YidC Is Strictly Required for Membrane Insertion of Subunits a and c of the F_1F_0ATP Synthase and SecE of the SecYEG Translocase†

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ABSTRACT: YidC was previously discovered to play a critical role for the insertion of the Sec-independent M13 procoat and Pf3 coat phage proteins into the *Escherichia coli* inner membrane. To determine whether there is an absolute requirement of YidC for membrane protein insertion of any endogenous *E. coli* proteins, we investigated a few representative membrane proteins. We found that membrane subunits of the F_0 sector of the F_1F_0ATP synthase and the SecE protein of the SecYEG translocase are highly dependent on YidC for membrane insertion, based on protease mapping and immunoblot analysis. We found that the SecE dependency on YidC for membrane insertion does not contradict the observation that depletion of YidC does not block SecYEG-dependent protein export at 37 °C. YidC depletion does not decrease the SecE level low enough to block export at 37 °C. In contrast, we found that protein export of OmpA is severely blocked at 25 °C when YidC is depleted, which may be due to the decreased SecE level, as a 50% decrease in the SecE levels drastically affects protein export at the cold temperature [Schatz, P. J., Bieker, K. L., Ottemann, K. M., Silhavy, T. J., and Beckwith, J. (1991) *EMBO J. 10,* 1749−57]. These studies reported here establish that physiological substrates of YidC include subunits of the ATP synthase and the SecYEG translocase, demonstrating that YidC plays a vital role for insertion of endogenous membrane proteins in bacteria.

A major problem in cell biology is understanding how membrane proteins assemble into the membrane and obtain their functional membrane conformation. Membrane proteins function as receptors, channels, pumps, transporters, respiratory complexes, and ATP synthases. One of the best-studied ATP synthases is the F_1F_0ATP synthase. The F_1F_0ATP synthase is comprised of eight subunits with five subunits (α, β, γ, δ, and ϵ) within the F_1 domain (in a stoichiometry $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$) and three subunits (a, b, and c) in the F_0 domain (in an $a_1b_2c_{10-12}$ stoichiometry) (*1*). The crystal structure of the F_1 domain has been solved at high resolution, but little information is available for the membrane structure and biogenesis of the F_0 domain (*2*).

In bacteria, the membrane insertion of the majority of proteins is catalyzed by the Sec translocase. The SecYEG translocase complex is comprised of the peripheral protein SecA, and the integral membrane polypeptides SecYEG and SecDFYajC (for a review see, refs *3* and *4*). SecY and SecE are absolutely critical for membrane protein insertion (*5*−*11*) and are believed to form with SecG an hetero-oligomeric channel within the membrane (*12*−*15*). SecA, the translocation ATPase, seems to be needed for translocation only of large loops of membrane proteins (*16*−*18*).

A membrane protein, YidC, was recently identified (*8*, *19*) and shown to be in close proximity to Sec-dependent proteins such as FtsQ and leader peptidase during membrane insertion (*8*, *19*, *20*). YidC stimulates the membrane insertion of these Sec-dependent proteins (*10*, *19*). A portion of YidC is found to be directly bound to the SecDFYajC subcomplex (*21*) of the Sec translocase complex (*8*).

The requirement of YidC for membrane insertion is strongest for SecYEG translocase-independent proteins. YidC was found to be critical for the membrane insertion of the M13 procoat (*19*, *22*, *23*) and Pf3 coat phage proteins (*23*, *24*). Since the presence of YidC is essential for the uninfected bacteria to grow (*19*), other vital membrane proteins besides phage proteins most likely also absolutely require YidC for insertion.

To examine whether any endogenous *Escherichia coli* membrane protein strictly depends on YidC for insertion and why YidC is essential in membrane protein insertion, we investigated its requirement for a few representative polytopic membrane proteins in *E. coli*.[1] We found that YidC is strictly needed for the membrane insertion and maintaining the intracellular membrane levels of the subunits a and c of the

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[1] Abbreviations: *E. coli*, *Escherichia coli*; OmpA, outer membrane protein A; lep, leader peptidase; IMV, inner membrane vesicles; Ara, arabinose; Glc, glucose; PAL, peptidoglycan-associated lipoprotein; SDS−PAGE, sodium dodecylsulphate−polyacrylamide gel electrophoresis.

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F_0 sector of the enzyme F_1F_0 ATP synthase. Also, YidC is required for the membrane insertion of SecE, an essential component of the SecYEG translocase. These studies demonstrate that YidC plays a key role for membrane insertion of *E. coli* membrane proteins in accordance with the essential role of YidC.

EXPERIMENTAL PROCEDURES

Bacteria, Plasmids, and Materials. The *E. coli* JS7131 YidC depletion strain and MC1060 strain is from our laboratory collection. CM124, the SecE depletion strain, was generously provided by Jan William de Gier. Subunits a, a-P2, Pf3-b-P2, c-6his, and TatC-6his were expressed by IPTG induction from the pMS119 vector, which contains the IPTG-inducible *tac* promoter and the *lac*I^q gene. SecE-tag was expressed by another IPTG-inducible vector, pEH1 (*6*). Amino acids and lysozyme were from Sigma. Proteinase K was from Qiagen. *Trans*-[^{35}S] label, a mixture of 85% [^{35}S]-methionine and 15% [^{35}S]-cysteine, 1000 Ci/mmol, was from ICN.

Protease Accessibility Studies. JS7131 cells were grown to the mid-log phase in LB media for 2 h and 30 min supplemented with glucose (0.2% final concentration) or arabinose (0.2% final concentration). The cells were then spun down and resuspended in M9 minimal media containing glucose or arabinose and grown for an additional 30 min. Expression of the plasmid-encoded membrane protein being tested for YidC dependency was induced by IPTG (1 mM) for 5 min. The culture was treated with *trans*-[^{35}S] label for 2 min, or where indicated, 20 s, and then converted to spheroplasts and incubated in the presence or absence of proteinase K, as described previously (*25*). The samples were acid-precipitated and immunoprecipitated (*26*) with antibody against the leader peptidase (Lep) (which precipitates subunits a-P2 and Pf3-b-P2), SecE, araB (which precipitates Band X, a cytoplasmic marker), GroEL (cytoplasmic protein), outer membrane protein A (outer membrane marker), and the 6-His tag (which precipitates subunits c-6his and TatC-6his). Samples were then analyzed by SDS−PAGE and phosphorimaging.

Construction of Plasmids. To examine the YidC dependence of membrane protein insertion, we made six constructs. The first construct, subunit a (encoded by the *UncB* gene) was subcloned into the *HindIII-XmaI* site of the expression vector pMS119. To do this, *uncB* was excised from pRPG54 (*27*) (bearing the ATP synthase genes) by digestion with *HindIII* and *Xma1* following the introduction of the *XmaI* site into the 3′ flanking region of the *uncB* gene. In the second construct, a-P2 fusion was created by fusing the P2 domain of Lep (containing the C-terminal 103 residues of leader peptidase) to the very C-terminus of subunit a. The third construct, Pf3-b-P2, has the first 18 residues of Pf3 coat protein fused to the amino-terminus of subunit b and the P2 domain of *lep* attached to the C-terminus of the protein. The fourth construct, c-6his, has six consecutive histidine residues attached using site-directed insertion mutagenesis to the C-terminus of the subunit c protein. The fifth construct, TatC-6his, was subcloned from the arabinose-inducible pBAD24 vector into the IPTG-inducible pMS119 vector. The sixth construct, SecE-tag, was expressed in the IPTG-inducible pEH1 vector. The *E. coli* SecE gene from pET610 (containing the SecYEG genes) (*28*) was subcloned into the pEH1 vector and was subsequently modified by adding an amino acid stretch, GVQDFFST, to the C-terminus of SecE. The modified SecE is named SecE-tag.

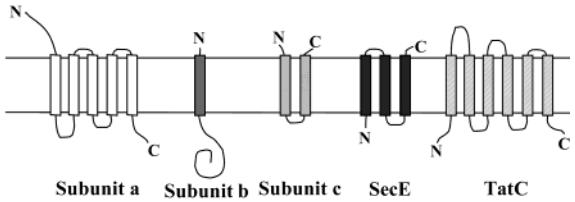

FIGURE 1: Membrane topology of F_1F_0 ATP synthase subunits a, b, and c; SecE; and TatC. Apolar domains are represented by rectangles. It should be noted that a recent study using PhoA and UidA fusions of TatC suggest that TatC has only four transmembrane helices with the same N_{in}/C_{in} orientation (*54*).

Immunochemical Detection of F_0 Sector Subunits a, b, c, SecE, SecY, and YidC in the Inner Membrane. JS7131 or CM124 cells were grown in LB medium with 0.2% arabinose or 0.2% glucose for various times after 1:50 back dilution of an overnight culture containing arabinose. The inner membrane vesicles (IMVs) were isolated, as described (*29*), and the normalized IMV protein concentration was 2.3 mg/mL by the BCA method. Rabbit antisera specific to F_0F_1-ATP synthase subunits a, b, and c were used in Western blots at 1:2000, 1:5000, and 1:5000, respectively (*30*). Both SecE and YidC antiserum from rabbit were used at 1:5000 dilutions, and SecY antiserum from rabbit was used at 1:10 000 dilution. The immunoblotting was done using the enhanced chemiluminescence Western blotting kit (from Amersham Bioscience).

RESULTS

F_0 Sector Components Are Strictly YidC-Dependent for Membrane Protein Insertion. We recently demonstrated that the integration of the SecYEG-independent membrane proteins M13 procoat and Pf3 coat phage proteins is strongly inhibited in the absence of YidC (*19*, *22*, *24*). In contrast, the insertion of the Sec-dependent Lep (*19*) and FtsQ (*10*) was only retarded in YidC's absence. Therefore, since Lep and FtsQ are only slightly dependent on YidC for insertion, we wanted to determine whether there exist some endogenous proteins in *E. coli*, which strictly depend on YidC for insertion as the phage proteins, M13 procoat and Pf3 coat.

To address whether YidC plays a critical role in assembly of inner membrane proteins and assists in membrane protein assembly of membrane protein complexes, we first investigated whether YidC is critical in the membrane integration of the subunits of the F_0 sector of the F_1F_0 ATP synthase. The integral membrane protein subunits a, b, and c of the F_0 sector have a different topology (Figure 1).

We initially analyzed the membrane insertion of subunit a, which spans the membrane five times (*1*). To deplete YidC, we used the strain JS7131, where the chromosomal copy of *yidC* is inactivated and another copy of the gene was introduced under the control of the *araBAD* promoter (*19*). Growth in arabinose allows expression of the *yidC* gene, while growth in the presence of glucose represses *yidC* expression and leads to depletion of YidC in growing cells. To facilitate the immunodetection of subunit a, we added the Lep P2 tag (residues 222−323) to the C-terminus of the

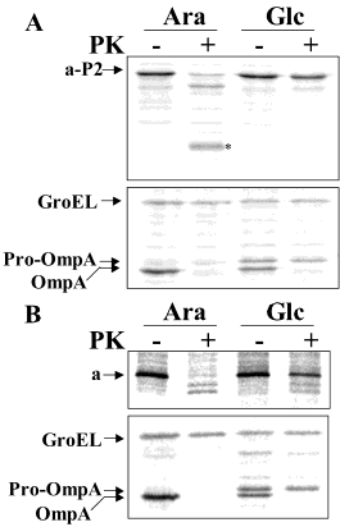

FIGURE 2: Subunit a is strictly dependent on YidC for membrane insertion. JS7131 bearing the plasmid expressing subunit a-P2 (A) or wild-type subunit a (B) was grown under YidC depletion or YidC expressing conditions, treated with *trans*-[^{35}S] label for 2 min and analyzed for protease accessibility, as described in the Experimental Procedures. The asterisk depicts the subunit a-P2 degradation fragment.

protein (the hybrid construct is called a-P2). JS7131 cells were grown under conditions where YidC is produced in sufficient (arabinose media) or deficient (glucose media) amounts and analyzed by protease-accessibility studies (*25*). Cells were pulse-labeled with [^{35}S]-methionine for 2 min, converted to spheroplasts, and treated with or without proteinase K for 60 min on ice. Figure 2A shows that when YidC is present (Ara), a-P2 inserts into the membrane, as it is degraded by the externally added protease. Proteinase K most likely cleaves a-P2 in the short periplasmic loop between transmembrane segments 4 and 5 to generate the C-terminal protected fragment containing the Lep P2 domain that is immunoprecipitated with Lep antibody. Under YidC deficient conditions (Figure 2A, Glc), a-P2 does not insert across the membrane, and it is protected from the protease by the cell membrane. We used OmpA, a positive control, to assay the efficiency of spheroplast formation. GroEL, a cytosolic protein, was used as a negative control to monitor the integrity of spheroplasts. To make sure adding the P2 does not change YidC dependency of insertion, we also tested the wild-type subunit a without immunoprecipitation. This was possible because it was highly expressed and there were not any strong background bands at the position of subunit a (Figure 2B). The position of wild-type subunit a was confirmed by ±IPTG induction of the plasmid-encoded subunit a (data not shown). When we added protease externally to these spheroplasts, subunit a was degraded only when YidC was present (Ara; compare +/− PK lanes) but was not degraded when YidC was deficient (Glc; compare +/− PK lanes). These latter data show that subunit a is dependent on YidC for membrane protein insertion and confirms the strict YidC requirement of the a-P2 protein.

To examine the membrane insertion of the F_1F_0 ATP synthase subunit b polypeptide, a protein with a single transmembrane domain (*1*, *31*, *32*), it was necessary to make two modifications. We added the Pf3 coat periplasmic domain (residues 1−18) to the N-terminus of subunit b and

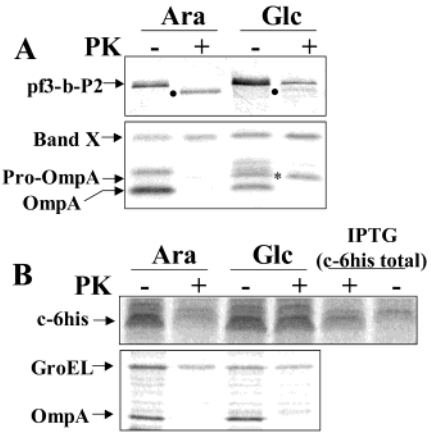

FIGURE 3: Subunits b and c constructs require YidC for membrane insertion. *E. coli* JS7131 synthesizing Pf3-b-P2 (A) or c-6his (B) was grown, labeled, and analyzed for membrane insertion, as described in the Experimental Procedures. JS7131 cells expressing Pf3-b-P2 were pulse-labeled for 20 s, while cells expressing c-6his were labeled for 2 min. The subunit b hybrid protein called Pf3-b-P2 was constructed by adding the Pf3 peptide to the subunit b amino-terminus and fusing the Lep P2 domain (see Experimental Procedures) to the cytoplasmically exposed C-terminus of subunit b. The subunit b derivative was immunoprecipitated with Lep antiserum. c-6his represents subunit c with a 6-His tag added to the C-terminus of the protein. The c-6-His protein was immunoprecipitated using an antibody to the 6-His tag. The solid circle represents the shifted Pf3-b-P2 band produced by protease degradation of the protein in the N-terminal region; the asterisk depicts the proOmpA band. The ± induction by IPTG for the c-6his study is included in panel B as a molecular weight control.

added the Lep P2 tag (residues 222−323 of leader peptidase) to its C-terminus. This allowed us to monitor the translocation of the N-terminus using protease mapping by looking for a shift in the molecular weight of the Pf3-b-P2 upon immunoprecipitation with Lep antiserum. In cells grown in the presence of arabinose, essentially all of the [^{35}S]-methionine-labeled Pf3-b-P2 is converted into a slightly shorter form in the 20 s pulse-label, indicated by the closed circle (see Figure 3A, right panel, Ara). In contrast, in YidC-deficient cells, very little of the shifted band is observed, while the full-length Pf3-b-P2 is detected (Figure 3A, right panel, Glc). Band X, a cytosolic control, was used as a negative control to confirm that the spheroplasts had not lysed. These studies show that YidC is required for efficient membrane insertion.

Next, we tested whether subunit c of the *E. coli* F_1F_0 ATP synthase depends on YidC for membrane insertion. Subunit c is a polytopic membrane protein that spans the membrane twice with both its N- and its C-termini facing the periplasmic space (*1*). To precipitate the radiolabeled protein, we added a 6-His affinity tag to its C-terminus (c-6his). To assess membrane integration, we radiolabeled wild-type (arabinose-grown) or YidC-depleted (glucose-grown) cells with [^{35}S]-methionine for 2 min and analyzed the cells by protease accessibility. When YidC is present, the majority of the c-6His is degraded by proteinase K and therefore is not precipitated with the His-tag antiserum (Figure 3B, Ara; compare −/+ PK lanes), establishing that the His-tag region (at the C-terminus) was translocated and proteolyzed (Figure 3B). In contrast, when YidC is depleted in the cell, c-6his is not proteolyzed (Figure 3B, Glc; compare −/+ PK lanes), indicating that its C-terminus requires YidC to insert across the membrane. We should note that the JS7131 strain

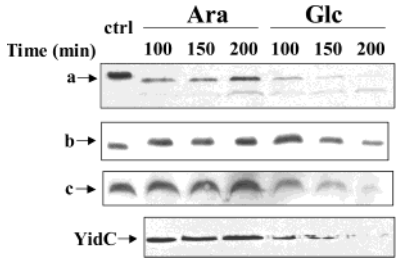

FIGURE 4: Steady-state membrane levels of F_0 sector subunits a and c are strongly reduced when YidC is depleted. JS7131 are grown for 100, 150, or 200 min in LB with either 0.2% arabinose or 0.2% glucose after a 1:50 back-dilution of an overnight culture containing 0.2% arabinose. IMVs were isolated, and the steady-state amount of F_0 sector subunits a, b, and c were determined by Western blots using antisera against subunits a, b, and c (*30*). Membrane proteins in the IMVs were normalized to the same protein concentration. The same volume of each sample was loaded. YidC in the IMVs was determined by Western blots using antisera against YidC. Solubilized membranes prepared from cells overproducing subunits a, b, and c were used as the source of the positive control for subunits a and b. Purified His-tagged subunit a, which runs slightly higher than the wild-type subunit a on the SDS polyacrylamide gel, was used as the positive control for subunit a on the Western blot.

overproducing the c-6His was somewhat sensitive to lysis either with or without YidC depletion.

Intracellular Membrane Levels of F_0 Subunits a and c Are Severely Reduced When YidC Is Depleted. Since the F_0 components subunits a, b, and c are dependent on YidC for membrane insertion, we tested whether the steady state levels of these components decreased in the membranes when YidC is depleted by Western blots (Figure 4). JS7131 was grown in LB media containing arabinose (0.2%) or glucose (0.2%) for 100, 150, or 200 min. Membrane vesicles were isolated and then analyzed for the chemical amounts of YidC and subunits a, b, and c by immunoblotting using antiserum directed against these proteins. Figure 4 shows that during the time of growth in glucose there is a large reduction in the amount of YidC in membrane vesicles, as compared to vesicles expressing YidC. Concurrently, there is a significant reduction in subunits a and c and a measurable reduction in subunit b. The position of subunits b and c was determined using solubilized membranes prepared from cells overproducing a, b, and c. For subunit a, we used a purified His-tagged subunit a, which runs slightly higher on the SDS−PAGE gel. The lower band below subunit a (Figure 4, top panel) serves as a loading control. Taken together, the immunoblot data as well as protease mapping data show that YidC plays an important role in membrane insertion as well as maintaining membrane levels of the F_0 sector subunits.

SecE Requires YidC for Membrane Insertion. Because the F_0 data indicate that there are endogenous proteins in *E. coli*, whose membrane insertion is highly dependent on YidC, we further investigated the YidC dependency by testing the membrane insertion of another membrane protein. Specifically, we tested the YidC dependency of *E. coli* SecE, a polytopic membrane protein that spans the membrane three times with its C-terminus in the periplasm (Figure 1) (*33*). SecE is an essential protein for all bacteria. Since the wild-type SecE was very resistant to proteinase K degradation, we extended the C-terminal tail by eight residues (SecE-tag; see Experimental Procedures) so we could observe a shift in the molecular weight when the C-terminal region of SecE is digested by externally added proteinase K. The SecE-tag complements the SecE depletion strain CM124 (data not shown), showing that the SecE-tag is functional and assumes the same membrane topology as the wild-type SecE protein. Figure 5A shows that the radiolabeled SecE-tag from cells that were pulsed for 2 min is efficiently inserted across the membrane when YidC is present; the full-length SecE was completely clipped to a lower molecular weight band by proteinase K treatment. However, the depletion of YidC prevented membrane insertion of the SecE, as no radiolabeled SecE-tag was degraded by the protease (Figure 5A, Glc; compare −/+ PK lanes). We also checked using a Western blot whether the steady state membrane levels of SecE are affected by YidC depletion. The membrane vesicles were isolated from JS7131 cells grown for various times under YidC expressing or YidC depletion conditions and were probed with SecE antiserum. Figure 5B shows there is a measurable reduction in the SecE levels (JS7131, lower panel, right side), confirming the strict YidC dependence for membrane insertion. However, even with this reduction in SecE, there is not a similar reduction in SecY levels, suggesting that the SecYEG translocase levels were not perturbed too significantly (Figure 5B, right side). This is consistent with the fact that export of proOmpA is not significantly affected when YidC is depleted at 37 °C (Figure 5B; ref *19*).

Since it is has been shown that decreasing SecE causes a decrease in SecY and other Sec components (*34*), we wanted to check at what level of reduction of SecE causes a decrease in SecY and an inhibition in protein export. Therefore, we analyzed the export of proOmpA in CM124, the SecE-depletion strain that requires arabinose for cell growth (*6*). As can be seen in Figure 5B, the precursor form OmpA starts accumulating at 60 min of growth of the SecE-depletion strain on glucose, and a significant block is observed at 90 min. This is in contrast to JS7131, the YidC depletion strain, where little effect on OmpA export is detected even after 200 min of growth in glucose. To examine the chemical amounts of SecE and SecY of the Sec translocase present in the membrane, inner membrane vesicles were isolated from CM124 at the 30, 60, and 90 min time points. All the samples for the Western blot prepared from the CM124 and JS7131 cells were normalized to be at the same protein concentration (2.3 mg/mL). The amounts of SecE and SecY in the IMVs prepared from CM124 and JS7131 are shown on the same immunoblot, and the position of SecE and SecY was confirmed using purified SecE and SecY. As can be seen in Figure 5B (lower panels), the amount of SecE in the IMVs at the 30 min time point of growth in glucose in the SecE-depletion strain CM124 is about the same as the 200 min point in the YidC-depletion strain JS7131. At this level, no proOmpA accumulated in either strain (Figure 5B, top panel). However, the amount of SecE is reduced further in the 60 and 90 min sample in CM124. At the 90 min time point, SecY levels appear to be reduced in the SecE-depletion strain consistent with inhibition in proOmpA export. From the comparison of SecE levels in CM124 and JS7131, we can see the depletion of YidC cannot cause SecE to reduce to the level at which OmpA translocation is inhibited (Figure 3B; 60 and 90 min glucose lanes). These data explain why depletion of YidC cannot result in the block of OmpA translocation at 37 °C. As a control, we show that the growth

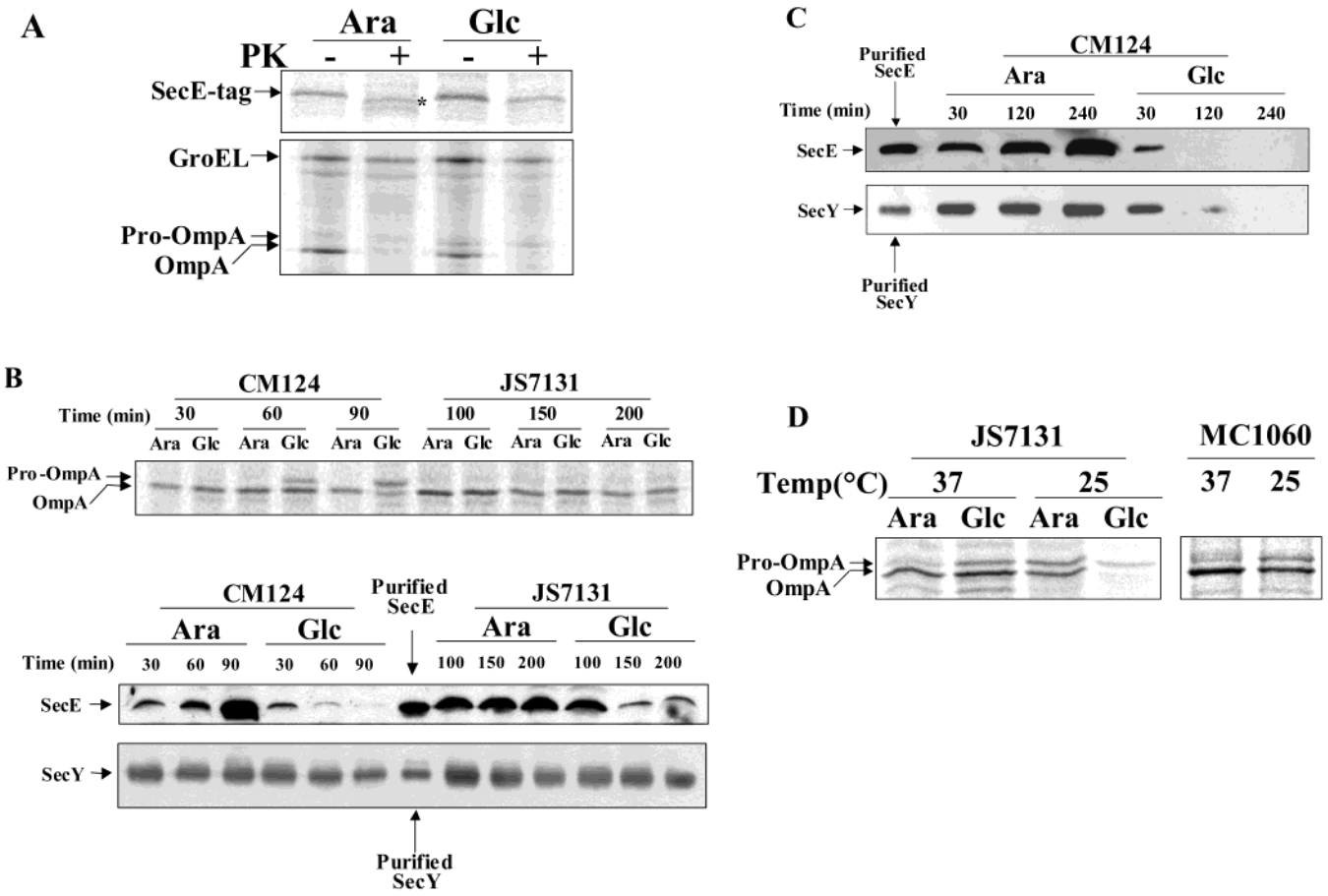

FIGURE 5: SecE is strictly dependent on YidC for membrane assembly. (A) Newly synthesized SecE with a short C-terminal tag is blocked in membrane insertion when YidC is depleted. *E. coli* JS7131 cells bearing a plasmid encoding SecE-tag were grown, pulse-labeled for 2 min with *trans*-[^{35}S] label, and analyzed for protease mapping, as described in the Experimental Procedures. The asterisk represents the SecE protein that has been degraded in the C-terminal tail region, leading to the shifted band. (B) The chemical levels of wild-type SecE are substantially reduced when YidC or SecE is depleted, in the JS7131 and CM124, respectively. The IMVs were isolated from CM124 and JS7131 grown in arabinose (0.2%, Ara) or glucose (0.2%, Glc) for the indicated times after a 1:50 dilution of an overnight culture with arabinose. The same normalized IMV protein concentration (2.3 mg/mL) was loaded onto the SDS−PAGE gel for the CM124 and JS7131 study. In addition, the two sets of samples from these two strains were analyzed on the same Western blot membrane, so that the Western blot conditions were the same for both sets of samples, and the level of SecE or SecY can be compared between the two strains. Purified SecE and SecY was used as a positive control. The top panel shows the effect of SecE and YidC depletion on processing of the outer membrane protein A precursor (proOmpA). CM124 or JS7131 cells grown for the indicated time in arabinose (0.2%) or glucose (0.2%) were pulse-labeled for 2 min with *trans*-[^{35}S] label. OmpA was immunoprecipitated using an antibody against OmpA. (C) The intracellular levels of SecE and SecY in IMVs prepared from CM124 cells that were grown in arabinose (0.2%) or glucose (0.2%) for the indicated times. The samples for immunoblot analysis were normalized and analyzed exactly as described in panel B. (D) The export of OmpA at 25 °C is strongly inhibited when cells are depleted of YidC. The JS7131 strain was grown in LB supplemented with arabinose (0.2%) (Ara) or glucose (0.2%) (Glc) at 37 °C. After growing the cells for 2.5 h at 37 °C (Ab_{600} of cells with arabinose, 0.65; Ab_{600} of cells with glucose, 0.4), the cells were switched to M9 media, and the growth continued at 37 °C for 0.5 h under arabinose or glucose conditions. Half of the cells were then shifted to 25 °C and grown for 0.5 h. The cultures were then radiolabeled with *trans*-[^{35}S] label for 20 s. Export of OmpA was analyzed by immunoprecipitation, and then subjected to SDS−PAGE, and phosphorimaging. As a control, the MC1060 strain with a wild-type *yidC* was grown at 37 or 25 °C and analyzed for export of OmpA, as described for the JS7131 strain.

of CM124, the SecE depletion strain, in glucose for 120 and 240 min causes a large reduction in SecE (Figure 5C, Glc) and also a large reduction in the SecY level (no SecE or SecY is observed at the 240 min time period), as SecY requires SecE for its stability.

Finally, it is well-known that a 40−50% reduction in the SecE level (by the *SecE501* promoter cold sensitive mutation) can result in a significant inhibition of protein export at cold temperatures as well as cold-sensitive growth (*35*, *36*). Therefore, we expect to see an effect on protein export in the YidC depletion strain at the cold temperature because we observe more than a 50% reduction of the Sec E level at the 200 min time point for JS7131 grown in glucose. Figure 5D shows that this is the case. Whereas processing of proOmpA is quite normal in JS7131 under YidC depleted conditions at 37 °C (37 °C, Glc), processing of proOmpA is completely abolished at 25 °C for glucose grown JS7131 cells (25 °C, Glc). Processing of proOmpA is nearly normal in MC1061 containing the wild-type YidC at 25 °C (MC1060, 25 panel) and processing of proOmpA is observed (greater than 50%) in the arabinose grown JS7131 strain at 25 °C (25, Glc). The delayed processing of the JS7131 strain at 25 °C in the presence of arabinose may be due to slightly decreased cellular levels of YidC in the JS7131 strain as compared to the wild-type strain (see ref *19*). Thus, our results show that protein export at 25 °C is strongly inhibited when YidC is depleted and is consistent both with the idea that SecE has been depleted by more than 50% and with the

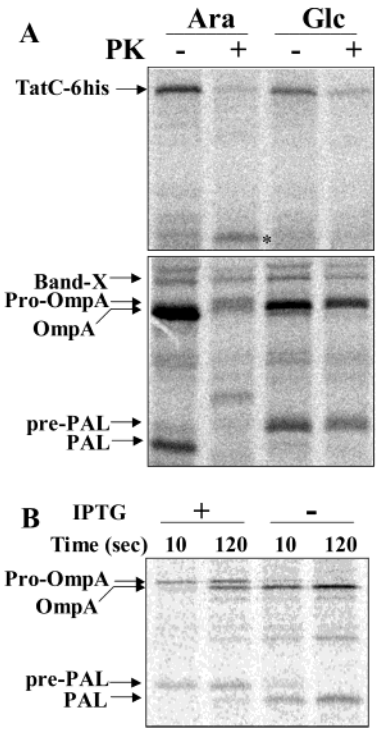

FIGURE 6: TatC insertion and protein export is enhanced by YidC. (A) JS7131 cells expressing TatC-6his were analyzed by protease mapping under conditions where cells were not depleted or depleted of YidC, as described in the Experimental Procedures. (B) The block in export of the precursor to OmpA and PAL is observed in YidC-depleted cells only when TatC is overexpressed. JS7131 cells bearing the pMS119 encoding TatC-6his were analyzed under a YidC depleted condition. The cells were treated with or without IPTG (1 mM) for 5 min prior to labeling with *trans*-[^{35}S] label for 20 s and chased with nonradioactive methionine for the indicated times.

idea that higher levels of the protein are needed for protein export at the lower temperatures than at higher temperatures (37 °C).

YidC Enhances TatC Insertion into the Membrane. Since we have observed that subunits a and c and SecE require YidC for insertion, we extended our investigation to another membrane protein. We then studied the insertion of TatC, which is one of the components of the bacterial Tat (twin arginine translocation) apparatus that is required for exporting folded proteins (*37*). It is also an integral membrane protein of the inner membrane and is predicted to span the membrane six times (*37*, *38*) (see Figure 1 for membrane topology). The insertion of TatC is only slightly inhibited when YidC is depleted (Figure 6). JS7131 cells synthesizing TatC with an appended C-terminal cytoplasmic 6-His tag were pulse-labeled and analyzed for insertion as before. When YidC is present in the cell membrane at wild-type levels, the majority of TatC-6his is inserted into the membrane and is digested by proteinase K producing a protease-resistant fragment with a lower molecular weight (Figure 6A, see asterisk). In contrast, there is a decrease in the amount of TatC-6his that inserts into the membrane when YidC is deficient in the cell. Figure 6A shows there is also an accumulation of the precursors to OmpA and the peptidoglycan-associated lipoprotein (PAL) when YidC is depleted. Figure 6B shows that the export block of pro-OmpA and Pre-PAL in YidC-deficient cells occurs only when IPTG is added to overexpress TatC (IPTG is added for 5 min). This indicates that the overexpressed TatC leads to the interference in protein export. This block in exported proteins such as OmpA and PAL was seen before when the membrane insertion of an overproduced Sec-dependent protein was analyzed under YidC depleted conditions (*22*). We believe this is due to the overproduced Sec-dependent protein causing a jamming of the Sec translocase under YidC-deficient conditions, thereby blocking protein export.

DISCUSSION

Previous studies showed that the Sec-independent M13 procoat and the Pf3 coat phage proteins are highly dependent on YidC for membrane insertion (*19*, *22*, *24*). In contrast, the Sec-dependent Lep and FtsQ are only slightly dependent on YidC for insertion into the membrane (*10*, *19*). This raised the question as to whether there are any nonphage proteins that are absolutely dependent on YidC for membrane protein assembly in *E. coli.*

In this paper, we report that YidC is strictly required for the membrane insertion of the F_0 sector membrane subunits a and c of the F_1F_0 ATP synthase and for SecE of the Sec translocase. Therefore, the physiological substrates of YidC include membrane components that fulfilll important cellular functions in *E. coli* for the generation of ATP and for protein translocation. It is very likely because of the strict in vivo requirement of YidC for the insertion of the membrane subunits of the F_1F_0 ATP synthase that YidC will be critical for the F_1F_0 ATP synthase activity. Indeed, the ATPase activity of the F_1F_0 ATP synthase is markedly lowered as YidC is depleted from the cellular membrane, as recently published (*39*). Thus, the essential role of YidC in *E. coli* could be due to the failure of assembling key membrane proteins or membrane protein complexes in the membrane, thereby preventing their functions in the cell and causing cell death.

The function of YidC is conserved for insertion of the F_0 sector subunits of the F_1F_0ATP synthase. For instance, in mitochondria, the YidC homologue Oxa1p is required for the F_1F_0 ATP synthase activity (*40*). Like the bacterial F_1F_0 ATP synthase, this ATP synthase also has three membrane protein subunits that are homologous to subunits a, b, and c. Oxa1p stimulates the insertion of the subunit a homolog, ATP6, in *Saccharomyces cerevisiae* (*41*) and is absolutely required for innermembrane integration of the subunit c counterpart called pSu9 in *Neurospora crassa* (*41*). Oxa1p is an essential protein in yeast (*42*), and inactivation of Oxa1p leads to respiratory deficiency (*43*). Moreover, the insertion of the Cox1p (*44*) and Cox2p (*45*, *46*) membrane subunits of cytochrome *c* oxidase is absolutely dependent on Oxa1p. These membrane protein insertion studies help explain why Oxa1p is required for the assembly of cytochrome *c* oxidase and the F_1F_0 ATP synthase (*40*). YidC is also required for the assembly of cytochrome *o* oxidase, the terminal respiratory chain complex, in *E. coli* (*39*).

The importance of our study is that it establishes that F_0 sector subunits and SecE are physiological substrates of YidC. YidC is strictly required for these endogenous membrane proteins. In a recent study by Froederberg et al. (*47*), the YidC dependence was examined using artificial protein constructs such as inverted leader peptidase, M13 phage procoat mutants, and various one transmembrane

ProW derivatives. They showed that the overall requirement of SecYEG or YidC varied for these tested model proteins and that small changes within the membrane protein can influence the assembly requirements. In our study, we show that the F_0 ATP synthase wild-type subunit a as well as subunit c and SecE that have small tags (of less than nine amino acids) were strictly dependent on YidC. Moreover, there is a correlation between the inhibition in the membrane insertion of the tagged subunit c and SecE (when YidC is depleted) with the decreased steady state membrane levels of the wild-type subunit c and SecE (without tags), strongly suggesting that the small tags added onto subunit c and SecE are not influencing the YidC dependency. It should be noted that in contrast to our results, Van Der Laan et al. reported that the level of SecE in the membrane does not decrease when YidC is depleted (*39*). However, our data from two perspectives, namely, the protease-mapping data (Figure 5A) and the Western blot data (Figure 5B), suggest that SecE insertion is dependent on YidC.

In line with our results reported here showing that the *E. coli* SecE protein requires YidC for insertion (Figure 5), we recently showed that the chloroplast SecE, when expressed in *E. coli*, is absolutely dependent on YidC for insertion (*48*). The chloroplast SecE is functional in *E. coli* as it can complement the SecE-depletion strain (*49*). Remarkably, the chloroplast YidC homolog, Alb3, which was shown to mediate insertion of several chloroplast proteins into the thylakoid membrane (*50*, *51*), could replace YidC in *E. coli* and was absolutely essential for insertion of the chloroplast SecE into the membrane (*48*). However, the role of YidC/Alb3 for SecE insertion is not conserved in the chloroplast, as SecE can insert into the thylakoid membrane by an Alb3-independent mechanism (*48*, *52*).

The requirement of YidC for SecE membrane insertion is consistent with a YidC depletion effect, albeit small, on Sec-dependent protein export, which is an indirect consequence of decreasing the SecE levels. This may explain why exported proteins such as OmpA, maltose binding protein, and β-lactamase are slightly affected when YidC is depleted. Also consistent with the finding that a 50% reduction in the SecE levels can cause a dramatic inhibition in protein export at cold temperatures (*35*, *36*), we found that protein export is strongly inhibited at the cold temperature (Figure 5D) when YidC is depleted where the SecE levels are reduced below 50% of wild-type levels.

It would be expected that the failure of SecE to insert properly into the membrane after YidC depletion (Figure 5B) would reduce the intracellular membrane levels of the SecYEG translocase given that SecY is degraded in the absence of SecE (*34*, *53*) and cause a profound inhibitory effect on protein export. We resolve this puzzle because the levels of SecE under YidC depletion conditions are still high enough to support the function of the Sec translocase (Figure 5B). We find that the reduction of SecE does not result in a significant depletion in SecY (Figure 5B) in the YidC depletion strain. In contrast, a much larger reduction in the SecE levels are observed in the SecE-depletion strain CM124 (Figure 5B), and a large decrease in SecY levels are observed at the 120 min time point (Figure 5C). A defect in protein export of the outer membrane precursor protein proOmpA is observed at 90 min of depletion of SecE in the CM124 strain (Figure 5B).

While there is an absolute YidC requirement for membrane insertion of subunits a and c of the F_1F_0 ATP synthase and SecE, YidC is not essential for TatC membrane insertion (Figure 6). However, YidC stimulates insertion and prevents the jamming of the Sec translocase by the overproduced TatC. Jamming was only observed when YidC is depleted and when TatC is overproduced.

In conclusion, we demonstrate that the physiological substrates of YidC include subunits a and c of the F_1F_0ATP synthase and SecE of the SecYEG translocase. We also show that protein export of OmpA is severely inhibited at cold temperatures when YidC is depleted, most likely because of the decreased SecE levels in the membrane.

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