DECRYPTING THE ROLE OF YIDC IN BACTERIAL MEMBRANE PROTEIN INSERTION

DISSERTATION

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Membranes contain proteins that catalyze a variety of reactions, which lead to the selective permeability of the membrane. For membrane proteins to function as receptors, transporters, channels, and ATPases, they must be targeted to their correct membrane and inserted into the lipid bilayer. The Sec complex (SecYEG/SecDFYajC) is the major translocase to mediate the protein membrane insertion in bacteria, but there also exists some proteins whose membrane insertion is independent of the Sec complex. What protein components mediate the Sec-independent protein membrane insertion was a mystery. In year 2000, a new membrane component called YidC was discovered that is essential for insertion of proteins into membranes in bacteria, especially for the Sec-independent proteins. Chapter 1 of this dissertation is an in-depth review of what is known about YidC in membrane protein assembly. YidC depletion strain has been constructed to study the role of YidC in bacterial cells. Depletion of YidC from the cells interferes with the insertion of membrane proteins that insert both dependent and independent of the Sec complex. YidC directly interacts with membrane proteins during the membrane protein insertion process and assists in the folding of the hydrophobic regions into the membrane bilayer. The chloroplast and bacterial YidC homologs are truly functional homologs because the chloroplast homolog Alb3 functionally complements the bacterial cells in which YidC is depleted.
In order to study whether YidC is directly involved in the protein membrane insertion, site-specific photocrosslinking was applied. In Chapter 2, I describe the site-specific photocrosslinking system that was successfully set up to study membrane insertion. In this system, the ligation of the amber suppression tRNA with the photocrosslinker amino acid (Tmd)Phe is critical. The choice of proper T4 RNA ligase (T4 RNA ligase from Boehringer Mannheim) was important to obtain the ligation with high efficiency, which resulted in good incorporation of (Tmd)Phe into the nascent polypeptide. Using the photocrosslinking system, we found YidC interacts with the first transmembrane domain of leader peptidase during its membrane insertion. More photocrosslinking products were found when the overexpressed level of YidC was present in the membrane, which supports the idea of YidC not being present as an integral, stoichiometric member of the Sec complex. These photocrosslinking data combined with the \textit{in vivo} depletion study of YidC (Jim Samuelson’s dissertation, 2000) provide strong evidence to support YidC is a newly identified translocase component in bacteria.

In Chapter 3, I report that the Sec-independent Pf3 coat protein requires the YidC protein specifically for the membrane translocation step. Using photocrosslinking techniques and ribosome-bound Pf3 coat derivatives with an extended carboxyl-terminal region, we find that the transmembrane region of the Pf3 coat protein physically interacts with YidC and the bacterial signal recognition particle Ffh component. We also find that in the insertion pathway, Pf3 coat interacts strongly with YidC only after its transmembrane segment is fully exposed outside the ribosome tunnel. Interaction between Pf3 coat and YidC occurs
even in the absence of the proton motive force and with Pf3 coat mutants that are defective for membrane insertion. Our study demonstrates that YidC can directly interact with a Sec-independent membrane protein and its role is at the stage of folding the Pf3 protein into a transmembrane configuration.

In Chapter 4, conditional lethal YidC mutants (temperature-sensitive or cold-sensitive mutants) have been isolated to decipher the role of YidC in the assembly of Sec-dependent and Sec-independent membrane proteins. We found incorporation of site-specific protease sites into YidC results in YidC temperature-sensitive (ts) or cold-sensitive (cs) mutants. YidC ts and cs strains have been constructed by replacing the wild type YidC gene in the E. coli chromosome with the YidC ts or cs mutants, respectively. We now show that the membrane insertion of the Sec-independent M13 procoat protein is inhibited in a YidC ts strain when the cells were grown at the non-permissive temperature (42 °C) for a short time (20 minutes). This provides the strongest evidence thus far that YidC plays a direct role in the insertion of the Sec-independent M13 procoat protein. Strikingly, using the cs YidC strain, we find the insertion of the Sec-dependent leader peptidase is inhibited at the non-permissive temperature (25 °C), whereas the insertion of the M13 procoat protein is nearly normal. The cs YidC mutant shows a reduced interaction with the SecDFYajC complex, one important component of Sec machinery. These data suggest that the cold-sensitive YidC mutant is blocked in the Sec-related function, while its activity for inserting procoat is functioning almost normally. These properties of the cold-sensitive mutant strongly support the idea that YidC can function in concert with the Sec machinery and on its own; the Sec-interacting domain is
most likely impaired in the cold-sensitive YidC mutant, but has a functional membrane
insertase domain that promotes insertion for both pathways.
Dedicated to my parents and my wife
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1.1 Introduction

Membrane proteins and exported proteins play vital roles for the cell. They participate in important processes for cell viability such as photosynthesis and oxidative phosphorylation as well as production of ATP using the energy of the transmembrane proton motive force. In order to function properly, membrane proteins must assemble into the membrane with their correct structure intact, and exported proteins must translocate across the membrane, and be delivered to appropriate destinations. Assembly of membrane proteins into membrane and translocation of exported proteins across the membrane require protein catalyst. Basically, the membrane protein assembly or protein translocation can be divided into two steps. The first step is protein targeting onto the...
membrane. In this step, signal recognition particle (SRP), molecular chaperone SecB, or the molecular motor SecA may be required. The second step is membrane protein inserting into the membrane or export protein translocating across the membrane, in which translocases such as Sec 61 or SecYEG complex are involved.

1.2 Protein membrane assembly in the endoplasmic reticulum (ER) and protein translocation across ER membrane.

In eukaryotic cells, signal recognition particle (SRP) targets membrane proteins and export proteins to the ER membrane (Figure 1.1). SRP, comprised of 6 protein subunits (SRP54, SRP68, SRP72, SRP19, SRP14, and SRP9) and a 7S RNA component (Walter and Blobel, 1980; Walter and Blobel, 1982) interacts with the hydrophobic domain of a membrane protein nascent chain (High and Dobberstein, 1991). SRP targets the nascent membrane protein-ribosome-mRNA complex to the membrane by interacting with the membrane-bound SRP receptor (SRα and SRβ) (Gilmore et al., 1982; Meyer and Dobberstein, 1980; Tajima et al., 1986). After targeting, the Sec61 translocon, the central component of the translocation machinery in ER, catalyze the integration of proteins into the ER membrane or catalyze the translocation of exported protein across the membrane (Figure 1.1 and 1.2a). The Sec61 translocon is comprised of the Sec61 α, β and γ subunits (Gorlich et al., 1992a; Gorlich et al., 1992b), and these subunits form a heterooligomeric channel (hanein et al 1996), the diameter of which is estimated to be 4 ~ 6 nm. The translocation associated membrane protein (TRAM), another component of the translocation machinery, has been proposed to help protein being inserted into the Sec61
translocon to laterally move out of the translocon channel and integrate into the lipid bilayer of the membrane.

1.3 Protein export and protein membrane assembly and in bacteria.

In bacteria, export proteins can be targeted onto membrane either co- or post-translationally (Powers and Walter, 1997; Randall, 1983). Co-translational targeting is the process in which a preprotein is targeted onto membrane as a ribosome-bound nascent polypeptide. In this process, bacterial SRP and SRP receptor (FtsY) are involved to help nascent protein target to the bacterial translocase, SecYEG translocon, on the inner membrane. The bacterial SRP consists of the 54 kD homolog Ffh and 4.5S RNA (Bernstein et al., 1989; Poritz et al., 1990). The SRP receptor, FtsY (Romisch et al., 1989), is the α subunit homolog SRP receptor in eukaryotic cells. Post-translational targeting refers to the process that a preprotein is first completely synthesized and then targeted onto the membrane. In the post-translational targeting process, SecB, a cytosolic chaperone, binds to the preprotein, keeps it in unfolded state (Kumamoto and Francetic, 1993). The SecB-preprotein complex is then targeted to SecYEG translocon via SecA protein, which is an ATPase and is proposed to function as a molecular motor to promote the polypeptide chain of the preprotein to enter the SecYEG translocase channel (Lill et al., 1989).

In the SecYEG translocase, SecY and SecE are homologous to Sec61 α and Sec61 γ, respectively (Gorlich et al., 1992b; Hartmann et al., 1994), while SecG does not show
any homology with Sec61β. Only SecY and E are essential for protein translocation. SecY, SecE and SecA are sufficient to promote protein translocation in vitro (Brundage et al., 1992). Although not essential, SecG can increase the protein translocation efficiency (Nishiyama et al., 1996). Like Sec61 complex, Sec YEG complex was revealed by electron microscopy to form a channel-like structure (Manting et al., 2000). In this structure, SecYEG is tetrameric. Recently, Bessonneau et al. (2002) provide evidence that SecYEG complex can exist in multimeric forms, but only the dimeric form is responsible for preprotein translocation (Bessonneau et al., 2002). Besides the SecYEG complex in bacterial translocation machinery, there is another complex, SecDFYajC. This complex can facilitate protein translocation by stimulating SecA cycling (Duong and Wickner, 1997; Economou et al., 1995; Pogliano and Beckwith, 1994).

For many bacterial membrane proteins, the SRP and FtsY play a critical role in targeting them onto membrane (de Gier et al., 1996; Macfarlane and Muller, 1995; Seluanov and Bibi, 1997; Ulbrandt et al., 1997), while some of membrane proteins were found to be SRP/FtsY-independent in their targeting (Figure 1.3). In the SRP/FtsY-dependent targeting pathway, SRP and FtsY delivers membrane proteins to SecYEG translocon, and SecA may be involved in the process (Neumann-Haefelin et al., 2000). After membrane protein targeting onto the membrane, SecYEG complex is the major translocase to mediate protein membrane insertion. These proteins that require SecYEG for membrane insertion are called Sec-dependent proteins. There also exist some proteins, such as Pf3 coat and M13 procoat proteins, which do not use SecYEG for their membrane insertion.
These proteins are called Sec-independent proteins (Rohrer and Kuhn, 1990; Wolfe et al., 1985).

What translocase mediates Sec-independent membrane insertion? Is it a spontaneous (without translocase) process? In year 2000, a protein named YidC was found to mediate such Sec-independent membrane insertion (Samuelson et al., 2000; Stuart and Neupert, 2000) (Figure 1.3 and 1.2b). YidC was also found to be involved in Sec-dependent membrane insertion, in which YidC may plays a role similar to TRAM in eukaryotic cells (to move the transmembrane domains of membrane proteins laterally out of the translocase tunnel and into lipid bilayer) (Figure 1.2c).

1.4 Oxa1p homologs are found in mitochondria, chloroplast, and bacteria.

The first clue that YidC could possibly be involved in the membrane insertion of bacterial proteins came from studies in the mitochondrial field investigating the membrane protein insertion into the mitochondrial inner membrane. The conservative pathway inserts proteins into the mitochondrial inner membrane from the matrix compartment in a manner dependent on the Oxa1p protein (Dalbey and Kuhn, 2000; Tokatlidis and Schatz, 1999). Proteins requiring Oxa1p for insertion are found to be either nuclear encoded (Hell et al., 1997; Hell et al., 1998) or mitochondrial encoded (Hell et al., 2001). Both types of proteins are inserted into the mitochondrial inner membrane from the matrix, although the nuclear encoded proteins are first imported from the cytoplasm. This finding was exciting because Oxa1p homologs called YidC and Alb3, respectively, exist
in both bacteria and chloroplasts (Figure 1.4). The Oxa1p proteins appear to be evolutionarily conserved. Whereas the mitochondrial Oxa1p and chloroplast Alb3 spans the membrane five times (Sundberg et al., 1997), the bacterial YidC spans the membrane six times with a large periplasmic domain (Figure 1.4; (Saaf et al., 1998)); the sequence homology among the homologs is found primarily within the C-terminal transmembrane regions.

1.5 YidC can function distinct of the Sec-translocase

To investigate the physiological role of YidC in membrane protein assembly in bacteria, a bacterial strain was constructed in which the expression of YidC is dependent on arabinose (Samuelson et al., 2000). The first protein that was examined for YidC dependence for membrane insertion was the M13 phage coat protein. The protein was known to insert into the membrane via a Sec-independent mechanism (Wolfe et al., 1985) and was believed to insert into the membrane by a spontaneous mechanism (Geller and Wickner, 1985; Soekarjo et al., 1996). The M13 coat protein is synthesized in a precursor form called procoat with an amino-terminal signal peptide (Figure 1.5). When cells are expressing YidC, M13 procoat is rapidly inserted and converted to the M13 coat protein by signal peptidase cleavage. In contrast, when YidC is diluted out by growth of the YidC-regulated strain in the absence of arabinose, procoat cannot insert into the inner membrane and is not processed by signal peptidase (Samuelson et al., 2000). Surprisingly, these studies proved that in vivo the M13 procoat protein does not insert spontaneously as was once thought; it requires the protein component YidC.
In addition to M13 phage procoat protein requiring YidC, YidC is also required for membrane insertion of the single-spanning Sec-independent Pf3 coat protein (Figure 1.5). The N-terminal tail region of Pf3 coat is inefficiently inserted across the membrane to the periplasmic space when YidC is depleted from the membrane (Chen et al., 2002). These studies are consistent with YidC playing an important insertion role, but it is possible that YidC does not play a direct role because depletion of YidC in the cell takes a long time and may have an indirect effect resulting in a block in membrane insertion. Therefore, the truncated mRNA/photocrosslinking approach recently used to study membrane insertion in bacteria (Beck et al., 2001b; Houben et al., 2000; Samuelson et al., 2000; Scotti et al., 2000; Urbanus et al., 2001) was employed out to prove that YidC interacts with the Pf3 coat membrane protein (Chen et al., 2002, and Chapter 3). Using this approach, membrane insertion intermediates can be generated (Gilmore et al., 1991) where the protein is trapped in the process of membrane insertion and a photocrosslinker (L-4’-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenylalanine, Tmd-Phe) is incorporated (Brunner, 1996) into the hydrophobic region of Pf3 coat at various positions. Shining ultraviolet light on the protein sample activates the photocrosslinker to create a very reactive carbene, which attacks nearby associated proteins. Ultraviolet radiation results in photocrosslinking of Pf3 coat nascent chains with YidC, demonstrating that YidC is directly involved in the membrane insertion step.

The function of YidC for Sec-independent proteins may be in the folding of protein into the membrane bilayer. This is based on two pieces of data (Chen et al., 2002). First,
carbonate extraction studies show that Pf3 coat can only partially integrate into the membrane in the absence of YidC. Second, photocrosslinking data shows that YidC can still interact with the Pf3 coat mutant without flanking charges, which cannot translocate its hydrophilic N-terminus across the membrane. This indicates that YidC interacts with non-transmembrane Pf3 coat protein. These results suggest that YidC may function as a membrane chaperone to help Sec-independent proteins fold into a transmembrane configuration. YidC makes contact with the hydrophobic segments of the inserting membrane protein (Chen et al., 2002; Urbanus et al., 2001). For the M13 procoat and Pf3 coat protein to integrate into the membrane, the proton motive force (pmf) is also required. The pmf stimulates the translocation of the acidic hydrophilic domain of procoat (Cao et al., 1995; Date et al., 1980) and Pf3 coat (Rohrer and Kuhn, 1990) across the bilayer.

1.6 YidC can function with the Sec translocase

The Sec-dependent protein leader peptidase (Lep) also requires YidC for efficient membrane insertion. Lep spans the membrane twice, with a large C-terminal domain in the periplasmic space (Figure 1.6). Previously, Wolfe et al. (1985) showed that SecY and SecA are needed for the translocation of the C-terminal domain across the membrane (Wolfe et al., 1985). Translocation also requires YidC for efficient insertion (Samuelson et al., 2000). In YidC depleted cells, the membrane insertion of the C-terminal domain was partially blocked. The requirement of YidC for optimal insertion is consistent with recent studies showing that YidC cooperates with the Sec translocase (Beck et al., 2001a;
Samuelson et al., 2000; Scotti et al., 2000; Urbanus et al., 2001). YidC interacts with the Sec translocase and copurifies with a 6His-tagged SecY, along with SecEG and SecDFYajC (Scotti et al., 2000). However, a portion of YidC does not interact with the Sec translocase as YidC is in higher concentration over the Sec machinery (Urbanus et al., 2001). This excess YidC may relate to its insertion function for Sec-independent proteins (Urbanus et al., 2001). YidC interacts with the first hydrophobic domain of Lep (Houben et al., 2000; Samuelson et al., 2000), suggesting that YidC may help in the insertion of this apolar region, leading to the translocation of the N-terminal domain. This direct interaction of apolar domain 1 of Lep (Figure 1.6) with YidC is consistent with protease mapping studies showing that YidC stimulates insertion of the N-terminus across the membrane (Samuelson et al., 2000). N-terminal translocation of Lep occurs efficiently when the SecY function is impaired in a temperature-sensitive SecY mutant (Lee et al., 1992). Lep nascent chains that contain apolar domains 1 and 2 (Figure 1.6) can also be chemically crosslinked to SecA and SecY (Houben et al., 2000). These studies are consistent with apolar domain 2 (Dalbey et al., 1987) functioning as an uncleaved signal to initiate translocation of the large C-terminal domain by engaging the Sec machinery.

Is there a YidC requirement for other membrane proteins requiring the Sec translocase? The answer is yes. Translocation of the C-terminal domain of FtsQ, a single-spanning membrane protein, is measurably affected when YidC is deficient in the cell (Scotti et al., 2000; Urbanus et al., 2001). Previous studies showed that the membrane insertion of FtsQ is mediated by SecYEG and SecA (Scotti et al., 1999; Valent et al., 1998). To
determine at which stage FtsQ interacts with the Sec components or YidC during insertion (Urbanus et al., 2001), ribosome-bound FtsQ nascent chains of different length were used. The inserting hydrophobic region of FtsQ first sees SecY and then moves to an environment that is in contact with YidC. The interaction of FtsQ first with SecY and later with YidC is also seen when SecYEG and YidC are reconstituted into lipid vesicles (van der Laan et al., 2001). The conclusion from these studies is that FtsQ interacts sequentially with SecY and YidC.

Additional information was obtained by studying mannitol permease (MtlA) which showed that YidC can function as an assembly site for helix packing of membrane proteins (Beck et al., 2001a). MtlA spans the plasma membrane six times with its N- and C-termini localized in the cytoplasm (Figure 1.6). To monitor membrane insertion, nascent chains of different lengths were used to probe the different stages of MtlA assembly (Beck et al., 2001b), as done for the above FtsQ study (Urbanus et al., 2001). The different nascent chains include MtlA102 (where 102 reflects the number of amino acids of the nascent chain) where two hydrophobic domains are exposed from the ribosome; MtlA130 with a 30 residue cytosolic loop exposed from the ribosome channel, in addition to the two hydrophobic segments; and MtlA189, with three hydrophobic domains and a cytosolic loop exposed from the ribosome. Using the MtlA102 construct, Beck et al. (2001) found that the first apolar domain makes contact with YidC during insertion, while the second apolar domain does not. Most likely this is because the second apolar domain is within the SecYEG channel. In contrast, in the longer nascent MtlA130 chain, the two hydrophobic domains both make contact with YidC and not with
SecY. Finally, the third hydrophobic domain contacts both SecE and YidC when the nascent chain is extended to 189 residues. The results indicate that the hydrophobic domain inserts into SecYEG channel and moves laterally toward YidC. This work also suggests that YidC forms an assembly site for hydrophobic domains prior to their assembly into the lipid bilayer.

Recently, the mechanism by which YidC is brought to the SecYEG channel was investigated (Nouwen and Driessen, 2002). YidC forms an heterotetrameric complex with SecDFYajC (Nouwen and Driessen, 2002), in which SecDFYajC functions as a bridge allowing YidC to interact with the SecYEG channel. YidC also complements the growth defect of SecDF depletion strain (Nouwen and Driessen, 2002). Together these results suggest that SecDFYajC is important for YidC to function with the Sec translocase. SecDF complex is also involved in the release of pre-proteins into the periplasmic space (Matsuyama et al., 1993). In addition, SecDF complex prevents the back movement of partly translocated preproteins into the cytoplasm (Duong and Wickner, 1997). In light of the importance of SecDFYajC in allowing YidC to interact with SecYEG channel (Nouwen and Driessen, 2002), it is likely that YidC also contributes to the release of preproteins from the channel and possibly plays a vital role in preventing backsliding. This hypothesis is currently under investigation.
1.7 YidC does not play a critical role in protein export

While the above studies show that YidC plays an important role in membrane protein topogenesis, studies have not shown that YidC plays a critical role for the export of proteins (Samuelson et al., 2000). The insertion of the outer membrane protein precursor pro-OmpA (Samuelson et al., 2000) and pre-peptidoglycan-associated lipoprotein (PAL) (Samuelson et al., 2001) are unaffected when YidC is depleted within the cell. Similarly, export of maltose binding protein to the periplasmic space is unaffected while β-lactamase is affected to a certain extent under YidC-limiting conditions (Samuelson et al., 2000). β-lactamase is an exported protein that seems to behave like a membrane protein in terms of its SRP dependence for translocation (Phillips and Silhavy, 1992).

In contrast, protein export is affected indirectly under YidC depletion conditions when a Sec-dependent membrane protein such as Lep is overproduced (Samuelson et al., 2001). This is in contrast to overproduction of the Sec-independent procoat protein or the exported protein maltose binding protein under YidC limiting conditions which does not cause this export defect (Samuelson et al., 2001). The hypothesis is that under YidC depletion conditions, the overexpressed Lep is getting stalled in the SecYEG channel causing exported proteins to be blocked. No observable effect on OmpA export is seen when YidC is not limiting under these Lep overexpressed conditions.

The specific function(s) of YidC for Sec-dependent proteins is not yet clear. Nevertheless, it appears that YidC function is necessary to clear the SecYEG channel
efficiently of the inserting membrane protein. As mentioned above, work on the FtsQ protein has shown that its hydrophobic region first interacts with SecY and then moves into contact with YidC. The role of YidC may be to catalyze the removal of the hydrophobic region of the membrane protein from the Sec channel, and assist in the integration of the hydrophobic segment into the lipid bilayer (Figure 1.7). This YidC catalyzed event in which a hydrophobic region is removed from a channel could occur sequentially (Figure 1.7, upper panel) or, after several hydrophobic domains form a helical bundle (Figure 1.7, lower panel). A similar function is proposed for TRAM in the integration of membrane proteins into the ER membrane (Do et al., 1996; Gorlich et al., 1992a).

1.8 Oxa1p family of proteins is evolutionarily conserved

As previously mentioned, the Oxa1p family of proteins is found in bacteria, chloroplast and mitochondria. Like the mitochondrial Oxa1p, the chloroplast homolog Alb3 was found to mediate the insertion of a membrane protein (Moore et al., 2000). Antibody directed to Alb3 inhibits the post-translational integration of the light harvesting chlorophyll-binding protein (LHCP) into the thylakoid membranes. Further studies by Robinson and coworkers (Mant et al., 2001; Woolhead et al., 2001) demonstrated that Alb3 is used for an additional three membrane proteins for integration, but does not appear to play a global role in membrane protein insertion in chloroplast.
Recently, it was investigated whether these evolutionarily conserved Oxa1p proteins are truly functional homologs (Jiang et al., 2002). The chloroplast Alb3, with or without a chloroplast transit peptide or a chimeric protein comprised of the first 57 amino acid residues of YidC, fused to residue 59 of Alb3 (H1Alb3), was tested to see if it could functionally complement the YidC depletion strain. The H1Alb3 could complement the growth defect in the YidC depletion strain, while the Alb3 protein with or without the chloroplast transit peptide was not capable of complementing the YidC depletion strain. H1Alb3 can functionally substitute for YidC in \textit{E. coli} as it stimulated the membrane insertion of the Sec-independent M13 procoat protein and the Sec-dependent Lep. Moreover, the chloroplast thylakoid SecE was found to insert into the bacterial inner membrane as long as YidC or the Alb3 chimeric protein was present. This emphasizes the importance of a YidC homolog for membrane integration in bacteria.

\textbf{1.9 Summary and open questions}

The current status of studies on the new YidC pathway in bacteria show that YidC is required for the membrane insertion of the M13 phage and Pf3 phage coat proteins into the bacterial inner membrane, proving that these Sec-independent proteins do not insert spontaneously \textit{in vivo}. For Sec-dependent proteins, YidC is necessary for efficient membrane insertion and, most likely, for the movement of the hydrophobic region of the membrane protein out of the SecYEG channel. The above studies demonstrate that YidC can function with and without the Sec translocase. YidC does not seem to play a role in protein export, although only a limited number of exported proteins have been tested so
far. The function of YidC seems to be as a membrane chaperone to integrate the membrane protein’s hydrophobic region into the lipid bilayer, and YidC possibly assists in helical bundle formation in polytopic membrane proteins.

A number of important questions remain to be answered. Does YidC play a global role in membrane protein insertion? What is the oligomeric state of YidC, and what is its structure? Which parts of YidC recognize the hydrophobic regions of the inserting protein substrate? How does YidC catalyze the removal of the hydrophobic region out of the SecYEG channel? How does YidC function as a translocase distinct from the SecYEG channel? Are there other novel components of the YidC pathway that are involved in membrane protein assembly? Finally, what are the features of a membrane protein that allow it to insert by the Sec-independent YidC pathway?
Figure 1.1  Membrane protein targeting and membrane insertion in the eukaryotic cells. SP, SRP, SR, Spase and ER depict signal peptide, signal recognition particle, signal recognition particle receptor, signal peptidase (leader peptidase), and endoplasmic reticulum, respectively.
Figure 1.2 The translocation machinery in the eukaryotic endoplasmic reticulum and bacterial cytoplasmic membrane. a) The Sec 61-αβγ translocon and TRAM mediate the integration of membrane proteins into the lipid bilayer. b) YidC mediates insertion of Sec-independent proteins into the membrane c). The SecYEG/SecDFYajC translocase and YidC catalyze the insertion of proteins into the membrane.
Figure 1. 3 Membrane protein targeting and membrane insertion in bacteria. There are sec-dependent and sec-independent membrane insertion pathways. YidC plays a critical role in sec-independent pathway. YidC is also involved in sec-dependent pathway, in which it may function similar to TRAM in eukaryotic cells. (Dalbey and Kuhn, 2000)
Figure 1.4  Membrane topologies of the Oxa1p family of proteins in bacteria, mitochondria and chloroplasts. The transmembrane segments are represented by solid rectangles.
Figure 1.5  Membrane orientation of the Sec-independent proteins: M13 phage procoat and Pf3 phage coat protein. The transmembrane segments are represented by solid rectangles. SP depicts the signal peptidase cleavage site.
Figure 1.6  Membrane orientation of the Sec-dependent Lep, FtsQ and MtlA in the plasma membrane. The transmembrane segments are represented by solid rectangles.
Figure 1.7 Proposed function of YidC for Sec-dependent proteins. Upper panel shows apolar domains entering and leaving the channel one at a time. Lower panel shows multiple apolar domains within the channel where they form a helical bundle. In both cases, YidC may be involved in catalyzing the removal of the apolar domains out of the channel.
CHAPTER 2*

CROSSLINKING STUDIES PROVE THAT YIDC INTERACTS WITH PROTEINS BEING INSERTED INTO THE MEMBRANE

2.1 Introduction

Membrane protein insertion in bacteria can be roughly divided into two classes: sec-dependent and sec-independent membrane insertions (Wolfe et al., 1985). As the name indicated, sec-dependent membrane insertion is mediated by the Sec translocase (SecYEG complex). It was not clear what proteins mediate Sec-independent membrane insertion or whether the Sec-independent membrane insertion is spontaneous (Soekarjo et al., 1996)? One possibility is that YidC was involved Sec-independent membrane insertion since there are around 30% homology between YidC and Oxa1p, the translocase in mitochondria (Bonnefoy et al., 1994). The Sec translocase does not exist in mitochondria, which indicates that membrane insertion in mitochondria is sec-_________________________

independent (Glick and Von Heijne, 1996). *In vivo* and *in vitro* studies were carried out to prove YidC is a translocase component and responsible for Sec-independent protein insertion. The *in vivo* approach was to knock out the YidC gene in the chromosome and introduce a new YidC gene under control of a tightly-regulated promoter, *araBAD* promoter, and to monitor the membrane protein insertion when YidC levels in the cells are depleted (Samuelson et al., 2000). The *in vitro* approach was to prove YidC physically interacts with the protein being inserted into membrane.

Membrane protein insertion is a dynamic process. The interaction between translocase components and the protein being inserted into membrane is transient; therefore crosslinking is the best way to study this kind of protein interaction. Crosslinking can “freeze” this transient interaction and make it detectable. Roughly, crosslinking can be classified into two types: chemical crosslinking and site-specific photocrosslinking. Chemical crosslinking is a process in which chemical crosslinkers is used to link the interacting proteins with a covalent bond so that the interaction is stabilized. Thiol and amine groups are the most commonly used chemical groups used for chemical crosslinking. Site-specific photocrosslinking is the technique, in which a photoactivatable group is incorporated into a specific site of a protein, and upon the ultraviolet (UV) activation, the photoactivatable group is converted to a highly reactive species such as nitrene, carbene, or radical, which attacks neighbor molecules to form a stable chemical bond (Brunner, 1996; Graf et al., 1998).
The key step of the site-specific photocrosslinking is to incorporate the photoactivatable group into a specific site of the newly synthesized polypeptide. One way to do this incorporation is to use a lysyl-tRNA modified at lysyl ε-amino group with 4-(3-trifluoromethyl)diazirino benzoyl-N-hydroxy-succinimido ester (εTDBA-lys-tRNA) to incorporate the photoactivatable TDBA group into the positions of lysine residues in the nascent polypeptide (Krieg et al., 1986; Kurzchalia et al., 1986) (Figure 2.1). The more versatile way to do the incorporation is to use an amber suppressor tRNA, which is charged with L-4’-(3-[trifluoromethyl]-3H-diazirin-3-yl)phenylalanine (abbreviated as (Tmd)Phe) (Baldini et al., 1988; Brunner, 1996; Cload et al., 1996; Noren et al., 1989; Noren et al., 1990) (Figure 2.2). The incorporation is mediated by a single amber codon mutation introduced at a chosen site in the gene to be expressed. In an in vitro translation system, the amber suppressor tRNA can recognize the stop codon and incorporate the (Tmd)Phe into the nascent polypeptide. Without the amber suppressor tRNA, the translation cannot proceed and stops at the stop codon position.

In order to utilize the photocrosslinking technique to study translocase-membrane-protein interactions during insertion, truncated nascent polypeptides of the inserting membrane protein need to be used (Gilmore et al., 1991). Membrane protein insertion is a dynamic process, in which the interaction between translocase and inserting protein is transient. Truncated nascent polypeptides are not released from the ribosomes and the ribosomes will prevent the insertion of nascent polypeptides to proceed. Therefore, the ribosome-bound nascent polypeptide can stop at intermediate steps in the insertion pathway, and the intermediates of translocase and ribosome-bound nascent polypeptides will form
(Gilmore et al., 1991) (Figure 2.2). Different lengths of the truncated nascent polypeptide can control how far the insertion proceeds (Laird and High, 1997). The shorter the nascent polypeptides, the earlier the insertion stage will be. When the insertion intermediate is formed, ultraviolet can be used to activate the photoactivatable group incorporated into the nascent polypeptide, and the nascent polypeptide will then be crosslinked to the translocase component bound to it. The crosslinking products can be identified by the molecular weights and immunoprecipitation.

In the chapter, I will describe the site-specific photocrosslinking system that was successfully set up in the lab. Improving the ligation of tRNA with (Tmd)Phe was the key to setting up the system. The photocrosslinking system was then used to study the interaction between YidC and protein being inserted into membrane, and to provide in vitro evidence that YidC is a new translocase component involved in membrane protein insertion.

2.2 Results and Discussion.

2.2.1 Setting up site-specific photocrosslinking system.

The components of the photocrosslinking system for the membrane insertion study include the truncated mRNA encoding the protein under study, the amber suppressor tRNA that is aminoacylated with a photocrosslinker, the in vitro translation system, and the inverted membrane vesicles (IMV). The photocrosslinker is an amino acid modified
with a photoactivatable group. Among these steps in the procedure, preparation of the amber suppressor tRNA aminoacylated with a photocrosslinker is the most difficult part, since aminoacylating tRNA with an unnatural amino acid is difficult. The aminoacylation method developed by Peter G Schultz (Cload et al., 1996; Noren et al., 1989; Noren et al., 1990) was adopted in this study. In the Schultz method (Figure 2.3), a tRNA lacking two nucleotides (C and A) at its 3’ end is ligated to the dinucleotide pdCpA that is aminoacylated with an unnatural amino acid. In this study, the photoactivatable amino acid, L-4’-(3-[trifluoromethyl]-3H-diazirin-3-yl)phenylalanine (abbreviated as (Tmd)Phe) was chosen as the photocrosslinker. Dinucleotide pdCpA aminoacylated with (Tmd)Phe ((Tmd)Phe-dpCpA) was synthesized by our collaborator, Josef Brunner. The E. coli Asn tRNA bearing CUA anticodon was used as the amber suppressor tRNA in the study. The reason to choose E. coli Asn tRNA is that the E. coli Asn tRNA-derived suppressor affords the best suppression efficiencies (Cload et al., 1996).

pEAsn, which harbors E.coli Asn tRNA gene with the “CTA” anticodon, was cut with the restriction enzyme, Fok I, to remove the dideoxynucleotide CA from the tRNA gene (Cload et al., 1996). The gene is under control of the T7 promoter. Therefore, the T7-MEGA shortscript RNA kit was used to generate the runoff tRNA.

The condition for the ligation of runoff tRNA with the (Tmd)Phe-dpCpA was optimized. The choice of the T4 RNA ligase was critical to improve the ligation efficiency. Figure 2.4 shows the ligation results that were obtained with the T4 RNA ligases from
Pharmacia Amersham and Boehringer Mannheim. These two RNA ligases were at the same concentration (10 units/µl). As seen, the ligation efficiency from the reaction using Boehringer Mannheim ligase is much better than the one using Pharmacia Amersham ligase. The ratio of aminoacylated tRNA (retarded band in Figure 2.4) to the non-ami-noacylated tRNA is around 3:1 in the ligation using Boehringer Mannheim ligase. In the ligation using Pharmacia Amersham ligase, although some aminoacylated tRNA was also obtained, almost no suppression of the *in vitro* translation was observed when the ligation product was used (data not shown). This may be due to too much non-aminoacylated tRNA in the ligation product, which competes with the aminoacylated tRNA for the binding to the A site of ribosomes, and inhibit the suppression.

After successfully obtaining the ligation reaction product with high efficiency (the reaction with Boehringer Mannheim ligase), we tested if this suppressor tRNA in the ligation product can efficiently incorporate the (Tmd)Phe into the nascent polypeptide. The ProW gene (Whitley et al., 1994) with an amber codon mutation at Ala13 or Phe113 was used in the *in vitro* translation to test if the amber mutation can be suppressed by the suppressor tRNA. The transcription and translation of the ProW mutant genes were done in a T7 *E. coli* S30 extract (for circular DNA, from Promega) in the presence or absence of the suppressor tRNA (Figure 2.5). When the amber suppressor tRNA was absent in the translation system, no full length of ProW (53 KD) was produced, while when the suppressor tRNA was added to the reactions, ProW translation read through the amber stop codon at the position of Ala13 or Phe113 and produced the full length ProW protein. The suppression efficiency is 10% ~ 20%, which was calculated by the amount of full
length ProW produced by the ProW mutant gene and the wild-type gene. This efficiency is typical for such suppression (personal communication with Matthias Muller). The successful suppression that we observed suggests that the ratio of aminoacylated tRNA and non-aminoacylated tRNA is critical for the efficient suppression. This suppression also indicates that the photocrosslinking system has been set up and is available to study the interaction between YidC and the protein being inserted to the membrane.

2.2.2 Crosslinking studies provide strong evidence that YidC interacts with proteins being inserted into membrane.

Three years ago, it was not clear whether YidC was involved in protein membrane insertion in bacteria. Many research groups, including our lab, in the world were trying to find evidence to support the idea that YidC plays a role in membrane protein insertion, since YidC is a homolog of Oxa1p. Oxa1p was shown to play a role in the insertion of protein into the mitochondrial inner membrane. Our lab finally provided the evidence from using two approach: *in vivo* depletion of YidC in cells and examination of the effect on the protein membrane insertion, and *in vitro* crosslinking to prove YidC interacts with a protein being inserted into membrane (Samuelson et al., 2000). The latter part is described below.

In the crosslinking studies, truncated messenger RNA is translated to produce truncated proteins bound to ribosomes and a photoactivatable amino-acid analogue, (Tmd)Phe, is introduced into the nascent protein by means of an amber suppressor tRNA (Baldini et
These nascent chain/ribosome complexes act as intermediates in the translocation process, allowing crosslinking of the nascent chains to translocation machinery proteins (Gilmore et al., 1991). Photocrosslinking experiments using leader peptidase (Lep) as a model substrate (Dalbey, 1991) demonstrate that YidC interacts with the transmembrane segment of an inner membrane protein during insertion. Lep is an inner membrane protein in *E. coli*, which spans the membrane twice with both its N- and C-termini on the periplasmic side of the membrane (See Figure 1.6 for topology). In order to facilitate monitoring the membrane translocation of N-terminus of Lep, a T7 epitope tag was added to the N-terminus of Lep. The hybrid protein is termed T7Lep. An amber codon replaced the codon encoding Phe5, Ile11, or Trp20 (the numbers after the amino acids designate their positions in the wild-type Lep protein), which are located in the first transmembrane domain of lep. PCR method was used to generate a truncated Lep gene fragment, containing the T7 promoter sequence for *in vitro* translation. The gene fragments encodes a truncated nascent polypeptides with 94 amino acid residues in the *in vitro* translation system.

(Tmd)Phe was first incorporated at position 11 (Ile11) in the middle of the first transmembrane segment (H1) of T7Lep. After translation of the truncated nascent protein (12 KD, indicated by an asterisk in Figure 2.6a) in the presence of inner membrane vesicles (IMVs) in the S100 translation system, the reactions were incubated at 4 °C with or without exposure to ultraviolet radiation. Shown in Figure 2.6a (left side) are the total radiolabelled proteins with an arrow indicating the location of a crosslinked complex that
is consistent with the combined molecular weights of T7Lep and YidC (72 KD). Immunoprecipitation with YidC antiserum (right side in Figure 2.6a) confirms the identity of the complex as T7Lep–YidC. The complex is detected at a low level with wild-type IMVs (YidC⁺), whereas the complex formation is greatly increased when IMVs containing an overexpressed level of YidC are added to the translocation reaction (YidC⁺⁺).

The amount of YidC immunoprecipitable complex was tested when IMVs are prepared from JS7131, MC1060 and the YidC overproducing cells MC1060 (pINGyidC). JS7131 is a YidC depletion strain (Figure 2.7), in which endogenous YidC gene in *E. coli* chromosome was disrupted, and a new YidC gene under control of arabinose promoter was introduced into chromosome at attB site, the λ phage lysogenic integration site (Samuelson et al., 2000). In the presence of glucose (to repress *araBAD* promoter), YidC in the JS7131 cells can be depleted to a non-detectable level by western blots (Samuelson et al., 2000). MC1060 is the parental strain, which contains wild-type level of YidC. pINGyidC is a plasmid, where YidC gene is under control of arabinose promoter. MC1060 harboring pINGyidC produced an overexpressed level of YidC when arabinose (to induce the *araBAD* promoter) was present. As displayed in Figure 2.6b, the amount of T7Lep–YidC complex is increased with IMVs (YidC⁺⁺) prepared from the YidC overproducer (MC1060 with pINGyidC), whereas no T7Lep–YidC complex is observed with YidC⁻ IMVs prepared from JS7131 in presence of glucose. The fact that increased photocrosslinking to the N-terminal region of Lep is detected in YidC⁺⁺ IMVs supports the idea of YidC that may also function distinct from the Sec complex (Figure 2.6a and
Crosslinking of YidC to other positions within H1 of T7Lep was analysed by creating amber mutations at positions 5 and 20 designated as Phe5Amber and Trp20Amber, respectively (Figure 2.6c). The amount of photocrosslinking to YidC was similar after correcting for the different suppression efficiencies.

Chemical crosslinking was also used to confirm the T7Lep–YidC interaction (Figure 2.8). In this study, the T7Lep gene without any amber codon was used to produce a gene fragment that encodes a truncated 94-amino-acid-residue nascent polypeptide of Lep. Chemical crosslinking was performed in the presence of 5 mM disuccinimidyl suberate (DSS). After crosslinking, the sample was immunoprecipitated with anti-YidC serum to identify whether YidC was crosslinked to the Lep nascent chain. Since chemical crosslinking is not very specific in some cases, therefore we used pre-bleed, anti-OmpA, and anti-ribulokinase sera as negative controls to monitor specificity of the chemical crosslinking. Figure 2.8 shows YidC was specifically crosslinked to the Lep nascent polypeptide, which provides further evidence to support YidC is involved in the membrane protein insertion.

In conclusion, photocrosslinking and chemical crosslinking data indicate YidC is directly involved in the membrane protein insertion. This data combined with the in vivo study of YidC (Samuelson et al., 2000) provides compelling evidence that YidC is a translocase component in bacteria.
2.3 Materials and Methods

2.3.1 Preparation of the protein and ribosome fractions of the S100 system, an *E. coli* cell-free and membrane-free *in vitro* translation system

The method was adopted from Martin Wiedmann’s lab. BB1553 strain, which is a RNase minus strain, was used to make the S100 system. 6 Liters of the BB1553 cells were grown to OD$_{600}$ 1.1 in LB media. The cells were harvested, washed with Buffer A (10 mM Tris.HCl, pH7.5, 10 mM Mg Acetate, 22 mM NH$_4$ Acetate and 1mM DTT) twice, quickly frozen in liquid nitrogen, and stored in –80 °C until being used in next step.

The cells were thawed and resuspended in 50 ml Buffer A supplemented with 1 tablet of complete protease inhibitor cocktail (EDTA free, from Roche). The cells resuspension was French-pressed at 1300 psi twice and 8000 psi once. This treatment lysed the cells and the solution became translucent and a little bit stringy. The cell lysate was then centrifuged at 45 K rpm (Ti 70 rotor, Beckman) for 20 min to remove the cell membrane. The supernatant was then centrifuged again at 60 K rpm (Ti 70 rotor, Beckman) for 90 min to pellet the ribosomes. Both pellet (ribosomes) and supernatant (protein fraction) were saved.

Protein fraction: a DEAE sepharose column (20 ml bed volume) was used to remove DNA from the protein fraction. The supernatant (around 50 ml) was applied to the column, which is buffered by Buffer A. The column was then washed with 50 ml Buffer
A, and then eluted with Buffer A supplemented with 0.35 M NH₄Cl. The protein fraction, which was light yellow, was collected and frozen in drops in liquid nitrogen.

Ribosome fraction: The ribosome pellet, which was light brown and translucent, was resuspended in 10 ml Buffer A using a homogenizer, and incubated at 37 °C for 90 min in order to remove those translating mRNA (still associated with the ribosomes) from the ribosomes. After incubation, the ribosome sample was first centrifuged at 12,000 g for 10 min to remove aggregates, then centrifuged at 198,000 g (60 K in Ti 70) for 90 min to pellet ribosome. The ribosome was then resupended in 4 ml of low Mg²⁺ Buffer A (10 mM Tris.HCl, pH 7.5, 5 mM Mg Acetate, 22 mM NH₄ Acetate and 1mM DTT), and frozen in drops in liquid nitrogen.

2.3.2 Procedure to prepare E.coli inverted membrane vesicle (IMV)

This method was adopted from Bruce Geller’s lab. 6 liters culture of E. coli, which were grown to OD₆₀₀ 0.5 to 0.6 in LB media, were harvested, resuspended in 300 ml buffer containing 0.25 M RNAase free sucrose, 0.2M Tris.HCl pH 8.0, 50 µg/ml chloramphenicol. 3.0 ml of 50 mM EDTA pH8.0, 1.5 ml of 1mg/ml lysozyme, 200 ml of water and 20mg of PMSF were sequentially added to the cell resuspension, which was then incubated at room temperature for 15 min to convert cells into spheroplasts. The spheroplasts were harvested by centrifuge (5 K rpm, JA-20, 10 min), resupended in 60 ml of cold DE buffer (1mM DTT and 20 mM EDTA, pH7.2) with 2 mg of PMSF, and French pressed twice at 8000 psi. The cell lysate was spun at 7K rpm for 10 min to
remove unbroken cells, loaded onto a 2-step sucrose gradient (1ml of 0.5 M and 1.4 M sucrose in DE buffer), centrifuged at 36 K rpm in SW41 Ti rotor for 3 hours. The band at the interface of the 0.5 M and 1.4 M sucrose gradients, which contains the crude membrane vesicles, was harvested and diluted with an equal volume of DE buffer. The crude membrane vesicle resuspension was then applied to a 4-step sucrose gradient (2 ml of 0.8, 1.0, 1.2, and 1.4 M sucrose in DE buffer), and centrifuged at 36 K rpm in SW41 Ti rotor for 15 hours. The membrane in the 1.2 M sucrose step, which contains the inverted membrane vesicle (IMV), was harvested, diluted with 20 ml TMD buffer (50 mM Tris.HCl pH7.5, 1 mM Mg Acetate and 1 mM DTT), and spun down at 50 K rpm for 1 hour in Ti 70 rotor. The membrane vesicles was resuspended with 0.75 ml TMD buffer, aliquoted into small volumes, frozen in liquid nitrogen, stored at –80 °C for future use.

2.3.3 Run-off reaction to make amber suppressor tRNA lacking CCA at its 3’ end

pEAsn, which was used to produce the amber suppressor tRNA, is a plasmid bearing the E. coli Asn tRNA gene with the original anticodon replaced by the anticodon of the amber suppressor (TAG) (Cload et al., 1996; Noren et al., 1990). pEAsn was completely digested with the restriction enzyme Fok I to remove the CCA sequence from the tRNA gene (Cload et al., 1996). The digested DNA was purified by phenol/Chloroform extraction and ethanol precipitation, and dissolved into 1µg/µl solution with water. In vitro transcription was performed to produce the amber suppressor tRNA using the T7-MEGA shortscript RNA kit (Ambion). The reaction was set up as followed.
10x Transcription buffer  12µl
ATP (75 mM)    12 µl
CTP (75 mM)    12 µl
GTP (75 mM)    12 µl
UTP (75 mM)    12 µl
H₂O      12 µl
pEAsn DNA fragments (1µg/µl) 30 µl
RNase Inhibitor (Promega)  6 µl
Transcription enzymes  12 µl

Total  120 µl

Table 2.1  tRNA in vitro transcription system using the T7-MEGA shortscript RNA kit.

After incubation at 37 ºC for 4 hours with constant shaking, the transcription reaction was digested with 6 µl DNase (Promega) for 30 min at 37 ºC. The reaction was then diluted to a final volume of 600 µl with RNase free water, and precipitated with 1600 µl ethanol and 60 µl 3 M Na Acetate (pH 5.2). The tRNA was recovered and dissolved in 400 µl RNase-free water. The total amount of tRNA, measured at the 260 nm wavelength with a UV spectrophotometer was 16.17 OD. RNA was analyzed by using polyacrylamide gel electrophoresis (PAGE) with a 9.5% polyacrylamide urea gel. The recipe for the 9.5%
polyacrylamide urea gel (total volume 5 ml for a Biorad minigel) is: 167 µl of 3M Na Acetate, 2.2 ml of H2O, 1.25 ml of the 41% acrylamide solution (39% acrylamide and 2% N,N’-Methylenebisacrylamide), 1.9 g urea, 33.5 µl of 10% ammonium persulfate (APS), 5 µl of TEMED. The RNA loading sample buffer is 100 mM Na Acetate (pH5.2), 8 M urea and 0.05% bromophenol blue. The running buffer is 100 mM Na Acetate (pH5.2). Running condition is 150 volts for 60 min. The gels were stained with 0.2% Toluidine Blue in 100 mM Na Acetate (pH 5.2) and destained with water. For quick analysis, RNA can also be analyzed using a 1.5 % agarose gel.

2.3.4 Ligation of the run-off transcript with the photoactivatable amino acid (Tmd)phe-pdCpA

(Tmd)phe-pdCpA is supplied as a N-Boc-protected compound. Before ligation of (Tmd)phe-pdCpA to the run-off transcript, the Boc protection group needs to be removed by treating with trifluoroacetic acid (TFA). The following is the procedure to perform the ligation. 10 OD_{260} 3′(2′)-Boc-(Tmd)Phe-pdCpA was dissolved in 200 µl TFA, and incubated in room temperature for 10 min. TFA in the sample was then removed by blowing a gentle stream of helium over the sample in a hood. Shielded with aluminum wrap, the sample then was spun under vacuum in a Speed-Vac for 30 min to remove any residual TFA. The samples were dissolved in 100 µl of 10% acetonitrile and used immediately for ligation reaction. The ligation reaction was set up as follows.
Run-off transcript (tRNA) 70 µl
(Tmd)Phe-pdCpA in 10% acetonitrile 100 µl
Ligation buffer 480 µl
DMSO 100 µl
RNA ligase (10 U/µl, Boehringer Mannheim) 36 µl
H₂O 200 µl
RNase Inhibitor (Promega) 8 µl

Total volume 1000 µl

Table 2.2  Ligation of tRNA lacking pCpA with (Tmd)Phe-pdCpA.

The recipe for the ligation buffer is 110mM HEPES, pH 7.5, 30mM MgCl₂, 6.6 mM DTT, 100µl/ml BSA (RNase-free) and 0.8 mM ATP (Li Salt). The 1000 µl reaction was split into 5 portions (5 x 200 µl) in eppendorf tubes, and incubated at 37 °C for 11 min with gently shaking. The reactions were stopped by adding 20 µl of 3 M Na Acetate (pH 5.2) into each aliquot. The samples were extracted once with the same volume of chloroform, precipitated with 3 volume of pure ice-cold ethanol, and frozen in liquid nitrogen for 30 min. The tRNA was then spun down, washed with 70% ethanol, dried in
a Speed-Vac, dissolved in the 200 µl of tRNA store solution (2 mM K Acetate, 5 mM Mg Acetate, pH4.5). The tRNA solution was aliquoted into 10 x 20 µl portions, quickly frozen in liquid nitrogen, stored in – 80 °C. In all of the steps, the samples were protected from direct sunshine and other ultraviolet sources. The ligation efficiency can be analyzed by running the ligation products on a urea polyacrylamide gel described in 2.3.3 section.

2.3.5 Preparation of mRNA for in vitro translation

T7 MEGA shortscript RNA kit was used to generate mRNA for in vitro translation. The in vitro transcription reactions were set up as follows.
Table 2.3 *In vitro* transcription system used to prepare mRNA.

In the reactions, the final concentration of DNA was adjusted to 150 nM. The reaction was incubated at 37°C for 4 hour with constant shaking and mixing. After incubation, 200 µl RNase-free H₂O and 25 µl 3 M Na Acetate (pH 5.2) were added to stop the reaction, and the transcription samples were extracted with equal volume of chloroform and precipitated with 3 volume of pure ethanol. The RNA were spun down, rinsed with 70% ethanol, dried, and dissolved in 70 µl RNase-free H₂O. The mRNA was aliquoted into 5 x 15 µl portions, quickly frozen in liquid nitrogen, and stored in -80°C.
2.3.6 *In vitro* translation using S100 cell-free system

The pre-mixture for the *in vitro* translation reactions in the S100 cell-free system can be set up as followed.

Table 2.4  The pre-mixture for the *in vitro* translation S100 system.
Mix I contains 250 mM Tris. Acetate, pH8.0, 250 mM K Acetate, 0.2 mM each of the 19 amino acids (minus Methionine), and 10 mM DTT. Mix II contains 40 mM ATP, 10 mM GTP, and 400 mM Na$_3$ PEP, pH6.5 (adjusted with 0.1 M KOH). The sequence of the anti-ssrA oligo is 5’-TTAAGCTGCTAAAGCGTAGTTTTCGTCGTTTGCGACTA-3’ (Hanes and Pluckthun, 1997; Keiler et al., 1996). The anti-ssrA oligo will bind to and block the ssrA RNA function, which is to release truncated nascent polypeptides from the bacterial ribosomes (Keiler et al., 1996). The pre-mixture can be aliquoted to smaller volumes for different reactions. 1 µl of mRNA (see Section 2.3.5), and 0.5 µl of Suppressor tRNA-(Tmd)phe (see section 2.3.4, only for photocrosslinking) are needed for each 10 µl of pre-mixture. After incubation at 37 °C for 4 min, a reaction is supplemented with 2 µl of IMV suspension (see Section 2.3.2) (per 10 µl pre-mixture), and the reactions continued at 37 °C for 30 min. The reaction then can be treated with ultraviolet light for photocrosslinking, or chemical crosslinkers for chemical crosslinking.

2.3.7 Photocrosslinking

Photocrosslinking was performed with 96-well plates using the portable ultraviolet lamp (20 W, 364 nm wavelength). After in vitro translation, the reactions were transferred to a 96-well plate, and an ultraviolet lamp was placed above the 96-well plate. The reactions were irradiated with 364 nm ultraviolet light for 30 at 4 °C. After irradiation, the reactions were transferred to eppendorf tubes, the wells in the plate were rinsed twice
with 60 µl of water, and the rinsed water was also transferred to the tube containing the same sample.

2.3.8 Chemical crosslinking

After \textit{in vitro} translation, 5mM DSS (disuccinimidyl suberate, dissolved in DMSO) (final concentration) was added to the reaction samples, and the crosslinking reaction continued at 37 °C for 20 min. After crosslinking, the chemical crosslinking reactions were quenched with 100 mM Tris.HCl (pH7.5) by incubation for 15 min at room temperature.

2.3.9 TCA precipitation and immunoprecipitation

After crosslinking, the reactions were precipitated with ice-cold 10% trichloroacetic acid (TCA) (final concentration), and incubated on ice for more than 30 min. The protein precipitate was spun down at 14,000 rpm for 10 min using a microcentrifuge, rinsed with 1 ml of ice-cold acetone, and dried in a heating block for 1 min or at room temperature for 5 min. For direct analysis (without immunoprecipitation), the pellet was dissolved in 45 µl of SDS sample buffer, and analyzed by SDS-PAGE (Sambrook and Russell, 2001) and phosphorimaging.

For immunoprecipitation, the protein pellets were dissolved in 100 µl SDS-Tris buffer (10 mM Tris.HCl, pH8.0 and 2% SDS). The protein solution was then treated with 1 ml Triton buffer (10 mM Tris.HCl, pH8.0, 5 mM EDTA, 150 mM NaCl and 2.5% Triton X-
100) and 25 µl Staph A cells, and incubated on ice for 20 min. The Staph A cells was spun down and the supernatant was transferred to a new tube, in which the appropriate amount of antiserum was added. The supernatant with the antiserum was incubated on ice for 1 hour, and then 25 µl Staph A cells were added and incubated on ice for another one hour. The Staph A cells were spun down (20 sec, 14,000 rpm in a microcentrifuge) and washed twice with 1 ml of Triton buffer, resuspended in SDS sample buffer (Sambrook and Russell, 2001), heated in a heating block for 5 min, and spun down again. 15 µl of supernatant was used for SDS-PAGE and phosphorimaging analysis.
Figure 2.1 Photocrosslinking using the Lys-tRNA method. The Lys-tRNA is modified at the epsilon-amino group with 4-(3-trifluoro-methyl)diazirino benzoyl-N-hydroxy-succinimido ester. The truncated mRNA is translated in the cell-free system in the presence of $[^{35}\text{S}]-\text{methionine}$ to produce nascent chains attached to the ribosome. In the nascent chains, the photoactivatable lysine derivative is incorporated at positions where lysines normally occur. Irradiation at 320 nm produces a carbene that reacts with neighboring proteins. Thus, crosslinks are formed between the nascent polypeptide and neighboring proteins. (Dalbey and von Heijne, 2002)
Figure 2.2  Photocrosslinking using an amber suppressor tRNA aminoacylated with (Tmd)Phe. The structure of (Tmd)Phe is shown. The amber suppressor tRNA is aminoacylated with (Tmd)Phe. A stop codon (UAG) is introduced into a specific position of mRNA. In an \textit{in vitro} translation system, the amber suppressor tRNA with (Tmd)Phe can suppress the stop codon mutation in the mRNA. Upon suppression, the translation proceeds, and (Tmd)Phe is incorporated into the nascent polypeptide at the location where the stop codon (UAG) is introduced. In order to tether the nascent polypeptide to the ribosome, truncated mRNA is used and inverted membrane vesicles are added. The ribosome-bound nascent polypeptide will not complete the full process of membrane insertion, and stop at a stage where the nascent polypeptide is interacting with a component of the translocation machinery (such as SecYEG or YidC). (Graf et al., 1998)
Figure 2.3 Schematic illustration of aminoacylation of amber suppressor tRNA with (Tmd)Phe. pEAsn, which harbors *E. coli* tRNA gene with an CTA anticodon, is cut with FokI, and *in vitro* transcribed to produce the amber suppressor tRNA lacking pdCpA at its 3’end. This tRNA product is ligated with (Tmd)Phe-pdCpA using T4 RNA ligase, and the amber suppressor tRNA that is aminoacylated with (Tmd)Phe is obtained. (Graf et al., 1998)
Figure 2.4  Ligation of tRNA (-pCpA) with (Tmd)Phe-pdCpA. The ligations were performed using T4 RNA ligase from two companies: Pharmacia Amersham and Boehringer Mannheim. The tRNA products were resolved using a 9.5% polyacrylamide urea gel.

Lane 1: tRNA without ligation (tRNA (-pCpA)), as a control;
Lane 2: Ligation products using T4 RNA ligase from Pharmacia Amersham;
Lane 3: Ligation products using T4 RNA ligase from Boehringer Mannheim.
Figure 2.5  Amber suppression of ProW in the S30 in vitro translation system in the presence of (Tmd)Phe-tRNA. An amber codon (TAG) is introduced into ProW gene at the positions encoding Ala13 or Phe113. The ProW amber mutants (A13amber and F113amber) were in vitro translated and radiolabeled with $[^{35}\text{S}]$-Methionine in the presence or absence of (Tmd)Phe-tRNA. Wildtype (wt) ProW was used as a positive control to show the position of ProW. There is also a background band in all the translation reactions.
Figure 2.6 Photocrosslinking of T7Lep to YidC. The arrow indicates the photocrosslinked product, T7Lep–YidC. The molecular weight of the complex is within the range of 60–90KD, which agrees with the expected molecular weight, 72KD. a, T7Lep (I11Amber) as numbered in the wild-type Lep sequence was used in a photocrosslinking reaction with IMVs prepared from YidC overproducing cells (YidC++) or from wild-type cells (YidC+). The total photocrosslinking reaction products are shown on the left side. Photocrosslinking products were immunoprecipitated with YidC antiserum or prebleed serum (right side). IP, immunoprecipitation; PB, prebleed. b, Immunoprecipitation of the T7Lep–YidC complex from photocrosslinking reactions with IMVs containing overproduced (YidC++), wild-type (YidC+), or depleted (YidC−) levels of YidC. T7Lep (Ile11Amber) was used in these reactions. c, T7Lep mutants Phe5Amber, Ile11Amber and Trp20Amber, which allow incorporation of the photoactivatable amino-acid analogue (Tmd)Phe in different locations of the first transmembrane segment, were used in photocrosslinking reactions. After correcting for different suppression efficiencies, the degree of photocrosslinking is similar in the three locations.
Figure 2.6
Figure 2.7 Construction of the YidC-depletion strain JS7131. Schematic showing the genetic makeup of *E. coli* strain JS7131 with a yidC deletion and a complementary wildtype yidC gene under control of the *araBAD* promoter/operator. (From Samuelson et al., 2000)
Figure 2.8 Chemical crosslinking of T7Lep with YidC. In this study, T7Lep without an amber mutation was used. The crosslinking reactions were immunoprecipitated with anti-YidC, anti-OmpA, anti-ribulokinase or prebleed serum. The arrow indicates the crosslinking product: T7Lep-YidC adduct.
CHAPTER 3*

UTILIZING SEC-INDEPENDENT PF3 COAT PROTEIN TO STUDY THE
FUNCTION AND MECHANISM OF YIDC

3.1 Introduction

Most bacterial membrane proteins insert into the membrane utilizing the Sec translocase. The Sec translocase is composed of the integral membrane core subunits SecY and SecE that function as a protein transporter, and the peripheral subunit SecA, an ATPase, that functions as a molecular motor to push the protein chain through the SecYE translocase by hydrolyzing ATP (Driessen et al., 2001; Economou, 1999; Mori and Ito, 2001). The other components of the Sec translocase, such as SecG, SecD, SecF and YajC, are not absolutely required for protein translocation. Prior to the membrane translocation step, the proteins need to be targeted to the membrane. In bacteria, this is achieved by two major pathways, one involving SecB (reviewed in (Manting and Driessen, 2000)) and the

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other involving the signal recognition particle (SRP) (Herskovits et al., 2000). In the SecB pathway, SecB, a small molecular chaperone, targets exported proteins post-translationally to the membrane-bound SecA subunit of the Sec translocase (Hartl et al., 1990). In the SRP pathway, SRP targets membrane proteins co-translationally to the membrane (Ulbrandt et al., 1997). In bacteria, SRP consists of a 48 KDa protein component, designated Ffh, and a 4.5S RNA component. SRP is thought to bind to FtsY in its targeting cycle. After targeting by SRP, SecA can also be required for translocation of periplasmic domains of some membrane proteins (Neumann-Haefelin et al., 2000).

YidC, a homologue of the mitochondrial Oxa1 (Hell et al., 2001) and chloroplast Alb3 (Moore et al., 2000), is a newly identified translocase component in bacteria (Dalbey et al., 2000; Dalbey and Kuhn, 2000). YidC is absolutely essential for the membrane insertion of the Sec-independent M13 coat protein (Samuelson et al., 2000; Samuelson et al., 2001). It also stimulates the membrane translocation of the C-terminal domain of the Sec-dependent proteins leader peptidase (Lep) and FtsQ (Samuelson et al., 2000; Scotti et al., 2000; Urbanus et al., 2001), as well as promotes the insertion of the N-terminal domain of Pf3-tagged leader peptidase (Pf3-Lep). For Sec-dependent membrane proteins, YidC has been shown to cooperate with Sec translocase and to play a possible role in the lateral movement of transmembrane segments out of the Sec translocase complex (Beck et al., 2001a; Luirink et al., 2001; Scotti et al., 2000; Urbanus et al., 2001; van der Laan et al., 2001). Therefore, a current model is that YidC functions in association with the Sec translocase or works independently to insert proteins into
membranes (Luirink et al., 2001; Stuart and Neupert, 2000). Whether YidC works with or without the Sec translocase depends on the membrane protein being inserted.

Pf3 coat, a 44-amino-acid membrane protein, is the major coat protein from \textit{Pseudomonas aeruginosa} phage Pf3. When Pf3 coat is expressed in \textit{Escherichia coli} (\textit{E. coli}), it inserts into the inner membrane with a single transmembrane segment adopting an N\textsubscript{out}/C\textsubscript{in} topology. Pf3 coat has been used as a model protein to study Sec-independent membrane insertion (Kuhn, 1995). It was widely believed that Pf3 coat inserts directly into the bilayer of the bacterial inner membrane without the assistance of proteinaceous factors (Kiefer and Kuhn, 1999; Ridder et al., 2000). However, since recent studies show that the Sec-independent M13 procoat protein requires YidC for membrane insertion (Samuelson et al., 2000; Samuelson et al., 2001), Pf3 coat protein might first contact YidC and require its function for translocation across the membrane.

In this chapter, we show that in a strain where YidC is depleted, the membrane insertion of wild-type Pf3 coat is severely affected. Using a photocrosslinking method and Pf3 coat mutants where the carboxyl-terminal regions are extended, we found that YidC, but not SecY, interacts with Pf3 coat protein after its transmembrane segment emerges from the ribosome. We also present data suggesting that YidC plays a specific role for membrane translocation. Furthermore, we present experimental evidence that Pf3 coat interacts with Ffh although SRP is not required for wild-type Pf3 coat membrane insertion \textit{in vivo}. However, Ffh becomes necessary for efficient Pf3 coat membrane
targeting when the positively charged residues in the C-terminal region are substituted by uncharged residues.

3.2 Results

3.2.1 YidC is required for wild-type Pf3 coat membrane insertion

We have demonstrated that M13 Procoat, a Sec-independent protein, requires YidC for membrane insertion (Samuelson et al., 2000). Therefore, we were interested in whether the single-spanning Pf3 coat may also require YidC for membrane insertion. Previously, Pf3 coat was shown to insert into the \textit{E. coli} inner membrane without the aid of the Sec translocase (Rohrer and Kuhn, 1990). \textit{E. coli} JS7131 cells (Samuelson et al., 2000) were grown in the presence of arabinose to express YidC or glucose to deplete YidC. Cells expressing Pf3 coat protein were labeled with trans-\[^{35}\text{S}\]-methionine for 20 sec and chased for various times. Protease mapping was performed to monitor the membrane insertion of Pf3 coat. Since the methionine at position one is the only methionine in the Pf3 coat protein, only Pf3 coat with intact N-terminus can be detected by autoradiography (see Figure 3.1a). When YidC was present (Figure 3.1b, YidC\(^{+}\)), Pf3 coat was inserted normally across the membrane with an N\(_{\text{out}}\)/C\(_{\text{in}}\) topology and was digested to a non-radiolabeled fragment by proteinase K added to the periplasmic side. In YidC deficient cells (Figure 3.1b, YidC\(^{-}\)), Pf3 coat was protected by the membrane from proteinase K digestion, indicating that it does not insert across the membrane even after a 2 min chase period. This demonstrates that Pf3 coat protein requires YidC for membrane insertion.
3.2.2 YidC and Ffh physically interact with the transmembrane region of Pf3 coat

Since Pf3 coat requires YidC for membrane insertion in vivo, we analyzed whether YidC physically interacts with Pf3 coat during insertion and whether other proteins interact with Pf3 coat. Site-specific photocrosslinking (Brunner, 1996) was applied to study Pf3 coat membrane biogenesis in vitro. With this technique, a truncated mRNA is generated that lacks the termination codon and therefore the nascent protein chains are not released from the ribosome. Ribosome-nascent chains trapped in the translocation process can then be subjected to crosslinking to identify interacting proteins. The length of wild-type Pf3 coat (44 amino acids) is too short for the photocrosslinking technique, since 35 to 40 amino acids are estimated to reside within the ribosomal tunnel upon arrest of translation. To make Pf3 coat suitable for photocrosslinking, we have lengthened the protein with a sequence of the leader peptidase (Lep) soluble P2 domain (the hybrid is called Pf3-P2, Figure 3.1a). The hybrid protein shows the same YidC dependence as Pf3 coat (Figure 3.1c). Pf3-P2 was expressed in the JS7131 strain and subjected to proteinase K mapping. In the cells grown with arabinose to express YidC (Figure 3.1c, YidC+), Pf3-P2 was digested to a smaller fragment, while in the cells grown with glucose to deplete YidC (Figure 3.1c, YidC−), Pf3-P2 was fully protected indicating that Pf3-P2 is also YidC-dependent.

Ribosome-attached Pf3-P2 nascent proteins were site-specifically modified with the photoactivatable crosslinking group, (Tmd)Phe (L-4'-(3-(trifluoromethyl)-3 H-diazirin-3-
yl)phenylalanine). Amber suppressor tRNA charged with (Tmd)Phe enabled incorporation of the photoprobe into the Pf3 coat transmembrane segment at positions T20, I27 and L34 (Figure 3.2a). A PCR method was employed to generate the truncated Pf3-P2 gene fragment for each amber mutant coding for a 91 amino acid protein. After synthesis, the Pf3 coat portion of 44 amino acids should be fully exposed from the ribosome, while the Lep P2 domain remains in ribosomal tunnel.

Each truncated mutant protein was synthesized in vitro in the presence of inverted membrane vesicles (IMV). During the synthesis, each reaction was exposed to ultraviolet (UV) radiation. Pf3 coat was crosslinked to YidC and also to Ffh when the (Tmd)Phe photoprobe was located within the transmembrane segment (Figure 3.2b) at positions T20, I27 and L34. UV irradiation resulted in two major crosslinking products with apparent molecular weights of 60 KDa and 70 KDa. Immunoprecipitation with antiserum to YidC or with antiserum to Ffh (Figure 3.2b) confirmed that the 70 KDa crosslinking product corresponds to the YidC-Pf3 coat adduct and the 60 KDa product is the Ffh-Pf3 coat adduct. The molecular weights of the crosslinked products (Pf3-YidC and Pf3-Ffh adducts) are consistent with the combined sizes of the individual components (the 91 amino acid Pf3 coat protein, approximately 10 KDa; YidC, 60 KDa; and Ffh, 48 KDa). We also introduced the photoprobe into the Pf3 coat amino tail (at position L12), and observed almost no crosslinking between Pf3 coat and YidC (data not shown). These studies are consistent with YidC promoting membrane insertion by physically interacting with the Pf3 coat hydrophobic segment and not with the hydrophilic region.
To test whether the interaction of the Pf3 coat to YidC occurs only during membrane translocation, photocrosslinking using the Pf3 coat L34 amber mutant (the total nascent chain length is 91 amino acids) was carried out after treating the translocation intermediates with puromycin/high salt (potassium acetate) (Figure 3.2c). Puromycin/high salt treatment releases the truncated nascent protein from the ribosome, so that no translocation intermediates will be formed (Urbanus et al., 2001). When the puromycin/high salt treatment was carried out for 5 minutes prior to UV-irradiation (Figure 3.2c, Puro+), the photocrosslinking between YidC and Pf3 coat was substantially reduced, as compared to the non-treated sample (Figure 3.2c, Puro-). This suggests that when Pf3-P2 is released from the ribosome, it cannot form translocation intermediates with YidC, which therefore indicates the Pf3 coat-YidC interaction occurs only during Pf3 coat membrane insertion. Taken together, the photocrosslinking data clearly demonstrate that YidC as well as Ffh can physically interact with Pf3 coat during its membrane insertion.

3.2.3 Ffh is important for Pf3 coat targeting when the two positively charged residues in the carboxyl-terminal are replaced by uncharged residues

Since we found that Ffh can physically interact with Pf3 coat, we tested whether Ffh is necessary for Pf3 coat targeting. To address this issue, we used the Ffh depletion strain, WAM121, in which the Ffh gene is under control of the arabinose promoter. Sodium carbonate extraction (Samuelson et al., 2001) was used to monitor the targeting of Pf3 coat to the membrane in the presence or absence of Ffh. More than 95% of wild-type Pf3
coat was pelleted with the membrane even in the absence of Ffh (Figure 3.3b, Ffh-), indicating that wild-type Pf3 coat is targeted to the membrane independent of Ffh \textit{in vivo}.

Gallusser and Kuhn had shown that the positively charged residues of M13 Procoat target the protein to the inner membrane, where the membrane surface is negatively charged (Gallusser and Kuhn, 1990). The positively charged residues of the Pf3 coat protein flanking the transmembrane segment may play a similar role and function for targeting to the membrane. To test this hypothesis, we used a Pf3 coat mutant, Pf3-NN (Figure 3.3a), where the two positively charged residues near the carboxy terminus were replaced by asparagines. Previously, this mutant has been shown to insert into membrane almost as efficiently as wild-type Pf3 coat (Kiefer et al., 1997). When Ffh was present (Figure 3.3b, Ffh+), we found 77% of Pf3-NN was in the pellet fraction in carbonate-treated cells. In contrast, when Ffh was depleted (Figure 3.3b, Ffh-), only 43% was found in the membrane pellet, indicating that Ffh is required for efficient targeting of Pf3-NN coat protein to the inner membrane. When the positively charged residues are present more than 95% were found in the membrane pellet. Consequently, for wild-type Pf3 protein, the targeting role of Ffh is redundant.
3.2.4 The YidC-Pf3 coat interaction is more efficient as the transmembrane segment of Pf3 coat is extended further from the ribosomal tunnel

To study at what stage Pf3 coat interacts with YidC during membrane insertion, we used truncated mRNA to synthesize truncated nascent Pf3 coat proteins of different length, i.e. 56-Pf3, 64-Pf3, 72-Pf3, 80-Pf3 and 91-Pf3, which have 56, 64, 72, 80 and 91 amino acids, respectively. In addition, the photoprobe (Tmd)Phe was incorporated by introducing an amber codon at position T20 in 56-Pf3 and in 64-Pf3, or at position L34 in 72-Pf3, 80-Pf3 and 91-Pf3 (Figure 3.4a). For nascent chains that remain bound to the ribosomes, about 35 amino acids will be trapped within the ribosomal tunnel. Therefore, as the nascent chains increase in length from 56-Pf3, 64-Pf3, to 72-Pf3, the Pf3 coat proteins gradually expose their transmembrane region (residues 19 to 36) from the ribosomal tunnel, whereas in 80-Pf3 or 91-Pf3 the transmembrane segment is fully exposed. The series of truncated nascent proteins should therefore represent insertion intermediates (Martoglio and Dobberstein, 1996) in the process by which the Pf3 coat protein leaves the ribosome to contact and insert into the membrane. Photocrosslinking was performed to investigate at which stage of the membrane biogenesis pathway YidC interacts with Pf3 coat. To do this, the photocrosslinking products of the nascent chains were analysed either directly (Figure 3.4b) or after immunoprecipitation with antiserum to YidC (Figure 3.4c, YC). The data show that the interaction between YidC and Pf3 coat is most intense when the transmembrane segment of Pf3 coat is fully exposed from the ribosome (80-Pf3 and 91-Pf3). This indicates that YidC interaction is more efficient when the C-terminal region of the Pf3 coat protein is exposed from ribosomal tunnel.
We also checked if YidC in the photocrosslinking described above was crosslinked to SecY. The photocrosslinking products of the different Pf3 coat truncated proteins (56-Pf3, 64-Pf3, 72-Pf3, 80-Pf3, and 91-Pf3) were immunoprecipitated with antiserum to SecY (Figure 3.4c, IP, SY). No SecY-Pf3 coat crosslinking products were detected among the series of the nascent proteins, which represent different stages of Pf3 coat membrane insertion (Figure 3.4c, IP, SY). As a positive control, we showed that the antiserum to SecY immunoprecipitated [35S]-labeled SecY, which was synthesized \textit{in vitro} in an \textit{E. coli} S30 translation system (Figure 3.4d). This data is consistent with Pf3 coat protein not contacting SecY. The fact that YidC mediates membrane insertion of Pf3 coat, a Sec-independent protein, implies that YidC can work independent of the Sec translocase.

### 3.2.5 YidC functions at the stage of protein translocation across the membrane

Pf3 coat membrane insertion can be understood as a process of three steps, i.e., the first step is targeting of Pf3 coat protein to the membrane, the second step is partitioning of the Pf3 coat hydrophobic domain into the membrane lipid bilayer, and the final step is translocation of the amino-terminal region across the membrane to achieve the correct orientation (Kiefer and Kuhn, 1999). In the third step, the proton motive force (pmf) is required for the electrophoretic transfer of the N-terminal tail which contains two negatively charged amino acid residues (Kiefer et al., 1997). Our data obtained by photocrosslinking suggests that YidC functions in the late stage of membrane insertion,
which might be the integration step (the second step) or the orientation step (the third step) to form the transmembrane form of the protein. Therefore, we dissected the membrane insertion of Pf3 coat to determine at which stage YidC functions.

First, we investigated whether YidC is important for the partitioning of the hydrophobic domain of Pf3 coat into the membrane. We used sodium carbonate extraction, which distinguishes peripherally bound proteins from integral membrane proteins. Pf3 coat was pulse labeled for 20 sec in YidC induced JS7131 cells (treated with arabinose) or YidC depleted JS7131 cells (treated with glucose). After converting the cells into spheroplasts, the cells were extracted with sodium carbonate (pH 11.5), and then subjected to ultracentrifugation to separate the membrane fraction (Pellet, P) and cytosolic fraction (Supernatant, S). As a control, we confirmed using proteinase K mapping that the insertion of Pf3 coat was essentially 100% blocked when YidC was depleted (Figure 3.5a, the protease mapping panel, YidC−). The carbonate extraction study showed that almost all the Pf3 coat was found in the membrane fraction when YidC was expressed by addition of arabinose (Figure 3.5a, the carbonate extraction panel, YidC+). In the YidC depleted cells, only around 40% of the Pf3 coat is in the membrane fractions and around 60% of Pf3 coat was extracted into the supernatant. This indicates, without YidC, more than half of the Pf3 coat cannot stably partition into the membrane and is extracted. The 40% of Pf3 coat detected in the membrane was still protected from proteinase K digestion, indicating that this portion of Pf3 coat is not correctly oriented in the N\text{out}/C\text{in} topology. This data suggests that YidC plays a role to orient Pf3 coat in the transmembrane configuration. Therefore, when YidC is absent, the hydrophobic region
of Pf3 coat does not span the membrane and can be more easily extracted by sodium carbonate.

The pmf is involved in membrane translocation of the negatively charged residues located within the amino-terminal region of Pf3 coat protein. What is the relationship between the function of YidC and the pmf? We applied photocrosslinking to investigate whether the interaction between Pf3 coat and YidC still occurs if the pmf is destroyed. The 91-amino acid truncated nascent Pf3 coat protein with amber mutation at L34, was used for photocrosslinking in the presence or absence of 140 µM carbonyl cyanide m-chlorophenylhydrazone (CCCP), a protonophore which dissipates the pmf. As previously shown by Kiefer and Kuhn (1999), CCCP can efficiently abolish the pmf under these \textit{in vitro} conditions (Kiefer and Kuhn, 1999). Figure 3.5b shows that there is no difference in the YidC-Pf3 coat photocrosslinking products between the non-CCCP-treated and CCCP-treated photocrosslinking reactions. This result indicates that the pmf is not necessary for YidC to interact with Pf3 coat and YidC does not function after the pmf-requiring step. Rather, YidC might act synergistically with the pmf to orient Pf3 coat in the membrane, whereby the pmf acts upon the negatively charged amino-terminal tail and YidC functions by transmembrane segment interaction.
3.2.6 The YidC-Pf3 coat interaction is independent of the charges flanking the transmembrane segment of Pf3 coat

Mutants of Pf3 coat protein have been described that insert with either an inverted orientation or they cannot insert at all (Figure 3.6a) (Kiefer et al., 1997; Kiefer and Kuhn, 1999). We asked whether these mutants can still interact with YidC by utilizing the photocrosslinking technique described above. Truncated nascent chains of 91 residues with the photoprobe at the L34 position were synthesized to study possible interactions with YidC. The photocrosslinking products were immunoprecipitated with YidC antibody and analyzed by SDS-PAGE/autoradiography (Figure 3.6b). The data show that the Pf3 coat mutants can interact with YidC with approximately the same efficiency, as wild-type Pf3 coat. In fact, Pf3-RD was crosslinked to YidC although its membrane insertion orientation (Cout/Nin) is opposite to wild-type Pf3 coat (Figure 3.6b). However, most striking is that YidC is efficiently crosslinked to Pf3-4N, a membrane insertion defective mutant (Kiefer and Kuhn, 1999) (Figure 3.6b). The reason why membrane insertion of Pf3 4N coat could not be observed is that Pf3 4N lacks negative charges in the N-terminal tail, on which the pmf acts, and therefore the pmf cannot drive the neutral N-terminal tail across the membrane. Taken together, the photocrosslinking data demonstrates that the Pf3 coat-YidC interaction occurs independently of the charges flanking the transmembrane segment of Pf3 coat. These findings are also consistent with the idea that the Pf3 coat-YidC interaction takes place prior to or at the same time as the pmf-requiring step.
3.3 Discussion

We have presented data showing, for the first time, that YidC interacts directly with a Sec-independent membrane protein, namely the Pf3 coat protein, and promotes its membrane insertion. Previously, we have shown that YidC promotes the membrane insertion of the Sec-independent M13 procoat protein (Samuelson et al., 2000). However, we could not rule out, that the YidC-depletion was causing an indirect effect, thereby inhibiting membrane protein insertion. In this paper, we find, using photocrosslinking, that ribosome-bound Pf3 coat nascent chains were crosslinked to YidC when the photoprobe is located either in the center or toward the amino- or carboxyl-terminal ends of the transmembrane segment (Figure 3.2). Moreover, we find that YidC binds to the non-translocated membrane protein as it is directly crosslinked to Pf3-4N which cannot insert across the membrane (Figure 3.6).

In our photocrosslinking studies, we extended the C-terminus of Pf3 coat such that we could fully expose the hydrophobic domain (residues 19 through 36) during synthesis with the protein still attached to the ribosome. This was necessary because Pf3 coat is too short when the ribosome is attached as around 35 ~ 40 amino acids residues are within the ribosome. The Pf3 coat with the extended C-terminal region, called Pf3-P2, was completely YidC dependent for membrane insertion (Figure 3.1). Efficient crosslinking to YidC was only observed when the carboxyl-terminal region of Pf3 coat had emerged from the ribosome. No crosslinking of YidC was observed to SecY. This data is consistent with Pf3 coat inserting by a Sec-independent mechanism (Rohrer and
Kuhn, 1990). Interestingly, our crosslinking results show some differences and similarities to those obtained with FtsQ, a Sec-dependent membrane protein, which has been investigated using the same photocrosslinking methodology (Urbanus et al., 2001). They are different because the hydrophobic domain of FtsQ first inserts in an environment around SecY and then moves toward YidC. The proposed function of YidC is to integrate the transmembrane regions into the membrane bilayer (Beck et al., 2001a). Our studies with the Sec-independent Pf3 coat are similar to those of FtsQ in that both these proteins contact YidC efficiently when the transmembrane segments are fully exposed from the ribosomal tunnel. This similarity suggests that YidC may function in a common way for Sec-dependent and Sec-independent proteins. The actual function of YidC might be that of a membrane chaperone to enable the transmembrane segments of either Sec-dependent or Sec-independent proteins to properly integrate into the lipid bilayer.

While the precise function of YidC is not known, it is required for the translocation of the hydrophilic domain of Pf3 coat across the membrane. We found that the Pf3-RD mutant, which inserts with the inverted topology also, interacts with YidC. Importantly, the Pf3-4N mutant, which does not translocate its hydrophilic domain across the membrane, still interacts with YidC (Figure 3.6), indicating that YidC recognizes the non-translocated coat protein. In addition, YidC still interacts with nascent Pf3 coat proteins that are in the process of inserting in the membrane even when the pmf is abolished by the addition of CCCP. We propose YidC and the pmf act synergistically to promote membrane insertion, whereby YidC associates with transmembrane segments and the pmf acts on negatively
charged residues. When YidC is depleted within the cell membrane, the hydrophobic region of wild-type Pf3 coat was only partially resistant to carbonate extraction suggesting that it cannot fully integrate into the membrane. This is in contrast to the M13 procoat protein, which still partitions into the membrane in YidC-depleted cells (Samuelson et al., 2001). However, M13 procoat protein has an additional hydrophobic segment that most likely promotes the hydrophobic interaction of the protein with the membrane.

In addition to YidC interacting with Pf3 coat with an extended C-terminal region, we also found that Ffh contacts the Pf3 coat protein in the membrane targeting and insertion pathway. This is in contrast to the YidC-dependent M13 procoat, which does not interact with Ffh and is targeted by an Ffh-independent mechanism (de Gier et al., 1998). However, while Pf3 coat interacts with Ffh, it does not require Ffh for insertion; the sodium carbonate extraction experiment showed that Pf3 coat is integrated in the membrane even in a Ffh-depleted strain. The Ffh-independent targeting is due to the presence of the two positively charged residues in the carboxyl-terminal region of Pf3 (Figure 3.3). The positively charged residues promote targeting to the membrane most likely by interacting with the negatively charged membrane surface, similar to the M13 procoat protein which is also targeted by positively charged residues (Gallusser and Kuhn, 1990). We propose that the charge interaction between Pf3 coat and the membrane is the major mechanism by which Pf3 coat is targeted to the membrane and the Ffh association is not necessary and is redundant. We used Pf3-NN, a Pf3 coat mutant which lacks the positively charged residues, to study this hypothesis. We found
that membrane targeting of the Pf3-NN mutant was only slightly affected \textit{in vivo} in the presence of Ffh. However, when Ffh was depleted, Pf3-NN membrane targeting was inhibited. This finding suggests that the photocrosslinking of the Pf3 coat to Ffh is not an artifact, and that Ffh interacts with Pf3 coat \textit{in vivo}, but this interaction is not essential. Another possibility is that the wild-type Pf3 coat protein may not strongly interact with Ffh because of its small size and that we see crosslinking to Pf3-P2 because these ribosome nascent chains have a better chance to productively interact with Ffh at the ribosome.

We propose a new model for Pf3 coat membrane biogenesis (Figure 3.7), based on the data in this paper and previously published work (Kiefer et al., 1997; Kiefer and Kuhn, 1999). Pf3 coat is targeted to the membrane with the aid of the bacterial SRP or by the interaction between the positively charged residues in the carboxyl-terminal region and the negatively charged membrane surface. After Pf3 coat is targeted to the surface of the membrane, its transmembrane segment partitions into the membrane and moves into an environment near YidC with the Pf3 coat polar amino-terminal tail region and positively charged carboxyl-terminal region located in the aqueous cytosol. Finally, the electrical potential, a component of the pmf, translocates the amino-terminal tail across the membrane by an electrophoretic mechanism and at the same time YidC mediates the transmembrane segment insertion.

In conclusion, we demonstrate for the first time using the photocrosslinking approach that YidC acts directly on a Sec-independent substrate to promote its membrane insertion.
YidC functions at a late stage of membrane insertion by associating with the membrane-bound hydrophobic region (Figure 3.4) and then helping the inserting protein to orient into the transmembrane form (Figure 3.5). The YidC-Pf3 coat interaction can even occur when the pmf is disrupted with CCCP and with a Pf3 coat protein mutant that cannot integrate across the membrane. Taken together, the data support the notion that the YidC-Pf3 coat interaction takes place during or before the pmf-requiring step of membrane protein insertion.

3.4 Materials and methods

3.4.1 Strains and plasmids

The *E. coli* YidC depletion strain, JS7131, is from our laboratory collection (Samuelson et al., 2000). WAM121, an Ffh depletion strain, was obtained from Greg Phillips’ lab. For *in vivo* studies, the genes of Pf3 coat, its mutants, and Pf3-P2 (Pf3 coat extended with Lep P2 soluble domain at its C-terminus) are under control of tac promoter in vector pMS119. pMS119 also carries the lacI<sup>q</sup> gene necessary for studies in JS7131. For *in vitro* studies, the truncated Pf3-P2 mRNA was transcribed from the T7 promoter in the pT7-7. pET610, harboring SecYEG genes under the control of the Trc promoter, was a generous gift from Arnold Driessen’s lab.
3.4.2 *In vivo* protease mapping

JS7131 strain bearing wild type Pf3 coat or its mutants, or Pf3-P2 in pMS119, was grown in LB medium with 0.2% arabinose overnight. The overnight culture was washed with LB medium twice and back-diluted 1 to 50 into fresh LB containing either 0.2% glucose or 0.2% arabinose. The cultures were grown for 2.5 h and shifted to M9 minimal medium supplemented with arabinose (to express YidC) or glucose (to deplete YidC) and grown for an additional 30 min. Ten minutes prior to labeling, 1 mM IPTG was added. The cells were labeled with trans-[\textsuperscript{35}S]-methionine for 20 s and chased for various times. *In vivo* protease mapping was performed to determine whether Pf3 coat protein was translocated across the membrane, as described (Whitley et al., 1994). Briefly, radiolabeled cells were resuspended in 33mM Tris HCl, pH 8.0, and a 40% sucrose solution, treated with 5 µg/ml lysozyme/1 mM EDTA (final concentrations), and incubated on ice for 15 min to convert the cells into spheroplasts. The spheroplasts was then treated with 0.5 mg/ml Proteinase K for 1 h on ice. The reaction was quenched with phenylmethylsulfonyl fluoride (PMSF) (0.33mg/ml, final concentration) and then precipitated with 10% trichloroacetic acid (TCA) and subjected to immunoprecipitation or directly analysed by SDS-PAGE/autoradiography. Pf3 coat can be analyzed directly without immunoprecipitation with anti-Pf3 coat serum using a 22% SDS-polyacrylamide gel containing urea (Boeke et al., 1980), since there is no background of radio-labeled proteins in the region of the gel less than 6 KDa. Pf3-P2 can be analyzed by immunoprecipitation with anti-Lep serum.
3.4.3 Sodium carbonate extraction

The cells expressing Pf3 coat were labeled with trans-[\textsuperscript{35}S]-methionine and converted into spheroplasts as described in the \textit{in vivo} mapping section. 0.2 M sodium carbonate (pH 11.5) was added to the spheroplast suspensions. The suspensions were then vigorously vortexed and incubated on ice for 30 min. After centrifugation for 1 min in the microfuge (14,000 rpm) to remove non-lysed cells and cell debris, the sample was subjected to ultracentrifugation (130,000 g) for one hour at 4 °C. The supernatant and membrane pellet were carefully separated and subjected to 10% TCA precipitation, and analyzed by SDS-PAGE and a phosphorimager.

3.4.4 \textit{In vitro} translation and photocrosslinking

The \textit{E. coli} S100 \textit{in vitro} translation system was made according to Gold and Schweiger (Gold and Schweiger, 1971). Site-specific photocrosslinking was performed based on the procedure as described (Graf et al., 1998). Amber codons were introduced into the Pf3 coat gene at various positions to allow the incorporation of the photoactivatable amino-acid analogue (Tmd)Phe (L-4'-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenylalanine) (Brunner, 1996). Using T4 RNA ligase (Boehringer Mannheim), (Tmd)Phe-pdCpA was covalently joined to the \textit{E. coli} suppressor tRNA\textsuperscript{Asn} (Cload et al., 1996) made using T7 Megashortscript \textit{in vitro} transcription kit from Ambion. PCR was used to generate the truncated gene fragments, from which the truncated mRNA transcripts were made using
the T7 Megashortscript in vitro transcription kit. The inner membrane vesicles (IMVs) were prepared according to the procedure (Rhoads et al., 1984). For photocrosslinking studies, the truncated mRNA and the (Tmd)Phe-charged tRNAAsn were added to the E. coli S100 in vitro translation system to produce [35S]-methionine labeled Pf3 coat nascent protein. IMVs were added to the translation reactions 4 min after the reactions were initiated at 37 °C, and the reaction continued to proceed for 40 min at 37 °C. The reactions were then irradiated with the 360 nm ultraviolet light for 30 min at 4 °C to perform photocrosslinking. The reactions were then subjected to TCA precipitation or immunoprecipitation. The SecY positive control was synthesized using pET610 (containing the SecY gene) with the T7 S30 circular DNA in vitro translation system (Promega). In the system, we took advantage of endogenous E. coli RNA polymerase, instead of T7 RNA polymerase. For Sec Y immunoprecipitation, the samples were not heated to avoid aggregation.
Figure 3.1 YidC is required for membrane insertion of Pf3 coat and Pf3-P2 coat proteins.  

a, Topologies of Pf3 coat and Pf3 P2 in the plasma membrane with the initiation Met (*) in the periplasm.  
b, Proteinase K mapping of the Pf3 coat protein in the YidC depletion strain, JS7131.  Cells were grown in the presence of arabinose (YidC+) or in the presence of glucose (YidC−), pulse labeled for 20 sec and chased for the indicated times.  The cells were then converted to spheroplasts and treated with or without proteinase K on ice for 1 h.  After quenching the reaction with PMSF, the samples were TCA precipitated, acetone washed, and analyzed by SDS-PAGE and fluorography.  
c, Proteinase K mapping of Pf3-P2 in the JS7131 strain.  JS7131 expressing Pf3-P2 were grown in the presence of YidC (YidC+) or under YidC depletion conditions (YidC−), and analyzed as described in Figure 3.1b, except there was no chase.  Pf3-P2 that is translocated across the membrane is cleaved by proteinase K to generate a resistant fragment (Digested Pf3-P2).
Figure 3.1

(a) Diagram showing the interaction of Proteinase K with Pf3 coat and Pf3 P2 proteins in the periplasm, membrane, and cytoplasm.

(b) Table showing the effects of YidC+ and YidC− on Pf3 coat digestion by Proteinase K at different chase times (10, 60, 120 seconds).

(c) Diagram illustrating the digestion of Pf3 P2 proteins by Proteinase K with and without YidC.
Figure 3.2 Photocrosslinking shows YidC and Ffh interacts with Pf3 coat during membrane insertion. a, Schematic illustration of Pf3 coat showing the positions where the photoprobe (*) were introduced. b, YidC and Ffh photocrosslinked to the Pf3 coat transmembrane segment. The 91-amino-acid nascent chains of Pf3-P2 coat with the photoprobe at the indicated positions were photocrosslinked (UV +) or not (UV -) (Totals). The sample with the photoprobe at position 34 was immunoprecipitated with antiserum to YidC (YidC IP) and Ffh (Ffh IP). The position of YidC-Pf3 coat adduct on the gel is indicated with *, and the Ffh-Pf3 coat adduct indicated with o. The positions of the molecular weight standards (MultiMark™ rainbow Marker, from Invitrogen) are marked on the leftside. c, Photocrosslinking of Pf3 coat to YidC is prevented by puromycin/high salt treatment. The 91-amino acid nascent Pf3 coat with the photoprobe at the position L34 was used. Samples were analyzed as in Figure 3.2b, except prior to photocrosslinking, 1 mM puromycin and 0.4 M potassium acetate were added to release the nascent chains from ribosomes (Puro+). Puro- corresponds to samples not treated with puromycin and high salt.
Figure 3.2

(a) Positions of photoprobes: T20 I27 L34

(b) Positions of photoprobes: T20 I27 L34

(c) Relative YidC Crosslinking: Puro Puro
Figure 3.3  Ffh is important for membrane targeting of Pf3-NN coat where the two positively charged residues are substituted with asparagines.  a, Schematic illustration of wild type Pf3 coat and Pf3-NN.  b, Sodium carbonate extraction shows that Ffh is important for Pf3-NN targeting but not for the targeting of the wild-type Pf3 coat protein.  WAM121 expressing wild-type (wt) Pf3 coat or Pf3-NN was grown with arabinose or glucose to express Ffh (Ffh⁺) or to deplete Ffh (Ffh⁻), respectively.  Cells were pulse-labeled for 20 sec, converted to spheroplasts, treated with sodium carbonate, and centrifuged to isolate the pellet (P, membrane fraction) and supernatant (S, cytosol fraction).  The percentage of Pf3 coats in each fraction was calculated from Phospholmager intensity.
Figure 3.3

a

N — — — ++ C  wt Pf3 coat
N — — — — C  Pf3-NN

b

<table>
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<th>Pf3-NN</th>
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<tr>
<td></td>
<td>Ffh⁺</td>
<td>Ffh⁻</td>
</tr>
<tr>
<td>S</td>
<td>P</td>
<td>S</td>
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<tr>
<td>&lt;5%, &gt;95%</td>
<td>&lt;5%, &gt;95%</td>
<td>23% , 77%</td>
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</table>

Figure 3.3
Figure 3.4 Efficient Pf3 coat interaction with YidC occurs after the transmembrane region has fully emerged from the ribosome and membrane insertion is independent of SecY. a, Schematic illustration of the different lengths of Pf3 coat nascent chains bound to ribosomes. The nascent chains with 56, 64, 72, 80, and 91 amino acid residues are termed 56-, 64-, 72-, 80-, and 91-Pf3, respectively. The photoprobe (*) was placed at position T20 in 56- and 64-Pf3, and at position L34 in 72-, 80-, and 91-Pf3. The junction between Pf3 coat and P2 in the cartoon is indicated by a “-”. b, Photocrosslinking of Pf3 coat with different lengths of nascent chains. The positions of the Ffh-Pf3 coat and YidC-Pf3 coat adducts on the gel are indicated with arrows. c, Photocrosslinking products shown in b were analyzed by immunoprecipitation with antiserum to YidC (YC) and to SecY (SY). d, In vitro synthesized [35S]-labeled SecY is detected with the SecY antiserum. SecY gene in pET610 was expressed, [35S]-methionine-labeled, and analyzed either directly (Tot) or immunoprecipitated with Sec Y antibody (IP).
Figure 3.4 (Continued)
Figure 3.4 (continued)

### c

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<th>Length (aa)</th>
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</tbody>
</table>

Pf3-YidC

### d

**Secl**  
**Secl /G**
Figure 3.5 YidC mediates Pf3 coat membrane insertion. **a, Upper panel.** Sodium carbonate extraction shows that YidC affects membrane partitioning under alkali conditions. JS7131 cells expressing Pf3 coat were grown in the presence of arabinose (YidC⁺) or in the presence of glucose (YidC⁻), pulse-labeled for 20 sec with [³⁵S]-methionine. The samples were split into two aliquots, one for the sodium carbonate extraction study (upper panel) and the other for protease mapping (lower panel). The supernatant (S) and pellet (P) fractions were prepared as described in the legend of Figure 3.3b. **a, Lower panel.** For the proteinase K mapping study, cells were analyzed as described in the legend of Figure 3.1b. **b, Pf3 coat interacts with YidC in the absence of a pmf.** Photocrosslinking was performed in the presence or absence of CCCP treatment with the 91-amino-acid Pf3 coat nascent chain containing the photoprobe at position L34. CCCP (140 µM, final concentration) was added to the photocrosslinking reaction 4 min after translation was initiated. The Pf3-YidC adduct on the gel is indicated with an arrow.
Figure 3.5

(a) Carbonate Extraction

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</tr>
</thead>
<tbody>
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<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pf3 coat

(b) Protease Mapping

<table>
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<th>YidC⁺</th>
<th>YidC⁻</th>
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<tr>
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</tr>
<tr>
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<td>+</td>
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<table>
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<th>YidC⁺</th>
<th>YidC⁻</th>
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Pf3 coat

<table>
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Pf3-YidC

Pf3 coat

Figure 3.5
Figure 3.6  Pf3 coat mutants with an inverted topology or with a defective insertion phenotype are photocrosslinked to YidC.  a, Schematic illustration of the wild-type (wt) Pf3 coat, Pf3-RD and Pf3-4N mutants.  b, Photocrosslinking of YidC with wild-type Pf3 coat and mutant Pf3 coat proteins containing the photoprobe at position L34 of the 91-residue nascent chain.  The in vitro translation and photocrosslinking was performed as described in the legend of Figure 3.2b. The YidC adducts (upper panel) and the Pf3 coat nascent proteins (lower panel) are indicated with an arrow.
Figure 3.6

<table>
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<th>Pf3 coat</th>
<th>Structure</th>
<th>Insertion Phenotype</th>
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</thead>
<tbody>
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<td>1: Pf3 wt</td>
<td>N – – ++ C</td>
<td>N\textsubscript{out} / C\textsubscript{in}</td>
</tr>
<tr>
<td>2: Pf3-RD</td>
<td>N ++ – – C</td>
<td>N\textsubscript{in} / C\textsubscript{out}</td>
</tr>
<tr>
<td>3: Pf3-4N</td>
<td>N – – – C</td>
<td>No insertion</td>
</tr>
</tbody>
</table>

b

Pf3-YidC

Pf3 coats

Figure 3.6
Figure 3.7 Proposed model of Pf3 coat membrane biogenesis mediated by YidC. Pf3 coat is synthesized and is then targeted to the inner membrane by the interaction of the positively charged residues in the carboxy terminal region with the negatively charged membrane surface. In addition, Ffh may also promote Pf3 coat membrane targeting. After Pf3 coat membrane targeting, the Pf3 coat hydrophobic region partially integrates, and then interacts with YidC. The pmf and YidC then cooperate together to promote translocation. The membrane electrical potential component (ΔΨ) of the pmf is responsible for translocating the negatively charged amino terminal region of Pf3 coat into the periplasm, and YidC mediates stable hydrophobic segment integration into a transmembrane configuration. The carboxy terminus of Pf3 coat is retained in the cytosol by interaction of its positive charges with the negatively charged membrane surface. P, M, and C stand for periplasm, membrane, and cytosol, respectively.
Figure 3.7
CHAPTER 4*

YIDC CONDITIONAL LETHAL MUTATIONS: EVIDENCE FOR SEPARATION OF THE SEC-INDEPENDENT AND SEC-DEPENDENT MEMBRANE PROTEIN INSERTION FUNCTIONS OF YIDC

4.1 Introduction

In prokaryotes and eukaryotes, exported proteins typically are synthesized with a cleavable N-terminal signal sequence and require a translocase to cross the membrane. In bacteria, the Sec translocase is located in the inner membrane, and is comprised of SecA, SecYEG and SecDFYajC (Driessen et al., 2001; Mori and Ito, 2001). SecB, an export-dedicated chaperone, interacts with a subset of exported proteins in the cytoplasm and keeps the fully synthesized exported protein in a translocation competent state (Randall and Hardy, 1995; Weiss et al., 1988). SecB then helps target the protein to the membrane by delivering it to the membrane-bound SecA (Hartl et al., 1990). SecA, the translocation

*Portions of this chapter were written into paper and submitted to *EMBO J.*

Chapter acknowledgement: Thanks to Kun Xie for his help on this project.
ATPase, uses the energy of ATP hydrolysis to promote membrane translocation of polypeptide segments of the exported proteins (Economou and Wickner, 1994; Hunt et al., 2002). SecA works in conjunction with the SecYEG, which most likely constitutes the translocation channel in which the exported protein is moved within as it crosses the membrane (Bessonneau et al., 2002; Breyton et al., 2002; Manting et al., 2000; Meyer et al., 1999). The SecD and SecF of the SecDFYajC complex facilitate protein export (Pogliano and Beckwith, 1994). After membrane translocation, the leader sequence of the exported protein is proteolytically removed by signal peptidase 1 (or leader peptidase).

The SecYEGDFYajC translocase plays center stage in membrane protein insertion as well. However, SecA seems only to be needed for membrane proteins that contain large translocated loops (Andersson and von Heijne, 1993; Kuhn, 1988; Neumann-Haefelin et al., 2000). The majority of membrane proteins require the bacterial signal recognition particle (SRP, Ffh and 4.5S RNA) and the signal recognition particle receptor (SR, FtsY) for membrane targeting (de Gier et al., 1996; Macfarlane and Muller, 1995; Seluanov and Bibi, 1997; Ulbrandt et al., 1997). In addition, a new membrane protein called YidC has been implicated in the membrane insertion step (Samuelson et al., 2000; Scotti et al., 2000). YidC was discovered to mediate the insertion event since cells that were depleted of YidC were found to insert membrane proteins less efficiently (Samuelson et al., 2000). YidC was found to be associated with the Sec translocase (Scotti et al., 2000) and can be cross-linked to membrane proteins that are in the process of inserting into the membrane (Houben et al., 2000; Samuelson et al., 2000; Scotti et al., 2000; Urbanus et al., 2001). The precise role of YidC in membrane insertion is not understood. However, it is
believed that YidC may assist in the folding of membrane proteins in the hydrophobic region into the lipid bilayer (Luirink et al., 2001). YidC is also suggested to function as a membrane chaperone during the Sec-independent protein membrane insertion (Chen et al., 2002). Moreover, YidC may form an assembly site for the folding of multiple hydrophobic regions of polytopic membrane proteins prior to their insertion into the lipid bilayer (Beck et al., 2001a).

For the membrane insertion of proteins that do not use the Sec translocase, YidC also plays an important role. Recently, YidC was found to be essential for the membrane insertion of the M13 phage procoat (Samuelson et al., 2000; Samuelson et al., 2001) and Pf3 phage coat protein (Chen et al., 2002). Up until these studies were reported, it was widely believed that these proteins inserted into the membrane by a spontaneous mechanism. Therefore, YidC most likely participates in two pathways, one involving the Sec translocase and one distinct from the Sec machinery (for review see (Luirink et al., 2001; Stuart and Neupert, 2000).

All current evidence showing YidC plays a role in protein membrane insertion are based on *in vitro* crosslinking data showing physical interaction between YidC and protein being inserted into membrane as well as *in vivo* studies examining membrane protein assembly where YidC is depleted in the cell (Samuelson et al., 2000). Since the depletion of YidC takes 2 to 3 hours, it has been argued that the role of YidC in membrane protein insertion may not be correct because the effects one observed (Samuelson et al., 2000) may be indirect due to the YidC depletion. The YidC depletion through several
generations of cell growth may cause the depletion of other translocase components, which may result in impaired membrane insertions. Therefore it is necessary to find a more straightforward approach to examine whether YidC plays a direct role in membrane protein assembly.

In this study, we have isolated temperature-sensitive (ts) and cold-sensitive (cs) YidC mutants to examine membrane protein insertion. We show that the membrane insertion of the Sec-independent M13 procoat protein is quickly inhibited in a temperature-sensitive strain when grown at the restricted temperature. This provides strong evidence that YidC plays a direct role and rules out that the membrane insertion block observed with YidC depletion strains were due to secondary effects. In a cs mutant strain, insertion of the Sec-independent M13 procoat protein was very efficient while the insertion of the Sec-dependent leader peptidase and outer membrane protein A precursor is inhibited. The YidC cs mutant seems defective in the Sec-related function of YidC while its activity is functioning quite normal toward the Sec-independent M13 procoat protein. This defective Sec-related function may be due to impaired interaction of the YidC with the Sec complex since there is impaired co-elution of YidC and SecDF in the c.s. YidC strain. The properties of the cs mutant provide compelling evidence that YidC can function both on its own and with the Sec machinery.
4.2 Results

4.2.1 Introduction of site-specific protease sites into YidC results in conditional lethal mutants

In order to elucidate the function of YidC in membrane protein insertion, we have isolated conditional lethal (cold and temperature sensitive) mutants of YidC. Incorporation of site-specific protease sites into YidC can result in temperature (ts) and cold (cs) sensitive mutants. In these studies, Factor Xa (FXa), PreScission Protease (PS) (Amersham Pharmacia Biotech), Thrombin (Thn), TEV Protease (Tev), and Genenase (Gase) (New England Biolabs) cleavage sites were incorporated into the cytoplasmic and periplasmic loops of YidC. Figure 4.1A and 4.1B show the positions of the protease sites in YidC and flanking amino acid sequences of the protease sites, respectively. The YidC mutants, which were under control of the YidC natural promoter, were cloned into a low-copy-number plasmid, pACYC184. These plasmids were transformed into the YidC depletion strain, JS7131, to test whether the YidC mutants can complement the growth defect of YidC depletion when JS7131 is grown in glucose media at the 30, 37 and 42 °C. When Factor Xa (FXa₁) and PreScission Protease (PS) sites are introduced into the N-terminal and C-terminal region of the first periplasmic loop of YidC (Figure 4.1), respectively, this YidC mutant (termed FP) becomes cold sensitive, i.e. it cannot complement the growth defect of YidC depletion in JS7131 at 30 °C or below (Figure 4.2). Incorporation of a Thrombin (Thn) site into the second cytoplasmic loop and a TEV protease (Tev) into the last periplasmic loop (mutant termed TTe) results in a YidC
temperature-sensitive phenotype, i.e. these mutants cannot function at 42 °C (Figure 4.2). The YidC mutants (TF and TG) also show temperature-sensitive phenotypes (data not shown) when the Thrombin (Thn) site in the second cytoplasmic loop is introduced in combination with Genenase (Gase) site (mutant termed TG) in the last periplasmic loop or with Factor Xa (FXa²) site (mutant termed TF) in the second periplasmic loop of YidC (Figure 4.1). All cs and ts YidC mutants can complement YidC depletion at 37 °C.

4.2.2 Construction of YidC ts and cs strains

To further analyze membrane protein insertion, ts and cs YidC strains were constructed by the gene replacement method (Hamilton et al., 1989). The yidC ts and cs mutant genes (TTe, TF, TG, and FP), described in Figure 4.1, were cloned into the integration vector pMAK705, in which the yidC mutant genes were flanked by the upstream natural yidC promoter sequence and the thdf gene, located downstream of the yidC gene in the chromosome (Figure 4.3). The replication origin of pMAK705 is temperature sensitive, which makes it suitable for gene replacement. The wt yidC gene in E. coli strain, MC1060, was replaced by either the yidC cs (FP) or ts genes (TTe, TG and TF) (Figure 4.3). The resulting E. coli strains, in which the yidC FP, TTe, TG, or TF gene has replaced the wt yidC gene, are named MYC-cs, MYC-Te, MYC-TG and MYC-TF strains. MYC-cs is the YidC cs strain, while MYC-Te, MYC-TG, and MYC-TF are YidC ts strains. The expression of the wt YidC from the pING vector complements the YidC cs and ts strains at their non-permissive temperatures (data not shown), confirming that the
yidC cs or ts mutant genes successfully replaced the chromosomal yidC gene, and the cs or ts phenotype of the strains is based on the mutation in the chromosomal yidC gene.

### 4.2.3 YidC ts and cs mutant proteins show different stability patterns at the permissive and non-permissive temperatures

We tested the stability of the YidC cs and ts mutant proteins in vivo by determining the protein level of YidC by immunoblotting using a YidC antiserum. Figure 4.4 shows that the ts YidC mutant in MYC-Te is stable at the permissive temperature (30 °C), while it is unstable and degraded at the non-permissive temperature (42 °C). Note that the upper band in the Western blot is a non-specific band that is recognized by the YidC antiserum. The other ts mutants (TG and TF) are also unstable at the non-permissive temperature (data not shown). In contrast, the YidC cs mutant is more stable at non-permissive temperature (25 °C) than at the permissive temperature (37 °C) (Figure 4.4). As a control, we show that wt YidC, in the parental strain, MC1060, is stable at 25, 30 and 37 °C, but less stable at 42 °C where there is a slight reduction in the full-length YidC levels (see degradation fragments in Figure 4.4). A comparison of the stabilities of wt, ts and cs YidC, suggests that introduction of the protease sites alters the conformation of the YidC protein and makes it sensitive to temperature changes. Such a conformational change in the ts YidC mutant at 42 °C could lead to degradation of the protein by proteases. On the other hand, FP, the YidC cs mutant, is very stable at 25 °C, but the protein cannot function normally at this temperature. This suggests that there is a structural change during the temperature switch from 37 to 25 °C. Notably, there is a reduced level of the
cs YidC at the permissive temperature while at the same time there is no growth defect of the cell. The level of YidC observed at 37 °C appears sufficient for cell growth, although it is lower than the wild-type level.

4.2.4 Procoat membrane insertion in the YidC ts strain can be blocked in a short time period in the non-permissive temperature

Since the ts or cs mutant proteins will be denatured quickly without affecting other translocase components, the use of the conditional lethal strains allows us to discriminate if the observed membrane protein insertion defects in the YidC depletion strain are due to a defective YidC function, or caused by secondary effects of the depletion. Therefore, we tested how quickly membrane protein insertion can be blocked in the YidC ts strains when YidC is inactivated. The membrane insertion of the M13 procoat protein, tagged at the C-terminus with the P2 domain of Lep (PClep), was tested in the YidC ts strain, MYC-Te, when the cells were treated at 42 ºC for 20, 40, 80 and 120 min. The cells were pulse-labeled for 20 s and the samples were analyzed for membrane insertion by examining the amount of PClep processed to the mature form by signal peptidase I cleavage. Processing indicates that PClep has successfully inserted across the membrane, as the signal peptidase I active site is on the periplasmic side of the membrane. Figure 4.5 shows that the membrane insertion of PClep can be blocked even after only a 20 min treatment at 42 ºC. As a control, we confirmed that PClep inserts efficiently in the wild-type strain at 42 ºC (data not shown) despite some degradation of YidC in the cells (Figure 4.4, MC1060, 42 ºC). We also tested the insertion of the wild-type procoat in
MYC-Te cells, and the results are similar to PClep. Membrane insertion of procoat is almost completely blocked in MYC-Te after 30 min at 42 °C (data not shown). Taken together, the data provide the most direct evidence yet proving YidC plays a direct role in the insertion of a Sec-independent membrane protein \textit{in vivo}.

4.2.5 The Sec-related function of YidC for leader peptidase membrane (Lep) insertion is impaired in YidC cs strain at the non-permissive temperature

Previous studies using the YidC depletion strain, JS7131, showed that the insertion of the C-terminal (P2 domain) of the Sec-dependent leader peptidase (Lep) is inefficient when YidC is depleted, which indicates that YidC plays a role in Sec-dependent membrane insertion (Samuelson et al., 2000). YidC is believed to catalyze the removal of hydrophobic regions of a membrane protein out of the SecYEG channel and into the lipid bilayer (Urbanus et al., 2001). Figure 4.6 shows that the insertion of the Lep P2 domain to the periplasmic space was inhibited in the cs YidC mutant at the non-permissive temperature (25 °C), but was not inhibited at the permissive temperature or in the parent strain containing a wild-type YidC grown at 37 or 25 °C. The cs YidC mutant was pulse-labeled with \[^{35}S\]-methionine for 20 sec after growth for 0.5, 1 or 2 hr at the permissive (37 °C) or non-permissive (25 °C) temperature. Cells were then converted to spheroplasts, and treated with or without proteinase K. At the permissive temperature, all the Lep is digested by the added protease, indicating the C-terminal domain inserts across the membrane. In contrast, at the non-permissive temperature, some of Lep is resistant to proteinase K degradation, showing that insertion is inhibited. Inhibition is quick and
occurs even after 30 min of growth at the non-permissive temperature (Figure 4.6A), but becomes stronger after 1 h or 2 h at 25 ºC (Figure 4.6B). Specifically, we observe 10% of Lep blocked when cells are at the restrictive temperature for 0.5 h (Figure 4.6A), whereas the amount of Lep blocked at 1 and 2 h at 25 ºC is ~20% and ~15% (Figure 4.6B), respectively. We score this inhibition as rather strong because previous studies showed the membrane insertion of the Sec-dependent proteins such as Lep and FtsQ are only partially inhibited (varying from 15 to 30% for Lep and ~15% for FtsQ) when YidC is depleted (Jiang et al., 2002; Samuelson et al., 2000; Urbanus et al., 2001) and the inhibition observed in MYC-cs is very similar to those effects. Therefore, we conclude that the Sec-related function of YidC for the membrane insertion of Lep is impaired. In all cases, there is also a block in the export of the outer membrane protein A (OmpA) precursor. In order to determine the reasons why proOmpA accumulated in the MYC-cs strain, we examined the export of OmpA in the MYC-cs and MC1060 strains harboring pMS119Lep at 25 and 37 ºC when IPTG is present and absent (Figure 4.6C). We observed that proOmpA accumulation resulted from two effects: One effect when YidC was defective in Sec-related function is due to the overexpression of Lep causing the proOmpA block, as we see ~90% of proOmpA accumulate when the plasmid-encoded Lep is overexpressed by IPTG induction for 5 min whereas only ~45% accumulate when IPTG is not added (compare 25 ºC/MYC-cs/“+” and “−” lanes in Figure 4.6C). This type of observation has been seen before when YidC is depleted and a Sec-dependent protein is overexpressed (Samuelson et al., 2001). The accumulation of OmpA precursors is likely due to the presence of overexpressed Lep that stalls in the Sec channel, thereby interfering with protein export by the Sec translocase. The other effect
resulting from a defect in the YidC Sec-related function at 25 °C is due to OmpA export being slightly inhibited even without induction of Lep (compare 25 °C/MC1060 IPTG “−” and MYC-cs IPTG “−” lanes in Figure 4.6C). This effect was further confirmed by testing OmpA export in the MC1060 and MYC-cs strains (without pMS119 Lep) at 25 and 37 °C. Figure 4.6D shows that OmpA export was delayed in the MYC-cs strain (~48% accumulation of proOmpA) as compared with the one in MC1060 strain (~24% proOmpA accumulation), when the cells were treated at 25 °C for 1 hour. This data raises the possibility that YidC directly stimulates the export of proteins when the temperature is low. This effect may also be indirect due to the jamming by endogenous membrane proteins expressed within the cell.

4.2.6 Procoat can insert into the membrane in the YidC cs strain at the non-permissive temperature

Since the cold-sensitive YidC mutant in the Myc-cs strain is defective in its function for the Sec-dependent Lep protein, we expected that the mutant cannot facilitate membrane insertion of M13 procoat protein, a Sec-independent protein. Surprisingly, we found that procoat tagged with P2 Lep domain (PClep) could insert quite efficiently in the YidC cs strain even within the same cells that were also defective at inserting the overexpressed Lep and which accumulated exported proteins (Figure 4.7). Figure 4.7A shows that processing of procoat is quite efficient (76% inserted) at the non-permissive temperature (25 °C), under conditions where the Sec-related function of YidC was strongly impaired (the insertion of Lep is ~40% inhibited, Figure 4.7B). PClep inserts into the membrane
very efficiently at 37 °C despite a reduction of the level of the cs YidC protein at this temperature (see Figure 4.4). Since PClep was almost completely blocked (<10% inserted) when YidC was depleted (Samuelson et al., 2001), we conclude that PClep can insert very efficiently with only a minor inhibition in the cold-sensitive strain at 25 °C. When PClep was expressed alone in the YidC cs strain, only slight inhibition of procoat membrane insertion occurs even after 4 hours of cell growth at the non-permissive temperature (data not shown). These data demonstrate that the YidC cold sensitive mutant can promote the efficient membrane insertion of the sec-independent procoat at its non-permissive temperature.

4.2.7 The YidC cs mutant is impaired in binding to the SecDF components of the Sec complex at the non-permissive temperature

Recently, YidC was shown to form a tetrameric complex with SecDFYajC (Nouwen and Driessen, 2002). It was suggested that this interaction allows YidC to associate with the SecYEG channel and perform its Sec-related function. Since the cs mutant is defective in the YidC Sec-related function, the possibility exists that it is impaired because it cannot associate with SecDFYajC. To test this hypothesis, we examined whether YidC copurifies with SecDF when isolated from the cs YidC strain. A His-tagged SecF was purified in detergent by the His-tag/Nickel affinity chromatography method (Smith et al., 1988). Figure 4.8A (right side) shows that SecD and SecF are the major proteins in the elution fraction profiles that were prepared from extracts from the cs strain grown at the non-permissive temperature. Similar elution profiles (left side) were observed from
MC1060 cells containing the wild-type YidC prepared at 25 ºC. When a Western blot analysis was performed using YidC antiserum (Figure 4.8B), dramatically less YidC appears to co-purify with SecDF in the cs strain compared to the wild-type (Figure 4.8B; see E1 and E2), even though the YidC level in the starting membrane fraction prepared from both strains is identical (Figure 4.8B, Lane M). These data suggest that the cs YidC mutant is largely defective in interacting with the SecDF complex at the non-permissive temperature. These data are also consistent with the in vivo data (Figure 4.6) which shows that the Sec-related function of YidC cs mutant is impaired at the non-permissive temperature.

4.3 Discussion

In this report, we describe the isolation of temperature- and cold-sensitive YidC mutants to study membrane protein insertion in E. coli. We found using the temperature sensitive mutant that even after 20 min at the non-permissive temperature, the insertion of PClep was inhibited (Figure 4.5). The ts mutant is inactivated quickly and does not have the problem that the YidC depletion strain has where it takes several hours to deplete the cell of YidC (Samuelson et al., 2000). Therefore, this result firmly establishes that YidC plays a direct role in membrane protein insertion. A direct role of YidC in membrane insertion of proteins was recently shown with the Pf3 coat protein. In this study, ribosome-bound Pf3 coat protein was trapped in the membrane insertion process and shown to interact with YidC using a photocrosslinking approach (Chen et al., 2002).
Remarkably, the YidC function for Sec-dependent membrane proteins is substantially inhibited in the cs mutant at the non-permissive temperature (Figure 4.6), while the Sec-independent PClep protein inserts very efficiently (Figure 4.7). At the non-permissive temperature, there was a significant inhibition in Lep insertion as well as an inhibition in the export of proOmpA, a secretory protein. This block in insertion at 25 °C takes place in a short time period (30 min and 1hr) in a cell where the cell doubling time is greater than 2 hours (in the M9 minimal media). This establishes that YidC plays a direct role for Sec-dependent membrane proteins. Nevertheless, procoat can efficiently insert into the membrane and be processed by leader peptidase even in the same cell where there is a significant defect in Lep insertion while non-translocated exported proteins accumulate (Figure 4.7). This result provides further *in vivo* evidence that the M13 procoat protein inserts via a distinct mechanism not involving the Sec translocase.

The block in the export of preproteins in the cold-sensitive mutant is most likely due to Lep getting stalled within the Sec translocase and being inefficiently transferred laterally out of the Sec YEG channel into the lipid bilayer when YidC is non-functional (Samuelson et al., 2001). This jamming of the Sec machinery would indirectly affect the export of Sec-dependent proteins. Supporting this idea is that the export defect on OmpA is much more pronounced when the plasmid encoded Lep is expressed by the addition of IPTG. Nevertheless, there is a measurable inhibition in the export of proOmpA at 25 °C in the cs YidC strain lacking the Lep plasmid (Figure 4.6D). Samuelson et al. (2000) reported that YidC is not required for protein export at 37 °C. The OmpA export in the MYC-cs strain suggests that YidC is important for efficient protein export at low
temperature. We propose that YidC may be required indirectly due to a defect in the insertion of endogenous amounts of Sec-dependent membrane proteins, which causes slight jamming of OmpA export. Another possibility we cannot rule out is that YidC is only required for protein export at low temperature such as 25°C, not at the normal growing temperature of *E. coli* (37 °C). Notably, Pogliano and Beckwith previously isolated cs mutants in SecD and SecF and suggested that the protein export pathway in *E. coli* was cold sensitive (Pogliano and Beckwith, 1994; Pogliano and Beckwith, 1993). The inhibitory effects on protein export in the cs mutants of SecD, SecF and SecY are much stronger than the inhibitory effects seen in the YidC cs mutant (Pogliano and Beckwith, 1994; Pogliano and Beckwith, 1993; Wolfe et al., 1985).

The emerging data (Chen et al., 2002; Samuelson et al., 2000; Samuelson et al., 2001; Urbanus et al., 2001) show that Sec-independent proteins are highly dependent on YidC for direct insertion into the lipid bilayer. In contrast, the requirement of YidC for membrane insertion for a Sec-dependent protein is small. This small effect for Sec-dependent proteins is consistent with our hypothesis that YidC is needed to remove Lep efficiently from the channel by catalyzing the transfer of the hydrophobic domains out of the SecYEG channel into the lipid bilayer. YidC plays no role in allowing a Sec-dependent membrane protein to insert into the Sec channel within the membrane. The block observed with the transfer of Lep P2 across the membrane would be indirect, since this Lep will not be able to insert into the Sec channel because the Sec translocases are jammed by the overexpressed Lep in the Sec channel. Therefore it does not play an insertion role, per se, like it does for Sec-independent proteins.
What might be the reason why the cs YidC mutant is impaired in its Sec function? This may arise because the introduction of the protease sites into the YidC molecule results in the protein being defective in interacting with the Sec machinery, while not perturbing its activity for insertion of the M13 procoat protein. Recently, it was demonstrated that YidC associates with SecDFYajC (Nouwen and Driessen, 2002), raising the possibility that YidC is brought to the Sec translocase by its interaction with the SecDFYajC heterotrimeric complex. Indeed, the SecDF interaction of the cs YidC mutant appears perturbed at the non-permissive temperature since little YidC cofractionates with the SecDF complex (Figure 4.8).

The isolation of a YidC mutant with the properties of the cs YidC mutant supports the proposal that YidC can function on its own or in conjunction with the Sec translocase. For the latter function, YidC needs to interact with the SecYEG complex and still be functional in membrane insertion. The YidC domain that catalyzes membrane insertion is probably the same for Sec-dependent and Sec-independent proteins. It seems plausible that the structural basis of the YidC mediated insertion of a Sec-dependent protein relates to its ability to associate with the Sec translocase. Since YidC is much more abundant than SecY (Urbanus et al., 2002), a major share of the YidC will be free of the Sec translocase. Therefore, one expects to isolate mutants that are functional in membrane insertion for Sec-independent proteins such as M13 procoat protein while being defective in the interaction with the Sec translocase. Such a mutant most likely would be impaired in Sec-related activity but still contain an active membrane insertase domain. The YidC
function for Sec-dependent and Sec-independent membrane proteins may be similar. In both cases, YidC helps promote the insertion of hydrophobic domains into the lipid bilayer. However, for Sec-independent proteins, YidC is used for direct insertion into the lipid bilayer. For Sec-dependent proteins, YidC assists in the lateral transfer of the hydrophobic regions out of the SecYEG channel in which the hydrophobic domains have inserted.

It is not known how the introduction of the site-specific protease-sites leads to the conditional lethal phenotypes. The cs YidC mutant had a Factor Xa site and PreScission Protease site introduced in the amino-terminal periplasmic loop of YidC, giving rise to its cs behavior. The combination of these protease sites is necessary for the cs phenotype, since the YidC mutants with single mutation site (Fxa1 or PS in Figure 4.1) incorporated do not display a cs phenotype (data not shown). The combined mutations may cause a structural change in the YidC molecule that is necessary for YidC to interact with the Sec complex. Possibly the large amino-terminal domain of YidC in which the protease sites were introduced may constitute part or all of the entire Sec-binding domain (Figure 4.9). This is currently being tested. Notably, the levels of the cs YidC mutant are reduced at the permissive temperature. Nevertheless, at this reduced level, the cs YidC mutant is functional for membrane protein insertion. For the temperature-sensitive mutants, the introduction of the protease sites into the YidC molecule could cause a conformational change that leads to significant perturbation of the protein at the non-permissive temperature. This structural change results in YidC being degraded within the cell. Most likely, it is the reduced intracellular levels of the ts YidC mutant (compared to wild-type
levels) that is the major reason that leads to the phenotype of the ts YidC mutant. Furthermore, the intact ts YidC that remains in the cell is most likely compromised in its activity. The endogenous proteases in the cells could cleave the ts YidC mutants at sites distinct from the introduced site-specific protease sites. This is supported by the observation that the introduction of one of the protease sites (Thn, Gase, or Fxa\(^2\) in Figure 4.1) into the YidC mutant, was insufficient to generate the ts phenotype (data not shown). The YidC mutant containing a single TEV protease site in last periplasmic loop (Tev in Figure 4.1) is one exception. This mutant was found to be temperature sensitive. Our explanation for this mutant is that the incorporation of the TEV protease site in the last periplasmic loop is enough to make the conformational change of YidC.

Currently, we have our working model for how YidC functions (Figure 4.9). YidC can be divided into two separate domains: the Sec-binding domain and the insertase domain. The Sec-binding domain may be comprised of the large loop in the periplasmic space (Figure 4.1). The Sec-binding domain interacts with SecDFYajC, and brings the YidC insertase domain to the Sec machinery. The insertase domain removes the protein being inserted (Sec-dependent proteins) laterally out of the Sec-channel and into the lipid bilayer. For insertion of the Sec-independent proteins, the Sec-binding domain is not necessary, and the insertase domain inserts the Sec-independent proteins from the cytosol into the lipid bilayer.

In the future it will be important to determine which structural regions of YidC are perturbed in the ts and cs mutant proteins. One possibility is to isolate intragenic
suppressors of the conditional lethal mutants since the suppressors could be useful for identifying regions that interact within the protein. Moreover, the isolation of the extragenic suppressors might shed light on additional components that interact with YidC, which may constitute part of the translocase or another complex in which YidC functions.

4.4 Methods and Materials

4.4.1 Strains and plasmids

Leader peptidase (Lep) and procoat-lep (PClep) are under the control of the IPTG inducible tac promoter in the vector pMS119. PClep, under control of the tac promoter, was also subcloned into pACYC184, a vector that can co-exist with pMS119 in E. coli cells. pMAK705, the vector for gene replacement, was kindly obtained from Dr. Sidney R. Kushner. E. coli strain MC1060, which contains wild type yidC gene, was used as the parental strain to construct the ts or cs YidC strains. JS7131, a YidC depletion strain, is from our lab collection. pINGYidC, in which YidC is under control of araBAD promoter, is also from our lab.
4.4.2 Construction of YidC ts and cs strains by homology recombination

*yidC* mutant genes, flanked by upstream and downstream sequences in the *E. coli* chromosome, were cloned into pMAK705 (chloramphenicol resistant), which has a temperature-sensitive replication origin. The construct was introduced into the MC1060 strain, and grown at 42 °C on chloramphenicol plates to select for integrants. The integrants were then grown at 30 °C, the temperature at which the plasmid can leave the chromosome and autonomously replicate, allowing an exchange between the wt *yidC* gene in the chromosome and the *yidC* mutant gene in the plasmid. Screening of the clones for a successful gene exchange was done by sequencing their *yidC* gene on the pMAK705 vector or by restriction enzyme digestions of the plasmid DNA. The positive clones carry pMAK705 with a wt *yidC* gene, instead of a *yidC* mutant gene. These bacterial strains were cured to remove pMAK705 by growing the cells in LB media with increasing amounts of coumermycin from 1 to 10 µg/ml. The growing culture with highest concentration of coumermycin was picked up, and screened for the colonies sensitive to chloramphenicol. Those chloramphenicol sensitive clones were the cells in which the *yidC* ts or cs genes have replaced the wt chromosomal *yidC* gene. The ts or cs phenotypes and gene sequences were confirmed.
4.4.3 Assay for Membrane Insertion by *in vivo* protease mapping and signal peptide processing

Cells were grown in LB media to OD$_{600}$ 0.4 at the permissive temperatures, and then pelleted and resuspended in M9 minimal media. The cells in M9 media (Miller, 1972) were incubated at the permissive temperature for 30 min. Half of each culture was shifted to the non-permissive temperature. Prior to labeling, 1mM IPTG was added to the cultures for 5 min to induce the expression of the plasmid-encoded protein. Cells were then labeled with $[^{35}S]$-trans methionine (100 µCi/ml cells) for 20 s and quickly chilled on ice. To analyze M13 procoat or PClep, the labeled cells were precipitated with trichloroacetic acid. For analysis of Lep, radiolabeled cultures were converted to spheroplasts and incubated in the presence or absence of proteinase K (0.5 mg/ml, final concentration) for 60 min at 0 °C, as described previously (Chen et al., 2002). Samples were precipitated with trichloroacetic acid (TCA). Immunoprecipitation with specific antisera as well as analysis by SDS-PAGE and phosphorimaging was carried out as previously described (Chen et al., 2002).

4.4.4 Co-purification study

SecF tagged with 6 histidines at its N-terminus was purified by nickel chelate affinity chromatography (Smith et al., 1988). The IPTG-inducible *SecD6hisFYajC* genes in pET606 were introduced into MC1060 (wild-type) and the cold-sensitive MYC-cs strains. The cells (1 liter) were grown to OD$_{600}$ 0.4 at 37 °C, induced with 1mM IPTG for
1 hour at 37 °C, and then incubated at 25 °C for 2 hours. Inverted membrane vesicles (IMV) were isolated from cells using sucrose gradients following lysing (Geller and Green, 1989). The co-purification was conducted as described (Nouwen and Driessen, 2002) with minor modifications. IMVs were solubilized with Buffer A containing 25 mM Tris·HCl (pH 8.0), 10% Glycerol, 100mM NaCl and 2% DDM (n-Dodecyl β-D-maltoside), and were applied to Ni-NTA agarose (Qiagen) columns. The columns were first washed with Buffer B (25 mM Tris·HCl (pH 8.0), 10% Glycerol, 100mM NaCl, 0.1% DDM and 10 mM imidazole), and then eluted with 100 mM imidazole added to Buffer B. The elution fractions were analyzed by SDS-PAGE and immunoblotting using YidC antiserum.
Figure 4.1  (A) The site-specific protease sites are incorporated into the periplasmic loops and cytoplasmic loops of YidC. FXa, Factor Xa; PS, PreScission Protease; Thn, Thrombin; Tev, TEV Protease; Gase, Geninase. P, M, and C refer to periplasmic space, inner membrane, and cytosol, respectively. The YidC transmembrane domains that are indicated by rectangles are numbered and the amino acid residues bordering the transmembrane domains are numbered according to (Saaf et al., 1998).  (B) The amino acid sequences in the YidC ts and cs mutants showing the location of the site-specific protease recognition sites. The site-specific protease recognition sites are underlined. The amino acid sequences flanking the protease sites are in gray. The amino acid residues bordering the protease sites are numbered to indicate their positions in the wt YidC.
Figure 4.1

A

FXa\(^1\) \[23 \ 343\] PS \[FXa^2\] Gase \[Tev\] \[513\] P

YidC \[1 \ 2 \ 3 \ 4 \ 5 \ 6 \ 6\] 370 417 481 494 509

B

cs: FP \[AW^{23}\] IEGRN\(^{28}\)P \[FXa^1\] \[WL^{333}\] LEVLFQGPL\(^{339}\)F \[PS\]

TTe \[MS^{482}\] LVPRGSD\(^{488}\)P \[Thn\] \[PS^{511}\] ENLYFOQG L\(^{513}\)V \[Tev\]

ts \[TG\] \[MS^{482}\] LVPRGSD\(^{488}\)P \[Thn\] \[WF^{509}\] PGAAHY L\(^{513}\)V \[Gase\]

TF \[VE^{445}\] IEGRQ\(^{448}\)A \[FXa^2\] \[Thn\] \[MS^{482}\] LVPRGSD\(^{488}\)P
Figure 4.2 The wild-type (wt) and mutant yidC genes (FP and TTe), under control of yidC natural promoter, were cloned into a low-copy-number plasmid, pACYC184, and were introduced into the YidC depletion strain, JS7131. The JS7131 cells harboring the constructs were streaked on LB plates containing either 0.2 % arabinose (Ara) or glucose (Glc) and incubated at 30 °C for 2 days, or at 37 or 42 °C overnight. Each half plate contains three streaks from three individual colonies bearing the same construct.
Figure 4.2
Figure 4.3  Construction of YidC ts and cs strains by gene replacement.  *yidC* ts or cs genes was subcloned into pMAK705 (chloroamphenicol resistant, cm<sup>r</sup>), in which the gene is flanked with upstream (*yidC* promoter) and downstream (*thdf* gene) sequences of wildtype *yidC* gene in the *E. coli* chromosome.  The origin of pMAK705 is temperature-sensitive.  Therefore only when the construct integrated into the chromosome, the *E.coli* cells became cm<sup>r</sup> at 44 °C.  The integrated plasmid left from the chromosome with an exchange of the *yidC* genes, when the cm<sup>r</sup> *E.coli* cells were grown at 30 °C, since the plasmid origin which is active at at 30 °C interfered the chromosome duplication.  After gene exchange, coumermycin was used to remove the plasmid from the *E.coli* cells, and the YidC ts or cs strains were obtained.
Figure 4.3
Figure 4.4  MYC-Te and MYC-cs strains were grown in LB to OD₆₀₀ 0.4 at their permissive temperatures, and then half of the cells were shifted to the non-permissive temperatures. The cells continued to grow for 2 hours. As controls, MC1060, the parent strain with the wild-type yidC gene, was grown under the same conditions as the MYC-Te or MYC-cs strains. MC1060 cells harboring pINGYidC, were grown under arabinose (A) and glucose (G) conditions, as a control to determine the position of YidC on the immunoblots. 25, 30, 37 and 42 indicates the temperature, in centigrade, of the cells in which they were grown. The YidC band is indicated. The band above the YidC band is a background band recognized by the YidC antiserum.
Figure 4.5  MYC-Te cells were grown, as described in the “Materials and Methods”, at 30 °C to OD_{600} 0.4, at which point, half of the cells were then shifted to 42 °C. The cells were then grown for an additional 20, 40, 80, or 120 min. After induction of the plasmid-encoded PClep for 5 min by the addition of 1mM IPTG (final concentration), the cells were radiolabeled with \[^{35}\text{S}\]-methionine for 20 sec. The cells were then subjected to immunoprecipitation with Lep antiserum and analyzed by SDS-PAGE and phosphorimaging. The precursor and mature forms of PClep are indicated as p and m, respectively.
Figure 4.6 MYC-cs cells were grown as described in the “Materials and Methods” to OD$_{600}$ 0.4 at 37 ºC. Half of the cells were then shifted to 25 ºC, and the growth continued for 0.5, 1 or 2 hr. After induction of the plasmid-encoded Lep for 5 min with 1 mM IPTG, the cells were radiolabelled with $^{[35}$S]-methionine for 20 sec and converted to spheroplasts. Aliquots were removed and treated with or without proteinase K (0.5 mg/ml, final concentration) for 1 hour on ice. Samples were then TCA precipitated and immunoprecipitated with Lep and OmpA antiserum, and subjected to SDS-PAGE and phosphorimaging. **Panel A** shows proteinase K (PK) mapping in MC1060 (wt) and MYC-cs (cs) cells grown for 0.5-hour at 25 or 37 ºC. Lep band is indicated. The precursor and mature forms of OmpA are indicated by p and m, respectively. **Panel B** shows proteinase K (PK) mapping of MYC-cs (cs) with a 1-or 2-hour treatment at 25 or 37 ºC. **Panel C** shows that overexpression of the plasmid-encoded Lep leads to strong inhibition in proOmpA export. MC1060 or MYC-cs cells bearing pMS119-Lep were grown to OD$_{600}$ 0.4 at 37 ºC as in Panel A. Half of the cells were shifted to 25ºC and the growth continued for 2 hr. The cells were treated with or without 1 mM IPTG (final concentration) for 5 min, and then radiolabeled, immunoprecipitated with OmpA antiserum, and subjected to SDS-PAGE and phosphorimaging as in Panel A. **Panel D** shows that the export of OmpA is inhibited at 25 ºC in the cold-sensitive MYC-cs strain. Cultures of MC1060 or MYC-cs cells were grown at 25 ºC for 1 hour and radiolabelled, as described in Panel A. The cells were TCA precipitated, immunoprecipitated with OmpA antiserum, and analyzed by SDS-PAGE and phosphorimaging. The precursor and mature forms of OmpA are indicated by p and m, respectively.
Figure 4.6 (Continued)
Figure 4.6 (Continued)

<table>
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C

D

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<tr>
<td>OmpA{P_m}</td>
<td>![Image]</td>
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**Figure 4.7** (A) PClep, under control of Tac promoter, was subcloned into pACYC184. This construct is compatible with the pMS119 vector containing the Lep gene. Both were then co-introduced into MC1060 (wt) or the MYC-cs strains. The cells were grown at 25 or 37 °C for 2 hours and analyzed as described in Figure 4.6. After 5-min induction of the plasmid-encoded Lep and PClep with 1 mM IPTG (final concentration) and labeling cells with \[^{35}S\]-methionine for 20 sec, one-fifth of the cells were directly precipitated with TCA, and immunoprecipitated with procoat antiserum to monitor PClep membrane insertion (A panel). (B) The remaining four-fifths of the cells were subjected to proteinase K mapping and immunoprecipitation with Lep or OmpA antiserum, as described in Figure 4.6B. Temp., PK, p, and m indicate temperature, proteinase K, precursor, and mature form, respectively. (C) shows efficient membrane insertion of PClep in the cold-sensitive strain when it is expressed alone. The cells were grown and radiolabelled, as described in Figure 4.6, except the cells were grown at 25 °C or 37°C for 1, 2, 3, or 4 hours. The radiolabelled cells were TCA precipitated, subjected to immunoprecipitation with lep antiserum, and analyzed by SDS-PAGE and phosphorimaging. p and m indicates the precursor and mature form of PClep.
### A

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![PClep](image)

### B

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![Lep](image)

![OmpA](image)

### C

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![PClep](image)

Figure 4.7
Figure 4.8 pET606 bearing SecD6hisSecFYajC genes was introduced into either MC1060 (wt) or the cold-sensitive MYC-cs strain. The cells were grown and the 6hisSecF was purified as described in the “Materials and Methods”. Briefly, isolated IMVs were solubilized with DDM (membrane, M) and the extract loaded onto a Ni-NTA column. The flow-through (F), wash (W) and elution fractions (E) were collected. Elutions 1 and 2 (E1 and E2) represent the first and second elution fractions collected after imidazole treatment; the elution fraction volume corresponds to one column bed volume. Samples were analyzed by SDS-PAGE (A panel) and by immunoblotting with YidC antiserum to determine whether YidC can be co-purified with SecDF (B panel). Panel A shows that SecD is also copurified. The position of YidC can be seen in the anti-YidC immunoblot (B panel) by the induced band in the arabinose-grown samples of MC1061 pING cells harboring the arabinose-inducible YidC protein (“Ara” lane). Less YidC corresponding to the chromosomally-induced level, is observed with samples prepared from MC1061 pING grown cells in Glucose (“Glc” lane).
Figure 4.8

A

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B

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**Figure 4.9** Working model for how YidC functions. YidC can be divided into two separate domains: the Sec-binding domain (B domain) and the insertase domain (I domain). The Sec-binding domain may be comprised of the large loop in the periplasmic space (Figure 4.1). The Sec-binding domain interacts with SecDFYajC, and brings the YidC insertase domain to the Sec machinery. The insertase domain removes the protein being inserted (Sec-dependent proteins) laterally out of the Sec-channel and into the lipid bilayer. For insertion of the Sec-independent proteins, the Sec-binding domain is not necessary, and the insertase domain inserts the Sec-independent proteins from the cytosol into the lipid bilayer.
Figure 4.9

SecDFYajC  YidC  YidC

Sec YEG  B  B

Sec-dependent proteins  Sec-independent proteins

Periplasm  Membrane  Cytosol
LIST OF REFERENCES


Bonnefoy, N., Chalvet, F., Hamel, P., Slonimski, P.P. and Dujardin, G. (1994) OXA1, a Saccharomyces cerevisiae nuclear gene whose sequence is conserved from
prokaryotes to eukaryotes controls cytochrome oxidase biogenesis. *J Mol Biol*, 239, 201-12.


