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Tim50 Is a Subunit of the TIM23 Complex that Links Protein Translocation across the Outer and Inner Mitochondrial Membranes

Hayashi Yamamoto,[1] Masatoshi Esaki,[1] Takashi Kanamori,[2] Yasushi Tamura,[1] Shuh-ichi Nishikawa,[1] and Toshiya Endo[1,3]
[1]Department of Chemistry
Graduate School of Science
Nagoya University
Chikusa-ku, Nagoya 464-8602
Japan
[2]Department of Biochemistry and Cell Biology
Institute of Gerontology
Nippon Medical School
Kosugi-cho, Nakahara-ku, Kawasaki
Kanagawa 211-8533
Japan

Summary

Based on the results of site-specific photocrosslinking of translocation intermediates, we have identified Tim50, a component of the yeast TIM23 import machinery, which mediates translocation of presequence-containing proteins across the mitochondrial inner membrane. Tim50 is anchored to the inner mitochondrial membrane, exposing the C-terminal domain to the intermembrane space. Tim50 interacts with the N-terminal intermembrane space domain of Tim23. Functional defects of Tim50 either by depletion of the protein or addition of anti-Tim50 antibodies block the protein translocation across the inner membrane. A translocation intermediate accumulated at the TOM complex is crosslinked to Tim50. We suggest that Tim50, in cooperation with Tim23, facilitates transfer of the translocating protein from the TOM complex to the TIM23 complex

Introduction

Mitochondria consist of the outer and inner membranes and two aqueous compartments, the intermembrane space (IMS) and the matrix. Most mitochondrial proteins are encoded by the nuclear genome, synthesized in the cytosol, and subsequently imported into mitochondria. Once within the mitochondria, the imported mitochondrial proteins are sorted to one of the four submitochondrial compartments. Targeting and sorting information is often contained in the N-terminal cleavable presequence of mitochondrial precursor proteins themselves, while it can also be contained in the mature part of mitochondrial proteins as internal signals. The complex processes of mitochondrial protein import and sorting are mediated by elaborate systems in mitochondria, including the translocator complexes in the outer and inner mitochondrial membranes (Neupert, 1997; Pfanner and Geissler, 2001).

The translocator of the outer mitochondrial membrane (TOM) mediates the entry of probably all nuclear encoded mitochondrial proteins into mitochondria. The TOM complex functions as a receptor for mitochondrial proteins (Endo and Kohda, 2002) and offers a protein conducting channel, mainly made of Tom40, through which mitochondrial proteins thread in unfolded conformations (Hill et al., 1998; Ahting et al., 2001). The TOM complex also contains a presequence binding site (the trans site) for mitochondrial proteins on the IMS side of the outer membrane, which consists of the IMS side region of Tom40 and is close to the IMS domain of Tom22 (Mayer et al., 1995; Rapaport et al., 1997; Kanamori et al., 1999).

The mitochondrial inner membrane contains two distinct translocator complexes, the TIM22 complex and the TIM23 complex, for protein insertion into and across the inner membrane. The TIM22 complex is required for insertion of presequence-less polytopic membrane proteins including mitochondrial carrier proteins into the inner membrane (Koehler et al., 1999; Bauer et al., 2000). The TIM23 complex mediates translocation across and insertion into the inner membrane of presequence-containing precursor proteins (Bauer et al., 2000; Pfanner and Geissler, 2001). The TIM23 complex consists of polytopic integral membrane proteins, Tim23 and Tim17, and a peripheral membrane protein, Tim44, associated with Ssc1p, Hsp70 in the matrix (mtHsp70). Tim23 can form a voltage-gated protein conducting channel (Bauer et al., 1996; Truscott et al., 2001) and functions as a receptor for presequences (Bauer et al., 1996; Komiya et al., 1998). Translocation of the presequence of mitochondrial precursor proteins requires the membrane potential ($\Delta\Psi$) across the inner membrane, whereas unfolding and transmembrane movement of the mature part require the ATP-dependent reaction cycle of mtHsp70 in cooperation with Tim44 and Yge1p (Mge1p) in the matrix (Pfanner and Geissler, 2001).

The translocation of precursor proteins across the inner membrane appears to be tightly linked to their translocation across the outer membrane, because no soluble intermediates in the IMS can be observed (Schleyer and Neupert, 1985; Kanamori et al., 1997). Nevertheless, the TOM complex in the outer membrane and the TIM23 complex in the inner membrane are not permanently linked, but they can interact only in the presence of a translocating precursor protein (Berthold et al., 1995; Horst et al., 1995; Dekker et al., 1997). Therefore, a precursor protein translocating through the TOM complex needs to engage the TIM23 complex in the boundary inner membrane, which is closely apposed to the outer membrane, to form a transient "translocation contact site" (Pfanner et al., 1992). Although the IMS domain of Tom22 and the N-terminal segment of Tim23 appear to facilitate the transfer of the translocating precursor protein from the TOM complex to the TIM23 complex (Court et al., 1996; Moczko et al., 1997; Kanamori et al., 1999; Donzeau et al., 2000), the molecular mechanism underlying the link of the translocation across the outer membrane and that across the inner membrane remains unclear.

In the present study, we identified Tim50, a subunit of

[3]Correspondence: endo@biochem.chem.nagoya-u.ac.jp

the TIM23 complex. Tim50 is an integral inner membrane protein and exposes the large C-terminal domain to the IMS, which interacts with the N-terminal domain of Tim23. Tim50 is directly involved in translocation of precursor proteins with a cleavable presequence, but not of presequence-less polytopic membrane proteins, across the inner membrane. Translocation intermediates lodged in the Tom40 channel crosslinked to Tim50. On the basis of these observations, a possible role for Tim50 in linking the protein translocation across the outer and inner membranes will be discussed.

Results

Identification of Tim50 as a Crosslinked Partner of the Translocation Intermediate Anchored to the Inner Membrane

The precursor of cytochrome b_2 (pCytb_2) possesses a bipartite presequence, which consists of the mitochondrial targeting signal followed by the sorting signal for the IMS and receives two-step cleavage by the matrix processing peptidase (MPP) in the matrix and by Imp1p in the IMS. When incubated with isolated yeast mitochondria in the presence of methotrexate (MTX), a high-affinity ligand for dihydrofolate reductase (DHFR), pb_2(220)DHFR, a fusion protein consisting of the N-terminal 220 residues of pCytb_2 and mouse DHFR, is arrested as a translocation intermediate spanning the mitochondrial outer membrane (Kanamori et al., 1997). While the C-terminal DHFR domain of the intermediate remains outside the mitochondria, the N-terminal pCytb_2 part has already crossed the outer membrane. The N terminus of pb_2(220)DHFR reaches the IMS to form the mature form, which received processing of the presequence by both MPP and Imp1p, or is anchored to the inner membrane to form the processing-intermediate form, which received only the first processing of the presequence by MPP (Esaki et al., 1999). The site-specific photocrosslinking experiments previously showed that benzoyl phenylalanine (BPA), at positions 94 and 114, which are located just downstream of the sorting signal for the IMS, of the processing-intermediate form of pb_2(220)DHFR, was crosslinked to an unidentified mitochondrial component to generate a 95 kDa crosslinked product (Kanamori et al., 1997). Since this crosslinked partner could be a new component involved in protein translocation across the inner membrane, we decided to identify the partner protein.

For this purpose, pb_2(220)DHFR-94C, a pb_2(220)DHFR derivative that has a unique cysteine residue at position 94 and a hexahistidine tag at the C terminus was prepared in large amounts from *E. coli* cells (Figure 1A). Modification of pb_2(220)DHFR-94C with 4-(*N*-maleimido)benzophenone (NMBz) directed introduction of a benzophenone moiety to Cys-94 of the fusion protein. Incubation of NMBz-modified pb_2(220)DHFR-94C with isolated yeast mitochondria in the presence of MTX resulted in the formation of a translocation intermediate and subsequent UV irradiation yielded a crosslinked product of ~95 kDa (Figure 1B, lane 4). The mitochondria were solubilized with detergent and the crosslinked product was purified by affinity chromatography for the hexahistidine tag of the fusion protein. Tryptic digestion of the purified crosslinked product followed by mass spectrometry analyses identified 4 peptides, whose sequences corresponded to the open reading frame (ORF) *YPL063w* on yeast chromosome XVI that encodes a 55.1 kDa polypeptide of an unknown function. We termed this ORF *TIM50*. Its predicted product, Tim50, consists of 476 amino acid residues and possesses a predicted N-terminal mitochondrial presequence (residues 1–43; Claros and Vincens, 1996) and a putative transmembrane segment (residues 113–132) (Figure 1C). Indeed, N-terminal sequencing of the 95 kDa crosslinked product showed that the mature part of Tim50 starts from residue 44 and has a MW of 50.4 kDa (not shown).

We raised antibodies against the C-terminal segment (residues 242–476) of Tim50 and tested if the crosslinked products arising from the MTX-arrested translocation intermediates of pb_2(220)-DHFR bearing BPA at positions 94 or 114 (Kanamori et al., 1997) contain Tim50. After solubilization of the mitochondria, the major crosslinked products with apparent MW of ~95 kDa or ~102 kDa were immunoprecipitated specifically with the antibodies against Tim50, but not with the preimmune serum, for BPA at residue 94 or 114, respectively (Figure 1D). These results indicate that the crosslinked partner responsible for the 95 kDa crosslinked product is Tim50.

Tim50 Is an Essential Inner-Membrane Protein of Yeast Mitochondria

To assess the functions of Tim50, the *TIM50* gene was disrupted in the diploid yeast strain by integration of the *HIS3* gene of *Candida glabrata*, and the cells were induced to sporulate and spores were subjected to tetrad analysis. Upon dissection, each tetrad yielded only two viable spores (Figure 2A). All viable spores were His^- and were confirmed to carry the non-disrupted *TIM50*. This indicates that *TIM50* is essential for vegetative growth of yeast.

Since Tim50 carries a putative mitochondrial targeting presequence, we analyzed its subcellular location by indirect immunofluorescence microscopy. Staining of yeast cells with the anti-Tim50 antibodies showed a tubular network in the cytoplasm (Figure 2B, left). This staining is typical for yeast mitochondrial proteins, and nearly identical staining was observed with the antibody against mitochondrial porin (Figure 2B, center). Thus, Tim50 is a mitochondrial protein.

We isolated yeast mitochondria and analyzed the suborganellar location of Tim50. Tim50 was inaccessible to protease added to intact mitochondria (Figure 2C, lanes 1 and 2), but was accessible to protease added to mitoplasts, where the outer membrane was selectively ruptured (Figure 2C, lanes 3 and 4). This behavior resembles that of Tim23, an inner membrane protein exposing a domain to the IMS, but is different from those of Tom70, an outer membrane protein exposing a domain to the cytosol, or subunit β of F_1-ATPase ($F_1\beta$), a peripheral inner membrane protein on the matrix side (Figure 2C). Tim50 was, like integral membrane proteins, Tom70 and Tim23, not extracted from the membranes with Na_2CO_3 at pH 11.5 while a peripheral membrane protein, $F_1\beta$, was extracted with Na_2CO_3 (Figure 2C, lanes 5 and 6). All these proteins were extracted from the membranes with Triton X-100 (Figure 2C, lanes 7 and 8).

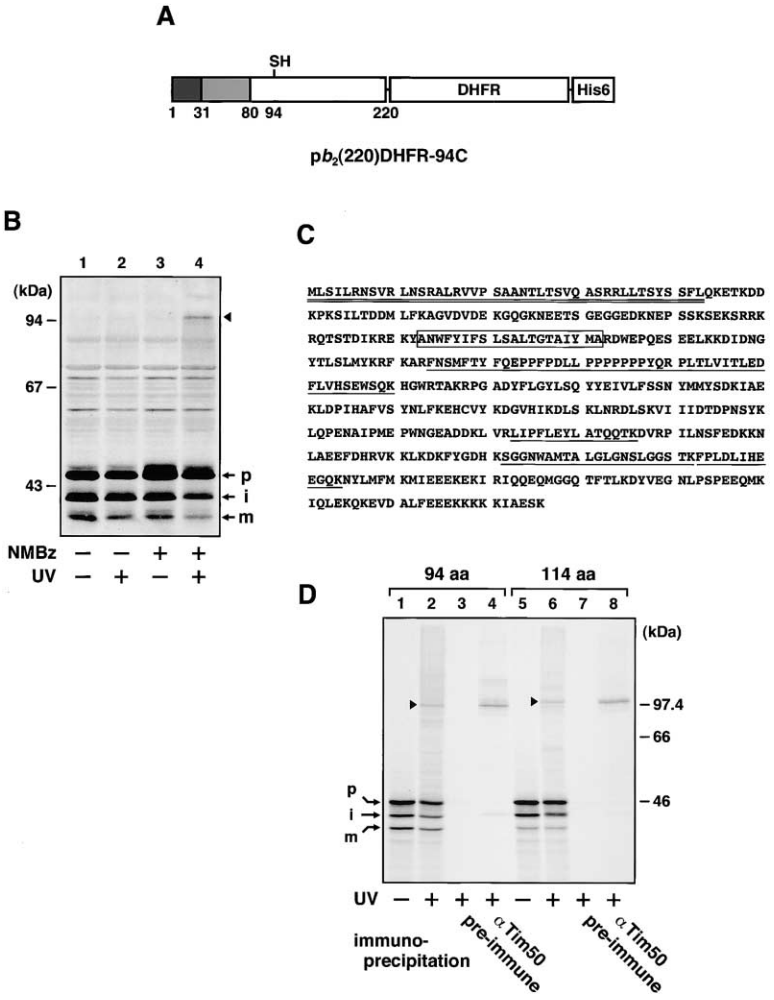

Figure 1. Identification of Tim50

(A) pb_2(220)DHFR-94C is schematically outlined. pb_2(220)DHFR-94C contains the first 220 residues of cytochorome b_2 precursor (with a unique Cys at residue 94), which is fused to a tandem sequence of the 7-residue linker fragment, DHFR, and a hexahistidine tag. The first 80 residues of cytochorome b_2 precursor represents a presequence, in which residues 1–31 function as the mitochondrial targeting signal and residues 32–80 the IMS sorting signal.

(B) The sulfhydryl group of Cys-94 of pb_2(220)DHFR-94C was modified with NMBz. Modified pb_2(220)DHFR-94C (lanes 3 and 4) and unmodified pb_2(220)DHFR-94C (lanes 1 and 2) were allowed to form mitochondrial translocation intermediates in the presence of 10 μM MTX, and then UV irradiated (lanes 2 and 4). Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with anti-DHFR antibodies. NMBz, modified with NMBz; UV, UV irradiation; p, i, and m, precursor, processing intermediate, and mature forms of pb_2(220)DHFR-94C, respectively. The arrowhead indicates the 95 kDa crosslinked product.

(C) The amino acid sequence of Tim50. The putative mitochondrial presequence (residues 1–43) and the potential transmembrane segment (residues 113–132) are double underlined and boxed, respectively. Four peptide sequences identified by tryptic digestion of the crosslinked product followed by mass spectrometry are underlined.

(D) Crosslinked products with pb_2(220)DHFR containing BPA at residues 94 (94 aa) or residue 114 (114 aa) were analyzed by immunoprecipitation with antibodies against Tim50 (αTim50) or the preimmune serum (preimmune). Site-specific photocrosslinking was performed as in Kanamori et al. (1997). The major crosslinked products are indicated with arrowheads. The amounts of the samples loaded for lanes 3 and 4 and lanes 7 and 8 are five times those for lanes 1 and 2 and lanes 5 and 6, respectively.

These results indicate that Tim50 is an integral membrane protein of the mitochondrial inner membrane. Tim50 became unrecognizable by the antibodies against the C-terminal segment of Tim50 after protease treatments of the mitoplasts (not shown). Since Tim50 has only a single putative transmembrane segment near the N terminus, Tim50 most likely takes the N_{in}–C_{out} topology in the inner membrane and exposes the large C-terminal domain (residues 133–476) to the IMS.

To further confirm that Tim50 is the inner-membrane protein of mitochondria, radiolabeled Tim50 synthesized in rabbit reticulocyte lysates was incubated with isolated mitochondria. Tim50 was efficiently converted to the mature form, but was only slowly sequestered to the compartment that was protected from protease added to the mitochondria (Figure 2D). The import of Tim50 strictly required $\Delta\Psi$ (not shown). This indicates that the N terminus of Tim50 can reach the matrix efficiently while the C-terminal domain of Tim50 moves across the outer membrane slowly.

The slow rate of the in vitro import of Tim50 into mitochondria might be due to the folded structure of the C-terminal domain of Tim50, which is resistant to moderate protease digestion (data not shown). Indeed, C-terminally truncated versions of the Tim50 precursor were, in contrast to the full-length Tim50 precursor, efficiently imported into mitochondria (not shown). In vivo, Tim50 is likely imported into mitochondria in a cotranslational manner because it is synthesized on membrane-bound polysomes (Diehn et al., 2000)

Tim50 Mediates Translocation of Presequence-Containing Precursor Proteins across the Inner Membrane

Since Tim50 is crosslinked to the pb_2(220)DHFR translocation intermediate, Tim50 may mediate protein import into mitochondria. We constructed a yeast strain GAL-TIM50, in which the galactose-inducible *GAL7* promoter was integrated into the chromosome in front of the *TIM50* ORF, allowing regulated expression of Tim50. Since Tim50 is essential for viability of yeast, GAL-TIM50 cells grew on galactose-containing medium (YPGal), but

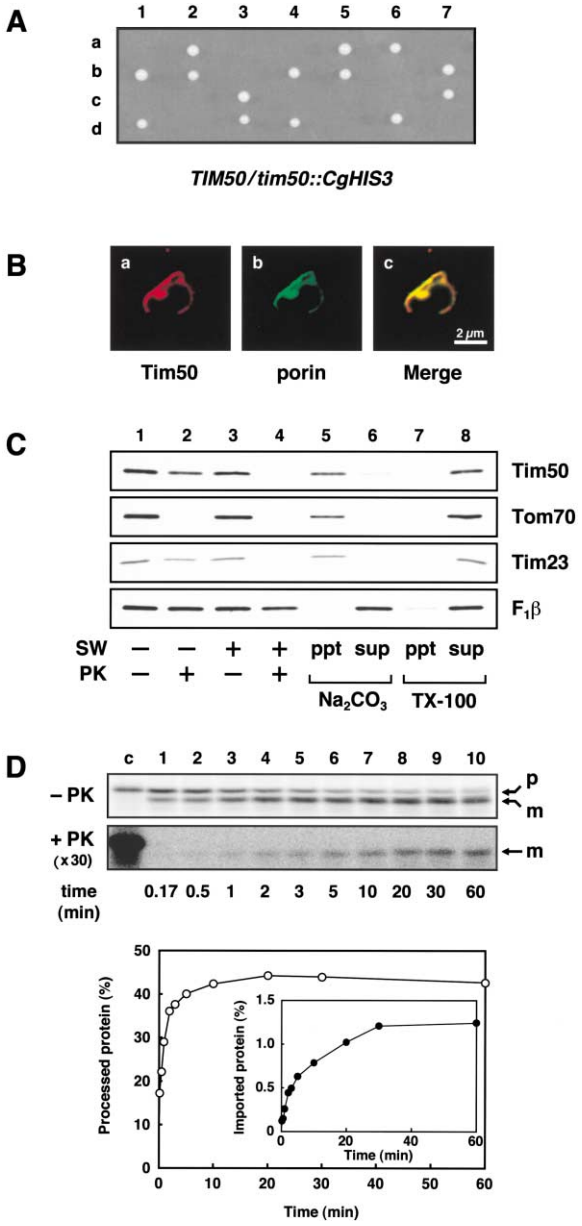

Figure 2. Tim50 Is Essential for Yeast Cell Growth and Is an Integral Membrane Protein of the Mitochondrial Inner Membrane

(A) One of the two chromosomal *TIM50* genes in a diploid *S. cerevisiae* strain was disrupted by the *C. glabrata HIS3* gene, the diploid was sporulated, and seven different asci were dissected. The four spores recovered from each of the asci were allowed to germinate and to grow for 3 days at 30°C on YPD.

(B) Localization of Tim50 by immunofluorescence microscopy. Cells of yeast strain W303-1A were analyzed by double label immunofluorescence microscopy using anti-Tim50 antibodies and the anti-porin antibody. Panels *a*, *b*, and *c* show the same field of the fluorescent images stained with anti-Tim50 antibodies (red) or the anti-porin antibody (green), and of the merged image, respectively.

(C) Submitochondrial localization of Tim50. Mitochondria (lanes 1 and 2) and mitoplasts (lanes 3 and 4) were treated with 200 μg/ml proteinase K for 20 min on ice (lanes 2 and 4). Mitochondria were treated with either 0.1 M Na_2CO_3 (lanes 5 and 6) or 1% Triton X-100 (lanes 7 and 8), and then pellets (lanes 5 and 7) and supernatants (lanes 6 and 8) were separated by centrifugation. SW, osmotic swelling; PK, proteinase K treatment; Na_2CO_3, extraction with Na_2CO_3; TX-100, extraction with Triton X-100; ppt, pellet fraction; sup, supernatant fraction.

(D) In vitro import of the precursor of Tim50 into isolated mitochondria. The radiolabeled Tim50 precursor was incubated with isolated mitochondria at 30°C for indicated times. After import, the samples were divided into halves, and one aliquot was kept on ice (−PK) and the other was treated with proteinase K (+PK). The mitochondria were reisolated by centrifugation and proteins were analyzed by SDS-PAGE and radioimaging with a Storm 860 image analyzer (Molecular Dynamics). The intensity for "+PK" is enhanced 30-fold as compared with that for "−PK." The mature form for −PK and that for +PK were quantified and plotted as "Processed protein" and "Imported protein," respectively against the incubation times. The amounts of the Tim50 precursor added to each reaction are set to 100%. c, 25% of the Tim50 precursor added to each sample; p and m, precursor and mature forms, respectively.

not on glucose-containing medium (YPD) (Figure 3A). Total lysates were prepared from wild-type cells or from GAL-TIM50 cells, various times after the shift from YPGal to YPD, and were analyzed by immunoblotting for various mitochondrial proteins (Figure 3B). When cultivated in the presence of galactose, the level of Tim50 in GAL-TIM50 cells was 2.5-fold higher than in wild-type cells (Figure 3B lane 3, Tim50). However, the levels of Tim50 decrease with the time of cultivation in YPD and reach 7.5% of that in wild-type cells 24 hr after the shift to YPD (Figure 3B, lane 6, Tim50). Depletion of Tim50 did not severely affect the expression levels of other proteins involved in the mitochondrial protein import, such as Tom40, Tom22, Tim23, Tim17, Tim44, and Ssc1p (Figure 3B). On the other hand, prolonged cultivation of GAL-TIM50 cells in the absence of galactose led to accumulation of uncleaved precursor forms of mitochondrial Hsp60 (mtHsp60) and Mdj1p (Figure. 3B, Hsp60 and Mdj1p). The level of phosphate carrier (PiC), a presequence-less polytopic inner membrane protein, was not significantly affected by depletion of Tim50 as compared with the wild-type strain. Therefore, reduction of the level of Tim50 apparently caused defects in import of presequence-containing mitochondrial proteins in vivo.

In parallel, the effects of depletion of Tim50 were tested on mitochondrial protein import in vitro. First, import of the radiolabeled presequence-containing precursor of mtHsp60 and radiolabeled pSu9-DHFR, a fusion protein between the presequence of subunit 9 of F_o-ATPase and DHFR, into mitochondria isolated from wild-type cells or GAL-TIM50 cells (Tim50↓ cells) after cultivation in galactose-free medium for 12 hr was monitored. The rates of import of the mtHsp60 precursor and pSu9-DHFR into Tim50↓ mitochondria were significantly decreased as compared with those into wild-type mitochondria (Figure 3C). We observed similar decrease in import rates of pb_2(220)DHFR and precursor of $F_1\beta$ into Tim50↓ mitochondria (not shown). $\Delta\Psi$ did not decrease in Tim50↓ mitochondria (not shown). Then import of ADP-ATP carrier (AAC), a presequence-less polytopic inner membrane protein, into Tim50↓ mitochondria was compared with that into wild-type mitochondria. In contrast to pSu9-DHFR and mtHsp60, neither translocation across the outer membrane nor insertion into the inner membrane for AAC was affected by depletion of Tim50 (Figure 3D). Import of another presequence-less polytopic membrane protein, the Tim23 precursor, into mitochondria was not affected by decrease in the level of Tim50, as well (not shown).

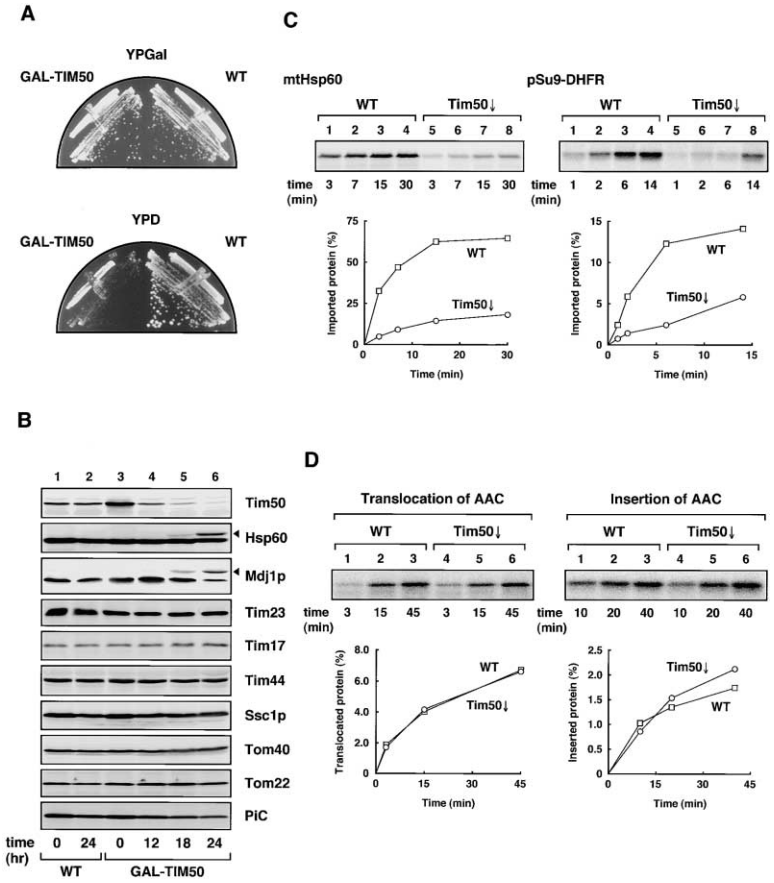

Figure 3. Tim50 Facilitates the Import of Presequence-Containing Precursor Proteins into Mitochondria Both In Vivo and In Vitro

(A) Yeast wild-type strain (W303-1A; WT) and the GAL-TIM50 strain, in which the promoter of *TIM50* is replaced by the inducible *GAL7* promoter, were streaked onto YPGal and YPD and incubated at 23°C for 3 days and for 5 days, respectively.

(B) Total lysates were prepared from yeast strains GAL-TIM50 and W303-1A (WT), which were grown at 23°C in YPGal, diluted, and then grown at 23°C for 0, 12, 18, and 24 hr in YPD. Total protein was isolated and analyzed by SDS-PAGE and immunoblotting with antibodies against the indicated proteins. The arrowheads indicate the accumulated precursor forms of mtHsp60 and Mdj1p.

(C) Mitochondria were isolated from yeast strains GAL-TIM50 (Tim50↓) and W303-1A (WT) after cultivation in glucose-containing medium (2% glucose) for 12 hr at 30°C. The amount of Tim50 in Tim50↓ mitochondria was 19% of that in WT mitochondria. The radiolabeled mtHsp60 precursor and pSu9-DHFR were incubated with Tim50↓ mitochondria (circles) and WT mitochondria (squares) at 23°C for indicated times. The mitochondria were treated with proteinase K, and the imported proteins were analyzed by SDS-PAGE and radioimaging. The amounts of precursor proteins added to each reaction are set to 100%.

(D) Radiolabeled AAC was subjected to the import reaction as (C). After import, mitochondria were treated with proteinase K to measure translocation of AAC across the outer membrane (left panel), or mitoplasts were generated and treated with proteinase K to measure insertion into the inner membrane (right panel). The amounts of AAC added to each reaction are set to 100%.

To confirm that Tim50 is directly involved in mitochondrial protein import, we tested the effects of antibodies against Tim50 on the protein import into mitochondria and mitoplasts. Mitochondria and mitoplasts were preincubated with immunoglobulins G (IgGs) prepared from an anti-Tim50 antiserum and from a preimmune serum, and subsequently incubated with radiolabeled mtHsp60, pSu9-DHFR, and Tim23. Like AAC, Tim23 is imported into mitochondria via the TOM complex and inserted into the inner membrane with the aid of the TIM22 complex instead of the TIM23 complex. While import of mtHsp60 or pSu9-DHFR into mitochondria was not affected by preincubation with anti-Tim50 IgG, the import rates of mtHsp60 and pSu9-DHFR into mitoplasts were significantly decreased by preincubation with anti-Tim50 IgG, but not with the control IgG (Figure 4). The amounts of anti-Tim50 IgG required for inhibition of the import of mtHsp60 and pSu9-DHFR into mitoplasts were <5 μg IgGs per 50 μg mitochondrial proteins. On the other hand, insertion of Tim23 into the inner membrane was not affected by preincubation of mitochondria or mitoplasts with anti-Tim50 IgG (Figure 4). These results indicate that Tim50 mediates the early step of translocation of mitochondrial precursor proteins with a cleavable presequence, but not the ones without a presequence, across the inner membrane.

Tim50 Is a Subunit of the TIM23 Complex

Since Tim50 is directly involved in the translocation of presequence-containing precursor proteins, but not of AAC or Tim23, across the inner membrane, Tim50 can be a component of the translocator for presequence-containing proteins, the TIM23 complex (Koehler et al., 1999; Bauer et al., 2000). We thus asked if Tim50 is associated with the subunits of the TIM23 complex in mitochondria. Yeast mitochondria were solubilized with 2% digitonin and subjected to immunoprecipitation with antibodies against Tim50, Tim23, and Tom40. The immunoprecipitates were then analyzed for Tim50 and Tim23 by SDS-PAGE and immunoblotting (Figure 5A). Tim50 was coprecipitated with anti-Tim23 antibodies, but not with anti-Tom40 antibodies. Conversely, anti-Tim50 antibodies immunoprecipitated Tim23 together with Tim50 (Figure 5A).

To obtain additional evidence that Tim50 is a subunit of the TIM23 complex, mitochondria containing hexahistidine-tagged Tim50 (Tim50-His6) were solubilized and subjected to incubation with Ni-NTA agarose resin. Tim50-His6 efficiently bound to Ni-NTA agarose and was eluted with 250 mM imidazole (Figure 5B, lanes 4–6). The subunits of the TIM23 complex, Tim23, Tim17, and Tim44 were also adsorbed to Ni-NTA agarose and were eluted with 250 mM imidazole, while Tom70 or AAC did

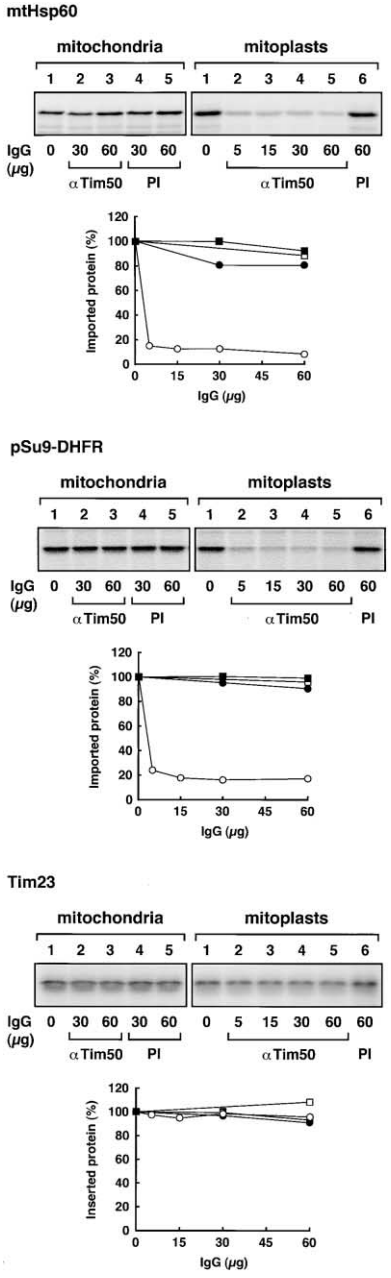

Figure 4. Protein Import into Mitoplasts, but Not into Mitochondria, Is Blocked by anti-Tim50 Antibodies

The mitochondria (filled symbols) and mitoplasts (blank symbols) (50 μg proteins) were preincubated with indicated amounts of anti-Tim50 IgG (αTim50; circles) or IgG prepared from the preimmune serum (PI; squares) in 250 μl import buffer for 30 min on ice. The radiolabeled mtHsp60 precursor, pSu9-DHFR and Tim23 were incubated with the IgG-treated mitochondria or mitoplasts for 6 min (mtHsp60 precursor and pSu9-DHFR) or 15 min (Tim23) at 23°C. For the import of mtHsp60 precursor and pSu9-DHFR, the mitochondria and mitoplasts were treated with proteinase K. For the import of Tim23, mitochondria were converted to mitoplasts, which were treated with 250 μg/ml trypsin for 20 min on ice (Paschen et al., 2000). Then the mitochondria and mitoplasts were recovered by centrifugation, and proteins were analyzed by SDS-PAGE and radioimaging. For the import of Tim23, the trypsin-resistant fragment, which represents Tim23 inserted in the inner membrane, is shown. The analysis was performed in the linear range of the import kinetics of the respective precursor proteins (not shown). The amount of protein imported in a control sample without IgGs was set to 100%.

not bind to the resin (Figure 5B, lanes 4–6). As a control, when solubilized mitochondria containing HA epitope-tagged Tim50 (Tim50-3HA) instead of Tim50-His6 were incubated with Ni-NTA agarose resin, neither Tim50 nor other subunits of the TIM23 complex bound to the resin (Figure 5B, lanes 1–3). These results indicate that Tim50 is a subunit of the TIM23 complex.

When mitochondria were solubilized and analyzed by glycerol density gradient centrifugation, Tim50 was recovered partly in the fractions corresponding to ~140 kDa and partly in those corresponding to ~250 kDa (Figure 5B). Since Tim23 was recovered in the fractions corresponding to ~250 kDa (Figure 5B), about one third of the Tim50 molecules are apparently associated with the TIM23 complex. By standardized immunoblotting in comparison with the purified Tim50 fragment expressed in *E. coli* cells, yeast mitochondria were found to contain ~30 pmol of Tim50 per mg of mitochondrial protein (not shown), while they were reported to contain approximately 17 to 20 pmol of Tim23 per mg of mitochondrial protein (Dekker et al., 1997; Sirrenberg et al., 1997). Therefore, the stoichiometry of Tim50:Tim23 in the TIM23 complex is roughly estimated to be 1:2.

We used a yeast two-hybrid analysis to further analyze the interactions between Tim50 and Tim23. To this end, we first made a fusion gene for the activator domain of yeast Gal4p and the IMS domain (residues 133–476) of Tim50 and those for the DNA binding domain of yeast Gal4p and three segments (residues 1–50, residues 51–96, and residues 1–96) of the N-terminal half of Tim23. After confirming the expression of the corresponding fusion proteins in yeast (not shown), we asked which combinations of the fusion genes expressed together with a reporter gene, *GAL2-ADE2*, in yeast cells give rise to Ade^+ colonies. Interactions between the two fusion proteins will lead to the localization of Gal4p to the DNA, thereby activating the *GAL2*-regulated allele of *ADE2* (James et al., 1996). The IMS domain of Tim50 fused to the Gal4p activator domain gave rise to Ade^+ colonies when Tim23(51-96) or Tim23(1-96), but not Tim23(1-50), fused to the Gal4p DNA binding domain was co-expressed (Figure 5D). This suggests that the IMS domain of Tim50 interacts directly with residues 51–96 of Tim23.

It has been reported that Tim23 takes an unusual transmembrane topology in which Tim23 spans both the outer and inner mitochondrial membranes (Donzeau et al., 2000); the N-terminal domain (residues 1–50) is integrated into the outer membrane and exposes the N terminus to the cytosol, while the C-terminal half (residues 100–222) is integrated into the inner membrane, leaving residues 50–100 between the two domains exposed to the IMS. Therefore, the results of the yeast two-hybrid analysis show that the IMS domain of Tim50 can interact with the possible IMS-exposed segment of Tim23. We next tested if protease accessibility of the N terminus of Tim23 is affected by overexpression or depletion of Tim50 in mitochondria. About half of the Tim23 molecules in wild-type mitochondria were clipped and a resistant fragment Tim23* was generated when intact mitochondria were treated with proteinase K (Figure 5E), as reported previously (Donzeau et al., 2000). The fraction of Tim23 that received the N-terminal cleavage decreased in mitochondria with decreased Tim50

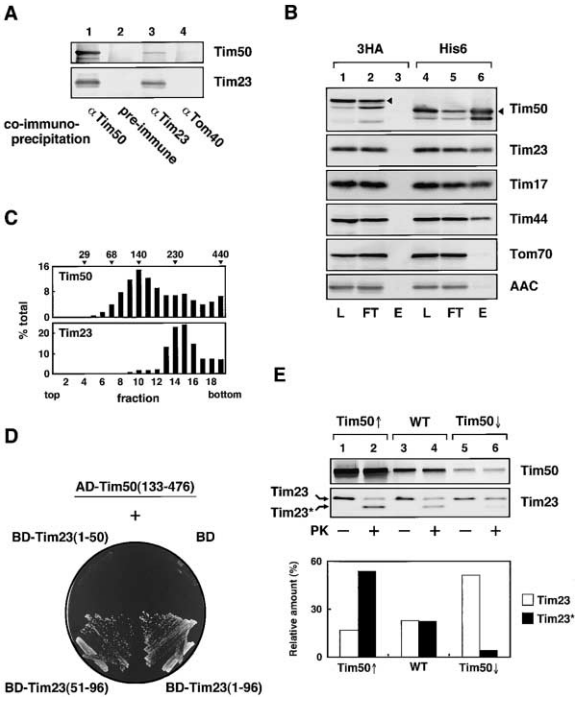

Figure 5. Tim50 Is a Subunit of the TIM23 Complex and Directly Interacts with Tim23

(A) Mitochondria were solubilized with 2% digitonin in 20 mM Tris-HCl (pH 7.4), 250 mM NaCl, 1 mM EDTA, and 10% glycerol, and subjected to immunoprecipitation with antibodies against Tim50 (αTim50), Tim23 (αTim23), and Tom40 (αTom40) and with preimmune serum (pre-immune). Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with anti-Tim50 antibodies and anti-Tim23 antibodies.

(B) Mitochondria were prepared from yeast strains expressing Tim50-3HA or Tim50-His6 instead of wild-type Tim50. The mitochondria were solubilized with 1% digitonin in 50 mM Tris-HCl (pH 8.0), 250 mM NaCl, and 20 mM imidazole and were incubated with the Ni-NTA resin (QIAGEN), which was subsequently washed with 20 mM imidazole, and proteins bound to the resin were eluted with 250 mM imidazole. Proteins were analyzed by SDS-PAGE and immunoblotting with antibodies against Tim50, Tim23, Tim17, Tim44, Tom70, and AAC. Tim50 had slight degradation during the analysis. L, loaded sample; FT, flow through fraction; E, eluted fraction with 250 mM imidazole; 3HA, Tim50-3HA; His6, Tim50-His6.

(C) Mitochondria were solubilized with 1% digitonin in 20 mM Tris-HCl (pH 7.5), 20 mM NaCl, 2 mM EDTA, and 10% glycerol, and were centrifuged at 100,000 × g for 30 min at 4°C. The supernatant was layered onto linear glycerol gradient (10%–40% glycerol in the same buffer as for the solubilization containing 0.2% digitonin), and centrifuged at 200,000 × g for 15 hr at 4°C. After centrifugation, fractions were collected from the top and analyzed by immunoblotting using antibodies against Tim50 and Tim23. Numbers indicate fractions (from top to bottom). Vertical arrowheads show the positions of apoferritin (440 kDa), catalase (230 kDa), alcohol dehydrogenase (140 kDa), BSA (68 kDa), and carbonic anhydrase (29 kDa).

(D) The IMS domain (residues 133–476) of Tim50 expressed as a fusion to the Gal4p activator domain (AD-Tim50(133–476)) was assayed for interactions with residues 1–50, residues 51–96, and residues 1–96 of Tim23 as fusions to the Gal4p DNA binding domain (BD-Tim23(1–50), BD-Tim23(51–96) and BD-Tim23(1–96), respectively) and with the Gal4p DNA binding domain (BD) as a control in yeast two-hybrid analysis. The plasmids for the BD fusions were individually co-transformed with the plasmid for AD-Tim50(133-476) into the yeast strain PJ69-4A and incubated on minimal medium lacking adenine at 30°C for 3 days.

(E) Yeast strain GAL-TIM50 was grown in galactose-containing medium or glucose-containing medium for 12 hr at 30°C to prepare mitochondria with overexpressed Tim50 (Tim50↑ or those depleted of Tim50 [Tim50↓], respectively). Wild-type mitochondria (WT) were prepared from the W303-1A strain (WT). The mitochondria were treated with 500 μg/ml proteinase K in 250 mM sucrose, 10 mM MOPS-KOH (pH 7.2), and 80 mM KCl for 20 min on ice (lanes 2, 4, and 6). The mitochondria were reisolated and proteins were analyzed by SDS-PAGE and immunoblotting with antibodies against Tim23 and Tim50. The amounts of Tim23 in lanes 1, 3, and 5 are set to 100% for those of Tim23 and Tim23* in lanes 2, 4, and 6, respectively. PK, proteinase K treatment; Tim23*, a proteolytic fragment of Tim23 in mitochondria.

(Tim50↓), whereas it increased in mitochondria with overexpressed Tim50 (Tim50↑). These results suggest that the integration of the N-terminal segment of Tim23 into the outer membrane is dynamic and is stabilized by Tim50. Since Tim50 excess over the wild-type level of Tim50 still affects the clipping of Tim23, interactions of Tim50 with the TIM23 complex may be also dynamic.

Tim50 Links Protein Translocation across the Outer and Inner Membranes

The N-terminal domain (residues 1–50) of Tim23 has been suggested to facilitate the transfer of precursor proteins from the TOM complex to the TIM23 complex (Donzeau et al., 2000). Since Tim50 has a large C-terminal domain exposed to the IMS, which interacts with the IMS-exposed segment of Tim23, Tim50 may also facilitate the transfer of precursor proteins between the TOM and the TIM23 complexes. To test such a possibility, we probed the interactions of the translocation intermediate accumulated at the TOM complex with Tim50 by a crosslinking approach.

pSu9-DHFR binds to the mitochondrial surface when incubated at 30°C with isolated mitochondria containing Tim50 or Tim50-3HA that have been treated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to prevent the presequence translocation across the inner membrane by dissipating $\Delta\Psi$ (Kanamori et al., 1999). In this intermediate, the presequence of pSu9-DHFR reaches the trans site of the TOM complex and the DHFR domain is unfolded and bound to Tom40 (Kanamori et al., 1999). Then the mitochondria were re-isolated, and treated with hetero-bifunctional noncleavable crosslinking reagents, MBS (*m*-maleimidobenzolyl-*N*-hydroxysulphosuccinimide ester) or GMBS (*N*-[γ-maleimidobutyryloxy] succinimide ester), for amino and sulfhydryl groups. The crosslinked partners were identified by solubilization of the mitochondria followed by immunoprecipitation with specific antibodies against the known components of the TOM and TIM complexes.

Figure 6 shows the crosslinked products and their assignments to components of the TOM and TIM complexes. The crosslinking partners for 91–92 kDa and 60 kDa crosslinked products were assigned to Tom40, and that for 44–46 kDa crosslinked products to Tom22. pSu9-DHFR contains a cysteine residue at positions 76 (residue 7 in the DHFR domain) and Tom40 at positions 165, 326, 341, and 355, but Tom22 does not have a cysteine residue. Therefore, while there are many possibilities for the cysteine residues involved in the crosslinking between pSu9-DHFR and Tom40, Tom22 should

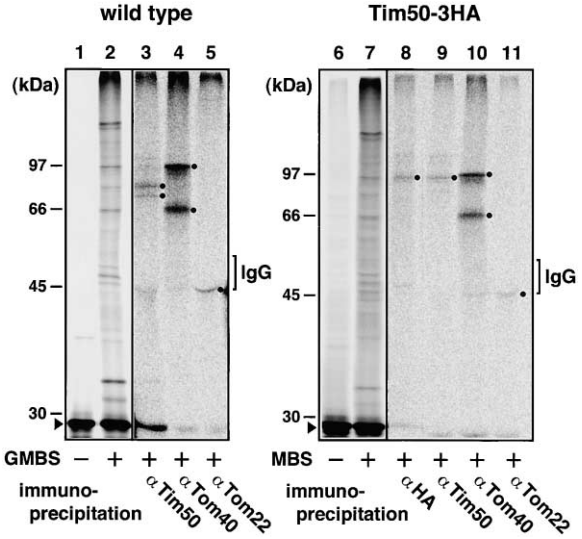

Figure 6. The Translocation Intermediate of pSu9-DHFR Lodged in the TOM Channel Is Crosslinked to Tim50

The translocation intermediate of radiolabeled pSu9-DHFR was generated by incubating with $-\Delta\Psi$ mitochondria containing wild-type Tim50 or Tim50-3HA for 10 min at 30°C (Kanamori et al., 1999). The mitochondria with wild-type Tim50 (left panel) and those with Tim50-3HA (right panel) were reisolated and subjected to crosslinking with 200 μM GMBS (Pierce) and MBS (Pierce), respectively, for 60 min on ice. After quenching the reaction with 50 mM Tris-HCl (pH 7.0), the mitochondria were reisolated, solubilized with 1% SDS, and subjected to immunoprecipitation with antibodies raised against Tim50 (αTim50), Tom40 (αTom40), and Tom22 (αTom22) and with the anti-HA monoclonal antibody. The immunoprecipitates were analyzed by SDS-PAGE and radioimaging. The intensities for lanes 3–5 and for lanes 8–10 are 25-fold and 40-fold higher than those for lanes 1–2 and for lanes 6–7, respectively. The faint bands of 44–46 kDa in lanes 3 and 10, but not in lane 5 or 11, vary with experiments and are not reproducible. Dots indicate the identified crosslinked products, and arrowheads pSu9-DHFR.

be crosslinked to a single cysteine residue near the N terminus of the DHFR domain of pSu9-DHFR.

Anti-Tim50 antibodies immunoprecipitated 75 kDa and 65 kDa crosslinked products for mitochondria containing wild-type Tim50 and a 90 kDa crosslinked product for those containing Tim50-3HA (Figure 6). Although the band of the 90 kDa crosslinked product for the mitochondria containing Tim50-3HA was partly overlapped by that of the crosslinked product involving Tom40, the 90 kDa product was immunoprecipitated with the anti-HA antibody, but not with anti-Tom22 antibodies. A single cysteine residue at position 268 of Tim50 is likely crosslinked to the presequence of pSu9-DHFR, which contains several amino groups. Therefore, the translocation intermediate lodged in the TOM channel is already interacting with Tim50 of the TIM23 complex, suggesting a role for Tim50 in linking translocation mediated by the TOM complex and that mediated by the TIM23 complex.

Discussion

In the present study, we identified Tim50 as a subunit of the TIM23 complex. Tim50 is an integral inner membrane protein and exposes the C-terminal domain to the IMS, which interacts with the N-terminal IMS domain of Tim23. Depletion of Tim50 led to accumulation of precursor forms of mitochondrial proteins in vivo and exhibited defects in import of precursor proteins with a cleavable presequence in vitro. Anti-Tim50 antibodies blocked the import of presequence-containing precursor proteins into mitoplasts. These results indicate that Tim50 is a subunit of the TIM23 complex that is directly involved in protein translocation across the inner mitochondrial membrane. Glycerol density gradient centrifugation of solubilized mitochondria showed that, while Tim50 is a subunit of the TIM23 complex, only one third of the Tim50 molecules are stably associated with the ~250 kDa TIM23 complex and the rest of the Tim50 molecules forms a pool that is distinct from the TIM23 complex. Blue-native PAGE analyses also showed that Tim50 is not tightly associated with the 90 kDa core assembly, which mainly consists of Tim23 and Tim17, of the TIM23 complex (not shown).

Database searches showed that ORFs encoding proteins with similarity to Tim50 and with a putative mitochondrial presequence are found in the genomes of evolutionarily distant organisms including *Caenorhabditis elegans* (*orf6.6573*), *Drosophila melanogaster* (*CG12313*), *Arabidopsis thaliana* (*F14J16.15*), mouse (*NP_079892*), and human (*XP_053074*). These Tim50 homologs in the other organisms have a possible transmembrane segment near the N terminus, so that they likely take the same N_{in}–C_{out} topology at the inner membrane as yeast Tim50. *S. cerevisiae* also has three other proteins that are homologous to Tim50 but exhibit different localizations, namely Nem1p in the nuclear/ER membrane (Siniossoglou et al., 1998) and Psr1p and Psr2p in the plasma membrane (Siniossoglou et al., 2000).

What is the role of Tim50 in protein translocation across the inner membrane? We provide evidence that Tim50 crosslinked to translocation intermediates lodged in the TOM channel in the absence of $\Delta\Psi$. This strongly suggests that Tim50 links translocation through the TOM complex to translocation through the TIM23 complex. Previously, the IMS domain of Tom22 and the N-terminal 50 residue segment of Tim23 have been suggested to facilitate the transfer of precursor proteins from the TOM complex to the TIM23 complex. Mitochondria containing a mutant Tom22 that lacks its IMS domain exhibit no defect in the accumulation of the translocation intermediate in the absence of $\Delta\Psi$, but impairs the chase of the intermediate into the matrix after regeneration of $\Delta\Psi$ (Court et al., 1996; Moczko et al., 1997; Kanamori et al., 1999). The mitochondria containing a mutant Tim23 that lacks the N-terminal 50 residues have defects in protein import into mitochondria, but not into the mitoplasts (Donzeau et al., 2000). We thus propose here that Tim50, in cooperation with Tom22 and Tim23, links protein translocation across the outer membrane and the inner membrane.

Tim50 crosslinks to the precursor form of translocating pb_2(220)-DHFR, not the mature form, and downstream of the IMS sorting signal. Therefore, it is possible that Tim50 is responsible for the sorting function, or, more specifically, the recognition of the IMS sorting signal of pCytb_2 as well as for protein translocation across the inner membrane. Such a role was previously assigned to Tim23 (Bömer et al., 1997) and Tim11 (Tokat-

lidis et al., 1996). Mitochondria containing a mutant Tim23 affect the sorting of pCytb_2 with an altered sorting signal (Bömer et al., 1997). Tim11 was crosslinked to the pCytb_2 sorting signal for the IMS efficiently (Tokatlidis et al., 1996). However, since Tim11 was found to be a subunit of F_1F_o-ATPase and was not included in the TIM23 complex (Arnold et al., 1997), it may not be involved in intramitochondrial sorting. The possible roles of Tim50 and Tim23 in sorting proteins to the IMS, e.g., recognition of the IMS sorting signal and discharge of the IMS proteins from the TIM23 complex, will be an essential subject of future studies.

Experimental Procedures

Plasmids and Yeast Strains

Construction of plasmids and yeast strains are described in detail in the Supplemental Data (available at http://www.cell.com/cgi/content/full/111/4/519/DC1).

Yeast strains were grown in YPD (1% yeast extract, 2% polypeptone, 2% glucose), YPGal (1% yeast extract, 2% polypeptone, 2% galactose), and SD (0.67% yeast nitrogen base without amino acids, 2% glucose) supplemented with nucleotides and amino acids according to Rose et al. (1990).

Identification of Tim50

The fusion protein pb_2(220)DHFR-94C expressed in *E. coli* cells from plasmid pET- pb_2(220)CA-94C was purified as an inclusion body, solubilized with 8 M urea in 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM EDTA, and 0.2 mM DTT, and incubated with 2 mM NMBz (Sigma) for 60 min on ice. After quenching the reaction by addition of 20 mM DTT, NMBz-modified pb_2(220)DHFR-94C was allowed to form a translocation intermediate with isolated mitochondria in the presence of 10 μM MTX, and was UV irradiated (Kanamori et al., 1997). The mitochondria were solubilized with 1% Triton X-100, and the crosslinked products were purified with the His-Bind resin (Novagen) and subjected to SDS-PAGE and CBB R-250 staining. The CBB-stained 95 kDa crosslinked product was excised from the gel and prepared for mass spectrometry analyses (Abe et al., 1998). Molecular mass information on the tryptic peptides was obtained with a MALDI-TOF mass spectrometer, Voyager Elite (Applied Biosystems). Abundant ions in the MALDI spectra were calibrated with the internal control and used for database searches using the software PeptideSearch (developed at the EMBL Bioanalytical Research Group at Heidelberg, Germany).

Antibodies and Immunoprecipitation

The C-terminal segment (residues 242–476) of Tim50 was purified as an inclusion body from *E. coli* cells and used to immunize rabbits for obtaining anti-Tim50 antibodies. The antibody against the HA epitope and the one against porin were purchased from Covance Research Products and Molecular Probes, respectively. IgGs were prepared using protein A-Sepharose chromatography (Amersham Pharmacia Biotech), and bound IgGs were eluted with 0.1 M glycine-HCl (pH 2.5), mixed with a 1/10 volume of 1 M Tris-HCl (pH 8.0), and dialyzed against 20 mM Tris-HCl (pH 7.2), 80 mM KCl. Immunoprecipitation was performed as described previously (Kanamori et al., 1997).

Import Assays and Miscellaneous

Preparation of mitochondria, protein import into mitochondria, generation of mitoplasts, alkaline extraction, and immunofluorescent microscopy are described in detail in the Supplemental Data (available at http://www.cell.com/cgi/content/full/111/4/519/DC1).

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