Different Regions of the Nonconserved Large Periplasmic Domain of *Escherichia coli* YidC Are Involved in the SecF Interaction and Membrane Insertase Activity†

Kun Xie,‡ Dorothee Kiefer,§ Gisela Nagler,§ Ross E. Dalbey,‡ and Andreas Kuhn*,§

Department of Chemistry, The Ohio State University, Columbus, Ohio 43210, and Institute of Microbiology and Molecular Biology, University of Hohenheim, Garbenstrasse 30, 70599 Stuttgart, Germany

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ABSTRACT: The YidC protein of *Escherichia coli* is required for inserting Sec-independent membrane proteins and has a supportive role for the insertion of Sec-dependent proteins into the membrane bilayer. Because a portion of YidC copurifies with the Sec translocase, this interaction might be necessary to assist in the membrane insertion of Sec-dependent proteins. This study describes a deletion analysis that investigates which parts of YidC are required for its interaction with the SecDF complex of the Sec translocase and for the function of YidC as an insertase for the Sec-dependent membrane proteins. The results suggest that the first periplasmic region, which includes residues 24−346, is required for the interaction of YidC with the Sec translocase, in particular with the SecF protein. Further studies showed that residues 215−265 of YidC are sufficient for SecF binding. Surprisingly, the interaction of YidC with SecF is not critical for cell viability as YidC, lacking residues 24−264, was fully functional to support the growth of *E. coli*. It was also observed that this YidC mutant was fully functional to insert the Sec-dependent subunit A of the F_1F_o ATP synthase and an M13 procoat derivative, as well as the Sec-independent M13 procoat protein and subunit C of the ATP synthase. Only when additional residues of the periplasmic region were deleted (265−346) was the membrane insertase function of YidC inhibited.

Proteins of the bacterial inner membrane use different pathways for reaching their final destinations in the cell. Some Sec-independent proteins are targeted directly to the membrane by interacting with the negatively charged headgroups of the phospholipids (*1*, *2*), whereas others use the signal recognition particle for targeting to the membrane surface (*3*). After membrane binding, the hydrophobic regions of a protein partition into the membrane bilayer and the periplasmic hydrophilic regions translocate across the membrane. This translocation step requires energy and, depending upon the length of the hydrophilic region to be translocated, may require the motor protein SecA, in addition to the integral SecYEG translocase complex (*4*). For Sec-dependent proteins, the integration of the hydrophobic regions into the bilayer likely occurs after the translocation of the flanking hydrophilic regions and then the hydrophobic regions leave the Sec translocase laterally (*5*) contacting YidC, prior to integration into the lipid bilayer (*6*). YidC has been proposed to play a role in the lateral integration (*6*, *7*) and folding of the Sec-dependent proteins (*8*).

The YidC protein of *Escherichia coli* belongs to a novel class of membrane assembly components that include homologues in mitochondria and chloroplasts (*9*−*11*). These proteins are dedicated to support the membrane insertion of newly synthesized membrane proteins so that they can adopt a transmembrane configuration (*12*). The newly synthesized Sec-independent proteins contact YidC at the membrane, presumably by binding the hydrophobic regions prior to their transmembrane insertion. Photoreactive probes introduced into the hydrophobic regions of the Pf3 coat protein or leader peptidase were found to cross-link these protein substrates to YidC during the membrane insertion process (*1*, *13*, *14*). Remarkably, YidC was also cross-linked to the Pf3 coat substrate even when membrane insertion was inhibited either by a mutation introduced into the substrate or by dissipation of the membrane potential (*1*). Recently, we purified the YidC protein and reconstituted the protein into functionally active proteoliposomes. When the purified Pf3 coat protein was added to these proteoliposomes, the coat protein was efficiently inserted and assumed its transmembrane configuration (*15*). Similarly, subunit C of the F_1F_o ATPase, a Sec-independent protein, was inserted into YidC proteoliposomes in contrast to a Sec-dependent protein (*16*).

Structure−function studies on the *E. coli* YidC showed that, when 90% of the large periplasmic domain is deleted, YidC remains functional for the membrane insertion of the Sec-independent procoat protein (*17*). Other studies revealed that the first transmembrane segment is not important for the YidC function per se (*18*) but is critical for translocation of the large periplasmic domain (*19*). When these data are taken together, they indicate that the C-terminal five transmembrane segments of the YidC protein, which are conserved in evolution, contain the region critical for its function in membrane protein biogenesis.

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* To whom correspondence should be addressed: Institute of Microbiology and Molecular Biology, University of Hohenheim, Garbenstrasse 30, 70599 Stuttgart, Germany. Telephone: +711-459-2222. Fax: +711-459-2238. E-mail: andikuhn@uni-hohenheim.de.

‡ The Ohio State University.

§ University of Hohenheim.

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Copurification studies demonstrate that YidC forms a heterotetrameric complex with SecDFyajC (*20*), which presumably allows YidC to associate with SecYEG (*21*). To investigate which parts of YidC are important for its interaction with the SecDF complex, we constructed various YidC deletion mutants. We found that a YidC mutant, where a large portion of the first periplasmic domain was deleted, showed an impaired interaction with SecF. Interestingly, this mutant is sufficiently active to promote the insertion of both Sec-dependent and Sec-independent proteins, suggesting that binding SecF is not vital for the membrane insertase function. Finally, we show that residues 215−265 of YidC include the SecF-binding region because a chimeric protein between the maltose-binding protein (MBP) and this YidC region still copurified with SecF by affinity chromatography.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Growth Conditions. E. coli JS7131 [MC1060: Δ*yidC*, *attB::R6Kori*, *ParaBAD-yidC*$^{+}$ (Spec^{R})] was from our laboratory stocks. To test for the membrane insertion of the M13 procoat protein, the cells were transformed with pMS-8 coding for M13 procoat protein (*14*) and pGZ-IM60 coding for YidC. pGZ-IM60 is a derivative of pGZ119 with the isopropyl-*β*-D-thiogalactopyranoside (IPTG)[1]-inducible *tac* promoter and the *lacI*^{q} gene (*22*). For the experiments investigating the YidC−SecF interaction, *E. coli* C43 (Avidis, Saint-Beauzire, France) was transformed with pET16b expressing YidC derivatives and pCDF encoding YajC, SecD, and SecF (*23*) kindly provided by Franck Duong (University of British Columbia, Vancouver, Canada). To deplete the JS7131 strain of the YidC protein, fresh overnight cultures [grown in Luria broth (LB) with 0.2% L-arabinose (ara)] were washed with LB, diluted 1:50 into LB with 0.2% glucose (glc), and grown for 3 h.

Generation of Mutants. To construct the mutant Δ24−264, a new *Bam*HI restriction site at position 24 was introduced and the region coding for residues 24−264 was removed. Using the endogenous *Bam*HI restriction sites, the regions coding for the amino acid residues 265−346 and 265−454 were removed, generating the Δ265−346 and Δ265−454 YidC mutants, respectively. This removes 82 of the 322 amino acid residues within the large periplasmic region in both mutants. In addition, the Δ265−454 mutant has transmembrane helices 2 and 3 deleted. To construct these deletion mutants, *Bam*HI restriction sites in the *yidC* gene at codons 24, 264, 346, and 454 were used for partial digestion of pGZ-IM60. The linear fragments were religated using T4 ligase (Fermentas).

Stop codons (TAG/TAA) were introduced by oligonucleotide mutagenesis (*24*) at codons 415, 493, and 527 to construct the C-terminal deletion mutants. This produces a YidC mutant lacking the carboxyl-terminal cytoplasmic tail (YidC Δ527−548) or YidC mutants missing transmembrane segments 5 and 6 (Δ493−548) or 3−6 (Δ415−548). The site-directed deletions of YidC were made with Quikchange (Stratagene) as described (*17*). All mutants were sequenced confirming the desired mutations.

[1] Abbreviation: ara, L-arabinose; DDM, dodecylmaltoside; ECL, enhanced chemiluminescence; glc, glucose; IPTG, isopropyl-*β*-D-thiogalactopyranoside; LB, Luria broth; M9, minimal medium; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

YidC Membrane Insertion Assay. E. coli JS7131 cells with pMS-8 encoding the M13 procoat protein and pGZ-IM60 encoding the respective YidC mutant were depleted for the chromosomally expressed YidC by growing the bacteria in LB medium with 0.2% glucose for 3 h. The cells were washed with minimal medium (M9) and resuspended into M9 with 0.2% glucose, supplemented with 20 *μ*g/mL of each amino acid, except methionine, and grown for an additional hour. The expression of M13 procoat and the plasmid-encoded YidC derivative was induced with 1 mM IPTG for 10 min; cells were pulse-labeled with [^{35}S]-methionine for 1 min and precipitated with 10% trichloroacetic acid. The growth conditions for PClep-3M, ATP synthase subunits A-P2 and C-His, were slightly different. The derivative of M13 procoat, PClep-3M, has the three negatively charged residues (EDD) shifted from positions 2, 4, and 5 to positions 11−13 within the periplasmic loop and, in addition, the P2 domain of leader peptidase at the C terminus (*24*). The growth time was 3 h in LB with 0.2% glucose and 30 min after the wash step. The induction with 1 mM IPTG was for 5 min, and labeling with [^{35}S]-methionine was for 30 s. PClep-3M was analyzed directly without immunoprecipitation because the plasmid-encoded protein is a highly expressed protein. The samples were then subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and analyzed by phosphorimaging. Quantification of the accumulation of procoat was measured by phosphorimaging and calculated by ($procoat_{glc}$ − $procoat_{ara}$)/total signal of $procoat_{glc}$ and $coat_{glc}$. For analysis of the membrane insertion of the ATP synthase subunit A-P2 and subunit C-His, cells were depleted of YidC as described above, pulse-labeled with [^{35}S]-methionine for 30 s, and then converted into spheroplasts (*25*). The spheroplasts were treated with or without proteinase K (0.5 mg/mL, final concentration) for 30 min on ice. The subunit A-P2 was immunoprecipitated with anti-leader peptidase serum (that recognizes the P2 domain). The subunit C-His was isolated using a BD TALON metal-affinity resin from BD Bioscience. After acid precipitation and acetone washing, the pellet was resuspended in 8 M urea buffer (8 M urea, 10 mM Tris-Cl, and 100 mM NaH_2PO_4 at pH 8.0). The sample was then shaken with the resin (30 *μ*L) for 20 min at room temperature. The resin was washed with the urea buffer, and then subunit C-His was eluted by adding the urea buffer containing 300 mM imidazole. The isolated subunit C-His and the immunoprecipitated subunit A-P2 were then analyzed by SDS−PAGE and phosphorimaging.

SecF Interaction Assay. To analyze for the interaction of YidC proteins with SecDF, we used the copurification procedure reported for the isolation of the wild-type YidC−SecDF complex, as described (*20*), with the following modifications. YidC with an amino-terminal 10×His tag was used to see whether coexpressed SecF coelutes after affinity chromatography. *E. coli* C43 cells transformed with pCDF, encoding SecDFyajC (*23*), and pET16-IM60 coding for the respective YidC mutant were grown at 30 °C to a cell density of 2 × 10^{8}/mL in 2×YT medium containing 25 *μ*g/mL chloramphenicol, 200 *μ*g/mL ampicillin, and 0.2% arabinose. The YidC mutants were expressed by adding 1 mM IPTG for 3 h. Cells from 100 mL of culture were disrupted in 25 mM Tris-Cl at pH 8.0, 10% glycerol, and 100 mM NaCl by two cycles in a French pressure cell at 8000 psi. The

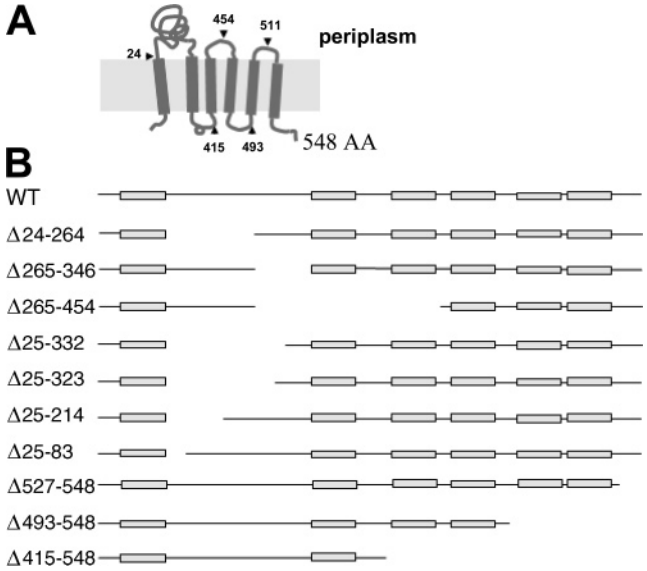

FIGURE 1: Mutants of YidC. Schematic representation of the membrane topology (A) of YidC and the deletion mutants (B). The boxes represent the transmembrane regions. Residues 24−264, 265−346, and 265−454 were deleted from YidC exploiting *Bam*H1 sites (see the Experimental Procedures). The constructs Δ25−83, Δ25−214, Δ25−323, and Δ25−323 were described in ref *17*. For the C-terminal deletion constructs, stop codons were introduced at positions 415, 493, and 527.

homogenate was centrifuged at 16 000*g* for 15 min, and the clear supernatant was solubilized by the addition of 2% dodecylmaltoside (DDM) for 1 h at 4 °C. The cleared extract was diluted to 0.1% DDM in 10 mM imidazole. Metal-chelating sepharose loaded with $CuSO_4$ was added, and the sample was incubated overnight at 4 °C while gently mixing. The beads were sedimented by centrifugation for 5 min at 3000 rpm in a microfuge and washed twice with buffer containing 10 mM imidazole and with buffer containing 500 mM imidazole. YidC−Sec complexes were eluted by buffer containing 50 mM ethylenediaminetetraacetic acid (EDTA).

For the isolation of the MBP−YidC fusion proteins, C43 cells (10 mL) were expressed for 3 h at 37 °C, harvested by centrifugation, and resuspended in 20 mM Tris-Cl at pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.1 mM PMSF. The cells were broken by freeze−thaw cycles and sonication and mixed with amylose resin for 30 min at 4 °C. The resin was washed with 10 column volumes of 20 mM Tris-Cl at pH 7.4, 200 mM NaCl, 1 mM EDTA, and 1 mM DTT and eluted with 10 mM maltose in the same buffer.

RESULTS

YidC Deletion Mutants. The 548 residue *E. coli* YidC protein spans the membrane 6 times (ref *19*, Figure 1). The periplasmic domain between the first two transmembrane regions is 322 amino acid residues in length, whereas the other two periplasmic regions are rather small and predicted to contain only 19 and 3 amino acid residues, respectively. In this study, the functional roles of the large periplasmic and the transmembrane regions of YidC were investigated by a defined set of site-directed deletion mutants (Figure 1B).

To test which regions of YidC are important for function, we first examined whether the various mutants can complement the growth defect of the YidC depletion strain, JS7131.

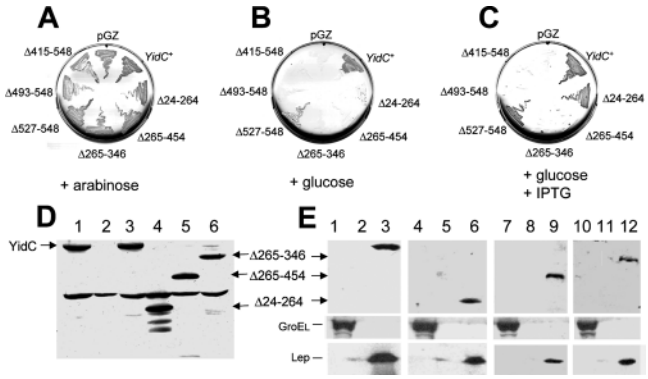

FIGURE 2: Complementation of the growth defect and inducible expression of plasmid-encoded YidC. (A−C) Fresh overnight cultures of JS7131 cells transformed with pGZ-IM60 encoding the respective mutant were grown for 3 h in LB supplemented with 0.2% glucose and streaked out on LB agar plates supplemented with 0.2% arabinose (A), 0.2% glucose (B), or 0.2% glucose and 1 mM IPTG (C). The empty plasmid (pGZ) and the plasmid coding for wild-type YidC ($YidC^+$) are shown as controls. (D) To test for expression levels of the mutant proteins, the cells were analyzed for their YidC content on a Western blot with antiserum directed to the C-terminal tail of YidC. Cultures (2 mL) encoding the wild-type YidC or the respective mutant were grown in 0.2% arabinose to a density of 2×10^8 cells/mL, washed, and resuspended in fresh medium containing 0.2% arabinose (lane 1), 0.2% glucose (lane 2), or 0.2% glucose and 1 mM IPTG (lane 3), respectively. The cells were grown for 3 h, and the protein was acid-precipitated and analyzed by SDS−PAGE and Western blot. Cells encoding Δ24−264 (lane 4), Δ265−454 (lane 5), or Δ265−346 (lane 6) were grown with 0.2% glucose and 1 mM IPTG under identical conditions. (E) Location of the mutant proteins in the cell was analyzed by cell fractionation. Wild-type YidC (lanes 1−3) and mutants Δ24−264 (lanes 4−6), Δ265−454 (lanes 7−9), or Δ265−346 (lanes 10−12) were expressed by the addition of 1 mM IPTG. High-speed supernatant (cytoplasmic and periplasmic fractions) (lanes 1, 4, 7, and 10), high-speed pellets (membrane fractions) (lanes 3, 6, 9, and 12), and the low-speed precipitates potentially containing inclusion bodies (lanes 2, 5, 8, and 11) were collected and analyzed by SDS−PAGE and Western blots.

E. coli JS7131 strain has a deletion in the original *yidC* gene and therefore depends upon a second chromosomal *yidC* gene under the control of the *araBAD* promoter (*14*). Growth of JS7131 in the presence of glucose causes depletion of YidC. After growth in glucose for 3 h, the plasmid-encoded mutant proteins were then analyzed for their ability to complement the growth defect of the YidC depletion strain. Strains containing specific YidC deletion mutants were plated on LB with arabinose, LB with glucose, and LB with both glucose and IPTG (parts A−C of Figure 2). IPTG induced the expression of the plasmid-encoded YidC variants. The cells expressing the wild-type *yidC* showed normal growth on arabinose medium (Figure 2A), but on the glucose medium, normal growth was only observed in the presence of IPTG (Figure 2C). In glucose medium without IPTG, the formation of colonies was restricted and occurred only in the heavy streak region (Figure 2B). Therefore, growth in glucose medium depends upon the induction of the plasmid-borne *yidC*. Interestingly, the Δ24−264 mutant, which lacks 240 residues of the large periplasmic domain, was viable on glucose with IPTG. The Δ265−454 mutant, missing the 82 carboxyl-terminal residues from the periplasmic domain and the transmembrane helices 2 and 3, and the mutant Δ265−346, where only the 82 residues of the periplasmic domain were deleted, did not grow under these glucose conditions in the presence of IPTG (Figure 2C). The expression levels

of both mutants (lanes 5 and 6 in Figure 2D) were comparable to the wild-type YidC (lanes 1 and 3 in Figure 2D), as determined by immunoblotting. The YidC Δ527−548, lacking the carboxyl-terminal cytoplasmic tail, was able to support growth on medium with glucose and IPTG (Figure 2C). However, when the last 2−4 transmembrane regions of YidC were removed, as with the Δ493−548 and Δ416−548 proteins, the YidC mutants were defective in supporting growth in glucose media in the presence of IPTG. These data suggest that the C-terminal cytoplasmic domain and most of the first periplasmic region of YidC have dispensable functions.

Membrane Fractionation of the YidC Deletion Mutants. The YidC mutants that have deletions in their periplasmic domain were further tested for their localization in the cell. Exponentially growing JS7131 cells expressing the YidC mutants were induced for 3 h with 1 mM IPTG and lysed using a French press. Potential inclusion bodies were spun down at 10 000*g* for 15 min (lanes 2, 5, 8, and 11 in Figure 2E), and the supernatant was subjected to high-speed centrifugation (100 000*g* for 90 min) to separate the membranes (lanes 3, 6, 9, and 12 in Figure 2E) from the soluble fraction that contains cytoplasmic and periplasmic proteins (lanes 1, 4, 7, and 10 in Figure 2E). The wild-type YidC (lanes 1−3) and the mutants Δ24−264 (lanes 4−6), Δ265−451 (lanes 7−9), and Δ265−346 (lanes 10−12) were found in the membrane fraction, together with the leader peptidase Lep control (Figure 2E). GroEL, the cytoplasmic control, was found in the soluble fraction. No YidC protein was found in the soluble fraction or in the low-speed pellet. Similarly, the mutants Δ493−548, Δ416−548, and Δ527−548 were found in the membrane fraction (data not shown). We conclude that these deletion mutants are localized to the membrane.

Interaction of YidC with the SecF Component Requires the First Periplasmic Region. The YidC protein interacts with the Sec translocase by direct interaction with the SecDF proteins (*20*). We investigated whether the YidC deletion mutants still show this interaction by testing whether YidC copurifies with SecDF. To express the YidC deletion mutants, they were first subcloned into the plasmid pET16b, which extends the protein with a 10×His tag at the N terminus. The His-tagged YidC proteins were expressed in C43 cells that overproduce and coexpress SecDF to ensure that the YidC proteins and SecDF are expressed in a similar amount. After metal-affinity chromatography of the solubilized membrane material (lanes 1, 4, and 7 in Figure 3A), the final wash (lanes 2, 5, and 8 in Figure 3A) and elution fractions (lanes 3, 6, and 9 in Figure 3A) were tested for their His-tagged YidC using anti-His antiserum (middle panels) and SecF content (lower panels). While the wild-type (lanes 1−3) and the Δ527−548 mutant lacking the C-terminal cytoplasmic tail (lanes 7−9) showed efficient coelution of SecF with YidC, the Δ24−264 mutant lacking 240 residues of the periplasmic domain, however, did not coelute (lanes 4−6). SecF and YidC Δ24−264 were found in comparable amounts in the load fraction (lane 1), but SecF was detected only in the flow through (data not shown). Therefore, we conclude that the Δ24−264 mutant is defective in the interaction with the SecDF of the Sec complex.

The YidC Periplasmic Region, Encoding Residues 24−345, Is Sufficient for Binding SecF. Next, we investigated

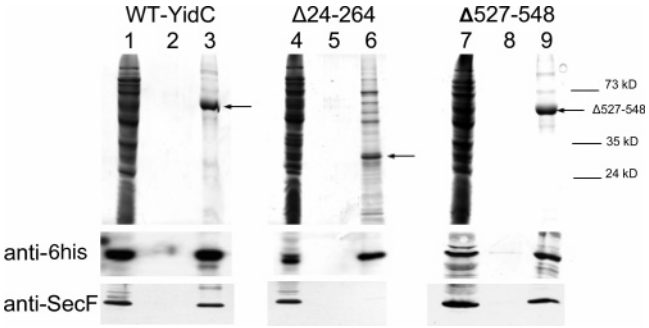

FIGURE 3: Interaction of YidC with SecF. *E. coli* C43 cells (100 mL cultures) overexpressing YidC-His-tagged mutants and SecDF were lysed, and the membrane fraction was gently solubilized by detergent. The Sec−YidC complexes were isolated by His-tag-affinity chromatography and analyzed on SDS−PAGE and Coomassie-stained gels (upper panels) or immunoblotted and visualized by enhanced chemiluminescence (ECL) detection (lower panels). Shown are the load fractions (lanes 1, 4, and 7), the fractions of the last imidazole wash (lanes 2, 5, and 8), and the EDTA-eluted fractions (lanes 3, 6, and 9). Indicated by an arrow are the positions of the wild-type YidC protein (lane 3), the Δ24−264 YidC mutant (lane 6), the Δ527−548 YidC mutant (lane 9). The middle panels show immunoblots of the respective bands with anti-His-tag antiserum, and the lower panels show immunoblots of the respective bands with anti-SecF antiserum.

whether the first periplasmic region of YidC is sufficient for SecF binding. To do this, residues 24−347 were fused to the carboxyl-terminal end of the MBP, creating the fusion protein MBP-P1. *E. coli* C43 cells expressing the fusion protein MBP-P1 or MBP as a control were used. These cells were induced for 3 h (lanes C = cells in Figure 4A), lysed by sonication, and incubated with amylose resin (lanes L = load). The nonbound material was washed off [lanes F = flow through (not shown) and W = wash], eluted (lanes E = eluent), and analyzed for the MBP binding (upper panel) and SecF coelution (lower panel). Whereas the cell extract expressing MBP (right panel) did not show SecF coeluting with MBP, the corresponding fraction from cells expressing the MBP-YidC fusion (left panels) did show SecF coeluting with the MBP-P1 protein. This indicates that residues 24−347 of YidC are sufficient for binding SecF.

Residues 215−265 Encompass the SecF-Binding Region. To determine which region of the periplasmic domain of YidC is required for SecF binding, deletions were made within the YidC portion of the MBP-P1 fusion protein (Figure 4B). The binding of YidC to the various MBP fusion proteins was analyzed by copurification studies, as described in the previous section for MBP-P1. All of the proteins containing YidC residues 215−265 had SecF in the coelution fractions, whereas the fusion protein with the YidC residues 265−347 did not coelute SecF. These results suggest that the SecF-binding motif is located within the YidC region 215−265.

Membrane Insertase Function for Sec-Independent Protein Substrates. To test whether the mutant YidC proteins are still functional for membrane insertion of Sec-independent proteins, the membrane assembly of M13 procoat protein (parts A and B of Figure 5) and subunit C of the F_1F_0 ATP synthase (Figure 7) were analyzed. In the YidC depletion strain JS7131, the Sec-independent procoat protein accumulates in a nontranslocated form at the cytoplasmic side of the membrane and is not cleaved by leader peptidase (*26*). Because the cleavage of procoat by leader peptidase can

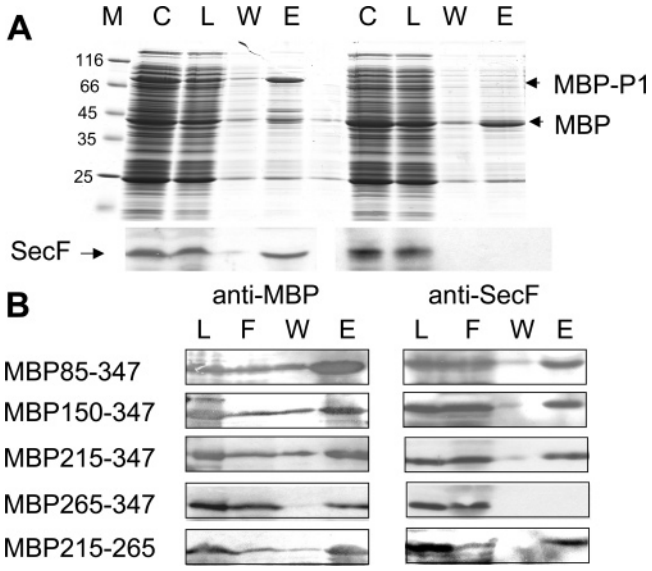

FIGURE 4: Residues 214−264 of YidC include the SecF-binding region. (A) Periplasmic region P1 of YidC (residues 24−347) was fused to the carboxyl-terminal end of the MBP, and the respective plasmid (pMalY) was transformed into *E. coli* C43 together with pCDF, encoding SecF, SecD, and YajC (left panels). As a control, cells expressing the nonfused MBP were analyzed (right panels). The cells were grown to exponential phase, induced with 1 mM IPTG, and grown for another 3 h. Then, the cells were lysed (lanes C = cells), and the cellular extract (lanes L = load) was passed over an amylose column. The flow through (F) was collected. The column was washed with 10 column volumes (lanes W = wash), and the bound material was eluted with 10 mM maltose (lanes E = eluent). The samples were analyzed by SDS−PAGE, Coomassie blue staining (upper panels), and Western blotting with antiserum to SecF (lower panels). (B) *E. coli* C43 cells overexpressing MBP fused to portions of the first periplasmic domain P1 of YidC. The numbers in the MBP construct refer to the residues of YidC that were added onto MBP. The samples were treated as described in A and analyzed by Western blot with anti-MBP (left panels) and anti-SecF (right panels) sera.

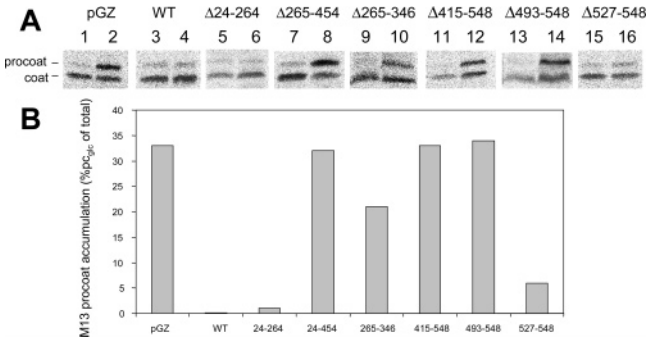

FIGURE 5: M13 procoat processing depends upon a functional YidC. *E. coli* JS7131 cells were transformed with pMS-8 coding for M13 procoat and pGZ-IM60 coding for the respective YidC mutants. The cells were depleted of YidC by growth in the presence of 0.2% glucose, as described in the Experimental Procedures. Plasmid-encoded proteins were induced with 1 mM IPTG for 10 min and pulse-labeled with [^{35}S]-methionine for 1 min. Samples were immunoprecipitated with an antibody to M13 coat and analyzed by SDS−PAGE and phosphorimaging (A). The accumulation of procoat was followed in cells grown in the presence of 0.2% glucose (even-numbered lanes) and compared to cells grown in the presence of 0.2% arabinose (odd-numbered lanes). (B) Quantification of the accumulation of procoat under glucose conditions when the cells contained an empty vector (pGZ), a respective plasmid coding for wild-type YidC (pGZ IM60), and the indicated YidC mutants was performed as described in the Experimental Procedures.

easily be visualized by phosphorimaging after SDS−PAGE, transformed JS7131 cells expressing the procoat protein were pulse-labeled for 1 min with [^{35}S]-methionine and analyzed by immunoprecipitation, SDS−PAGE, and phosphorimaging (Figure 5A). When the cells were grown with arabinose, to express the chromosomal copy of YidC, nearly all of the procoat protein was cleaved to the coat protein (odd-numbered lanes in Figure 5A). However, when the cells were grown in glucose to deplete the chromosomal YidC, uncleaved procoat accumulated in the cases where plasmid-encoded YidC was either not present (lane 2) or not functional (lanes 8, 10, 12, or 14). In cells bearing the plasmid pGZ-IM60, coding for the wild-type YidC, the procoat was efficiently cleaved under glucose conditions (lane 4). Cells expressing the deletion mutant Δ24−264 (lanes 5 and 6) also showed efficient cleavage of the procoat protein under glucose conditions. The YidC Δ265−454 (compare lanes 7 and 8) and the Δ265−346 (see lanes 9 and 10) mutants were defective in their membrane insertion function. Uncleaved procoat protein accumulated in both cases.

We also examined the effect of deleting the C-terminal tail of YidC and the region of YidC containing the either last 2 or 4 transmembrane segments (Figure 1B). As mentioned previously, the YidC Δ527−548, lacking the C-terminal tail, could functionally complement the YidC depletion strain (Figure 2C). YidC Δ493−548 and Δ415−548 mutants, lacking either 2 or 4 transmembrane segments, respectively, could not complement the growth defect of the YidC depletion strain (Figure 2C). As a control, we confirmed that these two C-terminal truncated mutants were expressed and localized in the membrane by fractionation studies (data not shown). These two mutants were also deficient in their membrane insertase function for M13 procoat protein (lanes 11−14 in Figure 5A), whereas the Δ527−548 mutant removing the C-terminal 22 residues was functional (lanes 15 and 16). Figure 5B summarizes the results with the accumulated procoat protein for each of the deletion mutants. JS7131 cells with the wild-type, Δ24−264, and Δ527−548 YidC proteins showed no or very little accumulation when depleted of the chromosomally encoded YidC, yet the other four mutants accumulated procoat to a level similar to the empty plasmid (pGZ). In conclusion, the deletion of the residues 265−346 in the periplasmic region of YidC severely affects the insertase function, whereas the removal of the residues 24−264 has no effect.

YidC Δ24−264 Mutant Is Still Functional To Insert Sec-Dependent Proteins. To test whether the impaired interaction of the YidC Δ24−264 with SecF affects the membrane insertase activity for Sec-dependent proteins, we examined the insertion of subunit A of F_1F_0 ATP synthase. The insertion of subunit A depends upon YidC and SecYEG, as was shown with subunit A-P2 with a C-terminally extended version derived from the P2 domain of leader peptidase (*25*). We used the proteinase protection assay to determine which residues in the periplasmic domain of YidC are required for inserting the subunit A-P2 protein (Figure 6). The wild type and the Δ24−264 and Δ25−323 YidC mutants allowed normal insertion of subunit A-P2, because it was accessible to the protease and generated the protease-protected fragments (PpfA and PpfB). Subunit A-P2 was largely resistant to proteinase K digestion when the Δ265−346 YidC mutant was present and therefore deficient for membrane insertion.

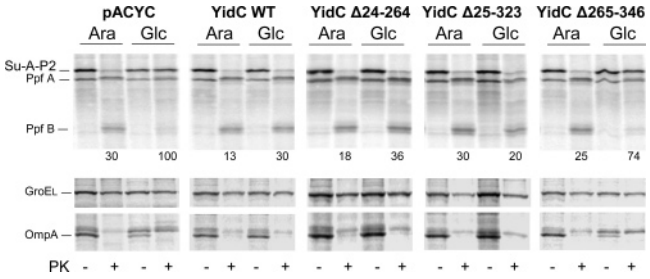

FIGURE 6: YidC Δ24−264 can promote the insertion of a Sec-dependent protein. *E. coli* JS7131 cells expressing YidC, YidC Δ24−264, YidC Δ25−323, or YidC Δ265−346 from pACYC and coexpressing the ATP synthase subunit A-P2 were grown in arabinose to express the chromosomally encoded YidC (Ara) or in glucose to deplete the chromosomal wild-type YidC (Glc). ATP synthase subunit A-P2 expression was induced for 5 min with 1 mM IPTG, and cells were pulse-labeled with [^{35}S]-methionine for 30 s. Cells were then converted to spheroplasts and analyzed by the protease accessibility assay, as described under the Experimental Procedures. The samples were then TCA-precipitated and subjected to immunoprecipitation with anti-Lep (recognizes P2 domain) serum. The amount of the nontranslocated subunit A-P2 was quantified by comparing the amounts of the intact protein in the protease-treated and nontreated samples. The percentage of accumulation is shown below the protease-treated lane. For controls, the accessibility of GroEL (a cytoplasmic protein) and OmpA (outer membrane protein) to externally added protease was tested (lower panels). The samples were analyzed by SDS−PAGE using a 15% polyacrylamide gel and phosphorimaging. PpfA and PpfB are protease-protected fragments generated by digestion of subunit A-P2.

In these studies, outer membrane protein A (OmpA), which serves as a positive control, is digested by externally added protease (lower panels in Figure 6). The cytosolic protein GroEL, the negative control, is resistant to the addition of protease.

We then tested a series of YidC mutants, with small to large deletions in the periplasmic domain, for the insertion of subunit C of the F_1F_0 ATP synthase. The insertion of this protein requires only YidC (*16*, *25*, *27*). The membrane insertion of the subunit C protein can be analyzed with a 10×His tag engineered into the cytoplasmic loop and an 8 residue tag added to the periplasmic tail. Both its N- and C-terminal tails are exposed in the periplasm (*25*). The addition of proteinase results in a small shift of the subunit C protein (left panels in Figure 7), if the protein was inserted into the membrane. The results confirm that residues 265−346 of YidC are functionally important for membrane insertion because the addition of the proteinase K did not result in a shift in the molecular weight of subunit C when the cells were grown in glucose to deplete the chromosomally encoded YidC (lowest panel). While the YidC Δ25−332 and Δ265−346 mutants could not promote the membrane insertion of subunit C-His, all of the other deletion mutants were able to stimulate membrane insertion of the protein. We then tested the procoat-Lep mutant 3M (*28*) and similar results for all of the YidC mutants were obtained (right panels in Figure 7). The membrane insertion and processing of the PClep-3M derivative strongly depends upon SecDF and YidC. Strikingly, the PClep-3M mutant was processed normally when expressed in the presence of the Δ24−264 mutant (right panel in Figure 7), despite impaired SecDF binding (Figure 3A). PClep-3M was inhibited for membrane insertion only when the cells were grown in glucose in the presence of the Δ25−332 and Δ265−346 mutants, respectively (Figure 7). We conclude that the region required for the interaction with SecF (residues 215−265) has no functional role in the membrane insertion of any of the substrates.

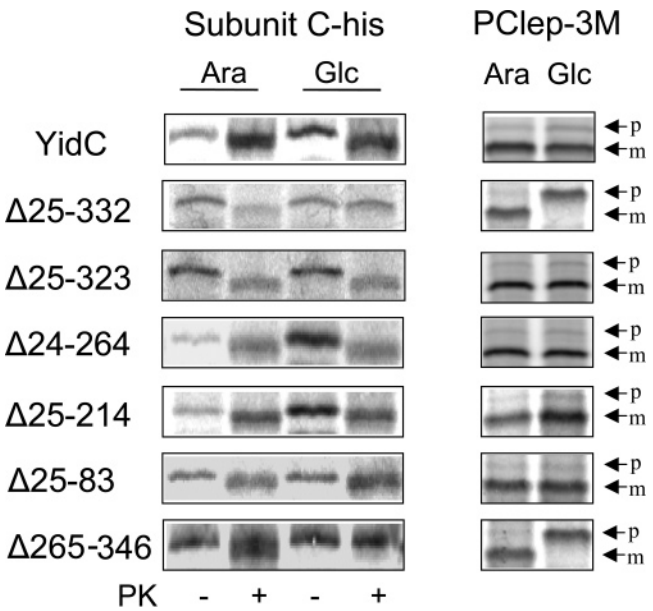

FIGURE 7: Residues 323−346 of the periplasmic domain of YidC are functionally important for membrane insertion of Sec-independent and Sec-dependent proteins. *E. coli* JS7131 cells expressing YidC, YidC Δ25−83, YidC Δ25−214, YidC Δ24−264, YidC Δ25−323, YidC Δ25−332, or YidC Δ265−346 from pACYC and coexpressing the ATP synthase subunit C-His (left panels) or procoat-Lep-3M (PClep-3M) (right panels), respectively, were grown in arabinose (Ara) or in glucose for 3 h to deplete the chromosomal wild-type YidC (Glc) as described in the Experimental Procedures. Expression of subunit C-His was induced by the addition of 1 mM IPTG for 5 min and then pulse-labeled with 60 μCi [^{35}S]-methionine for 30 s. The cells were converted to spheroplasts and digested with proteinase K for 30 min on ice, and then the subunit C-His was pulled down using BD TALON metal-affinity resin as described in Experimental Procedures and analyzed by SDS−PAGE and phosphorimaging (left panels). For PClep-3M, the cells were labeled with [^{35}S]-methionine for 30 s, TCA-precipitated, and analyzed by SDS−PAGE and phosphorimaging (right panels).

DISCUSSION

YidC is a novel participant in the protein-assisted pathway of membrane protein insertion in bacteria. It is a key player in the Sec pathway because conditional chromosomal mutants in *yidC* affect the translocation of certain Sec-dependent membrane proteins (*6*, *14*, *25*). YidC forms a complex with SecDF, allowing it to associate with the Sec translocase (*20*).

In this study, we analyzed a set of deletion mutants to identify the regions of YidC that are involved in the interaction with SecDF. In addition, we tested which of the YidC regions are critical for its membrane insertase function and for cell viability. We found that the YidC mutant Δ24−264, lacking 240 residues of the first periplasmic region of YidC, did not copurify with SecF, whereas the wild-type protein and the Δ527−548 YidC mutant lacking the C-terminal tail region were capable of binding SecF (Figure 3). Furthermore, we showed that YidC residues 215−265 are sufficient for SecF binding, because we found that a MBP fused to YidC Δ215−265 is capable of copurifying SecF by affinity chromatography (Figure 4). These observations

seem reasonable because Oxa1p, the mitochondrial homologue of YidC, lacks the region corresponding to the periplasmic residues 24−264. Accordingly, Sec homologues are not present in mitochondria (*29*).

Remarkably, the non-SecF-binding mutant Δ24−264 complemented the YidC depletion strain JS7131 for growth (Figure 2). This implies that its interaction with SecF is not an essential feature of YidC. In accordance with this finding, it was recently reported that only the Sec-independent function of YidC is conserved and essential for growth (*30*). When we analyzed the Δ24−264 mutant for its function to insert Sec-dependent and Sec-independent proteins, we were surprised to observe that the Δ24−264 mutant was fully functional and all substrate proteins tested were membrane-inserted (Figures 5−7). In contrast, when the region 265−346 was deleted from YidC, the function of YidC was inhibited for both Sec-dependent and Sec-independent substrates (Figures 5−7). The results suggest that the residues 24−264 of the first periplasmic domain of YidC are required for the interaction with SecF but that this region is not critical for the YidC function.

One possibility that explains these results is that the SecYEGDFyajC and YidC can operate sequentially and a direct molecular interaction is not crucial. Another possibility is that the high abundance of YidC over the SecYEG components allows for the interaction with the Sec machinery even with a low binding affinity. To address this, the low copy number plasmid pACYC was used for expressing the YidC mutant (Figure 6). Still, there was no observable effect in the YidC function for Sec-dependent proteins when residues 24−264 were deleted. In contrast, the deletion of the periplasmic residues 265−346 of YidC severely affected the insertase function (Figures 5−7) and also the growth of the cells (Figure 2), demonstrating that this region of YidC is essential. As a control, we confirmed that YidC Δ265−346 is expressed in the cell and localizes to the membrane (Figure 2E). However, deletion of residues 265−346 from YidC may cause a structural perturbation in another region of the YidC protein that is critical for the membrane protein insertion function. The requirement of this 265−346 region for YidC activity nicely complements previous data (*17*), showing that YidC is fully functional for a Sec-independent substrate when residues 25−323 of YidC are deleted, whereas YidC is not functional when residues 25−332 are deleted. Taken together, we suggest that the *E. coli* YidC C-terminal portion of the periplasmic region (residues 323−346) is of functional importance for inserting Sec-independent and Sec-dependent proteins.

As predicted for its role as a membrane insertase (*31*), the transmembrane regions of YidC are primarily important. Most likely, YidC binds its substrates by hydrophobic interactions. Site-specific cross-linking experiments have shown that hydrophobic regions of nascent chains are in proximity to YidC (*1*, *6*, *13*, *14*). The function of the large periplasmic region, which is more than half the size of the total protein, is still unknown. Intriguingly, in Gram-positive bacteria, the corresponding region of the YidC homologues is very short (*11*, *32*), although some of these bacteria have homologues of SecF and SecD.

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