YidC, an assembly site for polytopic *Escherichia coli* membrane proteins located in immediate proximity to the SecYE translocon and lipids

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Like its mitochondrial homolog Oxa1p, the inner membrane protein YidC of *Escherichia coli* is involved in the integration of membrane proteins. We have analyzed individual insertion steps of the polytopic *E. coli* membrane protein MtlA targeted as ribosome-nascent chain complexes to inner membrane vesicles. YidC can accommodate at least the first two transmembrane segments of MtlA at the protein lipid interface and retain them even though the length of the nascent chain would amply allow insertion into membrane lipids. An even longer insertion intermediate of MtlA is described that still has the first transmembrane helix bound to YidC while the third contacts SecE and YidC during integration. Our findings suggest that YidC forms a contiguous integration unit with the SecYE translocon and functions as an assembly site for polytopic membrane proteins mediating the formation of helix bundles prior to their release into the membrane lipids.

INTRODUCTION

The diverse molecular strategies that the Gram-negative bacterium *Escherichia coli* uses to target and insert proteins into the cytoplasmic (also termed inner) membrane have recently become evident in more detail. An inner membrane protein requires only the signal recognition particle (SRP) and its receptor for membrane targeting (MacFarlane and Müller, 1995; de Gier *et al.*, 1996; Seluanov and Bibi, 1997; Ulbrandt *et al.*, 1997; Koch *et al.*, 1999), unless it possesses large hydrophilic

domains whose translocation across the membrane renders it dependent also on SecA (Scotti *et al.*, 1999; Neumann-Haefelin *et al.*, 2000). The bacterial SRP, consisting of a protein termed Ffh or P48 and the 4.5S RNA, selectively recognizes inner membrane proteins via their long hydrophobic transmembrane segments (TMs) (Valent *et al.*, 1997; de Gier *et al.*, 1998; Beck *et al.*, 2000). Ribosome-nascent chain-complexes (RNCs) bound by SRP are then targeted via FtsY, the bacterial homolog of the SRP receptor, to the SecY translocon (Valent *et al.*, 1998; Beck *et al.*, 2000).

In addition, for a few inner membrane proteins harboring only one or two TMs, a participation of YidC in the insertion process has been shown (Houben *et al.*, 2000; Samuelson *et al.*, 2000; Scotti *et al.*, 2000). YidC is the *E. coli* homolog of Oxa1p from yeast which mediates insertion of nuclear and mitochondrially encoded inner mitochondrial membrane proteins (He and Fox, 1997; Hell *et al.*, 1998). Likewise, Albino3 which is a homolog of Oxa1p/YidC found in plant chloroplasts is required for the integration of membrane proteins into thylakoid membranes (Moore *et al.*, 2000). Here, we have examined the polytopic inner membrane protein, mannitol permease (MtlA), of *E. coli* with respect to a possible involvement of YidC in its membrane assembly. MtlA is predicted to span the cytoplasmic membrane of *E. coli* with six α -helical TMs following a 24 amino acid-long N-terminus located in the cytoplasm (Sugiyama *et al.*, 1991).

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RESULTS

Simultaneous contact between YidC and two neighboring TMs of a polytopic membrane protein

Recognition of MtlA by Ffh at the ribosome is shown in Figure 1. A 130 amino acid-long ribosome-associated chain of MtlA carrying a photoactivatable derivative of phenylalanine (Tmd-Phe) at position 37 within its first TM, is cross-linked by UV-irradiation to a 50 kDa protein (lanes 1 and 2) which is recognized by anti-Ffh antibodies (lane 3). When nascent Tmd-Phe 37-MtlA130, however, is synthesized in the presence of inside-out inner membrane vesicles (INV), cross-links to Ffh are lost (lane 6) in favor of a previously unidentified (Beck et al., 2000) adduct to an ~60 kDa membrane protein (lanes 4 and 5). This protein cross-reacts with antibodies directed towards YidC (lane 7). Using the chemical cross-linker DSS, RNCs of MtlA instead are found in close proximity to SecY (Beck et al., 2000).

The results shown in Figure 1 indicate that ribosome-associated MtlA130 has inserted into the membrane of INV at least with its first TM. Since MtlA130 is sufficiently long to also allow for integration of its second TM, we placed Tmd-Phe at various positions of both TMs to more systematically probe for their nearest neighbors. Figure 2A shows the prominent cross-link again between Tmd-Phe at position 37 of MtlA130 and YidC (lanes 14 and 15 compared with the control devoid of any Tmd-Phe in lanes 2 and 3). Remarkably, a similar, strong cross-link is obtained with Tmd-Phe at position 56 located in the second TM (lanes 18 and 19). Thus at an intermediate step of insertion represented by membrane-targeted MtlA130, both TMs are simultaneously in contact with YidC (Figure 2, left-hand scheme). Accordingly, amino acid 64 within the second TM is also found to be in contact with YidC (lanes 22 and 23) as are amino acids 27 and 32 of the first TM (lanes 6, 7 and 10,11). In general, the cross-links to YidC disappear after treatment of RNCs with puromycin, i.e. if MtlA130 is released from the ribosome (lanes 8, 12, 16, 20 and 24).

In addition to forming cross-links with YidC upon UV-irradiation, Tmd-Phe at all positions of MtlA130 tested also gives rise to adducts which are slightly bigger than MtlA130 itself (asterisks in the lower panel of Figure 2A, compared with each preceding lane showing non-irradiated samples). Most likely they represent cross-links of MtlA130 to lipids because their intensity decreases upon treatment with phospholipase A₂ (shown for Tmd-Phe27 in lanes 26, 27). As such they were expected to be preserved after release of MtlA130 from the ribosome which, however, is less clearly seen due to a poorer resolution by SDS-PAGE following treatment with puromycin (lanes 8, 12, 16, 20, 24, 29 and 30).

Collectively, the cross-links shown in Figure 2A demonstrate that both TMs of membrane-targeted MtlA130 RNCs are in contact with YidC and membrane lipids (left-hand scheme of Figure 2). When the various cross-links obtained for MtlA130 were quantified, the five selected positions of Tmd-Phe, however, turned out not to be sufficient to deduce a clear turn-specific interaction of the two helix surfaces with either YidC or lipids.

Fig. 1. Nascent chains of the inner membrane protein MtlA are targeted to YidC. A 130 amino acid-long nascent chain of MtlA carrying Tmd-Phe, a photoactivatable derivative of phenylalanine, at position 37 within the first transmembrane helix (Tmd-Phe 37-MtlA130) was synthesized by a reconstituted *in vitro* transcription/translation system made from *E. coli*. Newly synthesized proteins were resolved by SDS-PAGE and displayed by phosphoimaging. The position of MtlA130 (double bands) is indicated relative to those of molecular mass standards. Synthesis occurred in the absence or presence of INV as indicated. Ffh was added to all reactions. UV, irradiation to initiate cross-linking. Cross-links to Ffh (x) and YidC (arrowhead) are indicated. IPα, immunoprecipitation using antibodies against Ffh and YidC.

Insertion of a polytopic membrane protein stalls at YidC

In order to follow the association of the integrating MtlA chain with YidC in a more stage-dependent manner, we analyzed nascent chains that were shorter or longer than MtlA130, in which case about 30 amino acids lie between the exit site of the ribosome and the second TM (compare left-hand scheme in Figure 2). If this distance is shortened so that the second TM has just emerged from the ribosome (right-hand scheme in Figure 2), cross-linking analysis discloses no contact between amino acid 64 of the second TM and YidC (Figure 2B, lanes 22 and 23), and at best an extremely weak one for amino acid 56 (lanes 18 and 19). In contrast, cross-links between YidC and the first TM of the shorter MtlA chain are clearly detectable (Figure 2B, lanes 6, 7; 10, 11; 14, 15). At this length of nascent MtlA, the second TM obviously has not yet reached, or might have just started to reach, YidC. Although being less discrete than with MtlA130, presumed cross-links between nascent MtlA102 and lipids can be detected for all five positions of Tmd-Phe, both before and after treatment with puromycin (Figure 2B, asterisks, compared

Fig. 2. Stage-dependent contacts between nascent MtlA and YidC. Ribosome-associated MtlA chains of 130 (A) and 102 (B) amino acids were synthesized *in vitro* in the presence of INV. The positions of Tmd-Phe incorporated within the first two TMs of MtlA by *in vitro* suppression of the respective stop codon mutants are indicated on top of the panels (WT, wild type). The location of various amino acids within nascent MtlA is illustrated in the schemes. Puromycin was added as specified. UV-generated crosslinks are marked with an arrowhead (YidC) or asterisks (lipids). It is not clear why Tmd-Phe at position 27 gave rise to more than one YidC-cross-link. The two panels each of (A) and (B) are derived from the same gel with the intervening parts excised. The positions of MtIA130 and MtIA102 are indicated (note the slight increases in molecular mass due to covalent attachment of puromycin). The top right panel illustrates the decrease of lipid cross-links (asterisks) following digestion with phospholipase A₂ (PLA₂). PM, puromycin. The schemes depict ribosomes docked at the SecYE translocon (light grey ellipses) and the TMs of MtlA (black boxes) associated with YidC (dark grey ellipse) and membrane lipids.

with non-irradiated controls). Contacts between the second TM of MtIA102 and lipids would suggest that lipids might have access to the translocon even before a TM has fully reached YidC (Figure 2, right-hand scheme).

Next, nascent chains of MtlA of 189 amino acids were examined. As illustrated in the scheme in Figure 3, elongation-arrested MtlA 189 just exposes the third TM of MtlA outside of the ribosome. Even with the length of 189 amino acids, the first TM remains in contact with YidC (Figure 3, lanes 6 and 8). Surprisingly, Tmd-Phe placed at position 144 within the third TM of MtlA also cross-links to YidC (lanes 14, 16) indicating that a signal-anchor-type TM very early during its insertion gains access to YidC (compare scheme in Figure 3). If, however, Tmd-Phe is placed only three amino acids further downstream within the third TM of MtlA189, a smaller adduct of about 32 kDa (lane 21) is obtained. Co-immunoprecipitation identifies this cross-linking protein as SecE (lane 22). Although radioactive material of about the same molecular mass appears in almost every lane in Figure 3,

only the UV-generated adduct of MtlA189 carrying Tmd-Phe at position 147 was recognized by anti-SecE antibodies (compare lanes 3, 7, 11, 15 and 19 to 22).

DISCUSSION

We have analyzed various stages in the membrane assembly of a polytopic inner membrane protein of *E. coli*. Provided that ~30 amino acids are buried in the large ribosomal subunit, nascent MtlA102 would just allow the first pair of TMs to insert into the membrane (right-hand scheme in Figure 2). In this situation, only the first TM contacts YidC in what seems by cross-linking analysis to be an intimate association. However, when the nascent chain has grown such that the second TM has gained more lateral mobility it also moves into close proximity to YidC as reflected by the substantial cross-links to YidC now obtained (Figure 2A and B, lanes 18, 19 and 22, 23).

Notably, molecular proximity between membrane proteins of *E. coli* and YidC has so far become visible only by using Tmd-Phe

Fig. 3. Membrane insertion of MtdA occurring between SecE and YidC. 189 amino acid-long nascent chains of MtdA were synthesized *in vitro*. Tmd-Phe was incorporated at postion 37 within the first TM and at postions 144 and 147 within the third TM. Experimental details are outlined in the legends to the preceding figures. Note that in the absence of INV, the first TM (lanes 2 and 4) and the third TM (lanes 10, 12 and 18; immunoprecipitation not shown in the latter case) are recognized by Ffh. The scheme illustrates a possible insertion intermediate of a ribosome-associated nascent chain of 189 amino acids. There is no experimental evidence for a loop-like conformation of the third TM, extending from amino acids 135 to 154, nor for a cytosolic exposure of the loop between TM2 and TM3 during insertion.

as a cross-linker as opposed to cross-linkers with longer spacer arms (e.g. DSS). Vice versa, using Tmd-Phe as cross-linking probe we have observed contacts between a TM and SecE only for a single position of the photoprobe and so far not obtained any with SecY. One possible explanation would be that the aqueous pore, which the SecYE translocon, like its eukaryotic homolog Sec61, is thought to form (Simon and Blobel, 1992; Hamman *et al.*, 1997), leads to a rapid quenching of the UV-generated carbene by water molecules. If the different type of cross-links obtained between a TM and YidC as opposed to SecYE in fact reflects a more intimate contact with YidC probably excluding water molecules, it might suggest that YidC functions in providing an amphipathic environment for a TM on its transition from the bona fide channel-like translocon into the lipophilic core of the bilayer. This is consistent with the conclusions drawn from previous analyses of different membrane proteins (Samuelson *et al.*, 2000; Scotti *et al.*, 2000).

Quite different from recent results obtained with a eukaryotic single-spanning membrane protein (Heinrich *et al.*, 2000), the contact between the first TM of MtlA and YidC is maintained even in a situation where a long cytoplasmic loop of >60 amino acids spaced between TM2 and TM3 of MtlA189 would create

ample freedom for the first pair of helices to leave YidC and insert into the membrane lipids (Figure 3, scheme). In the present study, release from YidC has only been achieved by removal of the ribosome from the nascent membrane protein. Thus, in addition to providing amphipathic surfaces for individual TMs, YidC seems to function as an assembly site for multispanning membrane proteins. As such it might be required to first position adjacent helices in a manner such that they face their hydrophilic residues, thereby protecting them from the hydrophobic surface of the lipid core. At least in the case of MtIA studied here, this preassembly process seems to encompass more than only one pair of TMs. Based on experiments involving urea extraction of nascent P-glycoprotein chains, which is a polytopic protein of eukaryotic origin, it was also concluded that TMs first assemble within the translocation apparatus prior to membrane integration (Borel and Simon, 1996). Thus it seems likely that the mechanism of membrane integration of proteins harboring one and several TMs is distinctly different.

With a distinct amino acid (147), the signal-anchor-type TM3 of MtlA189 was found here to insert into the membrane in close vicinity to SecE. Whether or not this finding reflects a prominent function of SecE in the integration process cannot be answered

without having systematically probed the molecular environment of every amino acid present in this TM. Contacts between the mammalian homolog of SecE, Sec61 γ and the TM of a single-spanning membrane protein, however, have recently also been described (Heinrich *et al.*, 2000). In the experiment summarized in Figure 3, amino acid 147, more proximally located to the ribosome, is found close to SecE while the more distant amino acid 144 is already adjacent to YidC. Pending a complete Tmd-Phe scan of this TM, this finding nevertheless suggests that insertion of the third TM of MtlA189 proceeds via the SecYE translocon towards YidC. In addition, cross-linking of one and the same TM to both SecE and YidC at a mere distance of three amino acids can be taken as indication for YidC and SecYE being immediately juxtaposed when engaged in integration of a signal-anchor-type TM. Such an intimate contact between both components is also consistent with the reported copurification of YidC with the SecYEG complex (Scotti *et al.*, 2000). On the other hand, small phage proteins escaping targeting by SRP or SecA have recently been reported to directly insert via YidC into the membrane (Samuelson *et al.*, 2000). It therefore seems possible that membrane proteins can be targeted to YidC in different ways.

METHODS

Oligodeoxynucleotide-directed synthesis of elongation-arrested MtlA chains of 130 and 189 amino acids in length by programming a reconstituted transcription/translation system prepared from *E. coli* with plasmid p717mtlA-B, introducing TAG stop codons by site-directed mutagenesis, incorporation of Tmd-Phe (L-4'-[3-(trifluoromethyl)-3H-diazirin-3-yl] phenylalanine), preparation of INV, immunoprecipitation on 4-fold scaled-up reactions, and treatment with puromycin were performed as described recently (Beck *et al.*, 2000). Preparation of Ffh has been detailed in Koch *et al.* (1999). Synthesis of MtlA 102 in the presence of exogenous RNaseH was achieved by addition of 5'-GCC-GCCCAGCGGACCTGCAA-3'. Antibodies against SecE were raised against the peptide NH₂-KGKATVAFAREARTEVRKC-COOH coupled to KLH. Treatment with phospholipase A₂ was performed essentially as described (Beck *et al.*, 2000) using 5 U of the enzyme and incubating for 15 min at 42°C.

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