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Mia40, a novel factor for protein import into the intermembrane space of mitochondria is able to bind metal ions

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Abstract Many proteins located in the intermembrane space (IMS) of mitochondria are characterized by a low molecular mass, contain highly conserved cysteine residues and coordinate metal ions. Studies on one of these proteins, Tim13, revealed that net translocation across the outer membrane is driven by metal-dependent folding in the IMS [1]. We have identified an essential component, Mia40/Tim40/Ykl195w, with a highly conserved domain in the IMS that is able to bind zinc and copper ions. In cells lacking Mia40, the endogenous levels of Tim13 and other metal-binding IMS proteins are strongly reduced due to the impaired import of these proteins. Furthermore, Mia40 directly interacts with newly imported Tim13 protein. We conclude that Mia40 is the first essential component of a specific translocation pathway of metal-binding IMS proteins.
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Keywords: Protein import; Mitochondrion; Mitochondrial intermembrane space import and assembly 40; TIM protein; Metal-binding proteins

1. Introduction

Nearly all mitochondrial proteins are synthesized in the cytosol and are imported into mitochondria, a process mediated by proteinaceous machineries in the mitochondrial membranes. Proteins destined for the matrix typically carry N-terminal presequences and are imported in an ATP- and membrane potential-dependent process (for review, see [2–5]). In contrast, many proteins of the IMS typically lack presequences and traverse the translocase of the outer membrane, the TOM complex, by use of completely different energy sources. The import of one of these IMS proteins, Tim13, has been studied in detail [1]. Tim13 contains four conserved cysteine residues which are essential for import. Diffusion through the TOM channel of the unfolded protein followed by stable folding of Tim13 in the IMS was shown to trap the protein in mitochondria and thereby allow a vectorial translocation [6]. It was shown that the binding of zinc triggers the stable folding of Tim13 [1]. Although zinc binding to Tim13 and homologous small Tim proteins was shown [1,7], other studies suggest that the cysteine residues might form disulfide bridges in the IMS which likewise locks the protein in a folded conformation [8–10]. Recently, a factor for the assembly of small TIM complexes, Hot13p, has been described which might be involved in changes of the redox state of small Tim proteins [11]. This folding trap mechanism presumably drives the import of many IMS proteins, as they typically show conserved cysteines and very often coordinate metal cofactors. Well characterized examples are the copper chaperone Cox17 [12,13] and Cu/Zn-superoxide dismutase (Sod1) [14]. The machinery for import and folding of these IMS proteins is still elusive. Here, we report on the identification and characterization of Mia40, the first essential component of a specific import and folding machinery for metal-binding IMS proteins.

2. Materials and methods

2.1. Plasmids and strains

For purification of Mia40 from yeast, the sequence encoding the Mia40 precursor fused to eight histidine residues was inserted into the pVTU plasmid [15] and transformed into the wild type W303-1B. For expression in *Escherichia coli*, the sequence encoding residues 68–403 of Mia40 was amplified by PCR and subcloned into the plasmid pMAL-CRI (NEB, Beverly). For regulated expression of Mia40, a *GAL10* promoter was inserted upstream of the *MIA40* reading frame in the wild type strain YPH499. For depletion of Mia40, the resulting GAL-MIA40 cultures were shifted from lactate medium containing 0.1% galactose to lactate medium containing 0.1% glucose. Overexpression of Mia40 was obtained by growth of the same strain in the presence of 0.5% galactose.

2.2. Protein purification procedures and metal content determination

MBP-fusion proteins were expressed in the presence of 100 μM zinc acetate or copper sulfate. Protein purification and determination of the metal content was performed essentially as described using metal-free buffers [1]. Radiolabeled chemical amounts of Tim13 were prepared and used for import reactions essentially as described [1], with the exception that cells were grown in low sulfate medium (50 μM) in the presence of 5 mCi [^{35}S]sulfate.

2.3. Trypsin treatment of endogenous Mia40

Mitochondria were converted to mitoplasts by incubation in 60 mM sorbitol, 1 mM ATP, 4.5 mM β-mercaptoethanol, and 20 mM HEPES–KOH, pH 7.4, for 20 min on ice. Following further incubation for 10 min in the presence or absence of 10 mM EDTA and 2 mM bathophenanthroline-disulfonic acid, the mitoplasts were exposed to 5–25 μg/ml trypsin for 30 min on ice. After a clarifying spin, proteins

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Abbreviations: Mia, mitochondrial intermembrane space import and assembly; IMS, intermembrane space; MBP, maltose-binding protein; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; PK, proteinase K; ICP-AES, inductively coupled plasma atomic emission spectroscopy

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in the supernatant fraction were precipitated by addition of 12% trichloroacetic acid and analyzed by immunoblotting with antibodies against Mia40.

2.4. Miscellaneous

Import of proteins into isolated mitochondria, subcellular and submitochondrial fractionation, cross-linking of preprotein and co-immunoprecipitation were performed as described [1,16,17]. Antibodies were raised against a C-terminal peptide of yeast Mia40 (CVKKEPLNEESKP) in rabbits. For determination of the mature N terminus of Mia40, a C-terminal octahistidinyl-tagged form of Mia40 was expressed in yeast, purified by Ni–NTA chromatography [16] and analyzed by N-terminal sequencing at TopLab GmbH (Martinsried, Germany).

3. Results

3.1. Mia40 exposes a large domain in the IMS

Mia40/Tim40/Ykl195w was recently identified in a proteomic study on baker's yeast as an essential protein of mitochondria [18,19]. It codes for a protein of 403 amino acid residues and contains a typical mitochondrial targeting signal at the N terminus followed by a hydrophobic region. The mitochondrial location of Mia40 was confirmed by subcellular fractionation of yeast cells (Fig. 1A) and in vitro import experiments with radiolabeled Mia40 protein (data not shown). To assess the submitochondrial location of Mia40, mitochondria were fractionated and treated with protease (Fig. 1B). In intact mitochondria, Mia40 was protected against added proteinase K (PK). Selective rupturing of the outer membrane by hypotonic swelling made Mia40 accessible to protease leading to the loss of the C-terminal epitope of Mia40. Upon alkaline extraction of mitochondria, Mia40 partially fractionated with the membrane pellet. This fractionation behavior was reported before for other single-spanning membrane proteins like Tim14 [16]. We conclude that Mia40 is anchored into the inner membrane by an N-terminal transmembrane domain and exposes a large C-terminal domain to the IMS. To identify the N-terminal sequence of the mature Mia40, we purified an octahistidinyl-tagged version of Mia40 from mitochondria. Edman degradation revealed processing after amino acid residue 31 of the precursor protein (Fig. 1C), matching the consensus pattern of mitochondrial processing peptidase.

3.2. Mia40 is an essential copper- and zinc-binding protein

Homologs of Mia40 are present throughout the eukaryotic kingdom. They share a highly conserved domain of about 60 amino acid residues that contains six invariant cysteine residues forming a CXC-CX_9C-CX_9C- motif (Fig. 2A). This motif was found to be essential for Mia40 as a variant in which the first three cysteine residues were exchanged by serine residues was not able to rescue the *Δmia40* disruption strain (data not shown).

To assess a potential metal-binding capacity of Mia40, we recombinantly expressed and purified the IMS domain of Mia40 (amino acid residues 68–403) as a fusion protein with the maltose-binding protein (MBP-Mia40ΔTM). Induction coupled plasma atomic emission spectroscopy (ICP-AES) revealed significant amounts of zinc and copper ions in MBP-Mia40ΔTM (Fig. 2B). Other metals, like iron and nickel, were not detected. This indicates that Mia40 has the ability to specifically bind zinc and copper ions.

The presence of a metal cofactor in Mia40 was further supported by a significantly increased trypsin sensitivity of the endogenous protein in the presence of chelators (Fig. 2C). This suggests that, in vivo, the folding of the IMS domain of Mia40 is stabilized by metal ions.

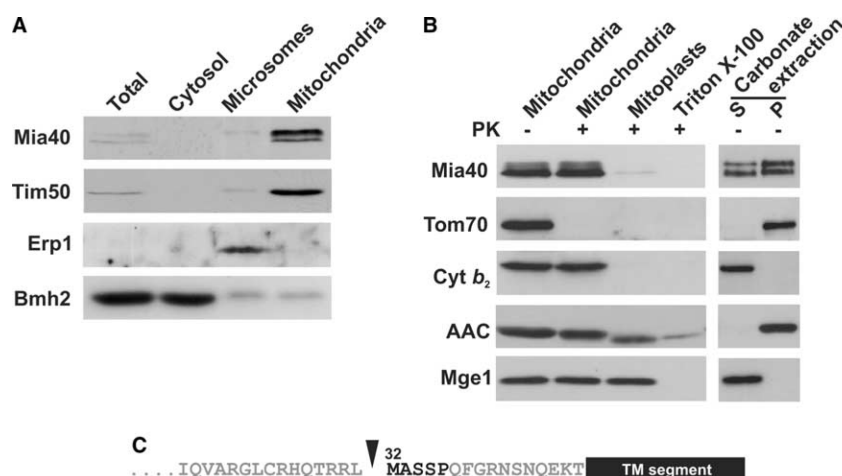

Fig. 1. Mia40 is a mitochondrial protein facing the intermembrane space. (A) Total yeast cell extract and subcellular fractions (each 50 μg protein) were analyzed by immunoblotting with antibodies against Mia40 and the marker proteins Tim50 (mitochondria), Erp1 (microsomes) and Bmh2 (cytosol). (B) Mitochondria, mitoplasts and a mitochondrial Triton X-100 extract were incubated in the absence or presence of 50 μg/ml PK. Mitochondria were fractionated by carbonate extraction into soluble (S) and membrane pellet (P) fractions and analyzed as in (A). Tom70, outer membrane; cytochrome b_2 (Cyt b_2), IMS protein; ATP/ADP carrier (AAC), inner membrane; Mge1, matrix protein. (C) Mia40 is processed (see triangle) between amino acid residues 31 and 32. The transmembrane (TM) segment present in the mature Mia40 protein is indicated. The sequence revealed by Edman degradation is presented in black. In half of the Mia40 molecules, the methionine on position 32 was absent. It should be noted that upon lysis of mitochondria in the presence of β-mercaptoethanol, Mia40 migrates as a double band (as in A and B). In the absence of β-mercaptoethanol (as used in C), Mia40 migrates as a single band. Presumably, the presence of β-mercaptoethanol leads to partial reduction of cysteine residues in Mia40 and the oxidized and the reduced species of Mia40 differ in their mobility. Comparable double bands have also been observed for other cysteine rich proteins like Mdj1 [17].

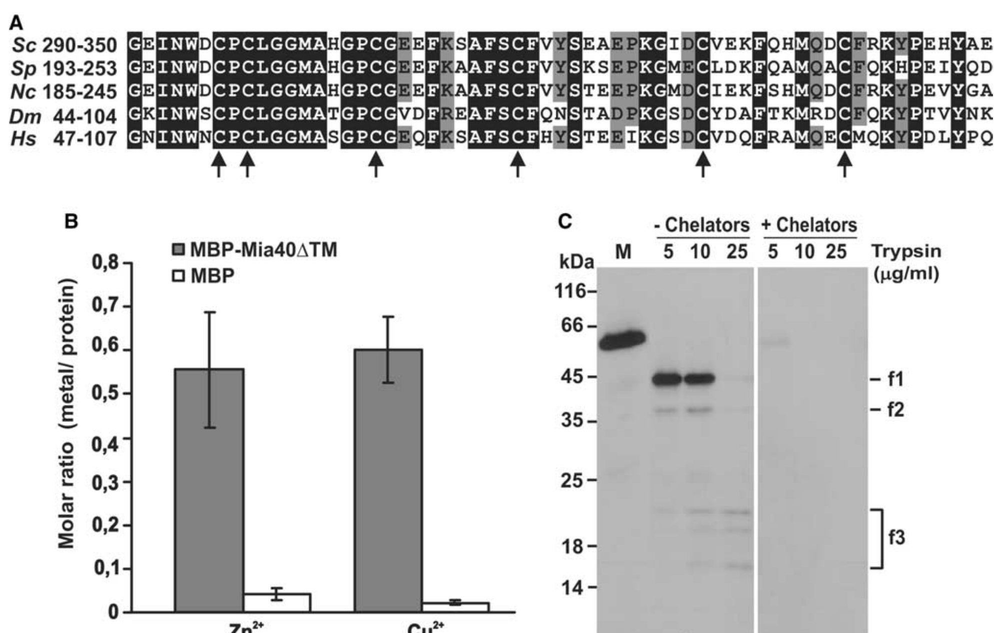

Fig. 2. Mia40 contains an essential conserved CXC-CX_9C-CX_9C motif which binds zinc and copper ions. (A) Alignment of the conserved CXC-CX_9C-CX_9C domain of Mia40 homologs. (B) A fusion protein of maltose-binding protein (MBP) and IMS domain of Mia40 (MBP-Mia40ΔTM) was purified. The content of zinc and copper ions was determined by ICP-AES. Shown is the molar metal to protein ratio. (C) Metal binding stabilizes Mia40 folding. Mitoplasts were treated with the indicated amounts of trypsin in the absence or presence of chelators (10 mM EDTA, 2 mM bathophenanthroline-disulfonic acid). Protease-resistant fragments of Mia40 in the soluble fraction (f1 to f3) were identified by immunoblotting.

3.3. Mia40 is required for the import of small IMS proteins

In order to identify the function of Mia40 in mitochondria, we constructed a strain harboring the *MIA40* gene under control of the glucose-repressible *GAL10* promoter. About 22 h after the shift from galactose- to glucose-containing medium, Mia40 was depleted from the mitochondria and the cells slowed down in their growth (Fig. 3A). Interestingly, the endogenous levels of the small metal-coordinating IMS proteins Tim13, Tim10 and Cox17 were severely reduced in mitochondria depleted of Mia40 (Mia40↓) (Fig. 3B). In contrast, IMS proteins that do not contain metal cofactors were not affected (cytochrome b_2, cytochrome *c* and cytochrome *c* heme lyase). The levels of mitochondrial proteins of the outer membrane (Tom40), the inner membrane (Tim23, AAC) and the matrix space (Tim44, Mge1) were likewise unaltered. The inner membrane protein Tim22 was decreased to some degree, presumably because the biogenesis of Tim22 depends on the presence of small Tim proteins in the IMS [7,20]. This suggests that Mia40 is specifically required for the biogenesis or the maintenance of metal-containing IMS proteins.

Next, we performed import experiments into isolated mitochondria to test for a role of Mia40 in this process (Fig. 3C). Import of Tim13 was strongly dependent on the presence of Mia40, regardless of whether Tim13 was purified from recombinant *E. coli* cells ('chemical amounts') or synthesized in reticulocyte lysate ('radiochemical amounts'). The import of Tim10 and recombinant Cox17 was also affected in Mia40↓ mitochondria. In contrast, import rates of cytochrome *c* heme lyase, an IMS protein that does not coordinate metal cofactors, and of proteins of outer membrane (porin) and the matrix (pSu9DHFR) were not reduced (Fig. 3C). We conclude that Mia40 is vital for the import of metal-binding proteins into the IMS. Interestingly, upon overexpression of Mia40, the import rates of Tim13 were significantly increased, suggesting that Mia40 represents a rate-limiting factor for import of Tim13 (Fig. 3D).

3.4. Mia40 interacts with small Tim proteins

Next, we tested by chemical cross-linking whether newly imported Tim13 directly interacts with Mia40. Radiolabeled Tim13 was imported into mitochondria in the presence or the absence of the cross-linking reagent 1,5-difluoro-2,4-dinitrobenzene (DFDNB). Following lysis of mitochondria under denaturing conditions, Mia40 was isolated by immunoprecipitation. As shown in Fig. 4A, a cross-linked product of Tim13 of about 75 kDa was precipitated with Mia40-specific antibodies. This product reflected a specific interaction of Tim13 with Mia40, as it was absent when no cross-linker was added or in control precipitations with preimmune serum (Fig. 4A, left panel). Moreover, no cross-linking to Mia40 was observed with the $Tim13^{SSSS}$ mutant in which the four conserved cysteine residues of Tim13 were replaced by serine residues (Fig. 4A, right panel). This Tim13 variant is unable to bind metal ions and, as a consequence, does not stably accumulate in the IMS of mitochondria [1]. The interaction between Mia40 and newly imported Tim13 was also detected by co-immunoprecipitation using antibodies against Mia40 (Fig. 4B). Endogenous Tim13 was not co-immunoprecipitated with Mia40 (data not shown). Thus, Mia40 physically interacts with Tim13 during or directly following its translocation into the IMS of mitochondria.

4. Discussion

We demonstrate that Mia40 exposes a large metal-binding domain into the IMS and is critical for the import of small metal containing proteins into the IMS. The intimate contact of

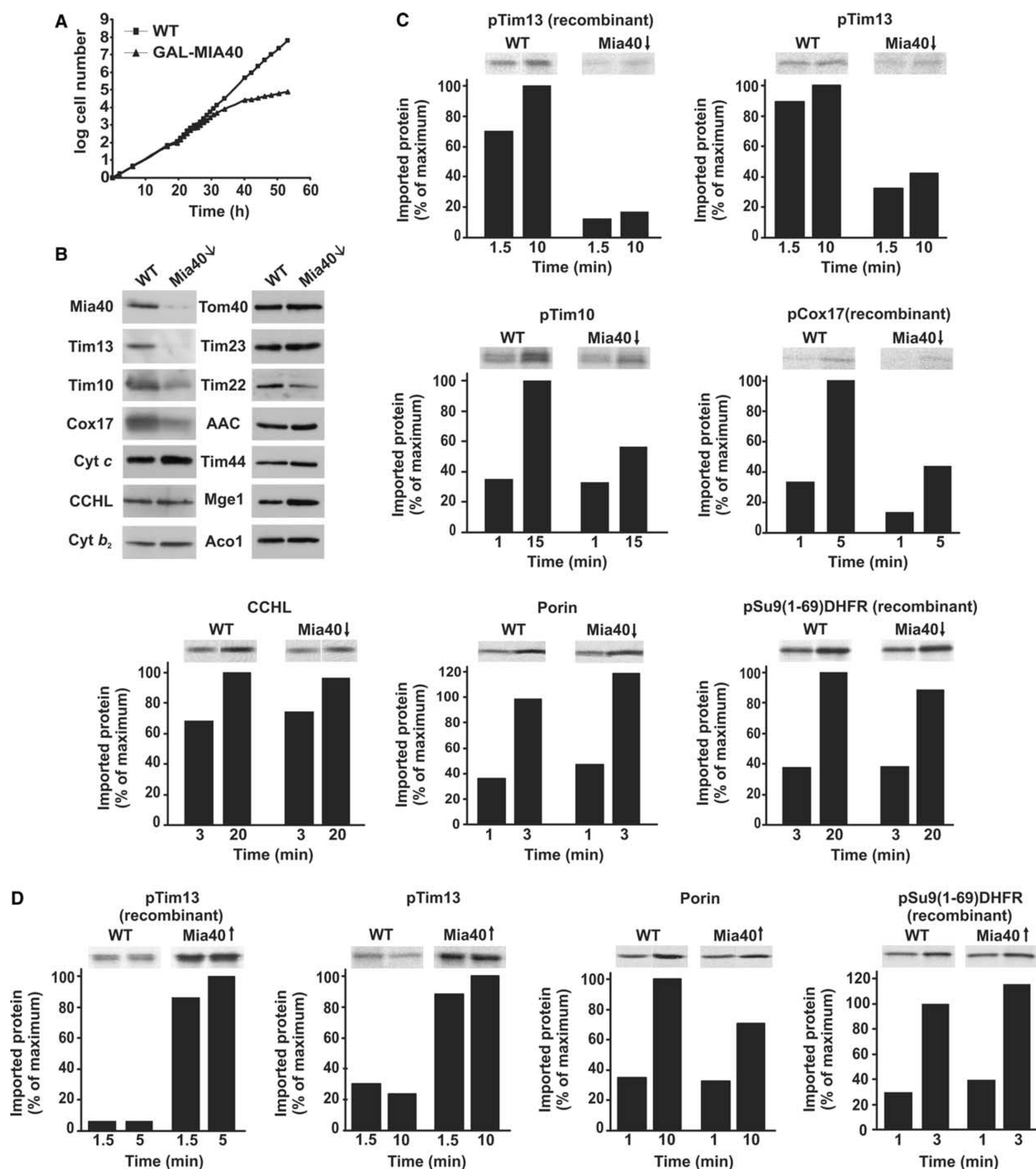

Fig. 3. Mia40 is required for the import of small IMS proteins. (A) Downregulation of Mia40 inhibits cell growth. Wild type (WT) or GAL-MIA40 cells were shifted from galactose to glucose containing medium. Cell number at time zero was set equal to 1. (B) Endogenous levels of the proteins indicated were analyzed by immunoblotting of extracts of wild type and of mitochondria from cells depleted of Mia40 for 16 h (Mia40↓). (C) Depletion of Mia40 strongly affects the import rates of metal-binding IMS proteins. Chemical (recombinant) or radiochemical amounts of preproteins were imported into wild type and Mia40↓ mitochondria. The amounts of imported, i.e., protease-resistant, proteins were analyzed by autoradiography and densitometric quantification. CCHL, cytochrome *c* heme lyase; pSu9(1-69)DHFR, presequence of subunit 9 of the F_1F_0-ATPase fused to dihydrofolate reductase. (D) Overexpression of Mia40 enhances import of Tim13. Import into wild type mitochondria and mitochondria containing about 10-fold increased Mia40 levels (Mia40↑) was performed and analyzed as in C.

Mia40 to newly imported Tim13 suggests a direct role of Mia40 in the trapping and/or folding of Tim13 in the IMS. Since metal-dependent folding and import of Tim13 appear to be mechanistically and kinetically coupled events [1], it is difficult to address the primary molecular function of Mia40 in this process. The observation that Mia40 itself has the ability to bind zinc and copper ions is intriguing and makes an active role of Mia40 in the metal transfer to newly imported IMS proteins possible. Alternatively, Mia40 may play a role as a receptor for a subclass of IMS proteins. Such a function would be in line with the observed binding of newly imported Tim13 to Mia40.

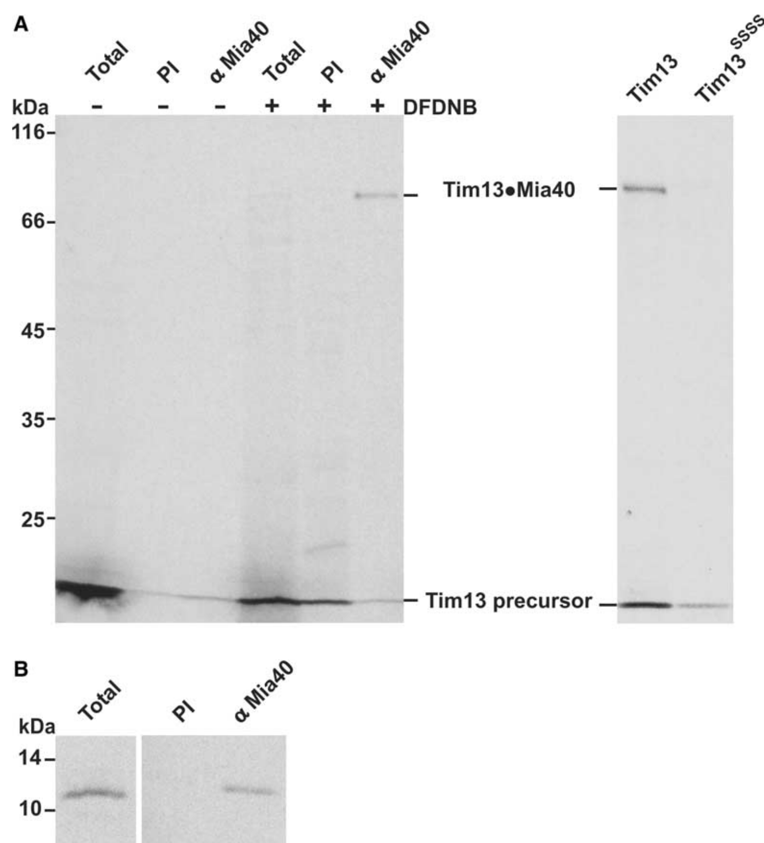

Fig. 4. Mia40 directly interacts with newly imported Tim13. (A) Radiolabeled precursor of Tim13 and of a cysteine-less mutant form of Tim13 ($Tim13^{SSSS}$) were incubated with mitochondria for 20 min at 12 °C and treated with the chemical cross-linking reagent DFDNB (200 μM). Mitochondria were lysed and the supernatants were subjected to immunoprecipitation with antibodies against Mia40 or preimmune serum. (B) Following import of radiolabeled Tim13, mitochondria were lysed using digitonin and subjected to co-immunoprecipitation. Total lanes represent 5% (A) and 20% (B) of the material used for the immunoprecipitations.

During preparation of the manuscript, two publications have reported on the identification of the Ykl195w/Mia40/Tim40 [21,22]. Their results and those presented here agree on a role of this protein in the biogenesis of small IMS proteins. The complex of Mia40 to newly imported small Tim proteins which we identified by cross-linking and co-immunoprecipitation experiments was also revealed in these studies, although by an alternative native gel approach. A metal-binding ability of Mia40 was, however, not reported before. Furthermore, we were able to clarify the topology and location of Mia40. There have been conflicting views presented in the two recent publications. One suggests that Mia40 is anchored to the inner membrane with a hydrophobic segment [22]. The other reports a second processing event which triggers release of Mia40 to the IMS of mitochondria [21]. We determined by Edman degradation the N-terminal sequence of Mia40 in mitochondria. This revealed that processing of the precursor protein occurs N-terminal to the hydrophobic segment, indicating that the transmembrane segment is present in the mature Mia40 protein.

The identification of Mia40 as an import component for Tim13 and other IMS proteins opens a new aspect of intracellular protein trafficking. Future studies will have to focus on the molecular mechanisms by which Mia40 mediates the vectorial translocation of IMS proteins across the outer membrane of mitochondria.

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