Identification of Tim40 That Mediates Protein Sorting to the Mitochondrial Intermembrane Space*

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Most mitochondrial proteins are synthesized in the cytosol, imported into mitochondria, and sorted to one of four mitochondrial subcompartments. Here we identified a new inner membrane protein, Tim40, that mediates sorting of small Tim proteins to the intermembrane space. Tim40 is essential for yeast cell growth, and its function in vivo requires six conserved Cys residues but not anchoring of the protein to the inner membrane by its N-terminal hydrophobic segment. Depletion of Tim40 impairs the import of small Tim proteins into mitochondria both in vivo and in vitro. In wild-type mitochondria, Tim40 forms a translocation intermediate with small Tim proteins prior to their assembly in the intermembrane space in vitro. These results suggest the essential role of Tim40 in sorting/assembly of small Tim proteins.

Mitochondria are essential organelles in eukaryotic cells that consist of four compartments, the outer membrane, intermembrane space (IMS), inner membrane, and matrix. Since most mitochondrial proteins are encoded by the nuclear genome and synthesized in the cytosol, mitochondria contain an elaborate system to take up these proteins from the cytosol and to sort them to specific intramitochondrial compartments. Recently, evidence has accumulated that the import/sorting pathways for mitochondrial proteins are much more complex than previously envisaged and involve the TOM40 (TOM, the translocase of the outer membrane) and the TIM23 or TIM22 complexes in the inner membrane, the TIM23 (TIM, the translocase of the mitochondrial inner membrane) and the TIM22 complexes in the inner membrane, small Tim proteins in the IMS, and the mitochondrial Hsp70 system in the matrix (1–3). The mitochondrial IMS contains many soluble, small size proteins including small Tim proteins and cytochrome c. They are synthesized without a cleavable presequence and enter the IMS with the aid of the TOM40 complex but independently of the TIM23 or TIM22 complex. Since there is no membrane potential across the outer membrane and the IMS lacks an ATP-dependent chaperone system, vectorial import of small IMS proteins should be driven by a unique mechanism (2, 4). One possible scenario to achieve this is the attachment of ligands such as heme (for cytochrome c) or zinc ion (for small Tim proteins) to the imported proteins in the IMS (5, 6). This will result in their folding/assembly preferentially in the IMS so that their translocation back to the cytosol will be prevented, leading to their accumulation in the IMS. However, it is still unclear whether proteinaceous factors in the IMS are further required for the small IMS protein biogenesis, which is discharged from the TOM40 complex, specific ligand binding, and assembly in the IMS etc.

In the present study, we looked for a component, if any, that mediates protein sorting to the mitochondrial IMS in yeast. Our approach relied on the fact that many mitochondrial proteins mediating mitochondrial protein assembly/import are essential or important for yeast cell viability (7, 8). We systematically analyzed the localization of yeast proteins that are indicated in the data base to have essential but unknown functions and identified a novel inner membrane protein Tim40 facing the IMS. We found that Tim40 is involved in the sorting/assembly of small Tim proteins to the IMS.

EXPERIMENTAL PROCEDURES

Plasmids and Yeast Strains—Wild-type Saccharomyces cerevisiae strains used in this study are D273-10B (MATa, ATCC 25657), W303-1A (MATa ade2-1 his3-11,15 ura3-1 leu2-3,112 trp1-1 can1-100), and W303-AB (MATa ade2-1 his3-11,15 his3-11,15 ura3-1ura3-1 leu2-3,112 leu3-1112 trp1-11rpr1-1 can1-100/ can1-100).

The Tim40 gene was cloned from the yeast genomic DNA by PCR using primers YKL195F-F (5’-CCC GGA TCC ATT TTT AAT AGT GTG TAT T3’) and YKL195F-R (5’-CGG GAA TTC AAA CGA ACC CCT AGG GTA TAG T3’). The amplified DNA fragment was digested with BamHI and EcoRI and inserted into the yeast single-copy plasmids pRS316 (URA3 selection marker) and pRS314 (TRP1 selection marker) (9) to give pRS316-Tim40 and pRS314-Tim40, respectively.

GAL-TIM40, a yeast strain expressing Tim40 under the control of the GAL7 promoter, was constructed as follows. A DNA fragment containing the GAL7 promoter was amplified from plasmid pGPHIS3-GAL7 (10) by PCR using primers GAL195F (5’-GCC GAC CAC CCA ATA AGA AAA AAA GGG AAA AGG AGC AGG GTA GTT TAA AAA CAT ATA AAC CCT GGG TCT GCT TTT GGA GC3’) and GAL195R (5’-ATA CAC TCT GTT GAT GGC CCC TAC TCT CAG TGT CAG TGT CTT CCA AAA GAA CAG GAG TTA AAC CAT TAA GAG TCA ATT ACG GGC TTC TCT GCT GAA CGC CGC). The amplified DNA fragment was used to transform the wild-type haploid strain W303-1A to integrate into the upstream region of the TIM40 gene.

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Disruption of the TIM40 gene was performed as follows. A DNA fragment containing the Candida glabrata HIS3 gene was ampliﬁed from plasmid pCgHIS11 (11) by PCR using primers Del195-F (5′-CTG TATG CCG TTC AAC AAG GCC TCG CCG CTG TGT AAA ACG AGC GCG AGT AGT-3′) and Del195-R (5′-TTA GGG TTT GGA TTC TTC ATT CAA TTA TTG TTC TTT CAC ACA CAG GAA ACA GCT ATG ACC-3′). The ampliﬁed DNA fragment, ﬂanked by 40 base pairs of the sequences upstream and downstream of the TIM40 gene, was introduced into the wild-type diploid strain W303-AB, and the resulting strain was named TIM40 Δtim40. The strain Δtim40 was transformed with TIM40 depending on the plasmid, but not on the chromosomal TIM40 gene, was constructed by tetrad analysis of the strain TIM40 Δtim40 harboring pRS316-Tim40 after sporulation.

The C296S/C298S, C307S/C317S, and C330S/C340S mutations were introduced into the TIM40 gene by PCR using pRS314-Tim40 as a template using primer sets 195–889F (5′-GGT GGT CCA CCG CAT GGA CCC-3′/195–298F-3′) and 195–330F (5′-GGT GGT CCA CCG CAT GGA CCC-3′/195–330F-3′), respectively, followed by self-ligation. The resulting plasmids were named pRS314-Tim40ΔC296S/C298S, pRS314-Tim40ΔC307S/C317S, and pRS314-Tim40ΔC330S/C340S, respectively.

Replacement of residues 1–70 of Tim40 with residues 1–220 or residues 1–85 of the cytochrome b2 precursor with or without the AA mutation (N80A and E81A) at the DNA level was performed as follows. The region corresponding to 576 nucleotides upstream of the TIM40 gene was cloned from pRS314-Tim40 by PCR using primers KpnI-195pro-F and SacI-195pro-R (5′-CCG GAA TTC CTT TTC GCC TCA GGA GAC TGT TTC ACC-3′/H11032-195pro-SalI-R-3′) and KpnI-330pro-SalI-F (5′-CCG GAA TTC CTT TTC GCC TCA GGA GAC TGT TTC ACC-3′/H11032-330pro-SalI-F-3′) and amplified by PCR using primers SalI-b2-F (5′-CAG AGA GCT AAC AGA GTC GAT ACC T-3′/H11032-195pro-SalI-R (5′-CCG GTC GAC TTT ACC AAC AGA GTC GAT ACC-3′), and 195–330S/1002R (5′-AAA CTT TTC AAC AGA GTC GAT ACC T-3′), respectively, followed by self-ligation. The resulting plasmids were named pRS314-Tim40ΔC296S/C298S, pRS314-Tim40ΔC307S/C317S, and pRS314-Tim40ΔC330S/C340S, respectively.

**RESULTS AND DISCUSSION**

**Tim40 Is a Mitochondrial Inner Membrane Protein—**Tim40, the gene product of YKL195W, is deposited as an essential protein in the data base of the yeast deletion project (Ref. 17; www.deletion.stanford.edu/YDPM/YDPM_index.html) (Fig. 1A). We analyzed its properties in mitochondrial association by treatment of isolated yeast mitochondria with PK, Na2CO3, and Triton X-100, followed by immunoblotting with anti-Tim40 antibodies (Fig. 1B). Tim40 was inaccessible to protease added to intact mitochondria (Fig. 1B, lanes 1 and 2) but was accessible to protease added to mitoplasts, where the outer membrane was selectively ruptured (Fig. 1B, lanes 3 and 4). This behavior resembled that of Tim23, an inner membrane protein exposed a domain to the IMS, but is different from those of Tom70, a surface-exposed outer membrane protein, or the β subunit of F1-ATPase (F1β), a peripheral inner membrane protein on the matrix side (Fig. 1B). Tim40 was, like integral membrane proteins, Tom70 and Tim23, not extracted by alkali-treatment of mitochondria but was solubilized by treatment of mitochondria with Triton X-100 and 500 mM NaCl (Fig. 1B, lanes 5–8). These results indicate that Tim40 is an integral inner membrane protein exposing a domain to the IMS.

The TIM40 protein comprises 403 amino acid residues (Fig. 1A). It is N terminus appears to possess a cleavable matrix-targeting presequence (Fig. 1A, underlined) followed by a stretch of hydrophobic amino acid residues (Fig. 1A, double underlined) that is reminiscent of the inner-membrane sorting signal, as found in the precursors to cytochromes b2 and c1, and subunit Va of cytochrome c oxidase (CoxA) (18). Precursors to cytochromes b2 and c1 have the inner-membrane-sorting signal in the form of a bipartite cleavable presequence, which receives two-step processing by mitochondrial processing peptidase in the matrix (for the N-terminal part) and by Imp1p/Imp2p on the IMS side of the inner membrane (for the C-terminal part). The CoxVa precursor has a regular matrix-targeting presequence, but not a bipartite presequence, and is anchored to the inner membrane by the N-terminal hydrophobic segment in the mature part. To test whether Tim40 has a bipartite presequence, we analyzed the in vitro import of Tim40 into isolated yeast mitochondria. The radiolabeled Tim40 precursor (44.5 kDa) synthesized with reticulocyte lysate exhibits unusually slow migration on the SDS-PAGE gel with an apparent molecular mass of 67.5 kDa (Fig. 1C, lane 9, p-form). This could be due to unusual distribution of acidic amino acid residues in Tim40 (Fig. 1A). When incubated with isolated mitochondria, the TIM40 precursor was converted to the lower molecular mass form (64.5 kDa; Fig. 1C, lanes 3–7, m-form), which was sequestered to the protease protected compartment (Fig. 1C, lane 1) and was of the same size as authentic Tim40 in isolated mitochondria as detected with anti-Tim40 antibodies (Fig. 1C, lane 10). Dissipation of the membrane potential
FIG. 1. Tim40 is a mitochondrial inner membrane protein and has three essential pairs of Cys residues. A, the amino acid sequence of Tim40 (YKL195W). The putative N-terminal cleavable presequence and the following hydrophobic stretch are single and double underlined, respectively. Asterisks indicate conserved Cys residues. B, mitochondria and mitoplasts generated by osmotic swelling (SW) were treated with 100 μg/ml PK for 30 min on ice (lanes 1–4). Mitochondria were treated with either 0.1 M Na2CO3 (lanes 5 and 6) or 1% Triton X-100 with 500 mM NaCl (lanes 7 and 8), and then pellets (ppt) and supernatants (sup) were separated by centrifugation (100,000 × g for 30 min at 4 °C). Proteins were detected by immunoblotting with antibodies against indicated proteins. C, in vitro import of the radiolabeled precursor of Tim40 into isolated yeast mitochondria at 23 °C for indicated times in the presence (lanes 2 and 8) or absence of 1 mM o-phenanthroline (o-phe) and 20 mM EDTA. The mitochondria were then treated with (lanes 1 and 2) or without (lanes 3–8) 100 μg/ml PK and were reisolated by centrifugation. Proteins were analyzed by SDS-PAGE and radioimaging. Lane 9, 10% of input; lane 10, authentic Tim40 in isolated mitochondria detected with anti-Tim40 antibodies. D, GAL-TIM40 carrying the TIM40 gene under the control of the GAL7 promoter was transformed with the genes for the indicated proteins on a single-copy plasmid of the TRP1 selection marker (pRS314) and were cultivated in SCD(-Trp) medium at 23 °C, and total cell extracts were prepared. Proteins in the extracts were analyzed by SDS-PAGE and immunoblotting with anti-Tim40 antibodies. E, growth of GAL-TIM40 cells shown in D were tested on a SCD(-Trp) plate at 23 °C for 3 days. Vector, pRS314 without an inserted gene was transformed. F, double point mutations for C296S/C298S or C307S/C317S or C330S/C340S in Tim40 were introduced into the TIM40 gene. Plasmids (pRS314) carrying these mutant or WT TIM40 genes or without an inserted gene (Vector) were introduced into the tim40 null mutant supplied with the TIM40 gene from a single-copy plasmid of the URA3 selection marker (Δtim40/pRS316-Tim40). The resulting transformants were grown on a SCD(-Trp) plate containing 0.15% 5-fluoroorotic acid at 30 °C for 2 days.
(ΔΨ) across the inner membrane inhibited generation of the m-form (Fig. 1C, lane 3). The presence of o-phenanthroline and EDTA, which inhibits the activity of mitochondrial processing peptidase in the matrix, prevented this processing, yielding a protease-protected precursor form (Fig. 1C, lanes 2 and 8, p-form). These results show that Tim40 does not have a bipartite presequence but is likely anchored to the inner membrane by its N-terminal hydrophobic stretch in the mature part.

**Conserved Cys Residues, but Not the N-terminal Anchor to the Inner Membrane, Are Essential for the Tim40 Functions—**

Next, we asked if the N-terminal anchoring of Tim40 to the inner membrane is essential for its function. For this purpose, we fused the IMS domain (residues 71–403) of Tim40 (Tim40\textsubscript{IMS}) to the N-terminal part (residues 1–220 or 1–85) of the cytochrome \textsubscript{b}2 precursor with or without the AA mutation (N80A and E81A), which removes the second processing site for Imp1p cleavage (19). Since Tim40 is essential for yeast cell viability as confirmed by tetrad analysis (data not shown), these cytochrome \textsubscript{b}2-Tim40 fusion proteins were expressed in yeast strain GAL-TIM40, in which the galactose-inducible \textit{GAL7} promoter was integrated into the chromosome in front of the \textit{TIM40} gene to achieve regulated expression of Tim40 by galactose. Immunoblotting with anti-Tim40 antibodies con-
firmed that pb2AA(85)-Tim40 IMS and pb2AA(220)-Tim40 IMS received only the first cleavage of the presequence (Fig. 1D, lanes 1 and 3), while pb2(220)-Tim40 IMS received the first and second cleavage of the presequence (Fig. 1D, lane 2). Since pb2(85)-Tim40 IMS lacks the folded heme-binding domain (residues 81–180 of cytochrome b2), which is required for the efficient cleavage by Imp1p, the second processing of pb2(85)-Tim40 IMS was retarded, so that both the intermediate-size and mature forms were detected (Fig. 1D, lane 4). The apparent molecular mass of authentic Tim40 was close to that of the processed form of pb2AA(85)-Tim40 IMS (Fig. 1D, lane 5), supporting that the Tim40 precursor receives only the first cleavage by mitochondrial processing peptidase. Therefore, pb2(220)-Tim40 IMS, pb2AA(85)-Tim40 IMS, and pb2AA(220)-Tim40 IMS are anchored to the inner membrane, whereas the N terminus of pb2(220)-Tim40 IMS is liberated from the inner membrane. When wild-type Tim40 was depleted by shifting cells to galactose-free medium, both wild-type and AA mutants of cytochrome b2-Tim40 IMS fusion proteins could functionally complement depletion of Tim40, without causing yeast growth defects (Fig. 1E). This suggests that anchoring of the N-terminal segment to the inner membrane is not essential for the function of Tim40 in vivo.

A data base search showed that open reading frames encoding possible Tim40 homologs including a putative mitochondrial targeting signal are present in the genomes of *Can-

* (59x186)
such as Drosophila melanogaster (NP_733343), Danio rerio (AAH77126), Xenopus laevis (AAH68790), Mus musculus (AAH19405), and Homo sapiens (BAB71132), although, since the encoded proteins lack a typical mitochondrial presequence, their mitochondrial localization is unclear. Tim40 proteins in various organisms consist of a rather divergent, N-terminal acidic domain and a conserved C-terminal domain containing three pairs of highly conserved Cys pairs, Cys^{296}/Cys^{298}, Cys^{307}/Cys^{317}, and Cys^{339}/Cys^{340}, the latter two of which form C-C motif (Fig. 1A) (20). Since Tim40 is essential for viability of yeast cells, we assessed the roles of these Cys pairs by testing the abilities of mutant Tim40 with replacement of one of the three Cys pairs with a pair of Ser residues to functionally replace wild-type Tim40 in vivo. Replacement of wild-type Tim40 with Tim40 mutants that lack one of the conserved Cys pairs was lethal (Fig. 1F), indicating that three pairs of conserved Cys residues of Tim40 are essential for its function.

**Tim40 Mediates Sorting of Small Tim Proteins to the IMS**—To analyze the functions of Tim40, we depleted Tim40 by shifting GAL-TIM40 cells to galactose-free medium; cell growth of GAL-TIM40 slowed down significantly 28 h after the shift (data not shown). Total cell extracts were prepared from wild-type (WT) and GAL-TIM40 cells at various times after shift from galactose-containing medium to galactose-free medium and were analyzed by immunoblotting for various mitochondrial proteins (Fig. 2A). The amount of Tim40 in GAL-TIM40 was decreased to <6% in WT cells 24 h after shift to galactose-free medium. Components of the TOM40 complex in the outer membrane (Tom70, Tom40, Tom20), those of the TIM23 complex (Tim50, Tim44, Tom23), those of the TIM22 complex (Tom22), mitochondrial matrix proteins (Hsp60, Mdh1p, Ssc1p), and the endoplasmic reticulum protein (Sec63p) were not affected by the depletion of Tim40 at all. However, the amounts of small Tim proteins in the IMS, Tim9, Tim10, and Tim12 were significantly decreased 24 h after shift to galactose-free medium. These results suggest that Tim40 is involved in biogenesis of small Tim proteins destined for the IMS.

Next, we tested the in vitro import abilities of mitochondria isolated from Tim40-depleted (Tim40 Δ) cells after 12 h cultivation in the absence of galactose for various radiolabeled precursor proteins. Mitochondria isolated from Tim40 Δ cells did not exhibit decrease in ΔΨ, which is essential for protein import via the TIM23 or TIM22 complex (data not shown). We analyzed the import of radiolabeled soluble matrix and polytopic inner membrane proteins: pSu9-DHFR, a precursor- containing precursor protein that uses the TIM23 complex to move across the inner membranes, and ADP/ATP carrier (AAC), a precursor-less polytopic inner membrane protein that uses the TIM22 complex to be inserted into the inner membrane. The import rates of pSu9-DHFR (Fig. 2B) and of AAC (Fig. 2C) were not affected by depletion of Tim40.

We then analyzed the import of small Tim proteins, Tim9 (Fig. 2D) and Tim13 (Fig. 2E), into WT and Tim40 Δ mitochondria. In contrast to pSu9-DHFR and AAC, import of Tim9 and Tim13 into mitochondria was impaired in Tim40 Δ mitochondria. These results suggest that Tim40 is involved in import of small Tim proteins into the IMS.

To understand more precise roles of Tim40 in import of small Tim proteins into the IMS, we followed the in vitro assembly of small Tim proteins in the IMS of WT mitochondria by BN-PAGE. When we incubated radiolabeled Tim9 with isolated mitochondria at 30 °C and analyzed the proteins by BN-PAGE, we observed its accumulation in three distinct bands with apparent molecular masses of 70, 140, and 300 kDa (Fig. 3A, lane 1). The bands at 70 and 300 kDa shifted to the higher molecular mass range upon incubation with anti-Tim9 antibodies (Fig. 3A, lane 4). This is consistent with the previous report that the 70-kDa complex represent the Tim9-Tim10 complex and the 300-kDa complex is the Tim9-Tim10-Tim12 complex associated with the TIM22 complex (21, 22). The 140-kDa complex shifted to the higher molecular mass range upon incubation with anti-Tim40 antibodies, suggesting that it contains Tim40 (Fig. 3A, lane 3). To demonstrate that the 140-kDa complex involving Tim40 is a transport intermediate on the correct Tim9 assembly pathway, we performed a chase reaction. Tim9 was incubated with mitochondria for 10 min at 16 °C, leading to formation of the 140-kDa complex alone. The mitochondria were then resolated and incubated for indicated times at 30 °C (Fig. 3B). The amount of the 140-kDa complex decreased with increasing time, whereas those of the 70-kDa Tim9-Tim10 complex and the 300-kDa complex with the TIM22 complex increased. These results indicate that Tim9 assembles into its final complexes with Tim10 alone and with the TIM22 complex together with Tim10 and Tim12 via the 140-kDa intermediate involving Tim40.

Finally, we analyzed the possible complex structure of Tim40. When mitochondria solubilized with digitonin were subjected to glycerol density-gradient centrifugation, Tim40, Tim22, and Tim23 were recovered in the fractions corresponding to 80, 320, and 270 kDa, respectively (Fig. 3C). Therefore Tim40 does not constitute the TIM22 or TIM23 complex. Nevertheless, it remains open whether Tim40 forms a homo-oligomer or a complex with other components involved in the small Tim protein assembly pathway in the IMS.

How can Tim40 facilitate import of small Tim proteins into the IMS? Since Tim40 can function at the inner membrane, not necessarily in the IMS, it may mediate zinc ion binding to or disulfide-bond formation of small Tim proteins (23) and/or assembly of small Tim proteins into hetero-oligomeric complexes on the IMS side of the inner membrane. Impairment of these processes will fail to provide small Tim proteins with a driving force for their efficient translocation across the outer membrane, as observed here with Tim40 Δ mitochondria. Elucidation of the precise roles of essential Cys residues of Tim40 will certainly help us to further understand the functions of Tim40.

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