YidC is a newly defined translocase component that mediates the insertion of proteins into the membrane bilayer. How YidC functions in the insertion process is not known. In this study, we report that the Sec-independent Pf3 coat protein requires the YidC protein specifically for the membrane translocation step. Using photocrosslinking techniques and ribosome-bound Pf3 coat derivatives with an extended carboxyl-terminal region, we found that the transmembrane region of the Pf3 coat protein physically interacts with YidC and the bacterial signal recognition particle Ffh component. We also find that in the insertion pathway, Pf3 coat interacts strongly with YidC only after its transmembrane segment is fully exposed outside the ribosome tunnel. Interaction between Pf3 coat and YidC occurs even in the absence of the proton motive force and with a Pf3 coat mutant that is defective in transmembrane insertion. Our study demonstrates that YidC can directly interact with a Sec-independent membrane protein, and the role of YidC is at the stage of folding the Pf3 protein into a transmembrane configuration.

Most bacterial membrane proteins insert into the membrane utilizing the Sec translocase. The Sec translocase is composed of the integral membrane core subunits SecY and SecE, which function as protein transporters, and the peripheral subunit SecA, an ATPase that functions as a molecular motor to push the protein chain through the SecYE translocase by hydrolyzing ATP (1–3). The other components of the Sec translocase, such as SecG, SecD, SecF, and YajC, are not absolutely required for protein translocation. Prior to the membrane translocation step, the proteins need to be targeted to the membrane. In bacteria, this is achieved by two major pathways, one utilizing SecA, the Sec translocase component, designated Ffh, and a 4.5S RNA component. SRP is thought to bind to FtsY in its targeting cycle. After targeting by SRP, SecA can also be required for translocation of the periplasmic domains of some membrane proteins (8).

YidC, a homologue of the mitochondrial Oxa1 (9) and chloroplast Alb3 (10), is a newly identified translocase component in bacteria (11, 12). YidC is absolutely essential for the membrane insertion of the Sec-independent M13 coat protein (13, 14). It also stimulates the membrane translocation of the carboxyl-terminal domain of the Sec-dependent proteins, leader peptidase (Lep) and FtsQ (13, 15, 16), as well as promoting the insertion of the amino-terminal domain of Pf3-tagged leader peptidase (Pf3-Lep). For Sec-dependent membrane proteins, YidC has been shown to cooperate with Sec translocase and to play a possible role in the lateral movement of transmembrane segments out of the Sec translocase complex (15–19). Therefore, a current model is that YidC functions in association with the Sec translocase or works independently to insert proteins into membranes (18, 20).

Whether YidC works with or without the Sec translocase depends on the membrane protein being inserted.

Pf3 coat, a 44-amino acid membrane protein, is the major coat protein from *Pseudomonas aeruginosa* phage Pf3. When Pf3 coat is expressed in *Escherichia coli*, it inserts into the inner membrane with a single transmembrane segment adopting an N_out/C_in topology. Pf3 coat has been used as a model protein to study Sec-independent membrane insertion (21). It was widely believed that Pf3 coat inserts directly into the bilayer of the bacterial inner membrane without the assistance of proteinaceous factors (22, 23). However, because recent studies show that the Sec-independent M13 procoat protein requires YidC for membrane insertion (13, 14), Pf3 coat protein might first contact YidC and require its function for translocation across the membrane.

In this study, we show that in a strain in which YidC is depleted, the membrane insertion of wild-type Pf3 coat is severely affected. Using a photocrosslinking method and Pf3 coat mutants in which the carboxyl-terminal regions are extended, we found that YidC, but not SecE, interacts with Pf3 coat protein after its transmembrane segment emerges from the ribosome. We have also shown that YidC can interact with Pf3 coat when it is partitioned into but not translocated across the membrane, suggesting that YidC plays a specific role for membrane translocation.
RESULTS

YidC Is Required for Wild-type Pf3 Coat Membrane Insertion—We have demonstrated that M13 Procoat, a Sec-independent protein, requires YidC for membrane insertion (13). Therefore, we were interested in whether the single membrane-spanning Pf3 coat may also require YidC for membrane insertion. Previously, Pf3 coat was shown to insert into the E. coli inner membrane without the aid of the Sec translocase (31). E. coli JS7131 cells (13) were grown in the presence of arabinose or glucose (YidC+ or glucose (YidC−)), pulse-labeled for 20 s, and chased for the indicated times. The cells were then converted to spheroplasts and treated with or without proteinase K on ice for 1 h. After the reaction was quenched with phenylmethylsulfonyl fluoride, the samples were trichloroacetic acid-precipitated, acetone-washed, and analyzed by SDS-PAGE and phosphorimaging.

YidC physically interacts with Pf3 coat. Site-specific photocrosslinking (28) was performed to monitor the membrane insertion of Pf3 coat. Because the methionine at position 1 is the only methionine in the Pf3 coat protein, only Pf3 coat with an intact amino terminus can be detected by autoradiography (see Fig. 1a). When YidC was present (Fig. 1b, YidC+), Pf3 coat was inserted normally across the membrane with an NoutCin topology and was digested to a nonradiolabeled fragment by proteinase K added to the periplasmic side. In YidC-deficient cells (Fig. 1b, YidC−), Pf3 coat was protected by the membrane from proteinase K digestion, indicating that it does not insert across the membrane even after a 2-min chase period. This demonstrates that Pf3 coat protein requires YidC for transmembrane insertion.

YidC physically interacts with the transmembrane region of Pf3 coat. Because Pf3 coat requires YidC for membrane insertion in vivo, we analyzed whether YidC physically interacts with Pf3 coat during insertion and whether other proteins interact with Pf3 coat. Site-specific photocrosslinking (28) was applied to study Pf3 coat membrane biogenesis in vitro. With this technique, a truncated mRNA is generated that lacks the
were analyzed as described for acting with puromycin/high salt treatment. The 91-amino acid nascent Pf3 coat (Tmd)Phe (L-4
ically modified with the photoactivatable cross-linking group
indicating that Pf3-P2 is also YidC-dependent.

FIG. 2. Photocrosslinking shows YidC and Ffh interact with Pf3 coat during membrane insertion. a, schematic illustration of Pf3 coat showing the position where the photoprobe (Tmd)Phe was introduced. b, YidC and Ffh photocrosslinked to the Pf3 coat transmembrane segment. The 91-amino acid nascent chains of Pf3-P2 coat with the photo-
probe at the indicated positions were photocrosslinked (UV+) or not (UV−) (Totals). The sample with the photoprobe at position 34 was immunoprecipitated with antiserum to YidC (YidC IP) and Ffh (Ffh IP). The position of YidC-Pf3 coat adduct on the gel is indicated by *, and the Ffh-Pf3 coat adduct indicated by an o. The positions of the molecular weight standards (MultiMark™ rainbow Marker, from Invitrogen) are marked on the left. c, photocrosslinking of Pf3 coat to YidC is prevented by puromycin/high salt treatment. The 91-amino acid nascent Pf3 coat with the photoprobe at position Leu-34 was used. Samples were analyzed as described for panel b, except prior to photocrosslinking 1 mM puromycin and 0.4 M potassium acetate were added to release the nascent chains from the ribosomes (Puro +). Puro− corresponds to samples not treated with puromycin and high salt.

termination codon and therefore the nascent protein chains are
not released from the ribosome. Ribosome-nascent chains
trapped in the translocation process can then be subjected
to cross-linking to identify interacting proteins. The length
of wild-type Pf3 coat (44 amino acids) is too short for the photo-
crosslinking technique, because 35–40 amino acids are esti-
mates to reside within the ribosomal tunnel upon arrest of
translation. To make Pf3 coat suitable for photocrosslinking,
we have lengthened the protein with a sequence of the leader
peptidase (Lep) soluble P2 domain (the hybrid is called Pf3-P2,
Fig. 1a). The hybrid protein shows the same YidC dependence
as Pf3 coat (Fig. 1c). Pf3-P2 was expressed in the JS7131 strain
and subjected to protease K mapping. In the cells grown with
arabinose to express YidC (Fig. 1c, YidC−), Pf3-P2 was digested
to a smaller fragment, whereas in the cells grown with glucose
to deplete YidC (Fig. 1c, YidC−), Pf3-P2 was fully protected,
indicating that Pf3-P2 is also YidC-dependent.

Ribosome-attached Pf3-P2 nascent proteins were site-spec-
ifically modified with the photactivatable cross-linking group
(Tmd)Phe—(L-4′-3-(trifluoromethyl)-3H-diazirin-3-y1)phenyl-
alanine). Amber suppressor tRNA charged with (Tmd)Phe
enabled incorporation of the photoprobe into the Pf3 coat trans-
membrane segment at positions Thr-20, Ile-27, and Leu-34
(Fig. 2a). A PCR method was employed to generate the trunc-
cated Pf3-P2 gene fragment for each amber mutant coding for
a 91-amino acid protein. After synthesis, the Pf3 coat portion
of 44 amino acids should be fully exposed from the ribosome,
the interaction between YidC and P3 coat is most intense when the transmembrane segment of P3 coat is fully exposed from the ribosome (80-P3 and 91-P3). This finding indicates that YidC interaction is more efficient when the carboxyl-terminal region of the P3 coat protein is exposed from ribosomal tunnel.

We also checked whether YidC in the photocrosslinking described above was cross-linked to SecY. The photocrosslinking products of the different P3 coat truncated proteins (56-P3, 64-P3, 72-P3, 80-P3, and 91-P3) were immunoprecipitated with antiserum to SecY (Fig. 3c, left panel, IP, SY). No SecY-P3 coat cross-linking products were detected among the series of the nascent proteins, which represent different stages of P3 coat membrane insertion (Fig. 3c, left panel, IP, SY). As a positive control, we showed that the antiserum to SecY immunoprecipitated 35S-labeled SecY, which was synthesized in vitro in an E. coli S30 translation system (Fig. 3c, right panel). These data are consistent with P3 coat protein not contacting SecY. The fact that YidC mediates membrane insertion of P3 coat, a Sec-independent protein, implies that YidC can work independently of the Sec translocase.

YidC Interacts with Membrane-partitioned P3 Coat to Promote Its Transmembrane Configuration—P3 coat membrane insertion can be understood as a process of three steps; i.e., the first step is targeting of P3 coat protein to the membrane, the second step is partitioning of the P3 coat hydrophobic domain into the membrane lipid bilayer, and the final step is the formation of a transmembrane helix with concomitant membrane translocation of the amino-terminal region (23). In the third step, the proton motive force (pmf) is required for the electrophoretic transfer of the amino-terminal tail, which contains two negatively charged amino acid residues (33). Our data obtained by photocrosslinking suggests that YidC functions at the stage of membrane insertion, which might be the membrane partitioning step (the second step) or the orientation step (the third step), to form the transmembrane form of the protein. Therefore, we dissected the membrane insertion of P3 coat to determine at which stage YidC functions.

First, we investigated whether YidC is important for the partitioning of the hydrophobic domain of P3 coat into the membrane. We used sodium carbonate extraction, which distinguishes peripherally bound proteins from integral membrane proteins. P3 coat was pulse-labeled for 20 s in YidC-induced JS7131 cells (treated with arabinose) or YidC-depleted JS7131 cells (treated with glucose). After converting the cells into spheroplasts, the cells were extracted with sodium carbonate (pH 11.5) and then subjected to ultracentrifugation to separate the membrane fraction (pellet) and cytosolic fraction (supernatant). As a control, we confirmed by using proteinase K mapping that the insertion of P3 coat was essentially 100% blocked when YidC was depleted (data not shown). The carbonate extraction study showed that almost all the P3 coat was found in the membrane fraction when YidC was expressed by the addition of arabinose (Fig. 4a, YidC*). In the YidC-depleted cells, only ~40% of the P3 coat is in the membrane fractions, and ~60% of P3 coat was extracted into the supernatant (Fig. 4a, YidC−). This indicates that without YidC, more than half of the P3 coat cannot stably partition into the membrane and is extracted. The 40% of P3 coat detected in the membrane was still protected from proteinase K digestion, indicating that this portion of P3 coat is not oriented correctly in the Nout/Cin topology. These data suggest that YidC plays a role to orient P3 coat in the transmembrane configuration. Therefore, when YidC is absent, the hydrophobic region of P3 coat does not span the membrane and can be more easily extracted by sodium carbonate.

The pmf is involved in membrane translocation of the negatively charged residues located within the amino-terminal region of P3 coat protein. What is the relationship between the function of YidC and the pmf? We applied photocrosslinking to investigate whether the interaction between P3 coat and YidC still occurs if the pmf is destroyed. The 91 amino acid-nascent P3 coat protein with amber mutation at L34 was used for photocrosslinking in the presence or absence of 140 μM carboxyl cyanide m-chlorophenylhydrazone (CCCP), a protonophore that dissipates the pmf. As previously shown by Kiefer and Kuhn (23), CCCP can efficiently abolish the pmf under these in vitro conditions. Fig. 4b (left panel) shows that there is no difference in the YidC-P3 coat photocrosslinking products between the non-CCCP-treated and CCCP-treated photocrosslinking reactions. This result indicates that the pmf is not necessary for YidC to interact with P3 coat and that YidC does not function after the pmf-requiring step. Rather, YidC might act synergistically with the pmf to orient P3 coat in the membrane, whereby the pmf acts upon the negatively charged amino-terminal tail and YidC functions by interaction with the
YidC-dependent Membrane Protein Insertion

Fig. 4. YidC mediates Pf3 coat membrane translocation. a, sodium carbonate extraction shows that YidC affects membrane partitioning under alkaline conditions. JST31 cells expressing Pf3 coat were grown in the presence of arabinose (YidC+) or glucose (YidC−) and pulse-labeled for 20 s with [35S]methionine. The samples were split into two aliquots, one for the sodium carbonate extraction study (upper panel) and the other for protease mapping (as a control; data not shown). The supernatant (S) and pellet (P) fractions were prepared as described under “Materials and Methods.” b, left panel, Pf3 coat interacts with YidC in the absence of a pmf. Photocrosslinking was performed in the presence or absence of CCCP treatment with the 91-amino acid Pf3 coat nascent chain containing the photoprobe at position L34 of the 91-residue nascent chain. The in vitro translation and photocrosslinking was performed as described in the legend for Fig. 2b. The YidC-Pf3 coat adducts and the Pf3 coat nascent proteins are indicated with an arrow. c, a schematic depicting the ribosome-bound Pf3 coat proteins interacting with YidC when the membrane potential is abolished by treating samples containing PF3-P2 with CCCP (left panel). The right panel shows the P3–4N mutant interacting with YidC even though the Pf3 coat cannot insert in a transmembrane form with the amino-terminal tail across the membrane. Truncated nascent chains of 91 residues with the photoprobe at the Leu-34 position were synthesized to study possible interactions with YidC. The photocrosslinking products were immunoprecipitated with YidC antibody and analyzed by SDS-PAGE/autoradiography. Fig. 4b (right panel) shows that strikingly P3–4N can interact with YidC with approximately the same efficiency as wild-type Pf3 coat. We believe that this Pf3 coat mutant, like the wild-type Pf3 coat that accumulates when the pmf is abolished, has an Nterm/Cterm topology. In both cases, the proteins are partitioned into the membrane and are able to interact with membrane-bound YidC (see Fig. 4c for proposed intermediates).

We also show that YidC can be cross-linked to another Pf3 coat mutant, Pf3–RD, although its membrane insertion orientation (Cterm/Nterm) is opposite to that of wild-type Pf3 coat (33) (Fig. 4b, right panel). Taken together, the photocrosslinking data demonstrate that the Pf3 coat-YidC interaction occurs independently of the changes flanking the transmembrane segment of Pf3 coat. These findings are also consistent with the idea that the Pf3 coat-YidC interaction takes place prior to or at the same time as the pmf-requiring step.

DISCUSSION

We have presented data showing for the first time that YidC interacts directly with a Sec-independent membrane protein, namely the Pf3 coat protein, and promotes its membrane insertion. Previously, we have shown that YidC promotes the membrane insertion of the Sec-independent M13 procot protein (13). However, we could not rule out the possibility that YidC depletion was causing an indirect effect, thereby inhibiting membrane protein insertion. In this study, we have found, using photocrosslinking, that ribosome-bound Pf3 coat nascent chains are cross-linked to YidC when the photoprobe is located either in the center or toward the amino- or carboxyl-terminal ends of the transmembrane segment (Fig. 2). Moreover, we found that YidC binds to a nontranslocated membrane protein, as it is directly cross-linked to Pf3–4N, which cannot insert across the membrane (Fig. 4).

In our photocrosslinking studies, we extended the carboxyl terminus of Pf3 coat such that we could fully expose the hydrophobic domain (residues 19–36) during synthesis with the protein still attached to the ribosome. This was necessary because Pf3 coat is too short when the ribosome is attached, as around 35–40 amino acids residues are within the ribosome. The Pf3 coat with the extended carboxyl-terminal region, called Pf3–P2, was completely YidC-dependent for membrane insertion (Fig. 1). Efficient cross-linking to YidC was observed only when the carboxyl-terminal region of Pf3 coat had emerged from the ribosome. No cross-linking of Pf3 coat was observed to SecY. These data are consistent with Pf3 coat being inserted by means of a Sec-independent mechanism (31). Interestingly, our cross-linking results show some differences and similarities to those obtained with FtsQ, a Sec-dependent membrane protein, which has been investigated using the same photocrosslinking methodology (15). They are different because the hydrophobic domain of FtsQ first inserts in an environment around SecY and then moves toward YidC. The proposed function of YidC is to integrate the transmembrane regions into the membrane bilayer (19). Our studies with the Sec-independent Pf3 coat are similar to those of FtsQ in that both of these proteins contact YidC efficiently when the transmembrane segments are fully exposed from the ribosomal tunnel. This similarity suggests that YidC may function in a common way for Sec-dependent and Sec-independent proteins. The actual function of YidC might be that of a membrane chaperone to enable the hydrophobic segments of either Sec-dependent or Sec-independent proteins to properly integrate into the lipid bilayer in a transmembrane configuration.

Although the precise function of YidC is not known, it is required for the translocation of the hydrophilic domain of Pf3 coat across the membrane. We found that the Pf3–RD mutant, which inserts also with the inverted topology, interacts with YidC (Fig. 4). Importantly, the Pf3–4N mutant, which does not translocate its hydrophilic domain across the membrane, still

hydrophobic segment. In addition, because YidC is a membrane protein, the cross-linking of Pf3 coat to YidC corroborates that Pf3 coat can partition into the membrane in the absence of the pmf.

We next examined a Pf3 coat mutant, Pf3–4N, which cannot translocate its hydrophilic domain across the membrane, as judged by protease mapping experiments (23). This mutant lacks negative charges in the amino-terminal tail on which the pmf acts, and therefore the pmf cannot drive the neutral N-terminal tail across the membrane. Truncated nascent chains of 91 residues with the photoprobe at the Leu-34 position were synthesized to study possible interactions with YidC. The photocrosslinking products were immunoprecipitated with YidC antibody and analyzed by SDS-PAGE/autoradiography. Fig. 4b (right panel) shows that strikingly Pf3–4N can interact with YidC with approximately the same efficiency as wild-type Pf3 coat. We believe that this Pf3 coat mutant, like the wild-type Pf3 coat that accumulates when the pmf is abolished, has an Nterm/Cterm topology. In both cases, the proteins are partitioned into the membrane and are able to interact with membrane-bound YidC (see Fig. 4c for proposed intermediates).

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Pf3 coat is synthesized and then targeted to the inner membrane by the interaction of the positively charged residues in the carboxy-terminal region with the negatively charged membrane surface. After Pf3 coat membrane targeting, the Pf3 coat hydrophobic region partially integrates and then interacts with Pf3 coat with an exo-dependent interaction of the protein with the membrane. In addition to YidC interacting with Pf3 coat with an extended carboxy-terminal region, we found that Ffh may contact the Pf3 coat protein in the membrane targeting and insertion pathway (Figs. 2 and 3). The Fh-Pf3 interaction might be enhanced because Pf3-P2 is bound to the ribosome and has a better chance to interact productively with Ffh at the ribosome. The wild-type Pf3 coat protein does not require Ffh for membrane insertion. We propose a new model for Pf3 coat membrane biogenesis (Fig. 5) based on the data in this study and on previously published work (23, 33). After Pf3 coat is targeted to the surface of the membrane, its hydrophobic segment partitions into the membrane and moves into an environment near YidC, with the Pf3 coat polar amino-terminal tail region and positively charged carboxyl-terminal region located in the aqueous cytosol. Finally, the electrical potential (ΔΨ) of the pmf, a component of the pmf, translocates the amino-terminal tail across the membrane by an electrophoretic mechanism, and at the same time YidC mediates the transmembrane segment insertion.

In conclusion, we demonstrate for the first time, using the photocrosslinking approach, that YidC acts directly on a Sec-independent substrate to promote its membrane insertion. YidC functions at a stage of membrane insertion by associating with the membrane-bound hydrophobic region (Fig. 3) and then helping the inserting protein to orient into the transmembrane form (Fig. 4). The YidC-Pf3 coat interaction can occur even when the pmf is disrupted with CCCP and with a Pf3 coat protein mutant that cannot integrate across the membrane. Taken together, the data support the notion that the YidC-Pf3 coat interaction takes place during or before the pmf-requiring step of membrane protein insertion.
Direct Interaction of YidC with the Sec-independent Pf3 Coat Protein during Its Membrane Protein Insertion
Minyong Chen, James C. Samuelson, Fenglei Jiang, Matthias Muller, Andreas Kuhn and Ross E. Dalbey

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