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The Integration of YidC into the Cytoplasmic Membrane of *Escherichia coli* Requires the Signal Recognition Particle, SecA and SecYEG*

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The integration of the polytopic membrane protein YidC into the inner membrane of *Escherichia coli* was analyzed employing an *in vitro* system. Upon integration of *in vitro* synthesized YidC, a 42-kDa membrane protected fragment was detected, which could be immunoprecipitated with polyclonal anti-YidC antibodies. The occurrence of this fragment is in agreement with the predicted topology of YidC and probably encompasses the first two transmembrane domains and the connecting 320-amino acid-long periplasmic loop. The integration of YidC was strictly dependent on the signal recognition particle and SecA. YidC could not be integrated in the absence of SecY, SecE, or SecG, suggesting that YidC, in contrast to its mitochondrial orthologue Oxa1p, cannot engage a SecYEG-independent protein-conducting channel.

Protein traffic in any living cell requires the specific recognition of the proteins to be transported and their selective transport across the lipid bilayer through an aqueous channel providing the polar environment for translocation. It is now evident that the core components of this protein-conducting channel, termed SecYEG in bacteria and Sec61 in the endoplasmic reticulum of eukaryotes, are conserved in all three kingdoms of life (1). The targeting of proteins to the SecYEG complex in *Escherichia coli* is mediated by two pathways exhibiting different substrate specificity. Largely hydrophobic membrane proteins are selectively recognized by the bacterial SRP[1] (signal recognition particle), consisting of the protein Ffh and the 4.5 S RNA (2–4). Binding of SRP to the nascent chains of membrane proteins initiates a cotranslational targeting to FtsY, the bacterial homologue of the SRP-receptor, and subsequently to the SecYEG translocon (5, 6, 7). The translocation of secretory proteins, on the other hand, involves the posttranslational binding of the preprotein by the chaperone SecB and its subsequent transfer to SecA, which then translocates the preprotein across the SecYEG channel in an ATP-dependent manner (8). While there is no general overlap between the SRP and SecA/B pathways, the integration of a subset of membrane proteins, *i.e.* membrane proteins with large periplasmic domains, requires the cooperative activity of both SRP and SecA (7, 9). This has been demonstrated for a single spanning membrane protein carrying a 320-amino acid-long periplasmic loop, which is cotranslationally targeted to SecY by SRP but remains untranslocated until SecA is present (7).

Recent evidence indicates that the translocase activity and the integrase activity of the SecYEG complex depend on different domains of SecY and different components of the translocon (10, 11). In particular, the activity of SecG seems to be dispensable for the integration of SecA-independent membrane proteins (11). The engagement of different components of the translocon during SRP-dependent integration of membrane proteins and SecA-dependent translocation of secretory proteins is further corroborated by recent results indicating that the integration of membrane proteins requires an additional component, the 60-kDa membrane protein YidC (12, 13). YidC seems to be functionally and structurally closely associated with the SecYEG translocon (14, 15) and is presumably involved in the lateral transfer of transmembrane domains (TMs) from the Sec complex into the lipid bilayer (13, 16). Even small phage-derived membrane proteins, previously thought to spontaneously insert into the *E. coli* membrane, depend on YidC for their correct insertion (17). Since the integration of these phage proteins occurs most likely in a SecYEG-independent manner, it is assumed that for some proteins YidC can mediate membrane insertion independently of the SecYEG translocon (18). Like YidC, its orthologues, Oxa1p in the inner mitochondrial membrane and Alb3 in the thylakoid membrane, are specifically involved in membrane protein assembly (18). Because mitochondria do not contain homologues to the bacterial SecYEG translocon, it has been suggested that Oxa1p represents a component of an individual integration machinery in the inner mitochondrial membrane through which membrane proteins, like the nuclear-encoded Oxa1p itself, are integrated from the matrix side into the inner membrane (19, 20).

Oxa1p consists of five transmembrane domains and a hydrophilic N-tail of about 100 amino acids, which is translocated into the intermembrane space of mitochondria (19). This topology is reminiscent of the bacterial protein ProW, which contains seven transmembrane domains and a periplasmic N-tail of about 100 amino acids (21). It has been demonstrated that the N-tail of ProW is translocated independently of the SecA/SecY system (21). The major topological difference between the *E. coli* YidC and Oxa1p/ProW is that it contains an additional N-terminal signal anchor sequence, which is connected by a 320-amino acid-long loop to the second TM (Ref. 22; Fig. 1*B*). In view of this special topology of YidC we wanted to analyze the mechanism of its integration. In particular, we asked whether the requirements for integration were comparable with those of single spanning membrane proteins, carrying large periplas-

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[1] The abbreviations used are: SRP, signal recognition particle; TM, transmembrane domain; INV, inside-out inner membrane vesicles; MPF, membrane-protected fragment; pmf, proton motive force.

mic domains. We demonstrate here that the integration of YidC into the inner membrane of *E. coli* requires a functional SecYEG translocon and the coordinated activity of both the signal recognition particle and SecA.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—The following *E. coli* strains were used: MRE 600 (23), XL1-Blue (Stratagene), TY1 (*ompT::kan*, *secY205*) (24), CU164 (*secY39*) (25), CM124 (*secEΔ19–111*, pCM22) (26), and KN553 (*ΔuncB-C::Tn10 ΔsecG::kan*) (27). For *in vitro* protein synthesis the following plasmids were used: pDMB (OmpA) (28), p717MtlA-B (mannitol permease) (29), and the YidC gene cloned in pROEX-HTB (Invitrogen), kindly provided by Dr. Ross Dalbey.

In Vitro Reactions—The composition of the reconstituted transcription/translation system of *E. coli* and the purification of its components, the preparation of INV, urea extraction of INV, and the protease protection assay employed in this study have been described previously (11, 28, 29).

YidC Purification and Production of Polyclonal Antibodies—Overexpression and purification of YidC-His was performed in a similar manner as described (15). His-tagged YidC was expressed from pROEX-HTB-yidC in *E. coli* XL1-Blue, grown to mid-logarithmic phase in LB medium supplemented with ampicillin (100 μg/ml), and 0.2% glucose. Expression was induced by adding isopropyl-β-D-thiogalactopyranoside (1 mM), and growth was continued for another 2 h. Cells were disrupted by several passages through a French pressure cell (8000 p.s.i.), and unbroken cells were removed by centrifugation for 30 min at 30,000 × *g* (S30). Membranes were collected by ultracentifugation (2.5 h, 45,000 rpm, Beckmann Ti50.2 rotor) of the S30 and solubilized in buffer A (10 mM Tris, pH 8.0, 20% glycerol, 100 mM KCl, 10 mM imidazole) containing 2% dodecylmaltoside. After removal of insoluble material (1 h, 100,000 × *g*) the solubilized proteins were applied to nickel-nitrilotriacetic acid-agarose (Quiagen, Hilden, Germany). The matrix was washed with buffer A containing 0.1% dodecylmaltoside, 40 mM imidazole, and bound material was eluted with buffer A containing 0.1% dodecylmaltoside and 400 mM imidazole. Polyclonal antibodies directed against purified and SDS-denatured YidC-His were raised in rabbits.

Sample Analysis and Quantification—All samples were analyzed on 13% SDS-polyacrylamide gels. Radiolabeled proteins were visualized by phosphorimaging using a Molecular Dynamics PhosphorImager and quantified using Imagequant software from Molecular Dynamics. The percentage of integration was calculated after correcting for the expected loss of methionine residues occurring during cleavage by proteinase K.

RESULTS AND DISCUSSION

In Vitro Synthesis and Integration of YidC—For analyzing its integration, a His-tagged version of YidC was *in vitro* synthesized in the presence and absence of INV of *E. coli* and subsequently treated with proteinase K. The *in vitro* synthesis resulted in a radioactively labeled band of about 65 kDa (Fig. 1*A*), which corresponds to the predicted size of YidC (22). In the absence of INV this band was almost completely digested by proteinase K. In the presence of INV a 42-kDa membrane-protected fragment of YidC (YidC-MPF) became detectable, which, like full size YidC, could be immunoprecipitated by polyclonal anti-YidC antibodies. According to hydropathy and PhoA fusion analyses YidC is predicted to comprise six TMs (Fig. 1*B*). In particular, the first TM is suggested to serve as an uncleaved signal anchor sequence, which is connected to the second TM by a large 320-amino acid-long periplasmic loop (22). The topology model proposed by Sääf *et al.* (22) suggests that the 42-kDa membrane-protected fragment of YidC consists of at least the first two TMs, connected by the large periplasmic domain.

The Integration of YidC Requires SecY, SecE, and SecG—The mitochondrial Oxa1p catalyzes its own integration as part of a Sec-independent integration machinery in the inner mitochondrial membrane (19). Its bacterial orthologue YidC seems to be closely associated with the SecYEG translocon (14). However, based on the observation that YidC is also required for the integration of SecY-independent phage proteins (17), it has been proposed that YidC can also function independently of SecYEG and as such might be sufficient for its own integration.

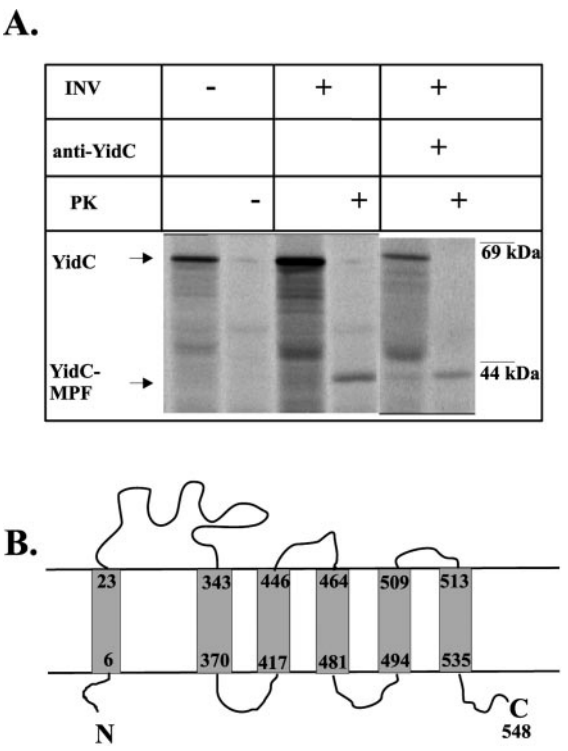

FIG. 1. **Integration of YidC into inside-out inner membrane vesicles of *E. coli*.** *A*, YidC was *in vitro* synthesized in a cell-free translation system in the presence of INV. ^{35}S-Labeled translation products were subjected to a protease protection assay (0.5 mg/ml proteinase K (*PK*), 20 min, 25 °C) or directly precipitated with trichloroacetic acid, separated by SDS-PAGE and visualized by phosphorimaging. *Arrows* indicate the position of full-length YidC and the membrane-protected fragment of YidC (YidC-MPF) resistant toward proteinase K. Immunoprecipitation using anti-YidC antiserum confirmed the identity of the membrane protected fragment of YidC. *B*, the predicted topology model for YidC (21). The YidC protein consists of an N-terminal transmembrane domain, a large periplasmic loop, and five C-terminal transmembrane helices. The N and C termini are located on the cytoplasmic side (22).

To address this, we analyzed the integration of YidC into INV prepared from different *E. coli* strains, carrying mutations within *secY*, *secE*, or *secG*. As controls we also tested the integration of the polytopic membrane protein mannitol permease (MtlA) and the translocation of the secretory protein OmpA. We have previously shown (11) that INV prepared from the *secY* mutants *secY39* and *secY205* were completely blocked in the transport of OmpA. The integration of MtlA on the other hand was not significantly influenced (Fig. 2). Similar to OmpA, both mutations also severely reduced the integration of YidC, which strongly argues for an involvement of SecY in YidC integration. The integration defect observed with the *secY205* mutant vesicles furthermore points to a SecA participation in the integration of YidC, since the *secY205* mutant is specifically impaired in the SecA-SecY interaction (24). This is why the integration of the SecA-independent membrane protein MtlA is not impaired by this mutation.

In SecE-depleted membranes, SecY is rapidly degraded by the FtsH protease (30), causing a pronounced transport defect for secretory proteins and membrane proteins (11). As for MtlA and OmpA (Fig. 2), YidC integration was completely blocked in SecE-depleted INV, which confirms that YidC is integrated into the membrane through the SecYE translocon.

We have previously shown that SecG is dispensable for the integration of those membrane proteins, which do not require

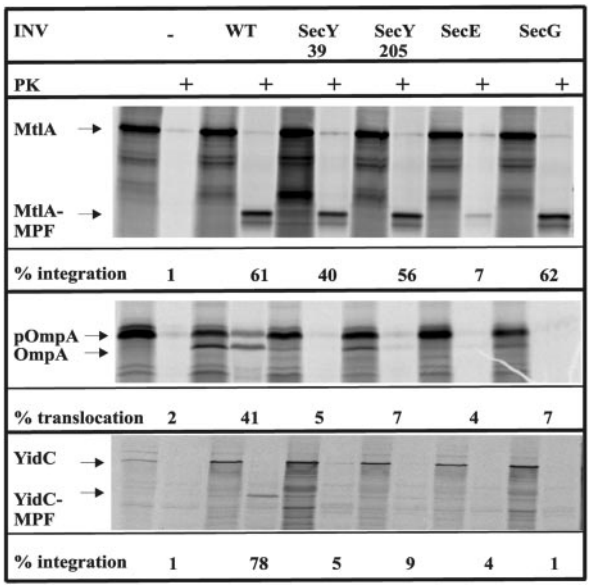

FIG. 2. **The integration of YidC requires SecY, SecE, SecG, and SecA.** The polytopic membrane proteins YidC and mannitol permease A (MtlA) and the secretory protein OmpA were synthesized *in vitro* in the presence of INV prepared from wild-type (*WT*) or different *secY*, *secE*, *secG* mutant strains. Integration or translocation of ^{35}S-labeled translation products was analyzed by protease protection assay. Indicated are the positions of full-length MtlA, the membrane-protected fragment of MtlA (MtlA-MPF), the precursor pOmpA and the mature form of OmpA, the full-length form of YidC, and the membrane-protected fragment of YidC (YidC-MPF). Efficiency of integration was calculated as the ratio between the signal of MPF and the full-length protein for MtlA and YidC. Translocation of OmpA was calculated as the ratio of signal present in proteinase K (*PK*)-resistant bands of pOmpA and OmpA and that recovered from corresponding bands before proteolytic digestion. Values were corrected for the expected loss of methionine residues occurring during proteinase K cleavage.

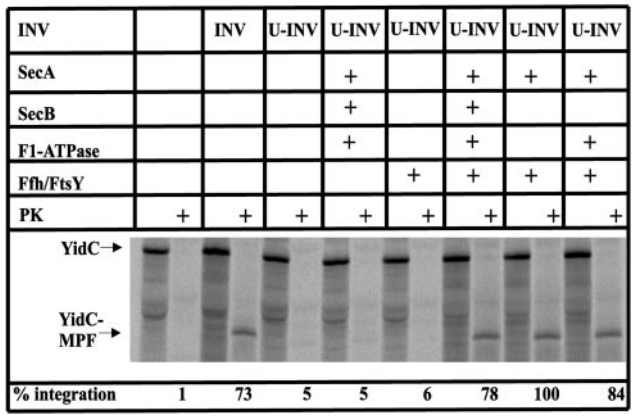

FIG. 3. **The integration of YidC requires SRP and SecA.** YidC was synthesized *in vitro* in the presence of the components indicated at the *top* (SecA (80 ng/μl), SecB (80 ng/μl), F_1-ATPase (40 ng/μl), Ffh (8 ng/μl), FtsY (20 ng/μl)). Integration of ^{35}S-labeled translation products was analyzed by protease protection assay. Percentage of integration was calculated as indicated in the legend to Fig. 2.

SecA for proper integration (11). Thus, the function of SecG is probably restricted to its role in assisting the insertion of the SecA-preprotein complex into the translocation channel (27). INV prepared from a *secG* deletion mutant did therefore not affect the integration of MtlA, but were completely blocked in the translocation of OmpA (Fig. 2). These INV were also unable to support the integration of YidC, which underlines the involvement of SecA in the integration of YidC. In summary, these data suggest a SecYEG- and SecA-dependent integration of YidC. Thus, in contrast to its mitochondrial orthologue Oxa1p, YidC appears to be unable to catalyze its own integration in the absence of SecYEG.

The Integration of YidC Requires SRP and SecA, but Is Independent of SecB—Based on the requirements for the translocation of secretory proteins across, and the integration of membrane proteins into the inner membrane of *E. coli*, three distinct groups of proteins using the SecY translocon can be discriminated: (i) polytopic membrane proteins requiring just SRP and FtsY, (ii) secretory proteins requiring just SecA/SecB and the proton motive force, and (iii) membrane proteins with large periplasmic domains requiring both SRP and SecA. Because of its structure YidC should belong to the third group of proteins.

To test the requirements for YidC integration, we used an *in vitro* system, which allows to independently assay the effects of SRP and SecA/SecB on YidC integration. This *in vitro* system utilizes a purified cytosolic extract, almost completely devoid of SecA, SecB, Ffh, and FtsY for transcription/translation and INV treated with 6 M urea (U-INV), which removes all peripherally associated translocation and integration factors. We have previously shown that the integration of membrane proteins devoid of large periplasmic loops, such as MtlA into these vesicles, can be restored by adding purified Ffh, 4.5 S RNA, and FtsY, while the restoration of OmpA translocation requires the addition of purified SecA, SecB, and F_1-ATPase (29).

Similar to MtlA and OmpA, treatment of INV with 6 M urea completely blocked the integration of YidC (Fig. 3). Adding SecA/SecB and F_1-ATPase failed to restore the YidC integration, as did adding Ffh and FtsY. However, by adding SecA/SecB/F_1-ATPase together with Ffh and FtsY the YidC integration activity of these vesicles could be fully restored. Full restoration was also possible in this *in vitro* system by adding just SecA, Ffh, and FtsY, suggesting that these components are essential for the integration of YidC.

The simultaneous dependence on SRP and SecA is reminiscent of single spanning membrane proteins with large periplasmic domains, like FtsQ or the fusion protein Momp2 (7, 11). A detailed analysis of Momp2 integration has provided a model on how SRP and SecA cooperate in the assembly of these membrane proteins: SRP mediates the cotranslational targeting of ribosome-associated nascent chains to the SecYEG translocon, but is unable to translocate the large hydrophilic domain, *i.e.* the protein is stably bound to the translocon but remains protease-sensitive until SecA is added (7). *In vitro*, Momp2 was found to be integrated even by SecA/SecB alone if SRP and FtsY were omitted. In this setup, however, SecA/SecB achieve both targeting and translocation in a posttranslational manner similar to the mechanism by which secretory proteins are translocated. The polytopic membrane protein YidC behaves clearly different from the single spanning Momp2, since it cannot be integrated by SecA/SecB alone in the absence of SRP. Presumably, if translocation would occur only after all TMs have been synthesized, a posttranslational binding of SecA would not lead to membrane assembly because of the tendency of the polytopic YidC to aggregate in solution. Thus, the occurrence of TMs following the large periplasmic loop of YidC seems to require a renewed binding of SRP probably to the third, signal-anchor type TM.

Most secretory proteins depend not only on SecA for their translocation but also on the chaperone SecB, which binds to the preprotein and stabilizes it in a transport competent conformation (8). In addition to its chaperone activity, SecB has also a targeting function, which is mediated by its ability to interact with SecA. Whether SecB is involved in the topogenesis of membrane proteins requiring a concerted action of SRP and SecA, such as Momp2, has not been addressed so far.

Under our experimental conditions, *i.e.* in the presence of SRP and SecA, the integration of YidC does not seem to require SecB (Fig. 3). In addition, we show that in the absence of SRP integration of YidC cannot be accomplished by SecA and SecB alone. Thus, the chaperone activity of SecB seems to be unable to maintain YidC in an integration competent conformation, suitable for a posttranslational transport by SecA.

Under our conditions, *i.e.* saturating concentrations of SecA, the integration of YidC occurs in the absence of the membrane potential, since F_1-ATPase is obviously not needed for this process (Fig. 3). The underlying mechanisms on how the proton motive force (pmf) effects protein translocation are mostly unclear (31). The pmf obviously influences the SecA reaction cycle, because high concentrations of SecA render the translocation reaction pmf-independent (32). The pmf presumably also effects the translocase and its pore size directly, and it might impose directionality to the translocation process (31). During the cotranslational targeting of SRP dependent proteins, a close contact between the translating ribosome and the SecYEG translocon is formed, and this seal should prevent any reverse translocation, therefore imposing directionality in the absence of the pmf.

In this paper we have analyzed the integration of YidC into the inner membrane of *E. coli*. Like single spanning membrane proteins with large periplasmic loops, its integration requires the coordinated activity of both SRP and SecA. Thus, the integration is probably initiated by a cotranslational binding of SRP to the signal anchor sequence of YidC and a subsequent targeting to the SecYEG complex. The activity of SecA is then required for the translocation of the large periplasmic loop. The SecA dependence of YidC is intriguing with respect to the so called N-tail phenomenon (33), which describes the Sec-independent translocation of periplasmic N-tails in bacterial membrane proteins. The major structural difference between YidC and N-tail membrane proteins such as Oxa1p or ProW is that in YidC a signal anchor sequence precedes the N-tail. Furthermore, the observation that YidC cannot be integrated in the absence of SecY, SecE, or SecG rules out the possibility that YidC, like its mitochondrial orthologue Oxa1p, catalyzes its own integration as part of a SecYEG-independent transport machinery.

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