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YidC and SecY Mediate Membrane Insertion of a Type I Transmembrane Domain*

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YidC has been identified recently as an evolutionary conserved factor that is involved in the integration of inner membrane proteins (IMPs) in *Escherichia coli*. The discovery of YidC has inspired the reevaluation of membrane protein assembly pathways in *E. coli*. In this study, we have analyzed the role of YidC in membrane integration of a widely used model IMP, leader peptidase (Lep). Site-directed photocross-linking experiments demonstrate that both YidC and SecY contact nascent Lep very early during biogenesis, at only 50-amino acid nascent chain length. At this length the first transmembrane domain (TM), which acquires a type I topology, is not even fully exposed outside the ribosome. The pattern of interactions appears dependent on the position of the cross-linking probe in the nascent chain. Upon elongation, nascent Lep remains close to YidC and comes into contact with lipids as well. Our results suggest a role for YidC in both the reception and lipid partitioning of type I TMs.

Most *Escherichia coli* inner membrane proteins (IMPs)[1] require the signal recognition particle (SRP) and its receptor FtsY for efficient routing to the inner membrane (1). *In vitro* cross-linking and *in vivo* depletion studies suggest that at least a subset of IMPs inserts at the Sec translocon (2). The Sec translocon was originally identified as the protein-conducting pore that receives and translocates secretory proteins. The core translocon consists of the integral IMPs SecY, SecE, and SecG, which constitute an oligomeric complex that is homologous to the Sec61 channel complex in the endoplasmic reticulum (ER) (3). The peripheral membrane ATPase SecA is translocon-associated and functions as the molecular motor that drives the translocation of secretory proteins and of large periplasmic loops of IMPs through the translocon (4, 5).

Recently, the 60-kDa IMP YidC was identified as a novel translocon-associated component that plays an important role in the biogenesis of IMPs. YidC interacts with the TMs of Sec-dependent IMPs during insertion (4–7). Depletion of YidC affects both Sec-dependent and Sec-independent membrane protein insertion (8, 9). Furthermore, YidC has homologues in both chloroplasts (Alb3) and in mitochondria (Oxa1p) that have been implicated in Sec-independent membrane insertion mechanisms (10, 11). Based on these findings, it has been suggested that the role of YidC in membrane protein biogenesis might be both in conjunction with and independent of the Sec translocon.

In the present study we investigated in detail the initial interactions of the IMP Lep during membrane insertion. The first TM of Lep (H1) has a type I topology in the native protein and is thought to insert independent of the Sec translocon (12). We show here that H1 interacts with YidC, SecY, SecE, and SecA very early in the insertion process even when H1 is not fully exposed outside the ribosome. The precise pattern of interactions appeared dependent on the position of the cross-linking probe in the nascent chain, suggesting an ordered insertion into an oligomeric YidC/Sec structure. These interactions were dependent on the context of the ribosome. Upon prolongation of the nascent chains, interactions with YidC persisted, whereas interactions with SecY and SecE were diminished. Together the results suggest a prominent role for YidC during membrane insertion of Lep starting very early in biogenesis.

EXPERIMENTAL PROCEDURES

Reagents and Sera—Restriction enzymes and the Expand Long Template PCR system were obtained from Roche GmbH. T4 DNA ligase was from Epicenter Technologies. MEGAshortscript T7 transcription kit was obtained from Ambion Inc. [^{35}S]Methionine and protein A-Sepharose were obtained from Amersham Biosciences. All other chemicals were supplied by Sigma. Antisera against YidC and Ffh have been described previously (5). Antisera against SecY and SecE were raised in rabbits using the synthetic peptides CSQYESALKKANLKGYGR (SecY) and KGKATVAFAREARTEVRKC (SecE) by Agrisera (Umeå, Sweden). SecA antiserum was a gift from W. Wickner.

Strains and Plasmid Constructs—Strain Top10F′ was used for the maintenance of plasmid constructs. Strain MRE600 was used to prepare translation lysate for suppression of TAG stop codons in the presence of (Tmd)Phe-tRNA^{Sup} (13). Strain MC4100 was used to obtain inner membrane vesicles (IMVs) (prepared essentially as described (14)). Plasmids pC4Meth40–90LepTAG10 were constructed by nested PCR using pC4Meth100LepTAG10 as a template (6). TAG codons were introduced at positions 3, 9–16, and 21 in pC4Meth50Lep using pC4Meth100Lep as a template in a nested PCR procedure, which resulted in pC4Meth50LepTAG3–21. The nucleotide sequences of the mutant genes were confirmed by DNA sequencing.

In Vitro Transcription, Translation, Targeting, and Cross-linking—Truncated mRNA was prepared as described previously (5) from *Hin*dIII linearized Lep derivative plasmids. *In vitro* translation, targeting to IMVs or proteoliposomes, photocross-linking, and carbonate extraction of nascent Lep derivatives were carried out as described pre-

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[1] The abbreviations used are: IMP, inner membrane protein; Lep, leader peptidase; TM, transmembrane domain; SRP, signal recognition particle; (Tmd)Phe, L-[3-(trifluoromethyl)-3-diazirin-3H-yl]phenylalanine; ER, endoplasmic reticulum; IMV, inner membrane vesicle.

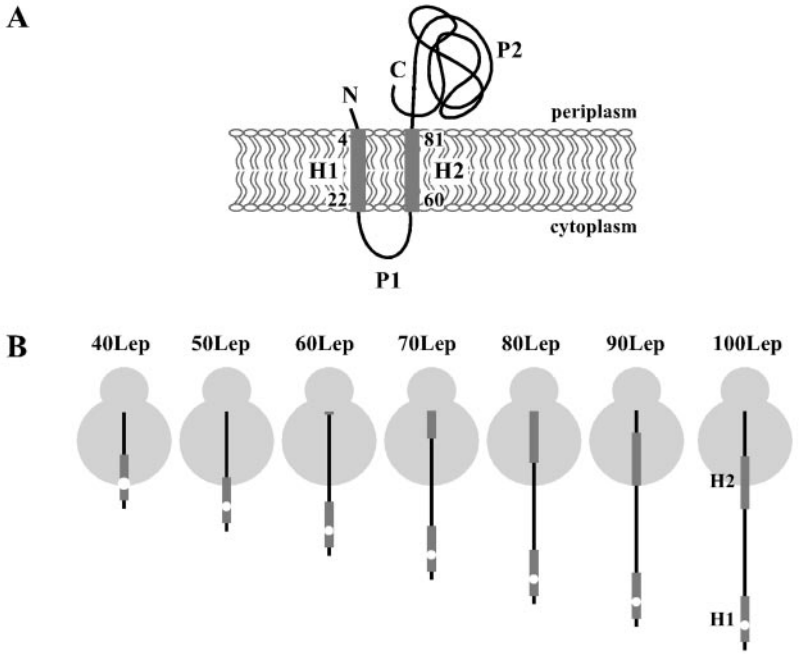

FIG. 1. **Schematic representation of Lep.** *A*, topology of Lep in the inner membrane. *B*, nascent Lep species used in this study. H1 is represented by a *thick line* with a *white dot* at the position of the photocross-linking probe (position 10).

viously (5, 15). Carbonate-insoluble fractions were either analyzed directly by SDS-PAGE and phosphorimaging or first immunoprecipitated as described previously (16) using 2-fold the amount used for direct analysis.

Phospholipase Treatment and Flotation Gradient Analysis—Phospholipase A_2 treatment was carried out essentially as described (5). To monitor membrane association, ribosome nascent chain complexes and IMVs were collected by centrifugation through a 100-μl sucrose cushion (0.5 M sucrose in 50 mM Hepes, pH 7.9, 500 mM KOAc, 5 mM $Mg(OAc)_2$) for 30 min at 90,000 rpm in a Beckman TLA100 rotor at 4 °C. The pellet material was then subjected to flotation centrifugation as described previously (17).

RESULTS

Lep H1 Interacts with YidC from the Initial Insertion Step—Lep, the major signal peptidase in *E. coli*, was used as a model protein to investigate the earliest stages of IMP insertion *in vitro*. Lep spans the membrane twice with a short translocated N-tail and a large translocated C-terminal catalytic domain (P2) (Fig. 1*A*). Lep has been shown to interact with SRP *in vitro* (18) and to depend on SRP for efficient targeting to the inner membrane *in vivo* (19). Furthermore, nascent Lep with a length of 100 amino acids (100Lep), in which H1 is well exposed out of the ribosome, has been shown to insert into the membrane in a carbonate-resistant conformation close to SecY, SecA, and YidC (6).

To investigate the membrane insertion of Lep as a function of chain length, the nature and order of interactions of H1 in the membrane were probed by site-specific photocross-linking using nascent Lep of different lengths. The insertion intermediates were generated by *in vitro* translation of truncated mRNA in a homologous cell-free translation system in the presence of inverted IMVs to allow co-translational targeting. The nascent chains were radiolabeled with [^{35}S]methionine. Through the addition of (Tmd)Phe-tRNA^{Sup} during the translation reaction, the photoreactive Tmd probe is incorporated into the nascent chain during biosynthesis by suppression of a specifically introduced TAG codon.

Lep nascent chains of 40, 50, 60, 70, 80, 90, and 100 amino acids were prepared containing a TAG codon at position 10 in the H1 coding region (Fig. 1*B*) and a C-terminal 4× methionine tag to increase the labeling efficiency. The TAG10 mutations were all efficiently suppressed by (Tmd)Phe-tRNA^{Sup} resulting in nascent Lep of the expected molecular mass (not shown). Following a targeting/insertion reaction using wild-type IMVs, 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 (Fig. 2*A*). The shortest construct, 40Lep, generated no cross-linking products (Fig. 2*A*, *lane 8*). Assuming that the ribosome covers ~35 residues, the cross-linking probe is located in the ribosomal tunnel and quenched in this aqueous environment. In contrast, 50Lep, in which H1 is almost fully exposed, gave rise to one major cross-linking product of ~68 kDa (Fig. 2*A*, *lane 9*). Immunoprecipitation identified YidC as the cross-linking partner (Fig. 2*B*, *lane 2*). Apparently, H1 is able to contact YidC even when it is not fully exposed outside the ribosome. 60- and 70Lep also showed a YidC cross-linking adduct of which the molecular weight increased with increasing nascent chain length (Fig. 2*A*, *lanes 10* and *11*). 80-, 90-, and 100Lep revealed slightly faster migrating cross-linking products (Fig. 2*A*, *lanes 12–14*), which could also be immunoprecipitated using YidC antiserum (Fig. 2*B*, *lane 4* and data not shown). These products might contain a different conformation of YidC or might represent cross-linking of nascent Lep to different parts of YidC. In addition, a weak ~100-kDa cross-linking adduct was detected, most prominent with 60Lep and 70Lep (Fig. 2*A*, *lanes 10* and *11*). This adduct could not be immunoprecipitated using antibodies against SecA (102 kDa) and remains to be identified.

In contrast to the longer constructs, 40Lep and 50Lep showed the same low carbonate resistance (Fig. 2*C*). 50Lep, however, was still found to be associated with inner membranes upon flotation under high salt buffer conditions, whereas flotation of 40Lep was not observed under these conditions (data not shown). These findings indicate that at the length of 50 amino acids, Lep starts to specifically contact the inner membrane integration site.

To investigate the possibility of interactions of nascent Lep with lipids, cross-linked samples were also analyzed on 15% SDS-PAGE for a better resolution in the lower molecular weight area (Fig. 2*D*). Indeed, cross-linking adducts were identified that migrated slightly slower than the non-cross-linked nascent chains of 70 amino acids and longer. The adducts were not detected after phospholipase treatment of the cross-linked samples, confirming that they represent cross-linking to lipids (shown for 70Lep in Fig. 2*D*, *lanes 15* and *16*).

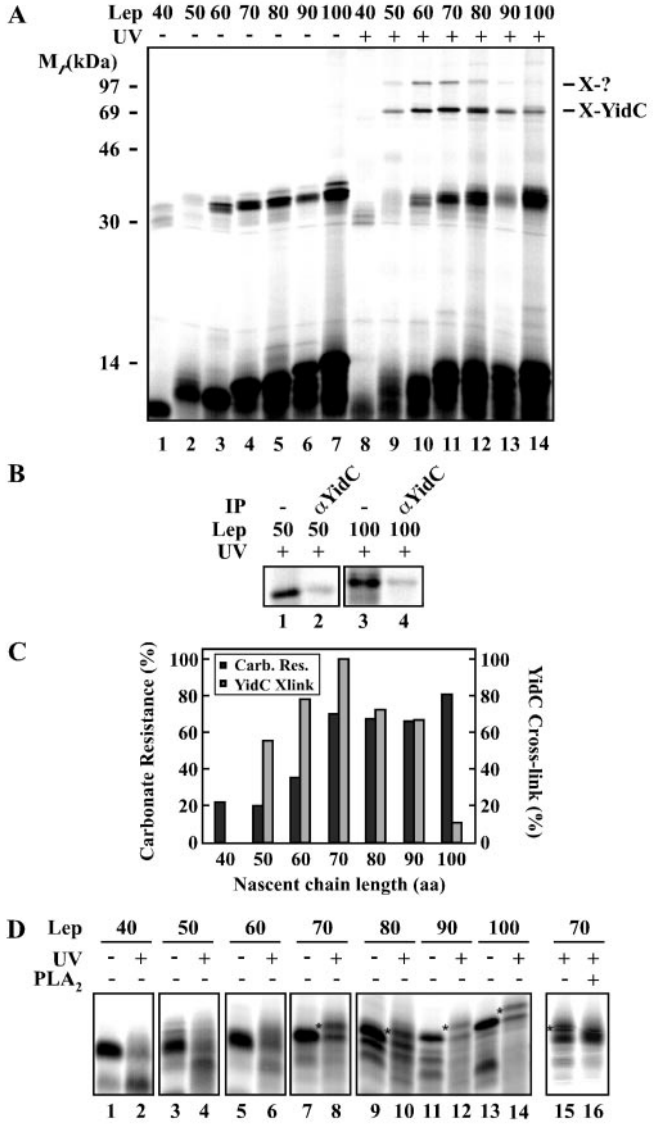

FIG. 2. **H1 interacts with YidC during the initial insertion step.** *A*, *in vitro* translation of nascent Lep 40–100mer (all with a TAG codon at position 10) was carried out in the presence of IMVs and (Tmd)PhetRNA^{Sup}. After translation, samples were irradiated with UV light to induce cross-linking or kept in the dark and extracted with carbonate. *B*, UV-irradiated pellet fractions were immunoprecipitated (*IP*) using antiserum against YidC. Immunoprecipitated YidC cross-links obtained with 50LepTAG10 and 100LepTAG10 are shown. *C*, quantifications of nascent chains present in carbonate pellets of non-irradiated samples after carbonate extraction (*panel A*, *lanes 1–7*) that were calculated as a percentage of the total amount of suppressed nascent chains (not shown), and quantifications of YidC cross-linking adducts (*panel A*, *lanes 8–14*) relative to the amount of non-irradiated carbonate-resistant nascent chains (*panel A*, *lanes 1–7*). The highest value for cross-linking efficiency was taken as 100%. *D*, Lep nascent chains were produced and cross-linked as described under *A* and analyzed by 15% SDS-PAGE. To identify lipid cross-linking adducts (indicated by *asterisks*), membranes of photocross-linked 70LepTAG10 samples were not carbonate-extracted but spun through a high salt sucrose cushion and incubated with bee venom phospholipase A_2 (*PLA_2*) or mock-treated with incubation buffer (*lane 15*).

Together the data suggest that nascent Lep inserts into the inner membrane very early during biosynthesis close to YidC, even before H1 is fully exposed and the nascent chains have acquired significant carbonate resistance. H1 remains in contact with YidC upon elongation of the nascent chains and acquires contact with lipids concomitant with an increased carbonate resistance.

Lep H1 Also Interacts with SecY during the Initial Insertion Step—Nascent Lep contacts YidC from amino acid position 10 in H1 when it has reached a length of 50 amino acids (see "Lep H1 Interacts with YidC from the Initial Insertion Step"). To investigate the molecular interactions of the entire H1 at this early insertion stage, a scanning photocross-linking experiment was carried out. TAG codons were introduced at positions 3, 9–16, and 21 in 50Lep (Fig. 3*A*). 50Lep nascent chains that carry Tmd(Phe)s at the indicated positions were prepared in the presence of IMVs (Fig. 3*B*). Upon UV irradiation, characteristic and reproducible cross-linking patterns were observed (Fig. 3*B*, *lanes 11–20*). Not only the ~68-kDa YidC cross-linking adduct was detected but also a ~45-kDa cross-linking product appeared. This cross-linking product could be immunoprecipitated using SecY antiserum (Fig. 3*C*, *lanes 3* and *9*, and data not shown). The SecY cross-links were most abundant with the photocross-linking probe at positions 12–16 (for quantifications, see Fig. 3*D*), whereas YidC was found to cross-link only weakly to this region. These data suggest that insertion of Lep H1 takes place at an SecY-YidC interface.

Furthermore on UV irradiation of 50LepTAG14 and 50LepTAG15, a prominent band appeared that migrated slightly faster than the non-irradiated nascent chains (Fig. 3*B*, *lanes 7* and *8 versus 17* and *18*; indicated by an *arrow*). Possibly, nascent 50Lep bends as a hairpin near these positions giving rise to internal cross-links. This might cause a more compact structure that withstands complete unfolding in SDS resulting in faster migration in SDS-PAGE (20).

Surprisingly, at amino acid position 21, only 29 amino acids from the peptidyltransferase center SecY were still cross-linked (Fig. 3*B*, *lane 20*). This indicates a close contact between the ribosome and the Sec translocon.

In contrast to the Tmd(Phe)s at positions 12–16 and 21, the cross-linking probes at positions 10 and 11 cross-linked more efficiently to YidC, whereas SecY cross-linked significantly at position 9 (Fig. 3*B*, *lanes 12–14*; quantified in Fig. 3*D*). The periodic pattern of SecY and YidC cross-linking at positions 9–12 might indicate that this region of H1 is structured and situated in between SecY and YidC.

YidC was most efficiently cross-linked at position 3, which is located just upstream from H1 (Fig. 3*B*, *lane 11*). Interestingly, weak cross-linking to SecA was also observed at this position as identified by immunoprecipitation (Fig. 3*B*, *lane 11* and Fig. 3*C*, *lanes 2* and *6*).

A weak ~25-kDa cross-linking product was detected at several positions, which could be immunoprecipitated using SecY antiserum (Fig. 3*C*, *lanes 3* and *9*, indicated by *X-SecY**, and data not shown). This product most probably represents a breakdown product of SecY as observed previously (5, 7). Using 50LepTAG9, -12, and -15, a small portion of the ~25-kDa product could also be immunoprecipitated using antiserum raised against SecE roughly consistent with the combined molecular mass of 50Lep (~6 kDa) and SecE (~14 kDa) (Fig. 3*C*, *lane 10* and data not shown). However, no SecE cross-links could be identified with the cross-linker at position 3 (Fig. 3*C*, *lane 4*).

In summary, the scanning photocross-linking of 50Lep suggests that H1 inserts into the membrane close to not only YidC but also to SecY and to a lesser extent, SecE and SecA. Contacts with these translocon components occur from different positions in H1. The N-terminal region appears close to both YidC and SecY, whereas the C-terminal region is more exclusively in contact with SecY.

Interactions of Lep H1 with YidC and SecY Require the Context of the Ribosome—Membrane-targeted 50Lep was found close to SecY and YidC, possibly in a hairpin conformation as

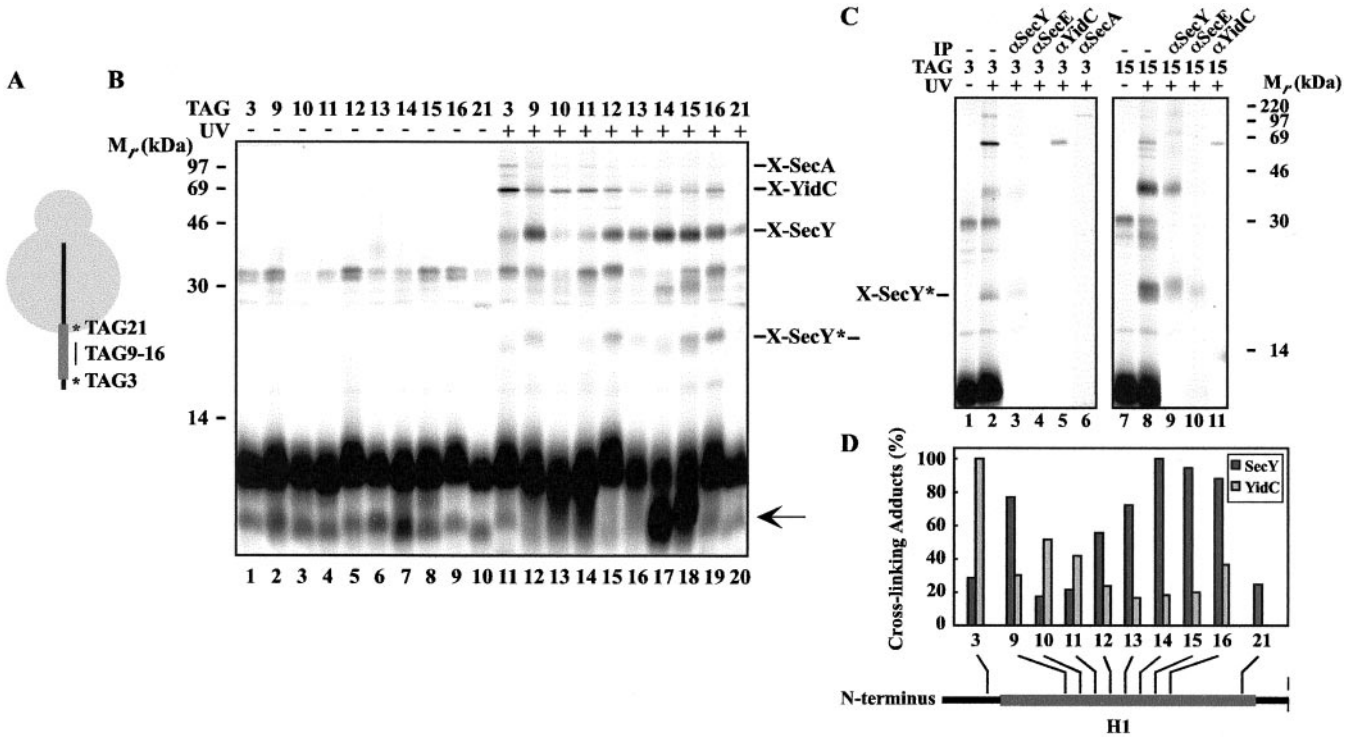

FIG. 3. **Scanning photocross-linking of membrane-inserted nascent 50Lep.** *A*, schematic representation of the position of the (Tmd)Phe in nascent 50Lep. *B*, 50LepTAG mutants were synthesized in the presence of IMVs and (Tmd)Phe-tRNA^{Sup}, UV-irradiated or kept in the dark, and carbonate-extracted. *C*, UV-irradiated membrane fractions of 50LepTAG3 and 50LepTAG15 were immunoprecipitated as indicated. *D*, quantifications of cross-linking adducts (*panel B*, *lanes 11–20*) relative to the amount of non-irradiated carbonate-resistant nascent chains (*panel B*, *lanes 1–10*). Highest values for cross-linking efficiency were taken as 100%.

described previously. Next, we investigated whether the context of the ribosome is required for these interactions using 50LepTAG10 (strong YidC cross-linking) and 50LepTAG14 (strong SecY cross-linking). After the translation/insertion reaction in the presence of IMVs, one fourth of each sample was treated with either puromycin (with or without high salt) or EDTA. These treatments provoke the release of the nascent chains from the ribosome in different ways. Puromycin breaks the tRNA bond, whereas EDTA disassembles ribosomes. High salt treatment extracts "empty" ribosomes from the membrane (Fig. 4). Upon UV irradiation, 50LepTAG10 and 50LepTAG14 were found to be cross-linked strongly to YidC (Fig. 4*A*, *lanes 5* and *9*) and to SecY (Fig. 4*B*, *lane 5*), respectively, whereas to the latter construct YidC cross-linking was inefficient (Fig. 4*B*, *lane 5*). After the puromycin treatment the YidC and SecY cross-links were severely reduced, and unexpectedly, another cross-linking product of ~62 kDa appeared (Fig. 4, *A* and *B*, *lane 6*). Treatment with puromycin in combination with high salt or treatment with EDTA resulted in an almost complete loss of YidC and SecY cross-linking. The ~62-kDa cross-linking product appeared even more intense under these conditions. This product could not be immunoprecipitated using YidC antiserum (Fig. 4*A*, *lane 10*) and did not disappear when using IMVs from a YidC-depleted strain (data not shown). These findings suggest the transfer of the nascent chains (~6 kDa) upon release from the ribosome from YidC and SecY to an ~56-kDa component.

The carbonate resistance of the non-irradiated nascent chains increased drastically upon all treatments (Fig. 4, *C* and *D*). Furthermore, the same percentage of nascent chains was found to be associated with membranes following puromycin treatment and flotation centrifugation under high salt buffer conditions (data not shown). This suggests that the released nascent chains do not aggregate but integrate more efficiently into the lipid bilayer.

Together, these results indicate that the interactions of nascent Lep with SecY and YidC require the context of the ribosome. Upon release from the ribosome, the nascent chains apparently move from the SecYEG/YidC translocon toward a novel, unidentified ~56-kDa protein and probably into the lipid bilayer. Identification of this novel cross-linking partner will be a topic for further studies.

Nascent Lep Inserts into Proteoliposomes That Contain Either YidC or SecYEG—Previously, the functional reconstitution of SecYEG and YidC into proteoliposomes has been reported (15). Using the model IMP FtsQ, it was shown that shorter nascent chains interact with SecY, whereas longer nascent chains interact with YidC, but only when SecYEG was co-reconstituted with YidC. This is consistent with the sequential interaction of nascent FtsQ in IMVs, first with SecY and then with YidC (7). To study which of the membrane components are required for the membrane interactions of Lep, which has a different membrane topology, we investigated the interactions of nascent Lep species in proteoliposomes.

50LepTAG10 (strong YidC cross-linking in IMVs), 100LepTAG10 (strong YidC cross-linking), and 50LepTAG15 (strong SecY cross-linking) were translated in the presence of proteoliposomes containing either SecYEG, YidC, or a combination of SecYEG and YidC (Fig. 5). The samples were UV-irradiated, carbonate-extracted, and analyzed. The cross-linking patterns in IMVs serve as a control (Fig. 5, *lanes 1*, *8*, and *15*). In all samples a cross-linking adduct was observed that could be immunoprecipitated using antiserum against Ffh, the protein component of the SRP (Fig. 5, *lanes 6* and *13*). This product resulted from the addition of extra SRP to improve the targeting efficiency.

50LepTAG10 yielded two cross-linking adducts of ~68 kDa in SecYEG/YidC proteoliposomes that were both immunoprecipitated with YidC antiserum, albeit with different efficiencies (Fig. 5, *lanes 4* and *7*). The difference in mobility of the YidC

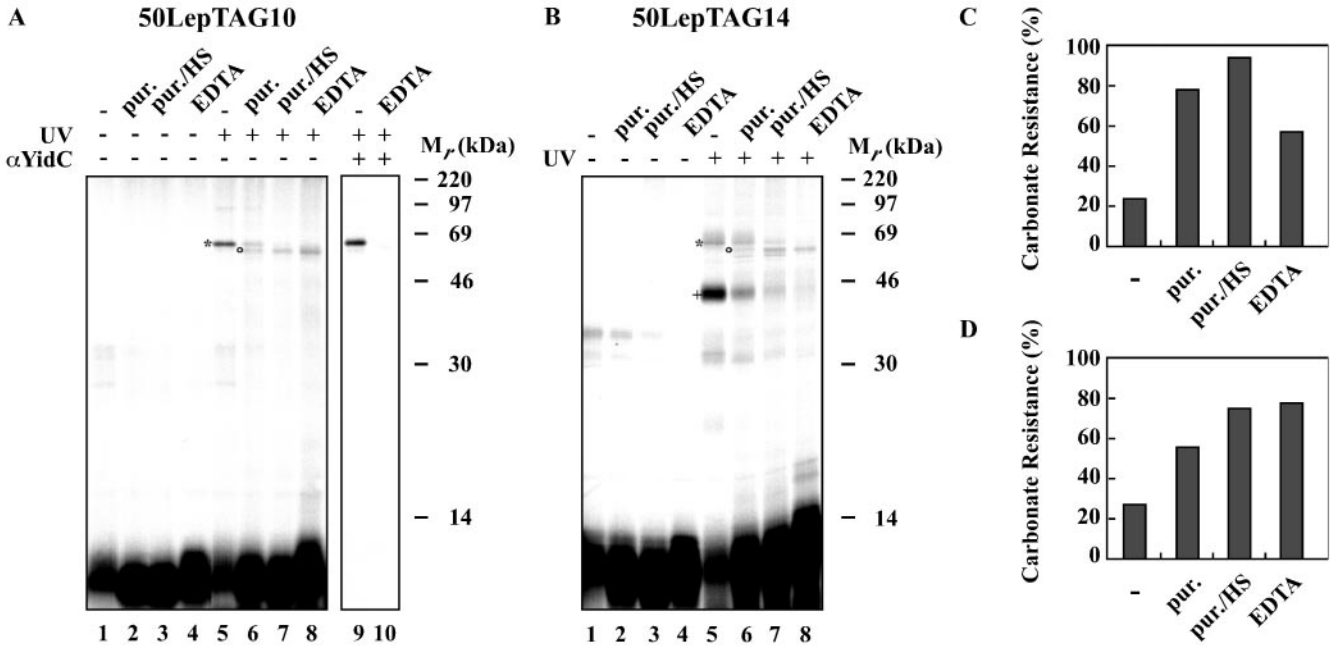

FIG. 4. **The interactions of Lep with SecY/YidC require the context of the ribosome.** 50LepTAG10 (*A*) and 50LepTAG14 (*B*) were synthesized in the presence of IMVs and (Tmd)Phe-tRNA^{Sup}. The samples were split into equal aliquots and treated with 2 mM puromycin (*pur.*), 2 mM puromycin and 0.5 M KOAc (*pur./HS*), or 25 mM EDTA (*EDTA*) or mock-treated with incubation buffer (−). After 10 min of incubation at 37 °C, half of each sample was UV-irradiated, while the other half was kept in the dark. All samples were carbonate-extracted, and the carbonate-resistant material is shown. UV-irradiated pellet fractions of 50LepTAG10, mock-treated or treated with EDTA, were immunoprecipitated using antiserum against YidC (*A*, *lanes 9* and *10*). YidC and SecY adducts and an unidentified ~62-kDa cross-linking adduct are indicated by *asterisk*, *plus*, and *open circle*, respectively. Non-irradiated carbonate-resistant 50LepTAG10 (*C*) and 50LepTAG14 (*D*) nascent chains (*A* and *B*, *lanes 1–4*) were quantified as a percentage of the total amount of suppressed nascent chains (not shown).

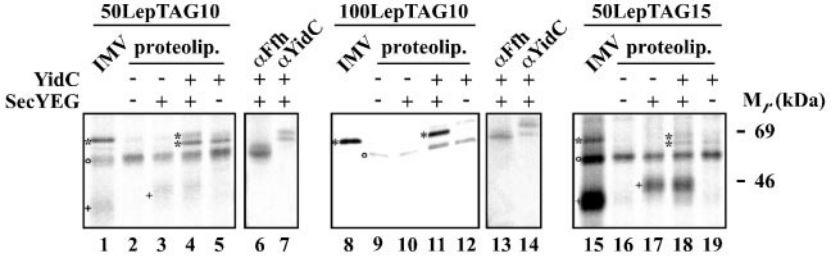

FIG. 5. **Reconstituted YidC and SecY can interact independently with 50Lep.** 50LepTAG10, 100LepTAG10, and 50LepTAG15 were synthesized in the presence of (Tmd)Phe-tRNA^{Sup} and IMVs or proteoliposomes. Reconstituted SRP and purified FtsY were added to the translation mixture to a final concentration of 0.26 and 0.5 μM, respectively. Samples were UV-irradiated or kept in the dark and carbonate-extracted. UV-irradiated pellet fractions of 50LepTAG10 and 100LepTAG10, targeted to the SecYEG/YidC proteoliposomes, were immunoprecipitated as indicated. YidC, SecY, and Ffh cross-linking adducts are indicated by *asterisk*, *plus*, and *open circle*, respectively.

adducts, as compared with the adduct in IMVs (Fig. 5, *lane 1 versus lane 4*), is possibly related to the His-tag that is present on YidC reconstituted in proteoliposomes. SecYEG was dispensable for these interactions, because cross-linking to YidC was also observed in the absence of SecYEG (Fig. 5, *lane 5*).

Using 100LepTAG100, a similar cross-linking pattern was observed in SecYEG/YidC proteoliposomes as was observed in IMVs showing predominant cross-linking to YidC (Fig. 5, *lanes 8*, *11*, and *14*). However, in contrast to 50LepTAG10, 100LepTAG10 showed almost no cross-linking to YidC in the absence of SecYEG (Fig. 5, *lane 12*), suggesting that SecYEG is necessary to bring or keep 100Lep in contact with YidC.

50LepTAG15 showed SecY cross-linking in SecYEG and SecYEG/YidC proteoliposomes, as in IMVs (Fig. 5, *lanes 15–17*), indicating that 50Lep interacts with SecY independent of the presence of YidC. The difference in mobility of the SecY adducts is most probably caused by the His-tag that is present on SecY in the proteoliposomes (15). With SecYEG/YidC proteoliposomes, 50LepTAG15 also cross-linked weakly to YidC in SecYEG/YidC proteoliposomes just as in IMVs (Fig. 5, *lanes 15* and *18*). Again, the cross-linking to YidC in proteoliposomes appeared independent of the presence of SecYEG (Fig. 5*A*, *lane 19*).

Taken together, in proteoliposomes, 50Lep appeared to be able to contact SecYEG independent of YidC and vice versa. In contrast, 100Lep did require SecYEG to contact or to remain in contact with YidC. Furthermore, the interactions detected in SecYEG/YidC proteoliposomes are consistent with those in IMVs.

DISCUSSION

In this study we have used Lep as a model IMP to investigate the early stages in membrane insertion. The protein has been used extensively as a model for *in vivo* inner membrane insertion (12, 19, 21, 22). Whereas translocation of the large P2 loop requires SecY, SecE, and SecA (12, 22, 23), translocation of the small N-terminal tail (fused to a reporter domain) appeared to be Sec-independent in a previous study (12). The data presented here suggest that discrete stages can be distinguished during the membrane integration process of the first TM (H1) in which YidC and SecY play a role (Fig. 6).

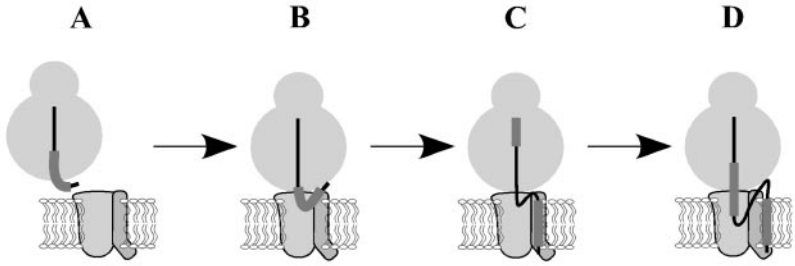

FIG. 6. **Model for the membrane insertion of H1 of Lep.** The model is described under "Discussion."

At the nascent chain length of 40 amino acids, most of H1 is covered by the ribosome, and the nascent chain is not targeted to the translocon or any other membrane components (Fig. 6*A*). In nascent chains of 50 amino acids, most of the hydrophobic core of H1 is exposed, and the nascent chain contacts the translocon (Fig. 6*B*). At this stage, SecY, SecE, and YidC were found close to H1 in a site-specific scanning photocross-linking approach. H1 can be divided into two regions according to the cross-linking patterns. The C-terminal region cross-linked predominantly to SecY and SecE. Even at position 21, which is 29 residues from the peptidyltransferase center, interaction with SecY was detected, suggesting an intimate contact of the translating ribosome with the membrane consistent with the observed affinity of the ribosome for the SecYEG complex (24). Furthermore, intramolecular cross-linking suggested that H1 might bend in this region as a hairpin. In contrast, residues in the N-terminal region of H1 were found to be close to either YidC or SecY suggesting that this region is structured.

These results illustrate that a close connection between SecYEG and YidC exists, at least during membrane insertion. The data indicate a very early role for YidC in the recognition of H1 and perhaps in the interaction with the targeted ribosome. Significantly, 50Lep does not require SecYEG to contact YidC in proteoliposomes, and YidC is dispensable for the interaction with SecY. This suggests that partial autonomous contacts are possible at this stage.

When short membrane-associated nascent Lep is prematurely released from the ribosome and especially when the ribosome is in addition removed from the membrane, the contact with YidC and SecY is lost. The released nascent chains further integrate into the membrane and are handed over to an ~56-kDa membrane protein. We have not yet been able to identify this component, but we suspect that it is involved in the quality control of membrane proteins.

At the length of 70 amino acids, when H1 is fully exposed, the nascent chains are more efficiently integrated and start contacting lipids while still being close to YidC (Fig. 6*C*). This situation remains at least until the nascent chains have reached a length of 100 amino acids (Fig. 6*D*). Then, H1 is close to YidC over its entire length. To reach this stage, SecYEG appears to be essential (concluded from the proteoliposome data) and is still associated with the hydrophilic region that follows H1 (6). Apparently, H1 inserts in a SecYEG/YidC translocon, moves toward a YidC/lipid interface upon elongation, and remains there perhaps until the second TM (H2) is integrated, translation stops, and Lep acquires its final lipid-embedded conformation. This type of model is in agreement with the interactions of the TMs of the polytopic IMP MtlA with YidC at different nascent chain lengths (25). It remains to be determined at which stage H1 reaches its final orientation in the membrane and whether YidC is required for this process.

Recently, a similar study was conducted using FtsQ, which has only one TM with the opposite orientation as compared with Lep H1. With FtsQ a sequential interaction of the TM, first with SecY then with YidC, was observed (7, 15). In addition, it seems that the FtsQ TM contacts lipids earlier during insertion than the H1 of Lep. It should be mentioned that these interactions were probed from only one position in the TM. The current study clearly illustrates that at this stage interactions may be very dependent on the position of the cross-linking agent in the TM. The features of a TM that determine its interactions with and requirements for SecYEG and YidC will be the focus of future research.

The early contact of H1 with YidC might explain why translocation of the N terminus of Lep occurs independent of SecY *in vivo* (12). Possibly, YidC is able to receive H1 and assists in the translocation of the N terminus on its own, whereas SecYEG is more important for the insertion of H2. Notably, YidC appears sufficient for the insertion and translocation of Sec-independent IMPs (8, 9, 26). However, YidC appears to have only a minor effect on the insertion of Lep H1 *in vivo* (8), indicating that Lep can use both YidC and SecYEG for proper insertion and positioning of H1 in the membrane.

Interestingly, the insertion of a hybrid Lep construct into the ER has also been investigated (20). As with *E. coli* membranes, H1 associated co-translationally with the ER and contacted Sec61α (homologous to SecY) when it was of similar length. H1 became lipid-embedded very soon after initial membrane insertion and lost its contact with Sec61α. In this context no extra factor appeared to be required to mediate the lipid partitioning of the H1 that rapidly escaped the translocon. Only when H1 was mutated to decrease its hydrophobicity did it contact the translocating chain-associating membrane (TRAM) that was proposed to keep the mutated H1 close to the Sec61 translocon and facilitate its lipid partitioning. It still seems possible that TRAM and YidC fulfill a similar function, but the timing of their action and their substrate specificity may differ, perhaps related to the different lipid composition of the ER and *E. coli* membrane.

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