YidCΔ307 integration into wild type and secY205 INVs; this was independent of whether SecA36 was added or not (Fig. 5B). Thus, in the presence of a downstream TM domain, the translocation of the 13-amino acid-long periplasmic loop of YidCΔ307 does not require SecA.

We wished to exclude the possibility that the use of RNCs (Momp2-60, Fig. 4A) as opposed to completed translation products (YidCΔ307, Fig. 5B) was the reason for the different SecA requirement of the 13-amino acid-long periplasmic loop in both constructs. Therefore we synthesized YidCΔ307-RNCs consisting of the first two TM domains and the 13-amino acid-long periplasmic loop. One aliquot from the translation mixture was directly trichloroacetic acid-precipitated to monitor protein synthesis (Fig. 5C, lanes 1, 4, 7, and 10). The remaining material was subjected to the reconstitution assay described above (c.f. Fig. 4B) to separate the cotranslational targeting from the translocation reaction. Only the membrane fractions of the flotation gradient were recovered and either directly trichloroacetic acid-precipitated (Fig. 5C, lanes 2, 5, 8, and 11) or only after proteinase K treatment (Fig. 5C, lanes 3, 6, 9, and 12). Efficient cotranslational targeting of YidCΔ307-RNCs was observed for both wild type and secY205 INVs (Fig. 5C, compare lanes 5 and 8). Importantly, independently of whether SecA36 was added or not, YidCΔ307-RNCs were efficiently integrated into both wild type and secY205 INVs. These data confirm that, in contrast to Momp2, the short periplasmic loop of YidCΔ307 is translocated independently of SecA.

Because the periplasmic loop in YidCΔ307 is sandwiched between two TM domains, whereas in Momp2 the periplasmic loop is connected to a single TM domain, we analyzed whether removing the downstream TM domains would render YidCΔ307 SecA-dependent. For this we constructed two YidCΔ307 derivatives consisting of either one TM domain (YidCΔ307-TM1) or two TM domains (YidCΔ307-TM1-2).

First we verified that both constructs were integrated into the membrane via the SecY translocon. In the presence of SecE-depleted CM124 INVs, both YidCΔ307-TM1 and YidCΔ307-TM1-2 showed significantly reduced protease protection in comparison to wild type INVs (Fig. 6A). Thus, the efficient integration of both truncated YidC constructs was dependent on the SecY translocon. The SecA dependence of YidCΔ307-TM1 and YidCΔ307-TM1-2 was analyzed in the secY205 INV/SecA36 system. The double spanning YidCΔ307-TM1-2 was efficiently integrated into both wild type and secY205 INVs independently of whether SecA36 was added or not (Fig. 6B). In agreement with the data presented above (c.f. Fig. 5), this confirms the SecA-independent translocation of YidCΔ307. In contrast, for the single spanning YidCΔ307-TM1 protease protection was significantly reduced in the presence of secY205 INVs unless SecA36 was added. These data clearly demonstrate that SecA is dispensable if a short periplasmic loop is followed by a downstream TM domain.

DISCUSSION

The biogenesis of bacterial membrane proteins is a multistep process requiring the SRP-dependent cotranslational targeting of ribosome-associated nascent membrane proteins to the SecY translocon and the subsequent insertion of the TM domains into the lipid bilayer. One particular feature of bacterial membrane proteins with extended periplasmic loops is that their assembly requires the ATPase SecA (9, 10). The current model suggests that SecA provides the driving force for the translocation of these hydrophilic domains across the membrane and that SecA is required only if periplasmic loops larger than 30 amino acids have to be translocated (for a review, see Ref. 12).

In agreement with this model, we show here that the SRP- and SecA-
dependent multiple spanning membrane protein YidC can be transformed into a SecA-independent protein by reducing the size of its large periplasmic loop. In our experimental system only YidC constructs with periplasmic loops larger than about 30 amino acids were found to depend on SecA for efficient translocation. In contrast, YidC/H9004307 in which the periplasmic loop was reduced to 13 amino acids was translocated independently of SecA. These observations fit well with in vivo studies showing that periplasmic loops of about 20 amino acids are efficiently translocated without the help of SecA but that larger loops become progressively more SecA-dependent (33–35). A threshold value of about 30 amino acids is also in agreement with studies showing that SecA binds to and translocates stretches of about 30 amino acids during its proposed ATP-dependent insertion and deinsertion cycle (6–8).

To our surprise, however, we were unable to render the single spanning membrane protein Momp2 SecA-independent by reducing the size of its periplasmic loop. Even periplasmic loops as short as 13 amino acids were still found to require SecA for complete translocation (Fig.
The hypothesis that the presence of a downstream TM domain influences the translocation mode of a periplasmic loop is also supported by our observation that for single spanning membrane proteins SecA executes its translocation activity only after the ribosome has been released (Fig. 3B). In contrast, a periplasmic loop that is followed by a downstream TM domain is translocated by SecA before ribosome release (Fig. 3B). These data suggest that at least for a membrane protein with...
more than one TM domain SecA can access the hydrophilic domain in the presence of the ribosome. Although the exact contact sites between the bacterial SecY translocon and the ribosome have yet to be mapped, extensive studies on the homologous eukaryotic Sec61 channel have indicated that RNC binding occurs at the cytoplasmic loop connecting TM8 and TM9 domains and at the C-terminal tail of Sec61 (50, 51). These domains are surface-exposed in the x-ray structure of the bacterial SecY and are also suggested to be involved in SecA binding (52). Thus, it is difficult to imagine that SecA and the RNCs bind simultaneously to a single SecY molecule. As the oligomeric state of the active SecYEG complex during translocation/integration is still a matter of debate (48, 53–55), a simultaneous binding of SecA and the RNCs to different SecYEG monomers is still a possibility. Alternatively the SecA-dependent translocation of a periplasmic loop in the presence of the ribosome could be explained by either a transient SecA-induced dissociation of the ribosome from the SecY translocon as shown recently for non-translating ribosomes (56) or by a rather flexible translocon-ribosome junction as observed for the eukaryotic translocon (57).

In summary, our data demonstrate a striking difference in the SecA dependence of single spanning and multiple spanning bacterial membrane proteins. Bacterial membrane proteins with more than one TM domain require SecA only if periplasmic loops larger than 30 amino acids have to be translocated. This is different from single spanning membrane proteins that, independently of the length of the periplasmic loop, always require SecA for efficient integration. This unexpected observation suggests that for single spanning membrane proteins SecA is not only involved in translocating periplasmic loops but has an additional function that needs to be further characterized.

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