THE IMPORT MOTOR OF THE YEAST MITOCHONDRIAL TIM23 PREPROTEIN TRANSLOCASE CONTAINS TWO DIFFERENT J PROTEINS, TIM14 AND MDJ2

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The import motor of the mitochondrial TIM23 complex drives translocation of presequence-containing preproteins across the mitochondrial inner membrane in an ATP-dependent manner. Tim44 is the central component of the motor. It recruits mtHsp70 which binds the incoming preproteins. The J protein Tim14 stimulates the ATPase activity of mtHsp70, and thereby enables efficient binding of mtHsp70 to preproteins. Tim16 is a J-like protein which forms a stable subcomplex with Tim14 and recruits it to the translocase. All but one subunits of the TIM23 translocase are essential for yeast cell viability. Yeast cells contain a close homologue of Tim14, Mdj2. In contrast to Tim14, its deletion leads to no obvious growth defect. In the present study we analyzed Mdj2 and compared it to Tim14. Mdj2 forms a complex with Tim16 and is recruited to the TIM23 translocase. It stimulates the ATPase activity of mtHsp70 to the same extent as Tim14 does. Mdj2 is expressed at a lower level compared to Tim14 and its complex with Tim16 is less stable. However, overexpressed Mdj2 fully restores growth of cells lacking Tim14. We conclude that Mdj2 is a functional J protein and a component of the mitochondrial import motor.

Translocation and sorting of precursor proteins into the various mitochondrial subcompartments are achieved by the concerted action of translocases present in both mitochondrial membranes (for reviews on protein import into mitochondria see (1-4)). After crossing the outer membrane via the TOM complex (translocase of the outer membrane), presequence-containing preproteins are directed to the TIM23 complex in the inner membrane. The TIM23 complex mediates their translocation across or insertion into the inner membrane. In doing so it uses two energy sources, the membrane potential and ATP. The TIM23 complex can be functionally and structurally subdivided into the membrane integrated translocation channel and the import motor which is bound to it at the matrix face. Tim50 is the first component of the TIM23 complex which interacts with the incoming polypeptides as soon as they emerge from the outlet of the TOM channel (5-7). They are then delivered to the translocation channel of the TIM23 complex which is most likely formed by Tim17 and Tim23. The amino acid sequences of the membrane integrated parts of Tim17 and Tim23 are related, suggesting a common evolutionary origin. Tim23 has an additional, N-terminal domain of about 100 amino acid residues that is exposed to the intermembrane space. It apparently cooperates with Tim50 in recognition of presequences. The N-terminal, intermembrane space exposed domain of Tim17 is much shorter but also appears to have an essential function in protein translocation, most likely in the gating of the channel (8). For completion of translocation into the matrix, the membrane integrated part of the translocase cooperates with the import motor.
The import motor is made up from at least five subunits, Tim14/Pam18, Tim16/Pam16, Tim44, Mge1 and mtHsp70. Tim44 is a peripheral membrane protein which binds both to mtHsp70 and to the translocation channel and thereby recruits the chaperone to the channel. MtHsp70 is the energy-transducing component of the import motor. It binds to the incoming polypeptides and prevents their backsliding. Like all members of the Hsp70 family, mtHsp70 binds unfolded polypeptides in a reversible, ATP-dependent manner. Upon hydrolysis of ATP, the peptide binding pocket closes and the polypeptide is bound tightly. Binding of new ATP then releases the polypeptide. Progression through the ATPase cycle is helped by various cochaperones. Tim14 is a typical J domain protein which stimulates the ATPase activity of mtHsp70 and thereby enables tight binding to the precursor. Mge1 is a nucleotide exchange factor which releases bound ADP enabling binding of ATP and the beginning of the new cycle. Tim16 forms a stable subcomplex with Tim14, its role is still not clear but it appears to recruit Tim14 to the translocase and modulate its function. Although the exact mechanism of the import motor remains under debate, it is clear that multiple cycles of binding and release of precursors from mtHsp70 lead to the complete translocation across the inner membrane.

All components of the TIM23 translocase, except Tim21, are essential for yeast cell viability and are usually present as unique genes in the yeast genome. One exception is Tim14 which shares 55% sequence identity with Mdj2. Tim14 and Mdj2 have the same overall structure. They are anchored to the inner membrane by a single, N-terminal transmembrane domain and expose their C-terminal J domains into the matrix. While Tim14 is essential for cell viability, no growth defect was observed upon deletion of Mdj2. We have set out to analyze the function of Mdj2 and its relationship to Tim14 and the TIM23 complex.

We report here that in wild type mitochondria all of Mdj2 is bound to Tim16 and this complex is recruited to the membrane-integrated part of the translocase. Mdj2 stimulates the ATPase activity of mtHsp70 to the same extent as Tim14 does. It differs from Tim14 in its membrane association and its complex with Tim16 is less stable. Nonetheless, overexpression of Mdj2 is able to completely rescue the deletion of Tim14. We conclude that Mdj2 is a functional J protein in the mitochondrial import motor.

**EXPERIMENTAL PROCEDURES**

**Plasmids, yeast strains and cell growth**

Wild type yeast strains YPH499 and YPH501 were used. TIM14 and TIM16 genes were deleted by homologous recombination of a disruption cassette in a diploid yeast strain YPH501. Complete reading frames coding for Tim14 and Tim16 were amplified from yeast genomic DNA using the following primers. For Tim14: BamYLRR008c (5´- CGGAATCCATGCCCATGAG TTCTCAGAATATCTGGA-3´) and YLR008cPst (5´- CCCTGCAGCTATTTGCTAAT ACCCTTTTTTTCC-3´) and for Tim16: BamMia1 (5´-CGGAATCCATGCCCATGACGCTCAAGCGGCCTTATC ATC-3´) and Mia1Pst (5´-GGGCTGCAGCTACTG ATGCTGCTTGCAAGCAC-3´). The resulting constructs were digested with BamHI and PstI and cloned into URA3 containing vector pVT-U. Constructs were transformed into corresponding diploid deletion strains. After sporulation and tetrad dissection, haploid deletion strains were obtained that carry a functional copy of the respective gene on the URA-containing plasmid.

A C-terminal His-tag was introduced into Tim16 by PCR. The fragment containing the endogenous promoter and the gene itself was amplified from the genomic DNA using primers BamTim16p (5´-CTCGGAATCTTAAACGTTAAAATCACCAGCAG-3´) and Mia-C8HisPst (5´- CCGAATCAGATGATGATGATGATGCTGATT GCTGCTTGCAACTATTAC ATC-3´). After digestion with BamHI and PstI, the fragment was cloned into pRS314. A yeast strain expressing only the His-tagged version of Tim16 was obtained after transformation of this construct into the haploid deletion strain of Tim16 and chasing out the URA3 plasmid by growth on 5-fluoroauric acid (5FOA).

A yeast strain expressing only the His-tagged version of Tim16 was obtained after transformation of this construct into the haploid deletion strain of Tim16 and chasing out the URA3 plasmid by growth on 5-fluoroauric acid (5FOA). A yeast strain expressing N-terminally His-tagged Tim14 was obtained in the essentially same manner. A construct in the pRS314 vector was cloned in the following way.
GGTGGTGG-3’) and Tim14pKpn (5’-AAAGGATCCATGCTCCCCTATACTAGGTTAT
TGG-3’) and cloned into pGEM4 vector (Promega) after digestion with SacI and KpnI. The
fragment containing the His-tagged version of Tim14 was amplified using primers NHis8Tim14
(5’-CCTCGGATCCATGCACCATCACCATCACCATCACCATGTTCTCAAAGTAATACTGG-
3’) and YLR008cPst and cloned behind the TIM14 promoter in pGEM4. The construct containing
the promoter and the gene for the His-tagged Tim14 was subcloned as a single SacI/PstI fragment into
pRS314.
To check for the complementation of TIM14 deletion, Tim14 and Mdj2 constructs were made in the overexpression TRP containing vector pVT-TRP. For amplification of Tim14, primers BamYLR008c and YLR008cPst were used and, after digestion with BamHI and PstI, the fragment was cloned into pVT-TRP. Mdj2 was amplified using primers KpnMdj2 (5’-CCCGGTACCATGGTTTTGCCTATAATAATTGG-3’) and Mdj2Pst (5’-AAACTGCAGTTATCTCTTTCTTAGTAAACAC-3’). The fragment was initially cloned into pQE30 vector (Qiagen) and then subcloned as BamHI/PstI fragment into pVT-TRP. To check for complementation under the control of the TIM14 promoter the same fragments were first cloned behind the TIM14 promoter in pGEM4 vector and then subcloned as SacI/PstI fragments in pRS314 vector. All constructs were transformed into the haploid deletion strain of Tim14 carrying TIM14 on URA3 plasmid and assessed for growth on 5FOA as described above.
Yeast strains in which expression of Tim14 is under the control of GAL10 promoter and the deletion strain of Mdj2 were described previously (12, 18). Yeast cells were normally grown in lactate medium containing 0.1% glucose.
Fusions proteins of Tim14 and Mdj2 with maltose binding protein were obtained by cloning full length genes as BamHI/PstI fragments (see above) into pMAL-cRI vector (New England Biolabs). Expression of recombinant proteins was induced by IPTG and proteins were purified according to the manufacturer’s instructions.
Tim16 lacking its first 25 amino acid residues was amplified using primers BamTim16_26 (5’-AAAGGATCCAGACAAGC
GGCTTCACAAATC-3’) and Mia1Pst. Obtained PCR fragment was digested with BamHI and PstI
and subsequently cloned into pQE30 vector. The resulting plasmid codes for an N-terminally His6-
tagged protein which was expressed in E. coli XI1Blue cells upon induction with IPTG. Protein
was purified under native conditions according to the manufacturer’s instructions.

**Antibodies against Mdj2**
Full length MDJ2 was cloned in pQE30 vector as described above. His-tagged protein was expressed upon induction with IPTG in the XL1 Blue cells, purified under denaturing conditions and injected into rabbits. Mdj2 specific antibodies were affinity purified from the serum before use.

**ATPase assay**
The ATPase activity of mtHsp70 was determined using a modified assay after Horst et al (22). ATP hydrolysis was measured at 25°C in 50
mM HEPES, pH 7.4, 100 mM KCl, 5 mM MgCl₂,
66 µM ATP and 0.1 mM γ-32P-ATP. 7.4 µg
mtHsp70 (24) were used per reaction. Where
indicated 2 µg Mge1 (22), 4.9 µg MBP-Tim14 and
4.8 µg MBP-Mdj2 were added. Aliquots were
removed at the indicated time points and the
reactions were stopped by addition of 50 mM
EDTA, 5 mM ATP and 5 mM ADP. Samples were
spotted on PEI-cellulose F TLC plates and
developed with 1 M formic acid, 0.5 M LiCl. The
chromatogram was analyzed by phosphoimaging.
Activity was expressed as the % of total
radiolabeled ATP which was hydrolyzed at the
indicated time point.

In the ATPase inhibition experiments,
MBP-Tim14 or MBP-Mdj2 were preincubated
with different amounts of Tim16 for 5 min at 25°C
before the ATPase assay was performed.

**Coimmunoprecipitations**
Wild type yeast mitochondria were
solubilized at 1 mg/ml with 1% (w/v) digitonin in
20 mM TRIS/Cl, 80 mM KCl, pH 7.5, 1 mM
phenylmethylsulfonyl fluoride at 4°C. After a
clarifying spin, solubilized material was added to
the affinity purified antibodies or preimmune IgGs
prebound to the Protein A-Sepharose beads.
Samples were gently rotated for 1.5 h at 4°C.
Beads were washed three times with the
solubilization buffer before bound proteins were
eluted with Laemml buffer. Samples were
analyzed by SDS-PAGE and immunodecoration.
Where indicated, mitochondria were solubilized

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with 1% (v/v) Triton X-100 which was then also used for washing steps.

**NiNTA-agarose pull down assay**

Wild type or mitochondria containing His-tagged Tim14 or Tim16 were solubilized at 1 mg/ml with 1% digitonin (w/v) in 20 mM TRIS/HCl, 80 mM KCl, 20 mM imidazole, pH 7.5, 1 mM phenylmethylsulfonyl fluoride at 4°C. After a clarifying spin, solubilized material was added to 50 µl NiNTA-agarose beads and gently rotated for 1 h at 4°C. After three washing steps with the solubilization buffer, bound proteins were eluted with Laemmli buffer containing 300 mM imidazole. Samples were analyzed by SDS-PAGE and immunodecoration.

**Crosslinking**

Manipulation of mitochondrial ATP levels followed by crosslinking was done as described previously (12). Mitochondria were preincubated for 10 min at 25°C either in the presence of apyrase (10 U/ml) and oligomycin (10 µM) to deplete mitochondrial ATP or in the presence of ATP (4 mM), NADH (5 mM), creatine-phosphate (10 mM) and creatine-kinase (0.1 mg/ml) to keep ATP levels high. Samples were cooled on ice and the crosslinker, diisuccinimidyl-glutarate (DSG), was added from a 100 fold stock in dimethylsulfoxide to a final concentration of 0.15 mM. After 30 min, crosslinker was quenched by addition of 0.1 M glycine, pH 8.8. Mitochondria were reisolated and analyzed by SDS-PAGE and immunodecoration.

**RESULTS**

**Mdj2 is part of the TIM23 translocase**

We used coimmunoprecipitations to analyze a possible association of Mdj2 with Tim16 and the TIM23 translocase. Wild type yeast mitochondria were solubilized with digitonin and subjected to immunoprecipitation with antibodies against Tim16 and Tim17. These two antibodies were able to precipitate all known components of the TIM23 translocase (15). Mdj2 was depleted from the mitochondrial extract by Tim16 antibodies, in the same manner as Tim14 was depleted (Fig. 1). This shows that Mdj2 interacts with Tim16 in wild type mitochondria. Furthermore, part of Mdj2 was coprecipitated with antibodies to Tim17. The same observation was made for Tim14. In conclusion, Mdj2 is a component of the TIM23 translocase, in particular of the import motor. In this assay, it cannot be distinguished from Tim14 in its interactions with the other components of the TIM23 complex.

**Mdj2 and Tim14 form two different complexes with Tim16**

Are Mdj2 and Tim14 part of the same complex or of separate complexes? We analyzed whether Tim14 can be coimmunoprecipitated with Mdj2 antibodies. Wild type mitochondria were solubilized in digitonin and subjected to immunoprecipitation with antibodies to Mdj2 prebound to ProteinA-Sepharose (Fig. 2A). Mdj2 antibodies immunodepleted Mdj2 from the detergent extract and they precipitated part of Tim16. Since antibodies to Tim16 were able to immunodeplete Mdj2, Tim16 appears to be present in excess over Mdj2. Interestingly, Tim14 was not coprecipitated along with Mdj2. Apparently these two proteins are not part of the same complex. Tim16 is likely involved in formation of at least two complexes, one with Tim14 and one with Mdj2.

To substantiate these results, pull down assays using NiNTA-agarose were performed with mitochondria isolated from yeast strains expressing a His-tagged version of either Tim16 or Tim14. These His-tagged versions of Tim14 and Tim16 are fully functional as normal growth rates were observed when they were introduced into the cells which completely lacked the wild type proteins (data not shown). Mitochondria were solubilized in digitonin and incubated with NiNTA-agarose beads (Fig. 2B). With His-tagged Tim16 mitochondria, virtually all of Tim16, Tim14 and Mdj2 were bound to the NiNTA-agarose. When mitochondria containing N-terminally His-tagged Tim14 were analyzed, only Tim14 itself was depleted from the supernatant and about half of Tim16 was bound to the NiNTA-agarose beads. This suggests an excess of Tim16 over Tim14. Mdj2 on the other hand was found completely in the supernatant. In summary, Tim14 and Mdj2 are not part of the same complex. In addition, not all of Tim16 is involved in the complex formation with Tim14, rather it forms at least one additional complex, namely with Mdj2.

Are the two complexes containing Tim16 dependent on each other? Mitochondria were isolated from three different strains: an **MDJ2**
deletion strain, a strain where the expression of Tim14 was under the control of the GAL promoter so that its level could be downregulated by growth on glucose (12), and a wild type strain as a control. Deletion of MDJ2 did not affect the levels of Tim14, nor did downregulation of Tim14 lead to reduced levels of Mdj2 (data not shown). We then analyzed the Tim16 complexes in cells lacking one J protein by immunoprecipitation with Tim16 antibodies (Fig. 2C). Deletion of MDJ2 had no influence on the complex of Tim16 with Tim14. On the other hand, depletion of Tim14 had no effect on the complex of Tim16 with Mdj2, again all of Mdj2 was present in a complex with Tim16. Taken together, Tim16 forms two separate, mutually not interfering complexes.

**Mdj2 stimulates the ATPase activity of mtHsp70 to the same extent as Tim14**

Mdj2 has a predicted fold of J domains with three helices and the conserved HPD motif in the loop between helices two and three. We analyzed the influence of Mdj2 on the ATPase activity of mtHsp70. Mdj2 was expressed recombinantly in bacteria as a fusion protein with maltose-binding protein and purified as a soluble protein to homogeneity (Fig. 3A). The same was done with Tim14 as a control. Their ability to stimulate the ATPase activity of mtHsp70 was tested. MtHsp70 was incubated at 25°C with radiolabeled ATP alone or in the presence of various cochaperones (Fig. 3B). In the presence of Mge1, Mdj2 stimulated the basal ATPase activity of mtHsp70 about 25 fold. Furthermore, no difference was observed in its cochaperone activity compared to Tim14. This shows that Mdj2 is a functional J protein. This result does, however, not explain why Mdj2 does not replace the essential J protein, Tim14 in yeast cells.

**Differences between Mdj2 and Tim14**

The observations presented so far open some important questions. What are the differences between Tim16 complexes with either Tim14 or Mdj2 and why is the latter one not sufficient to support growth of cells? We addressed these questions in several ways. The exact role of Tim16 in the import motor is still not clear but it was speculated that it modulates the function of Tim14. We compared the effect of Tim16 on the two J proteins. Tim16 was added in increasing amounts into the ATPase assay described above containing mtHsp70, Mge1 and either Mdj2 or Tim14 as a J protein. The reactions were stopped and analyzed for the amount of ATP hydrolyzed. Addition of Tim16 enhanced the stimulatory activity of Mdj2, in contrast to its effect on Tim14 which was inhibitory (Fig. 4A).

Next we analyzed the membrane attachment behavior of Mdj2 by carbonate extraction. Wild type yeast mitochondria were incubated with sodium-carbonate to extract soluble and peripheral membrane proteins. In such an experiment, Tim14 was previously observed to be largely extracted by carbonate suggesting that its transmembrane domain is in contact with other proteins, most likely hydrophobic segments of TIM23 components (12, 14). Mdj2, in contrast, was completely recovered in the membrane fraction, like a typical membrane protein (Fig. 4B). This labile interaction of Tim14 with the membrane may have functional significance.

Association of Mdj2 with Tim16 and the translocase was observed after solubilization of mitochondria with digitonin. We tested the stabilities of the Tim16 complexes with Mdj2 and Tim14 in a slightly harsher detergent, Triton X-100. Wild type yeast mitochondria were solubilized in Triton X-100 and subjected to immunoprecipitation with antibodies to Tim16. Whereas Tim16 interaction with both J proteins was stable in digitonin (see also Fig. 1), part of Mdj2 was not immunodepleted by Tim16 antibodies with Triton X-100-solubilized mitochondria (Fig. 4C). This is in contrast to the complex of Tim16 with Tim14 which remained completely stable in Triton X-100. This suggests a weaker interaction of Tim16 with Mdj2 as compared to its interaction with Tim14.

**Overexpressed Mdj2 restores the growth of cells lacking Tim14**

We asked whether the overexpression of Mdj2 is able to compensate the deletion of TIM14. To test this, we cloned MDJ2 into a multi copy yeast vector under a strong constitutive promoter. Under these conditions Mdj2 was able to compensate the deletion of TIM14. To test this, we cloned MDJ2 into a multi copy yeast vector under a strong constitutive promoter. Under these conditions Mdj2 was able to support growth of cells in the absence of Tim14 (Fig. 5A). This strongly suggests that whatever the differences between Mdj2 and Tim14 are, they can be overcome by the overexpression of the former. MDJ2 was also cloned in a single copy yeast vector under the control of the TIM14 promoter. Even under these conditions Mdj2 was capable of...
rescuing *TIM14* deletion (Fig. 5A). This suggests that in wild type cells Mdj2 is expressed at a lower level, compared to Tim14, which is apparently not sufficient to functionally replace Tim14. However, it should be noted that the *MDJ2* promoter, although probably not as strong as the *TIM14* promoter, is functional as Mdj2 itself is expressed in wild type cells. Furthermore, Tim14, when expressed from the *MDJ2* promoter, was able to rescue its own deletion (not shown). We analyzed the mitochondrial protein profiles of *TIM14* deletion expressing Tim14 or Mdj2 from different promoters by SDS-PAGE and immunodecoration. Wild type levels of both Mdj2 and Tim14 were observed in the strain expressing Tim14 under the *TIM14* promoter (Fig. 5B). In cells expressing Mdj2 from the same promoter, higher levels of Mdj2 were present and Tim14 was completely absent. Thus, Mdj2 is indeed capable of replacing Tim14. Even higher levels of Mdj2 were found when it was under the control of the *ADH* promoter. We asked whether the expression levels of Mdj2 correlated with growth rates. The same four strains were spotted in serial ten-fold dilutions on rich media containing either glucose or glycerol as a carbon source. Indeed, the strain expressing Mdj2 under *TIM14* promoter grew slightly slower than the strain expressing Tim14 from the same promoter (Fig. 5C). On the other hand, Mdj2 expressed from the *ADH* promoter fully restored growth of cells lacking Tim14.

Is Mdj2 functionally integrated into the import motor in these strains? To address this question we used a crosslinking approach. Mitochondria isolated from a *TIM14* deletion strain overexpressing Mdj2 and from wild type cells were pretreated in order to lower or increase mitochondrial ATP levels. Crosslinking was then performed and samples were analyzed by SDS-PAGE and immunodecoration with either Tim16 or Tim44 antibodies. In wild type mitochondria, the previously described crosslinks of Tim16 to Tim14, Tim44 and mtHsp70 were visible (Fig. 5D, left panel). In the *TIM14* deletion strain overexpressing Mdj2 the crosslinking pattern of Tim16 was virtually identical, with one exception. A crosslink to Tim14 was no longer visible and a crosslinking adduct of smaller molecular mass appeared. As the size difference corresponds to that between Tim14 and Mdj2, this crosslink most likely represents a crosslink of Tim16 to Mdj2. A corresponding result was obtained when Tim44 crosslinks were analyzed. In the strain overexpressing Mdj2, the Tim44-Tim14 crosslink disappeared and a crosslink to Mdj2 appeared. Notably, the Tim44 crosslink to Mdj2 showed the same dependence on the ATP level as the crosslink to Tim14 in wild type mitochondria. This demonstrates functional integration of Mdj2 in the import motor. To directly demonstrate the ability of Mdj2 to support protein translocation into mitochondria, we performed *in vitro* import experiments using radiolabeled precursor proteins and mitochondria isolated from a *TIM14* deletion strain overexpressing Mdj2. When complete translocation into the matrix via the TIM23 translocase was analyzed (exemplified here by Su9(1-69)DHFR), mitochondria containing Mdj2 as the only J protein in the import motor of the TIM23 translocase mediated import of precursors with the efficiency of about 90% as compared to those having Tim14 as the J protein partner of mtHsp70 (Fig. 5E). When the ability of the TIM23 translocase to insert preproteins into the inner membrane was analyzed (exemplified by DLD(1-72)DHFR), virtually the same efficiency as with the wild type mitochondria was observed. This confirms that Mdj2 is functionally integrated into the import motor of the mitochondrial TIM23 translocase.

**DISCUSSION**

The TIM23 preprotein translocase mediates the transport of presequence-containing precursors across or into the mitochondrial inner membrane. Two new essential subunits of the motor part of the translocase were recently identified, Tim14 and Tim16, a J and a J-like protein, respectively. In yeast, they form a stable heterodimeric complex. In the present study we have analyzed Mdj2, a yeast protein which shares 55% sequence identity with Tim14 but whose deletion led to no apparent growth defect under the conditions analyzed. We report here that Mdj2 forms a complex with Tim16 and is recruited to the membrane-integrated translocation channel. It stimulates the ATPase activity of mtHsp70 to the same extent as Tim14 does. Furthermore, the overexpression of Mdj2 enables the cells to live in the absence of Tim14. Taken together these data indicate that Mdj2 functions as the J protein in the mitochondrial TIM23 translocase. However,
Tim14 is apparently required as a J protein of the import motor in wild type cells because endogenous Mdj2 is expressed at a lower level compared to Tim14 and forms a less stable complex with Tim16.

The alignment of Tim14 and some of its homologues from higher organisms with Mdj2 provide some hints to an explanation of the observed differences between Tim14 and Mdj2 (Fig. 6). Yeast Tim14 and its homologues have one positively charged residue preceding and one negatively charged residue following the absolutely conserved GGF motif in front of the helix 1 of the J domain (amino acid residues 102-104 in the Sc Tim14 sequence). These charges are not conserved in Mdj2. It is tempting to speculate that they have a stabilizing effect on the interactions of Tim14 with other components of the import motor. Furthermore, helix 2 of the J domain, which is generally not very well conserved, contains a stretch that is conserved among Tim14 and its homologues but not in Mdj2. Helix 2 was proposed to be involved in the interaction of J proteins with their Hsp70 partners (25). Furthermore, the spacing between the transmembrane domain and the J domain is about 30 amino acid residues longer in Mdj2 than in the Tim14 homologues. The significance of these observations awaits further experiments. Tim14 has an N-terminal extension in the intermembrane space which is not present in Mdj2. However, it seems unlikely that it is responsible for the observed differences as it is present only in yeast Tim14 and even there its deletion has no effect on the function of the protein (our unpublished data).

Why does yeast have two homologous J proteins in the import motor when other fungi have only one? Tim14 in yeast is not the only component of the TIM23 translocase which has a close homologue. Beside the mtHsp70 protein Ssc1, a homologue called Ecm10 is present in yeast. Ecm10 is a mitochondrial matrix protein which shares 82% amino acids sequence identity with Ssc1p (25). With respect to the essential nature of the proteins, Ssc1p and Ecm10 behave like Tim14 and Mdj2. While deletion of Ssc1p is lethal, disruption of Ecm10 has no apparent mitochondrial phenotype. Ecm10 is expressed at a lower level compared to Ssc1p but even its overexpression did not rescue the deletion of Ssc1p (25, 26). In contrast, Mdj2 is obviously capable of replacing Tim14 if expressed at a level as high as that of Tim14. Even though the deletion of Mdj2 has no obvious phenotype it remains possible that Mdj2 has a distinct function which is, however, not essential for viability under the conditions analyzed. Indeed, Mdj2 was implicated in having a partially overlapping function with Mdj1, the J protein which helps mtHsp70 in folding of newly imported proteins (18). Furthermore, it seems possible that Mdj2 is part of a specialized TIM23 complex. This complex might differ also in other aspects from Tim14-containing TIM23 complex and might fulfill a distinct function, such as the translocation of a specific subset of proteins. In this respect it should be noted that overexpression of Tim14 does not displace Mdj2 completely out of the TIM23 translocase (data not shown).

While in fungal genomes TIM23 components are mostly present as single genes, in higher eukaryotes they are quite often present in multiple copies. For example, in human genome there are two genes for Tim17, Tim17a and Tim17b (27). They are both expressed and form separate complexes with hTim23. It is not known whether they can substitute for each other in forming the functional translocase or whether they are involved in formation of two translocases with different substrate specificities. We identified two genes in the human genome which code for proteins homologous to Tim14 ((12) and Figure 6). These proteins appear to be more closely related to Tim14 than to Mdj2, since they share the characteristics of Tim14 discussed above. They are both expressed as they are both present in the UniGene database of transcribed sequences on the NCBI server (28). Their ubiquitous expression argues against a tissue specific function. Whether these homologues have redundant functions or not remains to be determined. Interestingly, inactivation of DNADJ1 alone confers resistance to chemotherapeutic agents used in the treatment of ovarian cancer (29). This observation suggests that the two Tim14 homologues in the human genome either have different functions or that the second homologue is completely or partly nonfunctional so that DNADJ1 is the major J of the import motor, a situation which seems similar to that in yeast.

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REFERENCES

FOOTNOTES

The abbreviations used are: TOM, translocase of the outer membrane; TIM, translocase of the inner membrane; 5FOA, 5-fluoroorotic acid; IPTG, isopropyl-β-D-thiogalactopyranoside

FIGURE LEGENDS

Figure 1. Mdj2 is part of the TIM23 translocase. Wild type yeast mitochondria were solubilized in digitonin-containing buffer and subjected to immunoprecipitation with Protein A-Sepharose prebound antibodies against Tim17 and Tim16, or preimmune IgGs as a control. Bound proteins were eluted with Laemmli buffer and samples were analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. “Total” and “Supernatant” fractions contain 20% of the material present in “Pellets”. AAC, ATP/ADP carrier.

Figure 2. Tim16 forms two separate complexes with two J proteins. A. Wild type yeast mitochondria were solubilized with digitonin and subjected to immunoprecipitation with antibodies prebound to Protein A-Sepharose and directed against Mdj2 or preimmune IgGs as control. Samples were treated and analyzed as described in the legend to Figure 1. Total and supernatant (Sup) fractions represent 20% of the material present in pellets (Pel). B. Mitochondria from wild type and from cells containing His-tagged versions of Tim16 or Tim14 were solubilized with digitonin and incubated with NiNTA-agarose beads. Bound proteins were eluted with Laemmli buffer containing 300 mM imidazole. Samples were analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. Total (T) and supernatant (S) fractions represent 20% of material present in pellets (P). C. Mitochondria isolated from wild type (WT), from a MDJ2 deletion strain (∆mdj2), and from Tim14 depleted cells (Tim14Ψ) were solubilized in digitonin-containing buffer and subjected to immunoprecipitation with Tim16 antibodies as described in the legend to Figure 1.

Figure 3. Mdj2 stimulates the ATPase activity of mtHsp70. A. Coomassie Brilliant Blue stained gel of the purified proteins used in the ATPase assay. B. MtHsp70 was incubated with the indicated cochaperones in the presence of γ-32P-ATP. Samples were removed after the indicated time periods and analyzed for the amount of hydrolyzed radioactive ATP. The ATPase activity of mtHsp70 is depicted as the percent of ATP hydrolyzed at the indicated time point.

Figure 4. Differences between Mdj2 and Tim14. A. Tim14 and Mdj2 were incubated with the increasing amounts of Tim16 and then added to the ATPase assay. Tim14- and Mdj2-stimulated ATPase activities of mtHsp70 in the absence of Tim16 were set to 1. B. Wild type mitochondria were incubated with 0.1 M sodium carbonate to extract soluble and peripheral membrane proteins. Samples were centrifuged and soluble (S) and pellet (P) fractions were analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. C. Wild type mitochondria were solubilized with digitonin or Triton X-100 and subjected to immunoprecipitation with antibodies to Tim16 or preimmune IgGs prebound to the Protein A-Sepharose beads and further processed as in Figure 1. Total and supernatant (S) fractions contain 20% of the material present in pellets (P).

Figure 5. Overexpressed Mdj2 restores growth of cells lacking Tim14. A. A haploid deletion strain of TIM14 carrying the wild type copy of the gene on the URA plasmid was transformed with TRP plasmids carrying TIM14 or MDJ2 under either ADH or TIM14 promoter. Empty plasmids were applied as controls. Cells were plated on 5-fluoro-orotic acid medium to remove the URA plasmid and thereby checked for complementation by the genes carried on the TRP plasmids. B. Protein profiles of mitochondria isolated from TIM14 deletion strain rescued with TIM14 or MDJ2 under the ADH or TIM14 promoter. Decoration with antibodies against Tim44 is shown to confirm loading of equal amounts of protein. C. Ten-fold serial dilutions of different strains were spotted onto glucose- or glycerol-containing
medium and plates were incubated for 3 days at 30°C. D. Mitochondria were isolated from a wild type yeast strain and a TIM14 deletion strain rescued by overexpressed Mdj2. ATP levels in mitochondria were manipulated by incubation for 10 min at 25 °C in the presence of apyrase and oligomycin (-ATP) or in the presence of NADH and ATP (+ATP). Samples were placed on ice and the crosslinker disuccinimidyl glutarate (DSG) was added. After 30 min excess crosslinker was quenched by addition of glycine. Mitochondria were reisolated and analyzed by SDS-PAGE and immunodecoration with antibodies to Tim16 and Tim44. Identified crosslinks are indicated. E. Mitochondria were isolated from TIM14 deletion strains expressing either Tim14 or Mdj2 and incubated with radiolabeled precursor proteins consisting of either the presequence of subunit 9 of the F\(_0\)F\(_1\)-ATPase or the presequence of D-lactate dehydrogenase fused to mouse dihydrofolate reductase (Su9(1-69)DHFR and DLD(1-72)DHFR, respectively). Aliquots were removed after the indicated time periods and half of each sample was incubated with proteinase K (PK) to degrade nonimported material. Samples were analyzed by SDS-PAGE, autoradiography and densitometry. p, precursor form; m, mature form.

Figure 6. Alignment of Tim14 and its homologues with Mdj2. The alignment shows that certain amino acid residues (labeled with *) are conserved between Tim14 and its homologues but not in Mdj2. The transmembrane segment is underlined. H1-H3, helices 1 to 3 of the J domain. Sc, \textit{S. cerevisiae}; Ce, \textit{C. elegans}; Hs, \textit{H. sapiens}.
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**Figure 6 Mokranjac et al**
The import motor of the yeast mitochondrial TIM23 preprotein translocase contains two different J proteins, Tim14 and Mdj2
Dejana Mokranjac, Martin Sichting, Dušan Popov-Celeketic, Annette Berg, Kai Hell and Walter Neupert

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