Mitochondrial precursor proteins are directed into the intermembrane space via two different routes, the presequence pathway and the redox-dependent MIA pathway. The pathways were assumed to be independent and transport different proteins. We report that the intermembrane space receptor Mia40 can switch between both pathways. In fungi, Mia40 is synthesized as large protein with an N-terminal presequence, whereas in metazoans and plants, Mia40 consists only of the conserved C-terminal domain. Human MIA40 and the C-terminal domain of yeast Mia40 (termed Mia40core) rescued the viability of Mia40-deficient yeast independently of the presence of a presequence. Purified Mia40core was imported into mitochondria via the MIA pathway. With cells expressing both full-length Mia40 and Mia40core, we demonstrate that yeast Mia40 contains dual targeting information, directing the large precursor onto the presequence pathway and the smaller Mia40core onto the MIA pathway, raising interesting implications for the evolution of mitochondrial protein sorting.

Mitochondria contain two aqueous compartments, the intermembrane space (IMS) and the matrix. Most mitochondrial proteins are synthesized as precursors in the cytosol and recognized by the TOM (translocase of outer membrane) complex. After translocation through TOM, precursor proteins destined for the matrix employ the presequence TIM23 complex (translocase of inner membrane) and the associated motor. The precursors carry N-terminal precursors that specifically interact with TOM and TIM23 subunits and are proteolytically removed upon arrival in the matrix (1–6). Translocation of the precursors across the inner membrane requires the membrane potential (∆ψ).

Although the presequence pathway is used by virtually all proteins imported into the matrix, two different pathways have been characterized for protein transport into the IMS. (i) Precursors such as cytochrome b_2 contain bipartite targeting information in their N-terminal region, a presequence-type matrix targeting signal followed by a hydrophobic sorting signal, which arrests translocation in the TIM23 complex. The preprotein is laterally transferred into the inner membrane, and the IMS domain either can be released by proteolytic cleavage or remains attached to the inner membrane via the hydrophobic segment (4, 5, 7–9). (ii) Many IMS proteins are small (<15–20 kDa) and contain conserved cysteine residues that form disulfide bonds and/or bind metal ions (4, 10, 11). These proteins are synthesized without a presequence and are imported by the MIA (mitochondrial IMS import and assembly) machinery (12–16). Although the presequence-carrying precursors depend on ∆ψ for import, the MIA machinery does not require ∆ψ yet functions in a redox-regulated manner by forming transient disulfide bonds with precursor proteins. Two essential members of the MIA machinery have been identified, Mia40 (Tim40) and Erv1. Mia40 acts as a receptor in the IMS. After translocation across the outer membrane, the precursor proteins form mixed disulfides with Mia40 (17, 18). Erv1 is a sulfhydryl oxidase that functions in a disulfide relay to oxidize Mia40 and promote substrate release from Mia40 (14, 19–23).

Although most studies analyzing the MIA pathway used precursor proteins smaller than 12 kDa, a recent study revealed that the MIA machinery can handle larger substrates such as the IMS proteins Mic14 (14 kDa) and Mic17 (17 kDa) (24). Moreover, the components of the MIA machinery are encoded by nuclear genes, synthesized in the cytosol, and imported into mitochondria. Remarkably, the precursor of Erv1 with a molecular mass of 22 kDa is imported by the MIA machinery, representing the largest MIA substrate known (24, 25). In contrast, yeast Mia40 is synthesized with an N-terminal presequence. This preprotein of 45 kDa is imported via the presequence pathway in a ∆ψ-dependent manner (12, 13). A hydrophobic segment following the presequence arrests translocation of Mia40 in the inner membrane (13, 15). Mature Mia40 is bound to the inner membrane, although Naoé et al. (13) showed that...
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Mia40 is also functional as soluble protein when it is targeted to the IMS by the bipartite signal of cytochrome b$_2$.

Hofmann et al. (26) reported that human MIA40 (hMIA40) is considerably smaller than its yeast counterpart and not attached to the inner membrane. hMIA40 lacks an N-terminal presequence but is homologous to the C-terminal portion of yeast Mia40, including the conserved six cysteine residues. In a heterologous assay, hMIA40 was imported into yeast mitochondria, and several of its cysteine residues were required for import and/or stability of the protein (26).

hMIA40 has a molecular mass of 16 kDa and thus would fall in the size range of substrates that can be handled by the MIA pathway (24, 25), yet experimental evidence that hMIA40 is imported by the MIA system has been lacking so far. Grumbt et al. (22) reported that a recombinant C-terminal fragment of yeast Mia40 is competent in binding to Erv1 in vitro, raising the possibility that the C-terminal domain represents a functional domain. However, it has not been shown if the C-terminal domain alone can bind precursor proteins and is functional in vivo.

We report that not only hMIA40 but also the C-terminal domain of yeast Mia40 (termed Mia40core) can replace full-length Mia40 of yeast. Remarkably, hMIA40 and Mia40core were functional in vivo, demonstrating that the C-terminal half of yeast Mia40 is sufficient for function and import into mitochondria. We show that Mia40core is imported into mitochondria by the MIA machinery, and thus, yeast Mia40 contains dual targeting information for both the presequence and MIA pathways.

EXPERIMENTAL PROCEDURES

Yeast Strains—The Saccharomyces cerevisiae strains used in this study are derivatives of YPH499 (MATa, ade2-101, his3-D200, leu2-Δ1, ural3-52, trp1-Δ63, lys2-801). The conditional mia40-3, erv1-2, and ervl-5 mutants were characterized previously (12, 21, 23). hMIA40 was derived from the cDNA clone IRAIp970f0452D (RZPD). pFL39-derived plasmids with mia40 versions under the control of an endogenous promoter and terminator, pGB9334 (hMIA40) was derived from the cDNA clone IRAIp970f0452D (RZPD).

Phylogenetic Analysis—Mia40 homologs were identified by PSI-BLAST searches and confirmed by reciprocal PSI-BLAST analysis. 24 sequences from model organisms were selected and aligned with ClustalW2. From the resulting multiple sequence alignment, a subalignment was selected comprising residues 282–350 of S. cerevisiae Mia40 and used for the calculation of a PHYLIP tree.

Import of Recombinant Mia40—Recombinant Mia40core (pN-His10-Mia40Δ1–225) with a His$_{10}$ tag at the N terminus was expressed in Escherichia coli and purified as described for full-length soluble Mia40 (renamed due to the change in amino acid numbering by the Saccharomyces Genome Database from pN-His10-Mia40Δ1–94 (17) to pN-His10-Mia40Δ1–70). For import into mitochondria, Mia40core was reduced by 50 mM 1,4-dithiothreitol and denatured in 8 M urea, 30 mM MOPS/KOH, and 10 mM 1,4-dithiothreitol (pH 7.2). Mitochondria (50–75 μg of protein) were incubated with 1.25–2.5 μg of Mia40core in a 100-μl reaction at 30 °C. Where indicated, 50 mM iodoacetamide was added, or Δψ was dissipated by addition of 1 μM valinomycin, 8 μM antimycin, and 20 μM oligomycin. Non-imported material was removed by treatment with 50 μg/ml proteinase K. Import was assayed by immunodecoration with antibodies raised against intact Mia40. Nickel-nitrioltriacetic acid (Ni-NTA) affinity purification of imported Mia40core was performed according to the procedure described for endogenous Mia40-His$_{10}$(17).

Miscellaneous—To generate mitoplasts, mitochondria were subjected to hypo-osmotic swelling in 1 mM EDTA and 10 mM MOPS/KOH (pH 7.2), followed by 250 mM NaCl where indicated. Mitochondria and mitoplasts were subjected to Western blot analysis with different antibodies. The 35S-labeled precursors of Tim9 and Tim8 were synthesized and subjected to binding assays with purified Mia40 or imported into isolated mitochondria. After import of 35S-labeled Tim9, the samples were treated with 50 μg/ml proteinase K where indicated and analyzed by reducing or non-reducing SDS-PAGE to detect disulfide-bonded conjugates (12, 17, 21). Formation of the oxidized Tim8 monomer was analyzed as described previously (23). Briefly, following the

**FIGURE 1.** The C-terminal half of recombinant Mia40 is sufficient for the binding of precursor proteins. Recombinant forms of S. cerevisiae Mia40 and Mia40core were purified and incubated with 35S-labeled Tim9 at 15 °C for the indicated time periods. The samples were analyzed by nonreducing SDS-PAGE and digital autoradiography.
import of 35S-labeled Tim8, mitochondria were solubilized in digitonin solubilization buffer (1% (w/v) digitonin, 20 mM Tris-HCl, 0.5 mM EDTA, 10% (v/v) glycerol, 50 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride (pH 7.4)) supplemented with 50 mM iodoacetamide. The samples were analyzed by blue native electrophoresis and digital autoradiography.

**RESULTS AND DISCUSSION**

**The Essential Function of Mia40 Is Located in Its C-terminal Domain**—To determine whether the C-terminal domain of yeast Mia40 (termed Mia40core) is competent in the binding of precursor proteins, we expressed the domain (amino acid residues 226–403 of *S. cerevisiae* Mia40) in *E. coli* cells. For comparison, we also expressed the large form of Mia40 lacking the N-terminal targeting segments (17). Because disulfide-linked Mia40-precursor conjugates remain stable under nonreducing conditions (17, 18), we used nonreducing SDS-PAGE to directly monitor the binding of precursor proteins to the purified Mia40 constructs. Upon incubation with the 35S-labeled precursor of Tim9, both recombinant proteins formed Mia40-Tim9 conjugates with comparable efficiency (Fig. 1), indicating that Mia40core efficiently binds substrate proteins in vitro.

To address whether Mia40core is functional in vivo, we attached the bipartite presequence of cytochrome b2 to its N terminus (b2-Mia40core) (Fig. 2A) and expressed it in yeast cells that lacked the chromosomal copy of *MIA40* but expressed full-length Mia40 from a *URA3*-containing plasmid. Upon loss of the *URA3*-containing plasmid, the cells containing only b2-Mia40core were viable (Fig. 2B, right panel). The presequence of cytochrome b2 was also attached to the large form of Mia40 (b2-Mia40) (Fig. 2A) (13). The growth behavior of yeast cells expressing b2-Mia40core, b2-Mia40, or wild-type Mia40 was indistinguishable (Fig. 2B), demonstrating that the C-terminal domain contains the essential part of Mia40.

The presence of the Mia40 constructs in isolated mitochondria was analyzed by immunodecoration. Cytochrome b2 is first processed by the matrix processing peptidase and then by the inner membrane peptidase, leading to the mature form released to the IMS. The efficiency of the second cleavage depends on the presence of the heme-binding domain of mature cytochrome b2.

**FIGURE 2.** The C-terminal half of Mia40 is functional. A, a schematic representation of Mia40 constructs fused to the cytochrome (Cyt.) b2 presequence (plus linker amino acids) is shown. The numbering of amino acid residues is according to the current assignment in the *Saccharomyces* Genome Database. TM, transmembrane segment. B, the growth of yeast at 24 °C before and after removal of wild-type Mia40 by 5-fluoroorotic acid (5-FOA) treatment is shown. C and D, the immunodecoration of yeast mitochondria (Mitoch.) and mitoplasts (Mitopl.) is shown. i, intermediate; m, mature; CCHL, cytochrome c heme lyase. E, 35S-labeled Tim9 was imported into isolated yeast mitochondria at 30 °C for the indicated time periods. The mitochondria were treated with protease K (Prot. K) and analyzed by SDS-PAGE and digital autoradiography. F, 35S-labeled Tim9 imported into isolated mitochondria was analyzed by nonreducing SDS-PAGE and digital autoradiography. G, 35S-labeled Tim8 imported into isolated mitochondria was analyzed by blue native electrophoresis and digital autoradiography.
As the \( b_2 \) constructs contain only the presequence part, their processing was not complete, and two forms, intermediate and mature, were observed for \( b_2 \)-Mia40core (Fig. 2C), similar to the behavior of \( b_2 \)-Mia40 (13). Upon swelling of mitochondria, intermediate \( b_2 \)-Mia40core remained membrane-bound (Fig. 2C), like wild-type Mia40 and intermediate \( b_2 \)-Mia40 (12, 13, 15). The mature forms of \( b_2 \)-Mia40core and \( b_2 \)-Mia40 were released by swelling, like the soluble IMS protein cytochrome \( b_2 \) (Fig. 2C).

We performed several assays to determine whether mitochondria containing \( b_2 \)-Mia40core were functional in protein import to the IMS. The steady-state levels of IMS proteins, including the MIA substrate Tim10, were similar to those of mitochondria containing the large forms of Mia40 (Fig. 2, C and D). \(^{35}\)S-Labeled Tim9 was incubated with isolated mitochondria, and its import was assessed by protection against added proteinase K (12, 17). \( b_2 \)-Mia40core mitochondria efficiently imported the precursor (Fig. 2E). The formation of Mia40-Tim9 conjugates was monitored by non-reducing SDS-PAGE (Fig. 2F). The size difference of the Tim9 conjugates with intermediate Mia40core and mature Mia40core was sufficient for separation of the conjugates on the gel. Although the smaller mature \( b_2 \)-Mia40core was present in lower abundance than intermediate \( b_2 \)-Mia40core (Fig. 2C), it efficiently formed a conjugate with Tim9 (Fig. 2F), indicating that the soluble mature \( b_2 \)-Mia40core is functional.

To test whether \( b_2 \)-Mia40core mitochondria promoted oxidation of the imported substrate proteins, we used a blue native assay developed for the precursor of Tim8, which allows a direct separation of reduced and oxidized forms (23). \( b_2 \)-Mia40core mitochondria generated oxidized Tim8 in a time-dependent manner with the same efficiency as mitochondria containing large Mia40 (Fig. 2G). Thus, Mia40core targeted to mitochondria by the presequence of cytochrome \( b_2 \) fully replaced the essential function of wild-type Mia40.

**FIGURE 3. Mia40 homologs.** A, PHYLIP tree for Mia40. The tree was generated with ClustalW2 and visualized by TreeView (30). B, presequence prediction for Mia40 homologs. Mitoch., mitochondrial; aa, amino acid residues. C, complementation analysis of a yeast mia40 deletion mutant by hMia40. The growth of yeast at 24 °C before and after removal of wild-type Mia40 by 5-fluoroorotic acid (5-FOA) treatment is shown.

We systematically compared the primary structures of the available Mia40 homologs and constructed a phylogenetic tree (Fig. 3A). Fungal Mia40 proteins are large and possess N-terminal presequences (supplemental Fig. S1), whereas Mia40 proteins from higher eukaryotes usually correspond to the C-terminal half of fungal Mia40 and do not possess presequences. We asked whether hMia40 (26) is able to substitute for yeast Mia40. We expressed hMia40 in yeast, and by plasmid shuffling in a yeast strain with a chromosomal deletion of Mia40, we analyzed

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whether hMIA40 was able to substitute for the essential function of yeast Mia40. Fig. 3C shows that hMIA40 indeed rescued cell viability. Thus, the presequence-free human protein was able to complement yeast cells lacking endogenous Mia40.

As presequence-free hMIA40 is functional in yeast, we asked whether yeast Mia40 core may be correctly targeted and functional in vivo without attachment of a presequence. The plasmid shuffling approach indeed revealed that presequence-free Mia40 core fully rescued yeast viability (Fig. 4A). Mia40 core was released upon swelling of mitochondria (Fig. 4B, lanes 2 and 3), indicating that it is a soluble IMS protein. We performed a systematic analysis to determine whether Mia40 core mitochondria were functional: steady-state levels of IMS proteins (Fig. 4C, lanes 4 and 5), import of 35S-labeled Tim9 to a protease-protected location (Fig. 4D), formation of Mia40 core-precursor conjugates (Fig. 4E), and oxidation of imported Tim8 (Fig. 4F). In each case, Mia40 core mitochondria showed a similar activity as mitochondria with wild-type Mia40. Thus, the C-terminal half of yeast Mia40 can be targeted to mitochondria without a presequence and is functional, like full-length Mia40.

Mia40 core Is Imported via the MIA Pathway—To study the import of Mia40 core into mitochondria, we purified the recombinant protein via an N-terminal His tag. Mia40 core was transported to a protease-protected location by isolated yeast mitochondria (Fig. 5A, lanes 7–9), in contrast to the import of presequence-containing full-length Mia40 (12, 13). Blocking of sulfhydryl groups of Mia40 core by iodoacetamide completely inhibited its import (Fig. 5A, lanes 1–3). Upon import of Mia40 core, we lysed the mitochondria and performed Ni-NTA affinity chromatography. Mia40 core bound Erv1 but not control proteins like cytochrome c of the IMS (Fig. 5B). Thus, Mia40 core interacts with endogenous Erv1, like wild-type Mia40 (14, 21, 22, 25).

The Δψ-independent import of presequence-free Mia40 core raised the possibility that it was a MIA substrate. To address this directly, we used conditional yeast mutants of MIA40 and ERV1 (12, 21, 23). mia40-3 mitochondria imported Mia40 core with a diminished efficiency (Fig. 5C), and erv1-2 and erv1-5 mitochondria were strongly inhibited in its import (Fig. 5D). Because mia40 and erv1 mutant mitochondria import presequence-containing preproteins with wild-type efficiency (12, 21, 24), we conclude that Mia40 core is imported via the MIA pathway.

Taken together, these findings indicate that yeast Mia40 contains targeting information for both the presequence and MIA pathways. To directly demonstrate targeting via two pathways in vivo, we coexpressed presequence-containing full-length Mia40 as well as presequence-free Mia40 core in erv1-2 yeast cells. The amount of full-length Mia40, which is imported via the TIM23 pathway, was not affected in comparison with wild-type yeast, like Mia40 core.
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that of MIA-independent control proteins (Fig. 6). However, the level of Mia40core was strongly diminished in erv1-2 mitochondria, like that of Tim10 (Fig. 6, lanes 1 and 2). We concluded that yeast Mia40 contains dual targeting information.

Conclusions—To our knowledge, yeast Mia40 is the first protein that contains targeting information for both the presen- quence and MIA pathways. Mia40 proteins of fungi are large and contain N-terminal presequences that direct them onto the Δψ-dependent import route (12, 13), whereas Mia40 proteins of higher eukaryotes are small and do not contain cleavable presequences (this study and Ref. 26). Surprisingly, the N-terminal portion of yeast Mia40 including the presen- quence is dispensable for its targeting and function. Mia40 is essential for cell viability of yeast (12, 13). We have shown that this essential func- tion is located in the C-terminal half (termed Mia40core). Import of Mia40core into mitochondria does not require a membrane potential but depends on both Mia40 and Erv1, demonstrating that Mia40core is a substrate of the MIA pathway. Thus, the targeting information of Mia40 for the MIA pathway is located in the C-terminal half of the protein.

No prokaryotic homologs of Mia40 have been found, suggesting that the MIA machinery was developed by eukaryotic cells (12, 26). Because the import of small Mia40 forms via the MIA pathway will require the presence of pre-existing Mia40, we speculate that the large, presequence-containing Mia40 forms were established early in evolution to allow import of the central MIA component via the existing presequence pathway. Once the MIA system was established, it was then possible to import Mia40 also via this pathway, and various groups of eukaryotes, including animals and plants, do so. To switch from the presequence pathway to the MIA pathway in evolu- tion, the existence of a large Mia40 containing both a pre- sequence and targeting information in the C-terminal half (as in present-day yeast) was advantageous because our find- ings show that loss of the N-terminal half does not inhibit the biogenesis and activity of Mia40, at least under the laboratory growth conditions applied. It has not been determined for what purpose (or selective advantage) all species of fungi that we analyzed have kept the N-terminal domain of Mia40 and thus likely have retained the dual targeting information.

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