Targeting, Insertion, and Localization of *Escherichia coli* YidC

YidC was recently shown to play an important role in the assembly of inner membrane proteins (IMPs) both in conjunction with and separate from the Sec-translocon. Little is known about the biogenesis and structural and functional properties of YidC, itself a polytopic IMP. Here we analyze the targeting and membrane integration of YidC using *in vivo* and *in vitro* approaches. The combined data indicate that YidC is targeted by the signal recognition particle and inserted at the SecAYEG-YidC translocon early during biogenesis, unlike its mitochondrial homologue Oxa1p. In addition, YidC is shown to be relatively abundant compared with other components involved in IMP assembly and is predominantly localized at the poles of the cell.

In *Escherichia coli*, inner membrane protein (IMP)\(^{1}\) integration can occur via Sec-dependent or Sec-independent mechanisms (1). The majority of IMPS are targeted to the membrane by the signal recognition particle (SRP) and its receptor PtsY that mediates cotranslational targeting to the Sec-translocon (2). The SRP, which consists of the 4.5 S RNA and Ffh (for fifty-four homologue) is homologous to the eukaryotic SRP but less complex in composition. The core Sec-translocon consists of the integral membrane components SecY, SecE and SecG, which form a heterotrimer, and the peripheral subunit SecA (3). The translocon serves as a translocating pore for both secretory proteins and IMPS. SecA is an ATPase that functions as a molecular motor that drives the translocation of secretory proteins and large periplasmic domains of IMPS through the SecYEG pore.

Recent evidence shows that a novel component, YidC, is specifically involved in Sec-dependent IMP integration (4). Using a site-specific photocross-linking procedure, YidC was shown to interact with the nascent IMPS, PtsQ, Lep, and mannitol permease, as they move laterally from the Sec-translocon into the lipid bilayer (5–8). This interaction appeared to be specific for the transmembrane segments (TMs) in the nascent polypeptide. Moreover, YidC could be co-purified with the Sec-translocon suggesting a physical connection (7). Upon depletion of YidC, the assembly of Sec-dependent IMPS such as Lep and PtsQ is hampered, although the effect is relatively mild (8, 9).

In contrast to most IMPS, some small phase coat proteins (like Pf3 coat and M13 procoat proteins) insert into the inner membrane independent of the Sec-translocon (10). These proteins were considered to partition spontaneously into the lipid bilayer (i.e. without the requirement of any proteinaceous factor and only depending on the proton motive force to energize the process). However, it was recently demonstrated that membrane assembly of M13 coat protein is almost completely blocked upon depletion of YidC suggesting a crucial role for YidC in the integration of Sec-independent proteins (9, 11). YidC is homologous to the mitochondrial IMP Oxa1p and to the thylakoid membrane protein Alb3, which both have been implicated in membrane protein integration (4). Oxa1p is essential for the correct insertion of a subset of both mitochondrial-encoded IMPS (like pCoxII) and nuclear-encoded IMPS (like Oxa1p itself). Interestingly, mitochondria do not have an SRP-like targeting pathway or a Sec-like translocon suggesting that Oxa1p might function in a fashion similar to YidC in the Sec-independent route.

YidC is a polytopic IMP that spans the membrane six times with an N-in, C-in topology and a large, poorly conserved periplasmic domain between TM1 (signal anchor sequence) and TM2 (Fig. 2A) (12). As is true for most polytopic IMPS, hardly anything is known about the targeting, assembly, localization, and cellular abundance of YidC. Here, we present evidence that YidC is targeted co-translationally by the SRP to the Sec-translocon, which appears to be required for proper assembly. Nascent YidC is shown to contact SecA, SecY, and pre-existing YidC very early during biosynthesis. YidC is found to be present in excess over SecYEG. Finally, using GFP fusion technology we found that YidC accumulates at the poles of the cell.

**EXPERIMENTAL PROCEDURES**

*Materials—* Restriction enzymes, Expand long template PCR system, and Lumi-Light\(^{TM}\) Western blotting substrate were from Roche Molecular Biochemicals. Megashort script T7 transcription kit was from Ambion Inc. (\^{**})S)methionine and protein A-Sepharose were from Amersham Biosciences. T4 Ligase and T4 DNA polymerase were from Epicentre Technologies. All other chemicals were supplied by Sigma. Antiserum against a C-terminal peptide of YidC has been described previously (7). In addition, an antiserum against purified histidine tagged YidC (see below) was raised in rabbit by Agrisera (Umeå, Sweden). The antiserum directed against Trigger Factor (TF) and SecA were gifts from W. Wickner, and anti-SecY was a gift from A. Driessen.

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\(^{1}\) The abbreviations used are: IMP, inner membrane protein; SRP, signal recognition particle; PtsY, fifty-four homologue; TM, transmembrane segments; GFP, green fluorescent protein; TF, Trigger Factor; LB, Luria Bertani; IPTG, isopropyl-1-thio-β-D-galactopyranoside; IMV, inverted inner membrane vesicles.
Strain and Plasmids—E. coli strain MRE600 was used to prepare a lysate for translation of in vitro synthesized mRNA and suppression of UAG stop codons in the presence of (Tmd)Phe-tRNA
sup. Strain MC4100 grown in Luria Bertani (LB) medium was used to isolate inverted membrane vesicles (IMVs) and as an expression host for plasmid pEH1hisYidC/B. The C-terminus of YidC was fused to the N terminus of a GFP variant that has been selected to inhibit the protease. Finally, the samples were precipitated in 10% nyl fluoride was added to the spheroplast suspensions (0.33 mg/ml) to inhibit the protease. Furthermore, two less prominent cross-linking adducts of the photocross-linking group are just exposed outside the ribosome. The translation reaction was stopped by (Tmd)Phe-tRNA
sup (data not shown), resulting in a 35 amino acids, the 52-mer and 96-mers were identified as Ffh, the protein component of the pathway of targeting and membrane insertion of nascent YidC (a polycistronic IMP) using an in vitro translation targeting/photocross-linking assay that we initially developed for the analysis of molecular interactions during biosynthesis of the less complex IMPs, PtsQ and Lep (6, 7).

Radiolabeled nascent chains of YidC of 52 and 96 amino acids were synthesized by translation of truncated mRNA in a membrane-free E. coli extract in the presence of [35S]methionyl. Purified inverted IMVs were added from the start of the translation reaction to allow co-translational membrane targeting and insertion of the translation intermediates. To specifically probe the molecular environment of the first TM, a stop codon (TAG) was introduced at position 17 in the center of the TM region and suppressed during in vitro synthesis by the addition of (Tmd)Phe-tRNA
sup that carries a photoactive probe. After the translation/insertion reaction, one-half of each sample was irradiated with UV light to induce cross-linking whereas the other half was kept in the dark to serve as a control. The samples were extracted with carbonate to separate targeted from untargeted material from the membrane-integrated material, and cross-linking partners were identified by immunoprecipitation.

In both constructs, the TAG17 mutation was efficiently suppressed by (Tmd)Phe-tRNA
sup (data not shown), resulting in nascent YidC of the expected apparent molecular weight. Assuming that the ribosome covers ~35 amino acids, the 52-mer only partially exposes the TM outside the ribosome, and the photocross-linking group is just exposed outside the ribosome (Fig. 1A). Using this construct, a major cross-linking adduct of ~60 kDa was observed in the untargeted (carbonate soluble) nascent chains (Fig. 1B, lanes 1 and 2). Immunoprecipitation identified TF as the cross-linking partner (Fig. 1B, lane 3). Furthermore, two less prominent cross-linking adducts of ~55 and ~100 kDa were identified as Ffh, the protein component of the SRP, and SecA (Fig. 1B, lanes 4 and 5). Anti-Ffh also precipitated smaller adducts that might represent degradation products. The 52-mer was less efficiently integrated in the membrane than the longer YidC construct as judged by the criterion of carbonate resistance (~25% versus ~45%, not shown). Membrane-integrated 52-mers gave rise to distinct cross-linking adducts of ~40, ~60, and ~100 kDa (Fig. 1B, lanes 6 and 7), which were immunoprecipitated with anti-SecY, anti-YidC, and anti-SecA, respectively (Fig. 1B, lanes 8–10). For identification of nascent YidC-YidC adducts, antisera was raised against the C terminus of YidC was used to distinguish full-length YidC from nascent YidC. A fourth cross-linking adduct of ~55 kDa was not immunoprecipitated with any of the antisera used and remains to be identified.
In the longer 96-mer, the TM and ~38 residues of the first periplasmic domain are exposed outside the ribosome (Fig. 1A). Untargeted nascent chains still cross-linked to TF, giving rise to several distinct adducts possibly caused by the binding of TF at several positions in this longer nascent chain (23). Ffh and SecA were cross-linked but to a lesser extent than with the shorter nascent chains (Fig. 1C, lanes 1–5). UV-irradiated fractions were immunoprecipitated using antiserum against TF, Ffh, SecA, SecY, and the C terminus of YidC (lanes 3–5 and 8–10). The translation products at 30–40 kDa present in lanes 6 and 7 represent the peptidyl-tRNA form of nascent YidC.

Efficient Targeting and Assembly of YidC in Vivo Requires SRP, SecYEG, and SecA—We have shown that nascent YidC interacts with Ffh, TF, SecA, SecY, and YidC. To investigate whether these interactions reflect an in vivo dependence on the SRP targeting pathway or Sec-translocon for correct membrane assembly of the full-length protein, we monitored the requirements for in vivo assembly of YidC in a proteinase K accessibility assay. When YidC is correctly targeted and assembled in the inner membrane, the large periplasmic loop between TM1 and TM2 (Fig. 2A) is proteinase K-resistant except for a small region near TM1 (Fig. 2A) (12). Consequently, proteinase K treatment of spheroplasts derived from wild-type cells results in a small band shift of YidC in SDS-PAGE (Fig. 2B, lanes 1 and 2). OmpA and band X are periplasmic and cytoplasmic control proteins, respectively, used to monitor spheroplast formation (24). In addition, proOmpA processing was monitored to check SecA and SecE depletion.

Depletion of 4.5 S RNA, the RNA component of SRP, affected the assembly of YidC into the inner membrane as is evident from the appearance of full-length YidC in proteinase K-treated spheroplasts (Fig. 2B). Depletion of SecA had a similar effect whereas depletion of SecE had an even stronger effect (Fig. 2, C and D). The SecE depletion strain was used
because it enables the most efficient inactivation of the SecYEG core translocon thus far (15). Upon depletion of SecE, SecY is rapidly degraded by the protease FtsH (25). We also monitored the assembly of YidC in a SecG deletion background (Fig. 2).

The Sec-translocon component SecG also seems to be required for the efficient assembly of YidC. Unfortunately, we could not monitor the assembly of YidC in a YidC depletion background because the YidC background expression levels from the plasmid-borne copy of YidC were sufficient to alleviate chromosomal YidC depletion. Taken together, these data confirm the involvement of the SRP, SecA, and SecYEG in the proper targeting and assembly of YidC into the inner membrane.

**Abundance of YidC**—To determine the abundance of YidC in *E. coli* cells a semiquantitative Western blot procedure was employed. MC4100 was grown to the mid-exponential phase of growth and harvested for life cell counts and analysis by SDS-PAGE and Western blotting. A range of 0.05–0.0025 OD_{660} units of cells was analyzed (Fig. 3). To calculate the amount of YidC from Western blot, the chemiluminescent signal of a standard of purified hisYidC FL was quantified and fitted. A linear relationship between the amount of hisYidC and the chemiluminescent signal was observed throughout this range. The amount of YidC in the cell samples was calculated and correlated to the life cell counts. A MC4100 cell contains ~0.27 fg of YidC corresponding to 2700 copies of YidC per cell.

A duplicate experiment resulted in a similar amount of 2500 copies of YidC per cell (data not shown).

**YidC Is Localized at the Poles of the Cell**—Where the inner membrane assembly of IMPs occurs has not yet been studied. Because YidC appears to be exclusively involved in IMP insertion and assembly (9), we have studied the spatial localization of YidC in the inner membrane using GFP-tagging technology. GFP was fused to the C terminus of YidC, and cloned into the IPTG inducible expression vector pEH1. The YidC depletion strain JS7131, in which *yidC* is under the control of the araBAD operator/promoter, was transformed with pEH1 (empty control vector) and pEH1YidC-GFP.

To test if the fusion construct was functional, JS7131 (pEH1YidC-GFP) and its control strain JS7131 (pEH1) were plated onto LB-agar plates containing 0.2% L-arabinose (to
Biogenesis of YidC

**DISCUSSION**

So far, the biogenesis of *E. coli* IMPs has been studied using only a very limited set of model IMPs (1). Among these model IMPs there are hardly any complex polytopic IMPs. Here we analyze the biogenesis of YidC, a polytopic IMP that has recently attracted attention as a factor that might play a key role in the membrane integration and assembly of *E. coli* IMPs (7, 9). Using a combined *in vitro* and *in vivo* approach we provide evidence that the polytopic IMP YidC follows the SRP/Sec-translocon/YidC pathway for its membrane integration. In addition, we show that YidC is predominantly localized at the cell poles with an abundance of ~2500–3000 copies per cell.

Recently, we have analyzed the pathway of targeting and membrane insertion of the bitopic model IMP FtsQ by studying the sequential interactions of nascent FtsQ in the cytosol and membrane in an *in vitro* photocross-linking assay. This revealed a sequential interaction of the TM, first with SecY and then with YidC (8). The most prominent SecY interaction was found when the TM was only partially exposed outside of the ribosome, whereas the strongest YidC interaction was found when the TM and ~25–30 residues of the periplasmic domain were exposed outside the ribosome. In this study, two YidC constructs were used (52YidC and 96YidC) that resemble these two FtsQ constructs with respect to the distance between the two FtsQ constructs and the periplasmic domain.

In contrast to FtsQ, the cross-linking to YidC appears rather complex. The early interaction of nascent YidC with SecY that is almost absent in the longer construct is comparable with the transient interaction of nascent FtsQ with SecY. Different from FtsQ are the early interactions with pre-existing YidC and SecA that both persist and even increase in the longer construct. This might indicate that YidC inserts close to SecY/SecAVidC in a flexible environment that is shielded from water given the sensitivity of the cross-linking probe to quenching by water.

The mechanism of membrane insertion of polytopic IMPs may not be generic. The sugar co-transporter melibiose per-
mease has been claimed (26) to insert completely independ-
antly from the Sec machinery, whereas mannitol permease
inserts at SecYE but does not require SecA nor SecG (27, 28). It
has been suggested that SecA only plays a role in the translo-
cation of large periplasmic loops that are not present in man-
nitol permease (29). YidC does contain a large periplasmic loop
between TM1 and TM2, which might explain its dependence on
SecA (Ref. 12 and this study). Remarkably, SecA is cross-linked
to TM1 in 52YidC, which does not expose any periplasmic
sequence. It remains to be determined how the future require-
ment for SecA is sensed in such short nascent chains.

Interestingly, the absence of SecG strongly affected YidC
assembly, whereas the deletion of SecG only affects protein
secretion at low temperatures (19), and SecG has not previ-
ously been implicated in membrane protein assembly. The effect
on YidC assembly may be related to the proposed function of
SecG in the modulation of the SecA cycle of membrane
insertion and deinsertion (31). Alternatively, SecG may be spe-
cifically involved in connecting YidC to the core SecYE trans-
locon thus indirectly influencing YidC assembly.

Apparently, YidC inserts via the SRP/Sec-translocon/YidC
pathway. In contrast, the mitochondrial YidC homologue
Oxa1p assembles solely at Oxa1p itself in the absence of SRP
and Sec homologues (32). This may be related to the different
topology of Oxa1p, which lacks the first TM of YidC (4).

In the cytosolic fraction 52YidC is cross-linked to both SRP
and TF. This confirms the very early interaction of TF with nascent
sequences in the SecB/SecA targeting route (33). Our observations
are not consistent with a restricted role for TF in secretion. Apparent-
ly, TF is well able to interact with the TM sequence of a nascent IMP. We propose that TF is juxtaposed to all nascent sequences near the ribosome exit site,
whereas SRP displaces TF when the exposed region is of suffi-
cient hydrophobicity (Ref. 34 and this study).

Surprisingly, the E. coli strain MC4100 contains ~2500–
3000 copies of YidC per cell. The core translocon components
SecY and SecE occur with ~300–600 and 200–400 copies per
cell, respectively (30). Components of the accessory complex
SecDFyajC are even less abundant with ~30–100 copies per
cell (30). Assuming that SecYEG tetramers form functional
translocation units, a cell contains ~100–200 functional trans-
locons. Hence, YidC appears to be in excess, arguing against an
exclusive association with the SecYEG translocon even when
YidC would be dimeric (13). This may be related to the dual
function of YidC in conjunction with the Sec-translocon and as
a separate entity.

YidC is a bona fide IMP spanning the membrane six times
with short cytoplasmic N and C termini (12). Intriguingly,
fusion of the C terminus to GFP did not affect the functioning
of YidC and revealed a predominant localization at the poles of the
E. coli cell. At present it is too early to speculate whether the
cell poles function as dedicated IMP insertion sites perhaps
related to the high membrane curvature in this region. We will
investigate whether other Sec-translocon components colocal-
ize with YidC at the cell poles using the GFP-tagging and
Fluorescence Resonance Energy Transfer technology.

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