Conditional Lethal Mutations Separate the M13 Procoat and Pf3 Coat Functions of YidC

DIFFERENT YIDC STRUCTURAL REQUIREMENTS FOR MEMBRANE PROTEIN INSERTION*

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Conditional lethal YidC mutants have been isolated to decipher the role of YidC in the assembly of Sec-dependent and Sec-independent membrane proteins. We now show that the membrane insertion of the Sec-independent M13 procoat-lep protein is inhibited in a short time in a temperature-sensitive mutant when shifted to the nonpermissive temperature. This provides an additional line of evidence that YidC plays a direct role in the insertion of the Sec-independent M13 procoat protein. However, in the temperature-sensitive mutant, the insertion of the Sec-independent Pf3 phage coat protein and the Sec-dependent leader peptidase were not strongly inhibited at the restricted temperatures. Conversely, using a cold-sensitive YidC strain, we find that the membrane insertion of the Sec-independent Pf3 coat protein is blocked, and the Sec-dependent leader peptidase is inhibited at the nonpermissive temperature, whereas the insertion of the M13 procoat protein is nearly normal. These data show that the YidC function for procoat and its function for Pf3 coat and possibly leader peptidase are genetically separable and suggest that the YidC structural requirements are different for the Sec-independent M13 procoat and Pf3 coat phage proteins that insert by different mechanisms.

In prokaryotes, membrane proteins can insert into the membrane by a Sec-dependent and Sec-independent pathway. The best studied Sec-independent proteins are the M13 phage procoat and Pf3 phage coat proteins. Up until recently, it was widely believed that these proteins inserted into the membrane by a spontaneous mechanism. In the year 2000, it was found that the membrane insertion of the M13 phage protein requires a newly identified membrane protein called YidC (1). YidC was found also to be critical for Pf3 coat membrane insertion and is in close proximity with the Pf3 coat protein during its insertion into the membrane (2). YidC was suggested to function as a membrane chaperone to help in the folding and integration into the membrane (2, 3).

YidC was implicated also in the membrane insertion of Sec-dependent proteins (1, 4). YidC was found to be associated with the Sec translocase (4) and can be cross-linked to membrane proteins that are in the process of inserting into the membrane (1, 4–6). The function of YidC is not fully understood, but it may constitute an assembly factor for folding of hydrophobic domains of polytopic membrane proteins (7) or assist in the transfer of the hydrophobic segments from the Sec channel into the hydrophobic phase of the lipid bilayer (8).

All current evidence showing that YidC plays a role in protein membrane insertion is based on in vitro cross-linking data showing physical interaction between YidC and the protein being inserted into the membrane, as well as in vivo studies examining membrane protein assembly where YidC is depleted in the cell (1, 9). Because the depletion of YidC takes 2–3 h, it has been argued that the role proposed for YidC in membrane protein insertion may not be correct because the effects observed (1) may be indirect because of YidC depletion. YidC depletion through several generations of cell growth may cause the depletion of other translocase components, which may result in impaired membrane insertion. 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 insertion of the Sec-independent M13 procoat protein is quickly inhibited in a temperature-sensitive strain when grown at the nonpermissive temperature. In contrast, the membrane insertion of the Sec-independent Pf3 coat and Sec-dependent leader peptidase proteins was not strongly inhibited at the nonpermissive temperature in the ts YidC mutant. These results support the idea that YidC plays a direct role in procoat insertion, and the ts YidC mutation has distinct effects even on the membrane biosynthesis of two Sec-independent membrane proteins. Similarly, a discrimination of substrates was also observed with the cs mutant strain. Whereas the insertion of the Sec-independent M13 procoat protein was very efficient, the insertion of the Sec-independent Pf3 protein was blocked, and the Sec-dependent leader peptidase was inhibited.

MATERIALS AND METHODS

Strains and Plasmids—The genes of leader peptidase (Lep), M13 procoat, procoat-lep (PClep), and Pf3 coat are under the control of the IPTG-inducible tac promoter in the vector pMS119, which contains the lacIq gene. pMAK705, the vector for gene replacement, was kindly obtained from Dr. Sidney R. Kushner. Escherichia coli strain MC1060, which contains wild-type yidC gene, was used as the parental strain to construct the ts or cs YidC strains. J87151, a YidC deletion strain, is

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‡ The abbreviations used are: ts, temperature-sensitive; cs, cold-sensitive; Lep, leader peptidase; PClep, procoat-lep; IPTG, isopropyl-1-thio-β-D-galactopyranoside; wt, wild-type.
from our lab collection, pNGyidC, in which YidC is under control of araBAD promoter, is also from our lab.

Construction of YidC ts and cs Strains by Homology Recombination—
yidC mutant genes, flanked by upstream and downstream E. coli chromosome sequences 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. This allows 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 digests 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 medium with increasing amounts of coumermycin from 1 to 10 μg/ml. The growing culture with the highest concentration of coumermycin was picked up and screened for the colonies sensitive to chloramphenicol. These 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.

Assay for Membrane Insertion by in Vivo Protease Mapping and Signal Peptide Processing—The cells were grown in LB medium to A600 0.4 at the permissive temperatures, then pelleted, and resuspended in M9 minimal medium (10). The cells in M9 medium were incubated at the permissive temperature for 30 min. Half of each culture was shifted to the nonpermissive temperature. Prior to labeling, 1 mM IPTG was added to the cultures for 5 min to induce the expression of the plasmid-encoded membrane protein substrates. The cells were then labeled with [35S]trans-methionine (100 μCi/ml) 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 Pf3 coat and Lep, and, in certain cases, PClep, radioabeled cultures were converted to spheroplasts and incubated in the presence or absence of protease K (final concentration, 0.5 mg/ml) for 60 min at 0 °C, as described previously (2). The samples were precipitated with trichloroacetic acid. Immunoprecipitation with specific antisera as well as analysis by SDS-PAGE and phosphorimaging was carried out as previously described (2).

RESULTS

Introduction of Site-specific Protease Sites into YidC Results in Conditional Lethal Mutants—To elucidate the function of YidC in membrane protein insertion—To elucidate the function of YidC in membrane protein insertion, we isolated conditional lethal (cold- and temperature-sensitive) mutants of YidC. Incorporation of site-specific protease sites into YidC can result in ts and cs mutants. In these studies, Factor Xa, PreScission Protease (Amersham Biosciences), Thrombin, TEV Protease, and Genenase (New England Biolabs) cleavage sites were incorporated into the cytoplasmic and periplasmic loops of YidC. Panels A and B in Fig. 1 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 the 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 deletion when JS7131 is grown in glucose medium at 30, 37, and 42 °C. When Factor Xa and PreScission Protease sites are introduced into the N- and C-terminal region of the first periplasmic loop of YidC (Fig. 1), respectively, this YidC mutant (termed FP) becomes cold-sensitive, i.e. it cannot complement the growth defect of YidC deletion in JS7131 at 30 °C or below (Fig. 2). Incorporation of a thrombin site into the second cytoplasmic loop and a TEV protease site into the last periplasmic loop (mutant termed TTe) results in a YidC temperature-sensitive phenotype, i.e. these mutants cannot function at 42 °C (Fig. 2). The YidC mutants (TF and TG) also show temperature-sensitive phenotypes (data not shown) when the thrombin site in the second cytoplasmic loop is introduced in combination with Genenase site (mutant termed TG) in the last periplasmic loop or with Factor Xa site (mutant termed TF) in the second periplasmic loop of YidC (Fig. 1). All of the cs and ts YidC mutants can complement YidC deletion at 37 °C.

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 (11). The yidC ts and cs mutant genes (TTe, TF, TG, and FP), described in Fig. 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 thdG gene, located downstream of the yidC gene in the chromosome. 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). The resulting E. coli strains, in which the yidC FP, TTe, TG, or TF gene has replaced the wt yidC gene, are named the MYC-cs, MYC-Te, MYC-TG, and MYC-TF strains. MYC-cs is the YidC cs strain, whereas MYC-Te, MYC-TG, and MYC-TF are YidC ts strains. The expression of the wt YidC from the
Different YidC Membrane Protein Insertion Requirements

YidC is a bacterial membrane protein that plays a role in the insertion of other membrane proteins. The YidC protein has two periplasmic loops, and the protease sites are located in these loops. The protease sites are necessary for the YidC function, and the introduction of a multicopy plasmid overexpressing this protease site in the last periplasmic loop is enough to make the conformational change of YidC.

The YidC ts mutant is unstable at the non-permissive temperature (37°C), whereas it is stable at the permissive temperature (30°C). The YidC cs mutant is more stable at the non-permissive temperature (37°C), whereas it is unstable and degraded at the non-permissive temperature (42°C). Note that in the Western blot there is a band above YidC that is recognized nonspecifically by the YidC antiserum. The other ts mutants (TG and TF) were also unstable at the non-permissive temperature (data not shown).

The Sec-independent Pf3 Coat and Sec-dependent Leader Peptidase Are Not Strongly Inhibited at the Restricted Temperature in the ts YidC Strain—Next, we tested whether the ts mutation had a uniform effect on other membrane proteins. We examined the Sec-independent Pf3 coat protein and Sec-dependent leader peptidase because previous studies showed that the YidC stimulated their insertion (1, 2). MYC-Te, the ts YidC strain, was radiolabeled with [35S]methionine for 20 s after growth for 2 h at the permissive (30°C) or nonpermissive (42°C) temperature. As can be seen, the precursor of PClep that accumulates at 42°C is protected by externally added protease, and very little proteinase K-digested fragment (designated as d in Fig. 4A) was produced. This indicates that the precursor of PClep that accumulates at 42°C in MYC-Te does not insert into the membrane. We also tested the insertion of the wild-type coat protein in MYC-Te cells, and the results are similar to PClep. Procoat is almost completely blocked in MYC-Te after 30 min at 42°C (Fig. 4B). 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 in vivo.

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the ts YidC strain. In addition, there was inhibition in P3 coat membrane insertion at the permissive temperature in the ts YidC strain, in contrast to procot insertion. We then analyzed the Sec-dependent leader peptidase as to its behavior in the MYC-Te strain. There was little inhibition in membrane insertion at the nonpermissive temperature (25 °C) but was not inhibited at the permissive temperature (37 °C) (Fig. 5C) or in the parent strain (MC1060) containing a wild-type YidC grown at 37 or 25 °C (data not shown). The YidC cs strain bearing the plasmid-encoded Lep was pulse-labeled with [35S]methionine for 20 s after growth for 1 or 2 h at the permissive (37 °C) or nonpermissive (25 °C) temperature. The cells were then converted to spheroplasts and assayed by protease mapping. At the permissive temperature (37 °C), all of the Lep was accessible to protease digestion (Fig. 5C), demonstrating the C-terminal P2 domain inserts across the membrane. In contrast, at the nonpermissive temperature, some of Lep is resistant to proteinase K degradation, showing that insertion is inhibited. Inhibition occurs even after 1 h of growth at the nonpermissive temperature (Fig. 5C). Specifically, we observe that the amounts of Lep blocked at 1 and 2 h at the restrictive temperature (25 °C) are ~20 and ~15% (Fig. 5C), respectively. We score this inhibition as rather strong because previous studies showed the membrane insertion of Sec-dependent proteins such as Lep and FtsQ is only partially inhibited (varying from 15 to 30% for Lep and ~15% for FtsQ) when YidC is depleted (1, 6, 12), and the inhibition observed in MYC-cs is very similar to those effects. In all cases, there is also a block in the export of the outer membrane protein A (OmpA) precursor. We observed that proOmpA accumulation is due mainly to the overexpression of Lep causing
the proOmpA block in the cs YidC mutant, because 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 °C, MYC-cs, + and − lanes in Fig. 5D). This type of observation has been seen before when YidC is depleted and a Sec-dependent protein is overexpressed (9). The accumulation of OmpA precursors when IPTG is added is likely due to the presence of overexpressed Lep that stalls in the Sec channel when the YidC function is impaired, thereby interfering with protein export by the Sec translocase. There is an effect on OmpA export in the cs YidC at the 25 °C even when IPTG is not added, suggesting that YidC is needed to stimulate export when the temperature is low or that it is required to prevent jamming of endogenous membrane proteins that are expressed within the cell.

**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 a temperature-sensitive mutant that after only 20 min at the nonpermissive temperature, the insertion of PClep was inhibited (Fig. 4A). The YidC ts mutant is inactivated quickly and does not have the problem that the depletion strain JS7131 has where it takes several hours to deplete the cell of YidC (1). Therefore, the result provides more evidence that YidC plays a direct role in membrane protein insertion. However, we cannot rule out for certain that secondary effects could occur by the structural perturbations and the enhanced proteolysis of the YidC ts mutant at the nonpermissive temperature, which could as a result influence the insertion of membrane proteins. A direct role of YidC in membrane insertion of proteins was recently shown with the P33 coat protein. In this study, ribosome-bound P33 coat protein was trapped in the membrane insertion process and shown to interact with YidC using a photocross-linking approach (2).

Although the YidC function for the Sec-independent M13 procot is strongly inhibited at the nonpermissive temperature (42 °C) in the ts mutant, the function for Sec-independent P33 coat protein and Sec-independent leader peptidase is only marginally inhibited (Fig. 4, C and D). Even more surprising was that the membrane insertion of these proteins (P33 coat and leader peptidase) is inhibited more strongly at the permissive temperature (30 °C). Notably, procot inserts into the membrane efficiently at the permissive temperature (30 °C). Therefore, the mutation in the ts mutant has distinct effects depending on the membrane protein being analyzed.

Remarkably, the YidC function for the Sec-independent P33 coat protein (Fig. 5B) and the Sec-dependent leader peptidase (Fig. 5C) is substantially inhibited in the cs mutant at the nonpermissive temperature, whereas the Sec-independent PClep protein inserts very efficiently (Fig. 5A). At the nonpermissive temperature, there was a complete block in P33 coat membrane insertion and 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 (1 h) in a cell where the cell doubling time is greater than 2 h (in the M9 minimal media). Nevertheless, procot can efficiently insert into the membrane and be processed by signal peptidase I (Fig. 5A).

The contrasting effects of the cs or ts YidC mutant on the membrane insertion of the Sec-independent M13 procot and P33 coat protein may have to due with the facts that these YidC-dependent proteins insert across the membrane differently, and the structural requirements of YidC may be different for these different insertion mechanisms. For example, procot inserts across the membrane with two hydrophobic domains (13), whereas P33 coat inserts with only one hydrophobic domain (14). Both of the proteins require the proton motive force for insertion (14–16). The YidC cs mutant may be perturbed in a region such that it can no longer perform its function to promote P33 coat into the membrane, but it can with procot. The YidC cs mutant is also perturbed in its ability to promote the membrane insertion of the Sec-dependent leader peptidase at the cold temperature (Fig. 5). Therefore, this mutant does not discriminate between the Sec-independent and Sec-dependent pathways.

The ts and cs mutants described here are a result of protein
engineering and not due to spontaneous mutations or selection pressure, as is the case for the most frequently studied conditional lethal mutants. Both the ts and cs mutants have enhanced proteolysis at the higher temperature (Fig. 3). Therefore, it is not surprising that the MYC-Te mutant cannot complement the YidC depletion strain at 42 °C, because its proteolysis is so significant at this temperature. In contrast, it is surprising that the MYC-cs mutant is functional for membrane protein insertion at the 37 °C, the permissive temperature, where the level of the protein is reduced.

In the future it will be important to determine which structural regions of YidC are perturbed in the ts and cs mutant proteins. Additionally, these ts and cs mutants can be utilized to isolate extragenic suppressors that might shed light on additional components that interact with YidC, which may constitute part of the translocase or another complex in which YidC functions.

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