Mdj1p, a Novel Chaperone of the DnaJ Family, Is Involved in Mitochondrial Biogenesis and Protein Folding

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Summary

Mdj1p, a novel member of the DnaJ family, is a heat shock protein that is associated with the inner membrane of mitochondria of Saccharomyces cerevisiae. Disruption of the MDJ1 gene resulted in a petite phenotype, loss of mitochondrial DNA, and viability at 37°C. Import of precursor proteins was not affected by a lack of Mdj1p, but folding of newly imported proteins was markedly impaired. The efficiency of refolding of a test protein, dihydrofolate reductase, was significantly reduced in mitochondria lacking Mdj1p after incubation at elevated temperature. We conclude that Mdj1p is an important mitochondrial chaperone that participates in the folding of newly imported proteins and in the protection of proteins against heat denaturation and aggregation.

Introduction

Heat shock proteins of the Hsp70 and Hsp60 family have been shown to play essential roles as molecular chaperones in the translocation of proteins into mitochondria and in the subsequent folding and assembly of the newly imported precursor proteins within the organelle (Cheng et al., 1989; Ostermann et al., 1989; Scherer et al., 1990; Kang et al., 1990; Manning-Krieg et al., 1991; Craig et al., 1993; Ellis, 1993; Neupert and Pfanner, 1993). Cytoplasmic members of the Hsp70 class are required to maintain proteins in a transport-competent conformation during translocation across membranes (Chirico et al., 1968; Deshaies et al., 1988). One member of the family, Sec1p, is located in the mitochondrial matrix and mediates the vectorial transfer of precursor proteins across the two mitochondrial membranes (Kang et al., 1990). The partially unfolded polypeptide chain, bound to Sec1p, is then thought to be passed on to the chaperonin Hsp60, which facilitates protein folding and assembly (Cheng et al., 1989; Manning-Krieg et al., 1991; Martin et al., 1992).

Although considerable evidence has been accumulated in favor of these roles for Hsp70 and Hsp60, it remains unclear whether additional components are required in these complex processes. In fact, it seems likely that in mitochondria as in prokaryotic organisms, additional components may be necessary for the efficient regulation of heat shock protein activity. In Escherichia coli, the single Hsp70, DnaK, has been shown to be functionally active in a dynamic complex with two other heat shock proteins, DnaJ and GrpE. DnaJ stimulates the ATPase activity of DnaK, whereas GrpE acts as a nucleotide exchange factor (Liberek et al., 1991). In this manner, these three heat shock proteins participate in a variety of processes, such as bacteriophage λ DNA replication, protein folding, and regulation of heat shock response (Zylicz et al., 1989; Langer et al., 1992; Gamer et al., 1992).

In the yeast Saccharomyces cerevisiae, several genes of DnaJ homologs have so far been characterized: SCJ60, SCJ1, YDJ1, SIS1, ZU01, and XDJ1 (Rothblatt et al., 1989; Sadler et al., 1989; Blumberg and Silver, 1991; Caplan and Douglas, 1991; Atencio and Yaffe, 1992; Luke et al., 1991; Zhang et al., 1992; Schwarz et al., in press). Among these, Sec63p and Ydj1p have been shown to be involved in protein translocation across intracellular membranes (Rothblatt et al., 1989; Caplan et al., 1992). SCJ1 was identified as a gene whose overexpression restores the proper targeting of a fusion protein containing a nuclear localization sequence preceding the cytochrome c1 coding sequence to the mitochondria (Blumberg and Silver, 1991). Scj1p has been suggested to be localized within the mitochondria; however, its function and subcellular localization have yet to be demonstrated. Recent evidence suggests that Sis1p facilitates the formation of a specific protein complex within the translation machinery (Zhong and Arndt, 1993).

From what is known about the concerted action of heat shock proteins, especially in the prokaryotic system, one might predict that mitochondria have conserved the protein-folding apparatus of their endosymbiotic ancestors. Thus, the functional characterization of a mitochondrial DnaJ homolog in protein import and folding reactions should provide insight into the complex processes that contribute finally to organelle biogenesis. Here, we report a DnaJ homolog, Mdj1p, which is located in the mitochondrial matrix and is essential for respiration-dependent growth. Furthermore, our results demonstrate a role for Mdj1p in the folding of imported precursor proteins and the prevention of protein aggregate formation.

Results

Nucleotide and Predicted Amino Acid Sequence of MDJ1

The mitochondrial DnaJ (MDJ1) gene was identified during the DNA sequencing of a S. cerevisiae genomic library (a gift of M. V. Olson; Olson et al., 1986; Riles et al., 1993). MDJ1 lies head to head with HSP12/GLP1, and between the SUP9 and CDC4 genes, on chromosome VI. The 2457 bp sequence of the MDJ1 nucleotide sense strand and predicted protein sequence are shown in Figure 1. MDJ1 is a single copy gene, as demonstrated by hybridization
of a 1998 bp probe containing the complete MDJ1 coding sequences on a Southern blot (data not shown). Upstream of the MDJ1 open reading frame, at positions −28 and −88 with respect to the translational start codon, are two potential TATA boxes. Furthermore, the MDJ1 open reading frame is preceded by several weak matches to the heat shock element nGAAnnlXn (Amin et al., 1988; Young and Craig, 1993) and five copies of the CCCCT motif reported to be involved in heat shock factor-independent gene induction (Kobayashi and McEntee, 1993) (Figure 1). The heat inducibility of MDJ1 was tested by Northern blot analysis, which revealed a 2- to 3-fold transient increase of transcript abundance upon shift of the cells from 24°C to 37°C (data not shown).

The predicted amino acid sequence of Mdj1p consists of 511 residues with 33% identity to the E. coli DnaJ gene product. An alignment of these sequences with the other reported yeast DnaJ-like polypeptide sequences is presented in Figure 2. Highest similarity between Mdj1p and these other proteins is found in an 88 amino acid stretch (residues 59–127) conserved in all members of the DnaJ protein family, that is termed the J region (reviewed by Caplan et al., 1993; Silver and Way, 1993). In common with most other members of this protein family, Mdj1p possesses a glycine-rich stretch in the central portion of the molecule, followed by a series of CxxCxGxG motifs that are organized into four repeats (Figure 2).

**MDJ1 Is Essential for the Formation of Respiratory-Competent Mitochondria**

To investigate whether MDJ1 is an essential gene, the BsmI–SnaBI fragment (bases 173–1581, see Figure 1) of the cloned gene was replaced with a WAD encoding fragment and was transformed into the diploid strain a/a735. The presence of a disrupted and a wild-type copy of the gene was confirmed by polymerase chain reaction (PCR) and Southern blot analysis (data not shown). The heterozygous diploid (alaYNR3) was sporulated, and 39 tetrads were dissected. All of the tetrads showed a uniform 2:2 segregation into small and large colonies at a growth temperature of 30°C (Figure 3A). The small colonies were all Ura+, indicating that the mdj1 disruption cosegregated with the slow growth phenotype. Further replica-plate analysis showed that the Ura+ spores were inviable at 37°C on all carbon sources tested. They failed to grow on glycerol, a nonfermentable carbon source, at 24°C (Figure 3B), exhibiting a petite phenotype. The temperature-sensitive
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Figure 2. Amino Acid Sequence Comparison of Mdjlp with Other Yeast DnaJ Homologs and E. coli DnaJ

The alignments were performed with the Genetic Computer Group program (University of Wisconsin). The highly conserved J region is boxed, the glycine-rich stretches are underlined, and the conserved residues of the four cysteine-containing motifs (CxxCxGxG) are boxed. For Sec63p and Zuolp only, the J region is shown (residues 125–199 and 97–171, respectively) because only this region shows similarity to the other members of the protein family. DnaJ (Bardwell et al., 1986); SEC63 (Rothblatt et al., 1989); YDJ1 (Caplan and Douglas, 1991); SIS1 (Luke et al., 1991); SCJ1 (Slumberg and Silver, 1991); and ZUOl (Zhang et al., 1992).

growth defect was relieved by transformation with a plasmid (pMDJ315) containing the complete MDJ7 coding and regulatory sequences. However, the transformed strain (YNR5c) remained petite and failed to grow on nonfermentable carbon sources.

The cells containing the disruption in MDJ7 had lost their mitochondrial DNA (i.e., they were in the rho- state). This was concluded from the lack of restoration of respiratory competence after genetic crossing with mit+ tester strains (possessing point mutations in the mitochondrial genome) from fluorescence microscopy of DAPI-stained cells and the absence of mitochondrial DNA bands in CsCl gradients (data not shown).

We conclude that the inability of the mdj1-disrupted cells to grow at elevated temperatures is directly related to the lack of the MDJ7 gene product and that Mdjlp is required for the maintenance of mitochondrial DNA. The MDJ1 gene product is therefore essential for normal mitochondrial function.

Mdjlp Is Located on the Matrix Side of the Inner Mitochondrial Membrane

The predicted Mdjlp sequence contains an amino-terminal extension relative to the E. coli DnaJ gene product with the characteristics of a mitochondrial targeting sequence (Hartl et al., 1989). Two approaches were used to analyze the subcellular location of Mdjlp. First, antibodies were raised against the carboxy-terminal half of Mdjlp and were used for immunoblotting of yeast subcellular fractions. As shown in Figure 4A, Mdjlp was observed in the mitochondrial fraction and could not be detected in the cytosolic or nonmitochondrial membrane fractions.

In the second approach, radiolabeled Mdjlp was synthesized in vitro and was incubated with isolated yeast mitochondria. A major translation product with the apparent molecular mass of 55 kd was observed and was imported into a protease-protected location. Import was dependent upon a membrane potential and was accompanied by processing to a 49 kd protein (Figure 4B). The predicted amino acid sequence of the Mdjlp precursor contains a typical matrix processing site (Arretz et al., 1991) between residues 55 and 56 (IRNIN) that is consistent with the apparent 6 kd difference in molecular weight observed upon import and processing. Both the precursor and the mature Mdjlp...
Figure 4. Biochemical Localization of Mdj1p

(A) Subcellular localization. Markers for the different subcellular fractions were ADP/ATP carrier for mitochondria, Kar2p for microsomes (the monoclonal antibody against the HDEL peptide used preferentially recognized Kar2p), and fructose 1,6-bisphosphatase (FBPase) for cytosol.

(B) Import of in vitro-synthesized Mdj1p precursor into isolated mitochondria. Mdj1p precursor protein was synthesized in reticulocyte lysate and was incubated for 20 min at 25°C with isolated mitochondria from the wild-type S. cerevisiae strain, D273–10B. After the import reaction, samples were treated with proteinase K where indicated (PK). Dissipation of the membrane potential (ΔΨ) was achieved by the addition of carbonyl cyanide m-chlorophenylhydrazone (CCCP) to a final concentration of 30 μM prior to import. As a control, 40% of the precursor protein added to imports was loaded, p, precursor; m, mature Mdj1p.

(C) Submitochondrial localization. Lane 1, intact mitochondria; lane 2, mitoplasts; lane 3, proteinase K–treated mitoplasts; lane 4, membrane protein fraction after carbonate extraction of mitoplasts; lane 5, soluble protein fraction after carbonate extraction of mitochondria; lane 6, soluble protein fraction after sonication of mitoplasts; lane 7, soluble matrix protein fraction after sonication of mitochondria. Marker proteins for the different mitochondrial subcompartments were cytochrome b2 (cyt b2) for the intermembrane space; ADP/ATP carrier (AAC) for the inner membrane; and Ssc1p (mitochondrial Hsp70) for soluble matrix proteins. Note that proteinase K treatment of mitoplasts generated the typical degradation fragment of the AAC (Rassow and Pfanner, 1991).
30°C, and 37°C with the F$_{\beta}$-ATPase precursor (Figure 5A). Similar results were obtained with the precursors of cytochrome b$_2$, MOM38, cytochrome c heme lyase, ADP/ATP carrier, and Mdj1p, which were all imported with the same efficiency into mitochondria isolated from the ∆mdj1 and rho$^0$ strains (data not shown).

The only precursor whose processing pattern differed in mitochondria of the ∆mdj1 strain relative to the controls was the Rieske iron–sulphur (Fe–S) protein. This precursor was efficiently imported into mitochondria from the ∆mdj1 strain, but the second processing step (by the mitochondrial intermediate processing peptidase) was strongly reduced relative to the controls (Figure 5B). The same reduction in mature form was also observed after immunoblotting of ∆mdj1 mitochondrial protein with antiserum against the Fe–S protein (data not shown), indicating that the intermediate form of Fe–S protein accumulates in ∆mdj1 mitochondria.

Mitochondria Lacking Mdj1p Show a Deficiency in Protein Folding of Newly Imported Precursor

The possible involvement of Mdj1p in the processes of protein folding was investigated. The Su9–DHFR fusion protein, which consists of the presequence of Neurospora crassa F$_2$-ATPase subunit 9 preceding the complete mouse dihydrofolate reductase (DHFR) (Pfanner et al., 1987), was imported into mitochondria isolated from ∆mdj1 and from isogenic wild-type and rho$^0$ cells. As a measure of correct protein folding, protease resistance of the processed DHFR fusion protein was determined at various time intervals after import.

Urea-denatured precursor protein was efficiently imported into mitochondria from all three strains at 25°C (Figure 6A). After solubilization of mitochondria with digitonin, soluble and nonsoluble fractions were separated by centrifugation. Both fractions were then treated with 10 μg/ml proteinase K, as folded DHFR has been shown to be resistant to this concentration of the protease (Ostermann et al., 1989). A striking difference was observed between the levels of protease-sensitive insoluble protein in the wild-type and ∆mdj1 mitochondria; 20% of the imported protein was found in aggregates (Figure 6A). There was a markedly lower level of insoluble aggregates in mitochondria isolated from the isogenic rho$^0$ control strain, indicating that this accumulation was directly related to the absence of Mdj1p and was not due to the rho$^0$ state of the mitochondria.

A more pronounced difference was observed when import reactions were performed at 37°C (Figure 6B). After a 6 min import reaction, a negligible amount of aggregated DHFR was detected in mitochondria isolated from the wild-type strain, compared with 25% and 70% of the imported protein recovered in the insoluble fraction of the rho$^0$ and ∆mdj1 mitochondria, respectively. This aggregated form

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**Figure 5. Import of Radiolabeled Precursor Proteins into Isolated Mitochondria**

Mitochondria from the ∆mdj1 strain, the isogenic wild-type (WT), and the isogenic rho$^0$ strain were analyzed.

(A) Import of F$_{\beta}$ at 25°C, 30°C, or 37°C. Mitochondria were preincubated at the respective temperatures for 10 min prior to addition of the radiolabeled precursor protein. After 2.5 min or 5 min, respectively, the import reaction was stopped by addition of 4 vol of ice-cold SMKCI (250 mM sucrose, 80 mM KCl, 10 mM MOPS–KOH [pH 7.2]) and 1 μM valinomycin. One half of each sample was treated with proteinase K (PK), and the other half remained untreated. As a control, nontreated precursor protein is shown.

(B) Import of the Rieske Fe–S protein. The import reaction was performed at 25°C for 5 min, 10 min, or 25 min and was stopped as described above. Again, one half of each sample was proteinase K–treated, p, precursor; i, intermediate; m, mature form.
The ability to fold DHFR of mitochondria isolated from wild-type (WT), null mutant (Δmdj1), and null mutant complemented with MDJ1 (rho°) was analyzed at 25°C (A) and 37°C (B). Mitochondria were preincubated for 3 min (25°C) or 10 min (37°C) prior to the addition of urea-denatured radiolabeled Sus-DHFR precursor. After the indicated time periods, the import reaction was stopped, and nonimported Sus-DHFR precursor was digested by trypsin treatment. The reisolated mitochondria were then solubilized with 0.4% digitonin, and aggregated proteins were sedimented. The folding state of the soluble (supernatant) and insoluble (pellet) fractions of DHFR were analyzed by treatment with 10 μg/ml proteinase K. The amount of imported mature-form Sus-DHFR present in the pellet and the supernatant was measured by densitometry of SDS-PAGE fluorographs. The results are expressed in arbitrary densitometric units as the mean ± SEM of three independent experiments. The total amount of imported DHFR is indicated by closed circles, DHFR present in the pellet before proteinase K treatment is indicated by closed squares, and DHFR present after proteinase K treatment is indicated by open squares.

The folding of the DHFR protein was found to be completely imported into the matrix and did not represent an import intermediate as it cofractionated with matrix markers after digitonin extraction (data not shown). The observed increase in levels of insoluble protein in the Δmdj1 mitochondria at 37°C compared with 25°C, and relative to the controls at either temperature, is suggestive of a block at a specific stage in the protein folding process in Δmdj1 mitochondria at the elevated temperature.

To investigate the apparent block in protein folding with a protein whose native conformation can be directly tested by the enzymatic activity, a hybrid protein consisting of the Sus-ATPase presequence fused to firefly luciferase was imported into mitochondria isolated from the Δmdj1 and control yeast strains. After import at 25°C, folding of the imported luciferase protein to the native conformation was monitored by the level of enzymatic activity and was correlated with the total amount of protein imported. As shown in Figure 7, luciferase enzyme activity in Δmdj1 mitochondria was reduced by 70% relative to the controls. This result thus supports our conclusion that Mdj1p is actively involved in processes that mediate the folding of newly imported proteins.

Heat Treatment of Δmdj1 Mitochondria Leads to the Accumulation of Misfolded Protein Aggregates
Mdj1p is a heat shock protein, and therefore we investigated the possible involvement of Mdj1p in protecting heat-denatured protein from aggregation. Sus-DHFR fusion protein was imported into mitochondria of the Δmdj1, isogenic wild-type, and isogenic rho° strains. After an import time of 6 min at 25°C, nonimported precursor was digested by trypsin, and the mitochondria were incubated at 40°C for different intervals of time. Protease sensitivity of DHFR was determined as a percentage of the total imported material. The level of protease-resistant material decreased markedly in the isolated Δmdj1 mitochondria relative to the controls, which showed a decrease of approximately 20% over a period of 45 min (Figure 8A). Prolonged incubation of mitochondria at 25°C and 30°C did not result in an increase in protease sensitivity (Figure 8B). Therefore, the decrease in the levels of protease-resistant (native) DHFR at elevated temperature in the Δmdj1 mitochondria was a direct consequence of heat denaturation of the imported protein.

In summary, these results indicate that Mdj1p plays a
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Figure 6. Accumulation of Incorrectly Folded DHFR upon Heat Treatment

Role in the prevention of heat-induced protein aggregation after proteins have been folded to the native conformation.

Discussion

Mitochondrial Hsp70, Ssc1p, has been shown to play a central role in various processes of mitochondrial biogenesis. It is required for the translocation of many proteins across the mitochondrial membranes, and it is critical for the unfolding of precursor polypeptides outside the mitochondria during translocation (reviewed by Neupert et al., 1990; Neupert and Pfanner, 1993). However, it remains to be determined whether Ssc1p, in conjunction with ATP, is alone sufficient for these functions or whether additional components are involved in these rather complex reactions. The expectation that additional proteins are involved has been nourished by the observation that in E. coli the Hsp70 homolog, DnaK, acts in concert with two other heat shock proteins, DnaJ and GrpE (reviewed by Georgopoulos, 1992). Furthermore, in S. cerevisiae, the Hsp70 homologs Ssa1p and Kar2p are thought to interact with the DnaJ homologs Ydj1p and Sec63p, respectively (Caplan et al., 1992; Sadler et al., 1989; Feldheim et al., 1992).

The yeast heat shock protein described here, Mdj1p, is essential for cell viability at 37°C but not at 30°C. Furthermore, Mdj1p is necessary for maintenance of mitochondrial DNA. Thus, MDJ1 is essential for the formation of mitochondria competent for oxidative phosphorylation.

So what is the function of Mdj1p? Mitochondria, and consequently mitochondrial protein import, are indispensable in eukaryotic cells. In agreement with this notion, our experiments do not suggest an essential role for Mdj1p in assisting Hsp70 in its role of translocating polypeptide chains across the mitochondrial membranes into the matrix. Thus, it seems possible that mitochondrial Hsp70 can perform its role in mitochondrial protein import without the assistance of a DnaJ homolog. However, experiments with isolated mitochondria do not completely rule out some involvement of Mdj1p in these processes.

The lack of Mdj1p resulted in reduced competence of mitochondria to properly fold imported monomeric proteins. At 25°C, and especially at 37°C, imported DHFR had a propensity to aggregate in the null mutant mitochondria; the aggregated form was protease sensitive and, therefore, was in an improperly folded state. In contrast, almost no misfolded DHFR accumulated in wild-type mitochondria. Furthermore, when the Δmdj1 strain was complemented with the MDJ1 gene on a plasmid, the ability to correctly fold DHFR was restored to a large extent. Thus, the aggregation of imported DHFR in the null mutant is not due to the ρ0 state of the mitochondria, but is specifically related to the lack of Mdj1p.

Even more compelling evidence for a role of Mdj1p in protein folding reactions was obtained by assessing the amount of enzymatically active luciferase in Δmdj1 mitochondria after import in vitro. These mitochondria showed a 70% reduction in luciferase activity compared with the controls at physiological temperature. We therefore conclude that Mdj1p is involved in the folding of newly imported precursor proteins, and in particular, Mdj1p is important for folding at elevated temperature. Mdj1p does not only play a role in the folding of newly imported proteins, but it also appears to prevent aggregation of folded proteins.

The degree of Mdj1p-mediated protection against heat denaturation and its requirement for protein folding probably depends upon the protein. DHFR and luciferase are nonmitochondrial tester proteins, and therefore the observed effects in the null mutant mitochondria may be in-
termediate. Accordingly, the lethality of mdj1 disruption at elevated temperature may be a result of the dysfunction of a set of proteins that are critical for cell viability.

The means by which both protein folding and protection processes are mediated by Mdj1p remains to be analyzed. Very likely, Mdj1p cooperates with mitochondrial Hsp70 in these processes, although this leaves the intriguing question of why such cooperation is apparently unnecessary for the function of Hsp70 in the translocation of proteins into mitochondria. One could imagine that the binding of Hsp70 to the incoming unfolded precursor does not need to be promoted by a DnaJ-like protein, as components of the import apparatus could present the extended polypeptide chain to Hsp70. Furthermore, it cannot be excluded that a second DnaJ-like component may reside in the mitochondria and cooperate with Hsp70 specifically in the translocation process. It is very unlikely that this DnaJ homolog is Scj1p (Blumberg and Silver, 1991), since Scj1p is probably not localized in mitochondria and since Scj1, Mdj1 double mutants were found to be viable (P. Silver, personal communication; N. R., unpublished data).

Any discussion of protein folding has to consider the role of Hsp60 (the MIF4 gene product) in these processes. In a recent in vivo analysis of a temperature-sensitive mif4 allele, Martin et al. (1992) described a strikingly similar reduction in the levels of folded mitochondrial protein as that which we observe in vitro with mitochondria of the mdj1 disruption strain. Consistent with a model of the protein folding processes (Langer et al., 1992) where GroEL/GroES acts in a step subsequent to DnaK, DnaJ, and GrpE, the absence of Mdj1p could result in a similar phenotype as that of an Hsp60 mutation. Consequently, this would imply that Mdj1p itself is required for the action of Hsp60; this could be explained if Mdj1p mediates the passage of polypeptides to Hsp60.

The data presented here provide the first indication for a function of a DnaJ homolog in protein folding in eukaryotic cells. Thus, our conclusions conform with the findings by Langer et al. (1992) and by Schroder et al. (1993) which are very likely that this DnaJ homolog is Scj1p (Blumberg and Silver, 1991), since Scj1p is probably not localized in mitochondria and since Scj1, Mdj1 double mutants were found to be viable (P. Silver, personal communication; N. R., unpublished data).

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The observation that Mdj1 disruption causes loss of mitochondrial DNA is intriguing. Deficiency of protein folding in mitochondria lacking Mdj1p may indirectly influence mitochondrial translation and thereby DNA replication, as mitochondrial DNA replication has been shown to be dependent upon ongoing translation (Myers et al., 1985). Since the DnaJ homolog Sis1p is directly involved in cytosolic translation (Zhong and Arndt, 1993), Mdj1p might also play a direct role in mitochondrial protein synthesis, which could then explain the loss of mitochondrial DNA in the null mutant. Alternatively, it is also conceivable that Mdj1p plays a direct role in mitochondrial DNA replication, as its E. coli homolog, DnaJ, is involved in bacteriophage λ and phage P1 DNA replication (Zylitch et al., 1989; Wickner et al., 1991).

Mdj1p constitutes an additional mitochondrial protein that appears to have been conserved during the evolution of mitochondria from their endosymbiotic ancestors. Like its prokaryotic homolog in E. coli, DnaJ, Mdj1p is involved in processes such as protein folding and protection against heat denaturation. Its role in mitochondrial DNA replication and protein synthesis remains to be determined. It is however, tempting to speculate that similar to the situation in the prokaryotic system, Mdj1p exerts its functions in cooperation with Scj1p and possibly also with a mitochondrial homolog of bacterial GrpE.

**Experimental Procedures**

**Culture Conditions and Genetic Analysis**

Yeast cell growth conditions, sporulation, and tetrad dissection were performed according to Sherman et al. (1998). Yeast cells were made competent for transformation by the DMSO-enhanced method (Hill et al., 1991).

**Strains and Plasmids**

For biophysical analysis, mitochondrial and other subcellular fractions were prepared from yeast strain D273-108. For genetic analysis, yeast strain a/a735 (Newman and Norman, 1991) and its derivatives were used. The disrupted heterozygous diploid was named a/aYNS3 (a/a735 with Mdj1::URA3). A spore disrupted in Mdj1 derived from the tetrad analysis of a/aYNS3 and further investigated is referred to as YNS5 (MATa mdj1::URA3). The isogenic rho0 control strain was constructed by transforming strain YNS5, which had lost its mitochondrial DNA, with a CEN plasmid pMJD316, yielding strain YNP60. pMJD315 contains the Mdj1 coding and regulatory sequences on a 2.8 kb HindIII-SacI fragment of λ-cloned yeast genomic DNA inserted into pPH315 (Sisoe and Heeier, 1999). As confirmed by immunoblotting, the level of expression of Mdj1p in the complemented strain was similar to that in the wild-type (data not shown). The bacterial host strains used were TG1 (Gibson, 1994) and XL1 blue (Stratagene) for the overexpression of recombinant protein. To express the Mdj1p gene in vitro, a PCR fragment, Mj23, containing the coding sequence of Mdj1 was inserted into the expression vector pGEM4, placing the Mdj1 coding sequences under the control of the SP6 promoter.

**DNA Sequencing**

The mapped library of λ and oomycete clones of S. cerevisiae (strain AB972) was supplied by M. Olson (Olson et al., 1996; Riles et al., 1993). A random sequencing strategy (Bankier et al., 1987) was used to generate DNA sequence by the chain termination method (Sanger et al., 1977) using fluorescence-tagged dye primers and an ABI 370A fluorescent sequencer (Smith et al., 1993). The sequence was determined on both strands and compiled with the XBASE computer program (Dear and Staden, 1991). Nucleotide and protein comparisons were performed with the FASTA program (Pearson and Lipman, 1988) against the PIR (release 31, 1991), SWISS PROT (release 20, 1991), and the EMBL (release 29, 1991) databases.

**Southern Blot Analysis and Construction of PCR Fragments**

Total genomic DNA was isolated from yeast cells as described by Philipp et al. (1991), and Southern blot analysis was performed as described (Southern, 1975). DNA probes were prepared by PCR and labeled with 32P-dCTP (Amersham) by random priming (Promega). PCR fragments Mj13 and Mj23 were generated using 5' primers: Mj1, 5'-GGTCTAGAGAATGTCGCTAACG-3'; bases 231-211; and Mj2, 5'-CTTCCATGACTTCTAAATACATAC-3'; bases 19-44, respectively; and the 3' primer Mj3 (5'-CTTACACGCTTTCTTAAAATACATAC-3'; bases 1746-1766).

Hybridization of radiolabeled DNA probes and subsequent washing steps were performed under stringent conditions at 65°C (Church and Gilbert, 1984).

**Disruption of the Mdj1 Gene**

A disruption allele of Mdj1 was constructed in plasmid pDJ-1 by inserting a blunt-ended 1.1 kb Smal-ClaI fragment from YE24 containing the URA3 gene into the SmaI site of the Mj13 PCR fragment cloned into the SmaI site of pBS KSI'. This construct resulted in the deletion of almost the entire mature part of the protein. After
transformation of strain a/a735, the correct insertion of the URA3 gene and were further purified on a sucrose step gradient consisting of 20%, 30%, 40%, 50%, and 80% sucrose (w/v) in 10 mM MOPS-KOH (pH 7.4), 100 mM KCl, 1 mM EDTA, and 1 mM PMSF. The gradient was centrifuged at 940,000 × g for 15 min at 2°C in a Beckman SW41 rotor. Mitochondria were collected from the band between the 40% and 50% sucrose layers, were concentrated by centrifugation at 12,000 × g for 10 min at 2°C, were washed in SEM (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH [pH 7.2]), and finally were resuspended in SEM.

Preparation of microsomes and the cytosolic fraction was essentially carried out as described (Caplan and Douglas, 1991), with the modification that for the preparation of microsomes the micrococcal nuclease treatment was omitted. The membrane band from the 30% (v/v) Percoll gradient was directly concentrated by pelleting at 150,000 × g for 1 hr at 2°C in a Beckman Ti50 rotor and used for immunoblotting.

Subfractionation of Mitochondria

For generation of mitoplasts, mitochondria were resuspended in ice-cold hypotonic buffer (2 mg/ml mitochondrial protein, 20 mM HEPES-KOH [pH 7.4], 1 mM MgSO4), and were incubated on ice for 25 min with gentle vortexing every 5 min. As a control, mitochondria were resuspended in ice-cold isotonic buffer (0.6 M sorbitol, 20 mM HEPES-KOH [pH 7.4], 1 mg/ml BSA) and were incubated on ice without vortexing. For protease treatment of mitoplasts, 100 μg/ml proteinase K was added to the swelling buffer. Protease treatment was stopped by adding PMSF to a final concentration of 1.5 mM, followed by 5 min of incubation on ice. After swelling, mitoplasts were pelletted by centrifugation at 12,000 × g for 10 min at 2°C. For carbonate extraction, the mitoplasts were resuspended in 0.1 M Na2CO3 and 1 mM PMSF, were incubated for 30 min on ice, and were centrifuged at 226,000 × g for 1 hr at 2°C in a Beckman Ti50 rotor. The pellet was taken as the membrane protein fraction, and the supernatant was taken as the nonmembrane protein fraction. For sonication, mitoplasts were resuspended in 20 mM HEPES-KOH (pH 7.4), 100 mM KCl, and 1 mM MgSO4, were subjected to sonication with a Branson Sonifier 250 connected to a microtip (60% duty cycle, 15 pulses of 10 s [each]), were centrifuged at 226,000 × g for 1 hr at 2°C in a Beckman Ti50 rotor. The pellet was taken as the vesicle fraction, and the supernatant was taken as the soluble matrix protein fraction. All samples were precipitated with trichloroacetic acid (TCA) and were solubilized in 1% sodium dodecyl sulfate (SDS) buffer, were resuspended in SEM, 100 mM KCl, and were treated with 200 μg/ml proteinase K for 30 min on ice. Subsequently, 1 mM PMSF was added, and the mitochondria were washed in SEM containing 1 mM PMSF. Half of each sample was analyzed by SDS-PAGE to determine the amount of imported protein by densitometry. For the determination of luciferase activity, the second half was resuspended in the import buffer. Luciferase activity from resuspended mitochondria was determined in 30 μl of assay buffer (25 mM glycyl-glycin, 15 mM MgSO4, 5 mM ATP [pH 7.8]). The test tube was then placed in a luminometer (Berthold, Lumat LB9501), and the reaction was started by the injection of 0.1 μl luciferin. For each time point, the measured activity was related to the amount of imported luciferase. The specific luciferase activity determined in wild-type mitochondria after 60 min of import was set to 1.

Heat Denaturation of Imported DHFR

Urea-denatured Su9-DHFR was first imported into mitochondria for 6 min at 25°C as described above, except that the import reaction was then stopped by the addition of 1 μg/ml valinomycin. After trypsin digestion of the nonimported protein (see above), the mitochondria were resuspended in 40°C for 15, 30, and 45 min or at 25°C, 30°C, and 40°C for 30 min. Samples were then diluted with 4 vol of SEM, were resolated, and were lysed with 0.4% digitonin in SEM, 100 mM KCl. Proteinase K sensitivity of the samples was then determined as described above.

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