

## Mitochondrial GrpE Modulates the Function of Matrix Hsp70 in Translocation and Maturation of Preproteins

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**Mitochondrial GrpE (Mge1p) is a mitochondrial cochaperone essential for viability of the yeast *Saccharomyces cerevisiae*. To study the role of Mge1p in the biogenesis of mitochondrial proteins, we isolated a conditional mutant allele of *MGE1* which conferred a temperature-sensitive growth phenotype and led to the accumulation of mitochondrial preproteins after shifting of the cells to the restrictive temperature. The mutant Mge1 protein was impaired in its interaction with the matrix heat shock protein mt-Hsp70. The mutant mitochondria showed a delayed membrane translocation of preproteins, and the maturation of imported proteins was impaired, as evidenced by the retarded second proteolytic processing of a preprotein in the matrix. Moreover, the aggregation of imported proteins was decreased in the mutant mitochondria. The mutant Mge1p differentially modulated the interaction of mt-Hsp70 with preproteins compared with the wild type, resulting in decreased binding to preproteins in membrane transit and enhanced binding to fully imported proteins. We conclude that the interaction of Mge1p with mt-Hsp70 promotes the progress of the Hsp70 reaction cycle, which is essential for import and maturation of mitochondrial proteins.**

The majority of mitochondrial proteins are synthesized on cytosolic polysomes and are imported into the organelle in a multistep process (reviewed in references 12, 17, 22, 29, and 41). Cytosolic cofactors maintain the precursor proteins in a translocation-competent conformation. Receptor proteins on the mitochondrial surface and a general insertion pore mediate translocation of the preproteins across the outer membrane. Translocation across the inner membrane requires the membrane potential  $\Delta\psi$  and the mitochondrial inner membrane proteins Mim17, Mim23 (Mas6), and Mim44 (Isp45) (6, 11, 29). The translocating polypeptide chain interacts first with Mim44 and then with the 70-kDa heat shock protein in the matrix (mt-Hsp70; Ssc1p in *Saccharomyces cerevisiae*). mt-Hsp70, acting as a molecular chaperone, is essential to drive the movement of precursor polypeptides across the inner membrane (7, 14, 41). mt-Hsp70 reversibly binds to Mim44 in a nucleotide-sensitive complex (16, 33, 39), resulting in a tethering of mt-Hsp70 to the site of entry of the translocating polypeptide into the matrix. The preprotein is handed over from Mim44 to the Hsp70, which promotes complete import of the polypeptide chain by a combination of pulling and trapping (8, 30). In addition, mt-Hsp70 initiates the refolding of imported proteins that is typically completed in conjunction with the chaperonin system of Hsp60 (2, 14, 27).

Hsp70s are highly conserved molecular chaperones consisting of an N-terminal ATPase domain and a more C-terminal peptide-binding domain. The most highly evolved understanding of Hsp70 function has come from studies with DnaK, an Hsp70 of *Escherichia coli* (20, 21, 24, 28, 38, 42). Current models assert that the ATP-bound form of DnaK initially binds to substrates with high affinity and yet also rapidly releases the substrates and thus can be considered a “fast-binding/fast-

release” form of Hsp70. The ADP-bound form of DnaK is relatively slow in binding substrates, but it is also slow in releasing substrates, favoring more stable binding, and can be considered the “slow-binding/slow-release” form of DnaK. ATP hydrolysis of DnaK-ATP with bound polypeptide converts the fast-binding/fast-release form of Hsp70 to the slow-binding/slow-release form. DnaK cooperates with the two cochaperones DnaJ and GrpE. DnaJ is a chaperone in its own right and binds substrate proteins. In addition, DnaJ interacts directly with DnaK, stimulating its ATPase activity. GrpE acts to mediate nucleotide release from DnaK and is thereby essential for the reaction cycle of DnaK. In eukaryotic cells, Hsp70s have been detected in several compartments, including the cytosol, endoplasmic reticulum, and mitochondria. Numerous DnaJ-like proteins which cooperate with Hsp70 partners have been found in eukaryotes (3). Mitochondrial DnaJ of *S. cerevisiae* (Mdj1p) is involved in folding of proteins in the matrix and yet is dispensable for membrane translocation of preproteins (35).

A eukaryotic GrpE homolog has only recently been detected in the mitochondria of *S. cerevisiae* and *Neurospora crassa* (1, 13, 19, 45). This mitochondrial GrpE, termed Mge1p, forms a complex with mt-Hsp70 which is disrupted by ATP. Accumulation of precursor forms of preproteins has been observed in cells having smaller than normal amounts of Mge1p (19, 25). mt-Hsp70 and Mge1p have been found complexed with preproteins traversing the mitochondrial membranes (45). Together these results suggest a role for Mge1p in the import of mitochondrial proteins.

However, since *MGE1* is essential for viability of yeast cells, a characterization of its functional importance in deletion mutants was not possible. Therefore, we generated a temperature-sensitive mutant of *MGE1*, termed *mge1-100*, which we used to investigate Mge1p functions. Mge1p influences both membrane translocation of preproteins and maturation of preproteins in the matrix, presumably by modulating the reaction cycle of mt-Hsp70 in its interaction with preproteins.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype and/or phenotype	Reference or source
<b>Strains</b>		
PK81	<i>MAT<math>\alpha</math> ade2-101 lys2 ura3-52 leu2-3,112 <math>\Delta</math>trp1 ssc1-2::LEU2</i>	7
Y164	<i>MAT<math>\alpha</math> arg3 his4-519 leu2-3,112 mif1</i>	32
PJ53	<i>MAT<math>\alpha</math> trp1-1/trp1-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ade2-1/ade2-1 can1-100/</i> <i>can1-100 GAL2<sup>+</sup>/GAL2<sup>+</sup> met2-<math>\Delta</math>1/met2-<math>\Delta</math>1 lys2-<math>\Delta</math>2/lys2-<math>\Delta</math>2</i>	13a
SL18	<i>trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 GAL2<sup>+</sup> met2-<math>\Delta</math>1 lys2-<math>\Delta</math>2 mge1-<math>\Delta</math>2</i> pSL31	This study
SL20, SL23	<i>trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 GAL2<sup>+</sup> met2-<math>\Delta</math>1 lys2-<math>\Delta</math>2 mge1-<math>\Delta</math>2</i> pSL40	This study
SL22, SL26	<i>trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 GAL2<sup>+</sup> met2-<math>\Delta</math>1 lys2-<math>\Delta</math>2 mge1-<math>\Delta</math>2</i> pSL20	This study
<b>Plasmids</b>		
pSL20	<i>Amp<sup>r</sup> CEN6 TRP1 ARSH4 MGE1</i>	This study
pSL31	<i>Amp<sup>r</sup> CEN6 URA3 ARSH4 MGE1</i>	This study
pSL40	<i>Amp<sup>r</sup> CEN6 TRP1 ARSH4 mge1-100</i>	This study

## MATERIALS AND METHODS

**S. cerevisiae strains and plasmids.** Yeast strains and plasmids that were used in this study are listed in Table 1. SL23 and SL26 are YPG-adapted derivatives of SL20 and SL22, respectively, which maintain a temperature-sensitive growth phenotype on both fermentable and nonfermentable carbon sources. Standard methods were used for construction of recombinant plasmids (36) and maintenance and manipulation of yeast strains (9).

**Isolation of mutant alleles of MGE1.** A new *MGE1* disruption mutant allele, *mge1- $\Delta$ 2*, was constructed. The *HindIII* site in *MGE1* was rendered blunt with Klenow polymerase and religated. Subsequently, a 2.8-kb *LEU2* fragment was inserted into the *BglII* site of the modified *MGE1* gene to generate the *mge1- $\Delta$ 2* mutant allele. Transformants of the diploid strain PJ53 having a genomic copy of the disrupted allele were selected on leucine-omission plates. The integration of the *mge1- $\Delta$ 2* allele in this strain (SL11) was confirmed by Southern blot analysis. The plasmid pSL31 carrying a copy of the *MGE1* gene was transformed into the above-described strain, and transformants carrying the plasmid were selected on uracil-omission plates. The transformants were sporulated, and the tetrads were dissected to obtain a haploid strain (SL18) having a disrupted genomic copy of *MGE1* rescued by the plasmid pSL31.

For isolation of temperature-sensitive mutant alleles of *MGE1*, a plasmid (pSL20) carrying the *MGE1* gene and *TRP1* as a yeast selectable marker was mutagenized in vitro with hydroxylamine. The mutagenized DNA was amplified in *E. coli* and used for the transformation of the yeast strain SL18. Screening for temperature-sensitive alleles of *MGE1* was done by plasmid shuffling on 5-fluoroorotic acid (5-FOA) plates to select against the plasmid pSL31. *Trp<sup>+</sup>* transformants were replica plated on 5-FOA plates and incubated at 18, 30, 35, and 37°C. Plasmids were isolated from strains having a temperature-sensitive phenotype, and DNA fragments (*PstI-XhoI* and *BglII-XhoI*) containing possible *mge1-ts* alleles were subcloned into pSL20. After retransformation to SL18 the resistant strains were retested for temperature-sensitive growth on 5-FOA plates. The DNA sequence was determined by the dideoxynucleotide chain termination method using Sequenase (United States Biochemical). A strong temperature-sensitive mutant of *MGE1* was identified in this manner (*mge1-100*), and a haploid strain (SL20) that had lost the plasmid carrying the wild-type *MGE1* gene was obtained by counterselection against plasmid pSL31 with 5-FOA.

**Analysis of precursor accumulation in vivo.** Haploid yeast strains carrying either a mutant (SL20) or a wild-type (SL22) *MGE1* gene were cultured in liquid YPD medium (9) at 23°C to an optical density at 600 nm of 0.5 to 1. The cultures were divided into two parts; one was shifted to 37°C, and the other was maintained at 23°C. Cells were harvested at various time intervals after the shift (0.5, 1, and 1.5 h), and total cell lysates were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting) using Hsp60 antiserum as described previously (19).

**Import of radiolabeled precursors into isolated mitochondria in vitro.** Mitochondria were isolated from the mutant (SL23) and the isogenic wild-type control strain (SL26) grown at 23°C in YPEGlycerol medium (1% yeast extract, 2% peptone, 2% ethanol, 3% glycerol [pH 4.5]) as described previously (5, 10). Radiolabeled precursor proteins were synthesized by translating in vitro-transcribed RNA (with SP6 RNA polymerase) in a reticulocyte lysate translation system using [<sup>35</sup>S]methionine (Amersham). Mitochondria were preincubated at 37°C for 15 min prior to import at 25°C. The import assay mixture contained 2 to 6 mM ATP, 2 to 6 mM NADH, 5 mM methionine, and mitochondria (corresponding to a final concentration of 0.25 mg of mitochondrial protein per ml), and the assay was performed in P80 buffer (10 mM MOPS [morpholine-propanesulfonic acid; pH 7.2], 5 mM MgCl<sub>2</sub>, 80 mM KCl, 250 mM sucrose, 3% [wt/vol] bovine serum albumin [BSA]) in a final assay volume of 200  $\mu$ l. Import was terminated by addition of valinomycin (0.5  $\mu$ M), antimycin A (8  $\mu$ M), and

oligomycin (20  $\mu$ M) and transfer to ice. Half of each sample was treated with protease K (25 to 50  $\mu$ g/ml) for 15 min on ice. Protease was inactivated by addition of phenylmethylsulfonyl fluoride (PMSF; 2 mM). Mitochondria were reisolated, and import was analyzed by SDS-PAGE and digital autoradiography.

**Determination of the amount of Mge1p associated with mt-Hsp70-Mim44 complexes by coimmunoprecipitation.** Isolated yeast mitochondria (200  $\mu$ g) from strain SL26 or SL23 were preincubated for 15 min at 37°C in P80 buffer and pelleted. Lysis was performed by resuspending the mitochondrial pellet in 200  $\mu$ l of lysis buffer (0.3% [wt/vol] Triton X-100, 30 mM Tris-HCl [pH 7.4], 150 mM KCl, 5% glycerol, protease inhibitor mix [1.25  $\mu$ g of leupeptin per ml, 0.5  $\mu$ g of pepstatin per ml, 2  $\mu$ g of antipain per ml, 0.25  $\mu$ g of chymostatin per ml], 0.5 mM PMSF) and shaking the mixture vigorously for 5 min at 4°C with the addition of either 2 mM ATP-5 mM magnesium acetate or 5 mM EDTA. After a clarifying spin for 5 min at 15,000  $\times$  g and 4°C the supernatant was divided. Two-thirds was incubated with affinity-purified anti-Mim44 antibodies and one-third was incubated with anti-mt-Hsp70 antibodies (both coupled to protein A-Sepharose) for 1 h at 4°C with gentle agitation. After the mixture was washed three times with 200  $\mu$ l of lysis buffer, the bound proteins were eluted from the protein A-Sepharose by treatment with 200  $\mu$ l of 200 mM glycine, pH 2.5. The eluted proteins were recovered by trichloroacetic acid precipitation, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Millipore). The presence of Mim44, Ssc1p, and Mge1p was detected by immunodecoration with the respective antisera by using the ECL system (Amersham).

**Coimmunoprecipitation of b<sub>2</sub> $\Delta$ -DHFR with mt-Hsp70.** To induce the mutant phenotype, mitochondria of yeast strains SL26 and SL23 were preincubated for 15 min at 37°C at a concentration of 1.6 mg of mitochondrial protein per ml in P80 buffer before dilution to a final concentration of 0.25 mg/ml into the import mix. Imports were performed under standard conditions (44) for 30 min at 25°C. Membrane-spanning translocation intermediates were accumulated by the addition of 1  $\mu$ M methotrexate to the import reaction mixture and the preincubation of the precursor proteins in the presence of methotrexate for 10 min on ice. Import reactions were stopped by the addition of valinomycin (1  $\mu$ M) and by cooling on ice. Proteinase K digestion (0.1 mg/ml) of nonimported prepeptides was done for 15 min on ice and stopped by addition of PMSF (2 mM). Mitochondria were reisolated by centrifugation for 10 min at 16,000  $\times$  g and washed once with ice-cold SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH [pH 7.2]) containing 1  $\mu$ M methotrexate. Mitochondria were resuspended in lysis buffer (30 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM EDTA, 0.01% [wt/vol] BSA, 0.1% [wt/vol] Triton X-100, 1 mM PMSF) at 0.25 mg of mitochondrial protein per ml, and nonsolubilized material was removed by centrifugation for 10 min at 16,000  $\times$  g. Mt-Hsp70-associated proteins were coimmunoprecipitated for 1 h at 8°C by addition of protein A-Sepharose coupled anti-Ssc1p antibodies. Immune complexes were isolated by centrifugation, washed twice with 200  $\mu$ l of lysis buffer, and resolved by SDS-PAGE. Ssc1p-associated b<sub>2</sub> $\Delta$ -dihydrofolate reductase (DHFR) was visualized and quantified by digital autoradiography.

**Assessment of folding and aggregation of imported Su9-DHFR.** Import of radiolabeled Su9-DHFR into wild-type and *mge1-100* mutant mitochondria was performed either for 1 min at 25°C after urea denaturation of the precursor protein (27) or for 10 min at 37°C. Prior to the addition of the precursor protein, mitochondria were treated for 15 min at 37°C to induce the mutant phenotype. Import was terminated by dilution into 4 volumes of ice-cold SEM containing valinomycin (0.5  $\mu$ M), antimycin A (8  $\mu$ M), and oligomycin (20  $\mu$ M). Nonimported precursor protein was digested with trypsin (75  $\mu$ g/ml) for 15 min on ice. After addition of soybean trypsin inhibitor (1 mg/ml), mitochondria were reisolated by centrifugation and solubilized in SEM containing 100 mM KCl and 0.5% (wt/vol) Triton X-100. For the folding assay, proteins in the solubilized mitochondria were digested with proteinase K (10  $\mu$ g/ml) for 10 min on ice. After addition of 2 mM PMSF, the samples were centrifuged for 10 min at 18,000  $\times$

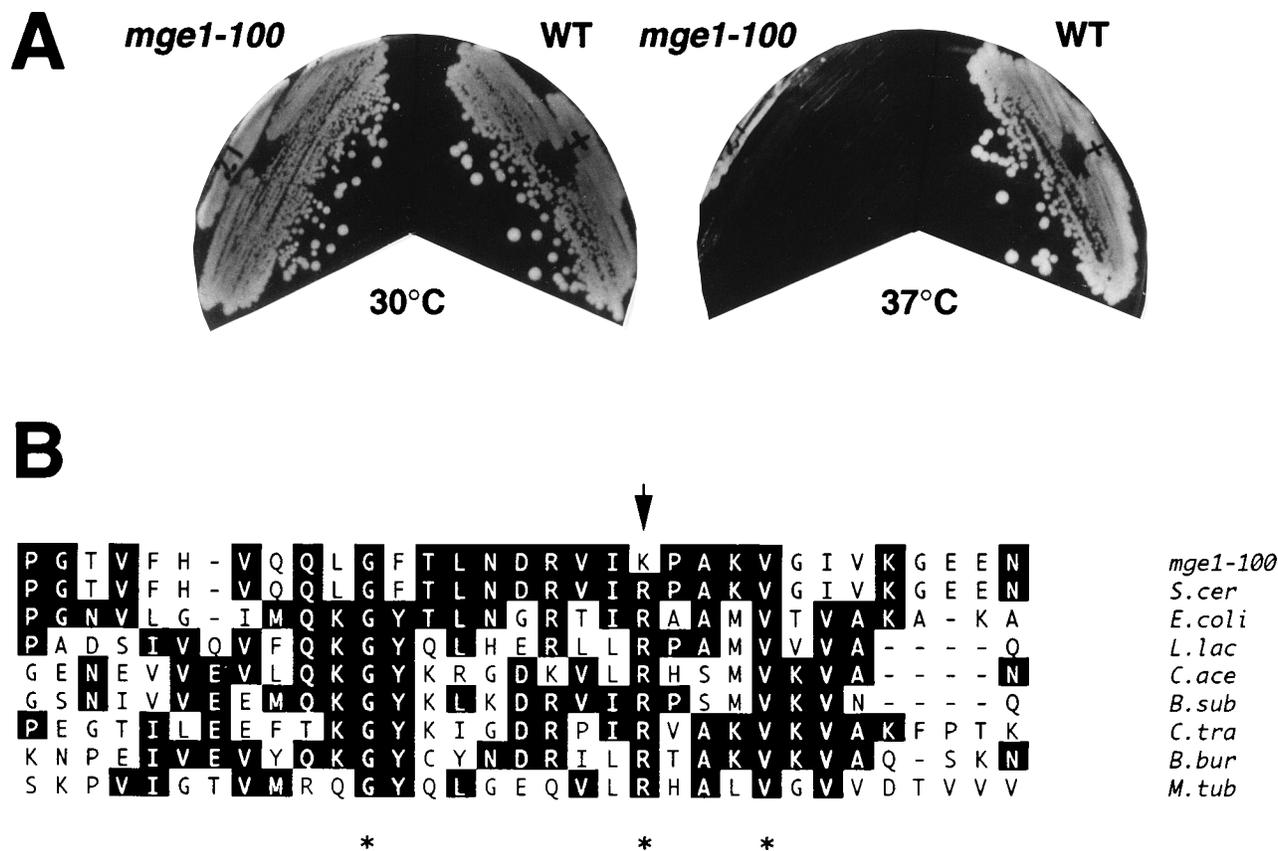


FIG. 1. Temperature-sensitive *S. cerevisiae* mutant, *mge1-100*, with a mutation in a highly conserved amino acid residue. (A) Temperature-sensitive growth of haploid *mge1-100* cells relative to wild-type cells. Cells from strains SL22 (wild type [WT]) and SL20 (*mge1-100*) were streaked on YPD plates (9) and incubated at 30 and 37°C for four days. (B) Alignment of the Mge1-100p sequence (32 C-terminal amino acids [residues 196 to 228]) with the deduced amino acid sequences of various GrpE proteins (wild-type Mge1p [*S. cer.*; U09565] and GrpEs of *E. coli* [X07863], *Lactococcus lactis* [*L. lac.*; Z19148], *Clostridium acetobutylicum* [*C. ace*; M74569], *Bacillus subtilis* [*B. sub*; M84964], *Chlamydia trachomatis* [*C. tra*; M62819], *Borrelia burgdorferi* [*B. bur*; M96847], and *Mycobacterium tuberculosis* [*M. tub*; X58406]; abbreviations used in the figure and GenBank accession numbers are given in brackets). The last 10 residues of the *C. trachomatis* protein and the last 43 residues of the *M. tuberculosis* protein are not included. Amino acid sequence identities are shaded. Hyphens denote gaps in the sequence that were inserted for optimal alignment. Asterisks denote conserved residues in all GrpE homologs. The arrow denotes the *mge1-100* mutation (R-216 to K) in a highly conserved residue.

g and 2°C to pellet aggregated material. The supernatants (folded material) were precipitated with trichloroacetic acid. For the aggregation assay, the treatment with proteinase K was omitted and the pellets (aggregated material) were dissolved in sample buffer. Analysis was carried out by SDS-PAGE and digital autoradiography.

## RESULTS

**A temperature-sensitive mutant allele of MGE1.** We isolated a temperature-sensitive mutant allele of *MGE1* (*mge1-100*) which is unable to complement a null allele of *MGE1* at elevated temperatures. A plasmid carrying the *MGE1* gene was mutagenized with hydroxylamine in vitro and introduced into a haploid yeast strain having a chromosomal null allele of *MGE1* and harboring a plasmid carrying wild-type *MGE1* and a counterselectable marker, *URA3*. Temperature-sensitive strains likely to have an *mge1*-ts allele were identified by plasmid shuffling, and subsequent characterization of the *MGE1* gene on the mutagenized plasmid was done as described in Materials and Methods. A strongly temperature-sensitive mutant which showed significantly reduced growth at 35°C and no growth at 37°C when compared with an isogenic control strain having a wild-type copy of *MGE1* instead of the *mge1-100* mutant was identified in this fashion (Fig. 1A). The temperature-sensitive growth defect of the mutant strain was observed on both fermentable and nonfermentable carbon sources. We observed an

increased frequency of petite colonies arising from different isolates of the mutant strain compared with the wild-type control strain. The studies reported here were performed only with [*rho*<sup>+</sup>] cells to avoid indirect effects due to deletions in the mitochondrial genome.

DNA sequencing of the *mge1-100* mutant allele revealed a G-to-A transition which results in the replacement of a highly conserved arginine residue (R-216) by a lysine residue. A comparison of the amino acid sequences of all known GrpE proteins reveals that R-216 is one of nine absolutely conserved amino acids in the GrpE gene family (Fig. 1B). The amounts of Mge1p in mutant and wild-type cells and isolated mitochondria were indistinguishable, (see below), indicating that the mutation affects the function of Mge1p and not its steady-state level in the cell.

**Accumulation of a mitochondrial preprotein in *mge1-100* cells in vivo.** Exponentially doubling cultures of a haploid yeast strain having the *mge1-100* mutant allele and an isogenic wild-type control strain were divided into two parts, one of which was maintained at 23°C and the other of which was shifted to the restrictive temperature (37°C). Total protein from cell lysates, prepared at various time intervals after the shift, was analyzed by Western blotting using antiserum directed against the matrix protein Hsp60. The mutant strain showed accumulation of the uncleaved precursor form of Hsp60 within 30 min

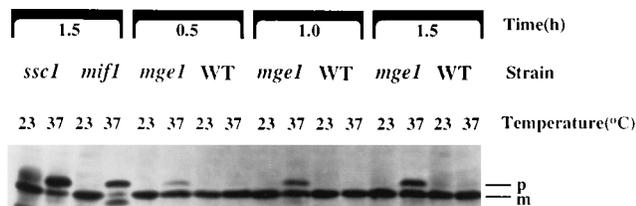


FIG. 2. Accumulation of mitochondrial precursor proteins at the nonpermissive temperature in *mge1-100* cells in vivo. Cultures of PK81 (with the *ssc1-2* mutation; *ssc1*), Y164 (with a temperature-sensitivity-conferring allele in the gene encoding the  $\beta$  subunit of matrix processing peptidase; *mif1*), SL20 (with the *mge1-100* mutation; *mge1*), and SL22 (wild type [WT]) grown at 23°C were split in half and shifted to 37°C or maintained at 23°C for 0.5, 1.0, and 1.5 h. Protein extracts corresponding to 0.12 optical density at 600 nm units were analyzed by immunoblotting with antisera against Hsp60. p, precursor; m, mature.

after the shift to the nonpermissive growth temperature (Fig. 2). The precursor form was not detected in the isogenic wild-type control strain after the shift to 37°C or in the mutant strain when it was maintained at 23 or 30°C.

**The mutant Mge1p is impaired in its interaction with mt-Hsp70.** Since Mge1p has been shown to quantitatively associate with mt-Hsp70 in wild-type cells (1, 45), we examined the association of the mutant Mge1p with the matrix Hsp70 (Ssc1p). Mitochondria were isolated from mutant and wild-type strains grown at the permissive growth temperature and preincubated for 15 min at 37°C to induce the mutant phenotype. The mitochondria were solubilized with nonionic detergent, and coprecipitations with antibodies directed against mt-Hsp70 were performed in the presence of EDTA. The amount of Mge1p coimmunoprecipitated with Ssc1p for the mutant strain was reduced by approximately 70% compared with that for the wild-type mitochondria (Fig. 3A; compare lanes 3 and 1). Upon addition of Mg-ATP, Mge1p was released from mt-Hsp70 (Fig. 3A, lanes 2 and 4). The total amounts of Mge1p were indistinguishable for mutant and wild-type mitochondria (Fig. 3B), excluding the possibility that the reduced amount of Mge1p found in complexes with mt-Hsp70 was due to a reduction of the mitochondrial content of Mge1p. Moreover, the amounts of mt-Hsp70 and of marker proteins for the inner membrane (ADP/ATP carrier), intermembrane space (cytochrome  $b_2$ ), and outer membrane (Mom38) were comparable for wild-type and mutant mitochondria (Fig. 3B), indicating that the protein composition of the isolated mutant mitochondria was not altered.

By coimmunoprecipitation with antibodies directed against Mim44, we determined if the mutation in *MGE1* influenced the binding of mt-Hsp70 to Mim44. Figure 3C demonstrates that the binding of mt-Hsp70 to Mim44 was not disturbed in *mge1-100* mitochondria.

**The amino acid alteration in Mge1p causes retardation of the membrane translocation of preproteins.** We asked if the impaired binding of Mge1-100p to mt-Hsp70 influenced the transport of preproteins across the mitochondrial membranes. The precursors of the  $\alpha$  subunit of mitochondrial processing peptidase (a matrix protein) and the  $\beta$  subunit of  $F_1$ -ATPase (destined for the inner side of the inner membrane) were synthesized *in vitro* in rabbit reticulocyte lysate in the presence of [ $^{35}$ S]methionine. The preproteins were then incubated with isolated energized mitochondria from wild-type and *mge1-100* cells. Cells were grown at the permissive temperature to minimize the chances of indirect effects due to the *mge1-100* mutation, and the isolated mitochondria were preincubated at 37°C to induce the mutant phenotype. The import reactions

were performed at 25°C in the kinetically linear ranges for import of the preproteins. Transfer of the N-terminal presequences into the matrix, evidenced by processing to the mature-sized forms, was only slightly reduced in the mutant mitochondria (Fig. 4A and B, upper panels, lanes 4 and 5) compared with the wild-type mitochondria (Fig. 4A and B, upper panels, lanes 1 and 2). However, complete import, measured by protease protection of the mature protein, was significantly retarded in the mutant mitochondria (Fig. 4A and B, lower panels; compare lanes 4 and 5 with lanes 1 and 2).

The degrees of stability of the mitochondrial membranes were indistinguishable for wild-type and mutant mitochondria, as assessed by the protease protection of marker proteins for the mitochondrial subcompartments (Mom38, cytochrome  $b_2$ , ADP/ATP carrier, Mim44, and mt-Hsp70; data not shown), excluding the possibility that the increased protease susceptibility of preproteins in the mutant mitochondria was caused by reduced integrity of the mitochondrial membranes. Moreover, we concluded that the retardation of membrane translocation

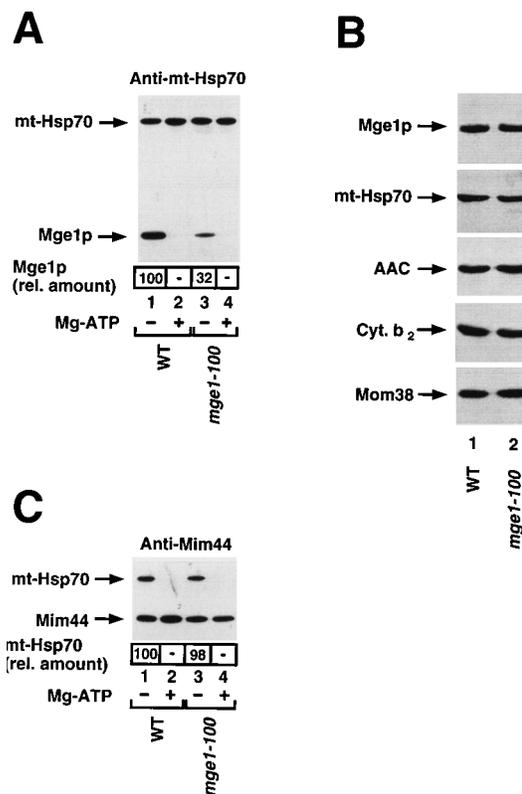


FIG. 3. Mutant Mge1p is impaired in binding to matrix Hsp70. (A) Cells were grown at the permissive temperature, and mitochondria were isolated and preincubated at 37°C for 15 min, a period sufficient to induce the mutant phenotype. After lysis with nonionic detergent, proteins interacting with mt-Hsp70 were coimmunoprecipitated with antibodies against mt-Hsp70 as described in Materials and Methods in the presence of 2 mM ATP–5 mM magnesium acetate (+) or 5 mM EDTA (–). The amounts of precipitated mt-Hsp70 and Mge1p were determined by Western blotting and quantified by densitometry. The amount of Mge1p coprecipitated for wild-type (WT) mitochondria was set at 100 rel. (B) Portions (10  $\mu$ g) of mitochondrial protein from WT and *mge1-100* cells were separated by SDS-PAGE and transferred to nitrocellulose. The indicated marker proteins were detected by immunodecoration with the specific antisera. AAC, ADP/ATP carrier. (C) Proteins associated with Mim44 were isolated as described for panel A except that affinity-purified antibodies against Mim44 were used and detected by immunodecoration with antisera against Mim44 and mt-Hsp70. The amount of mt-Hsp70 coprecipitated from wild-type mitochondria was set at 100.

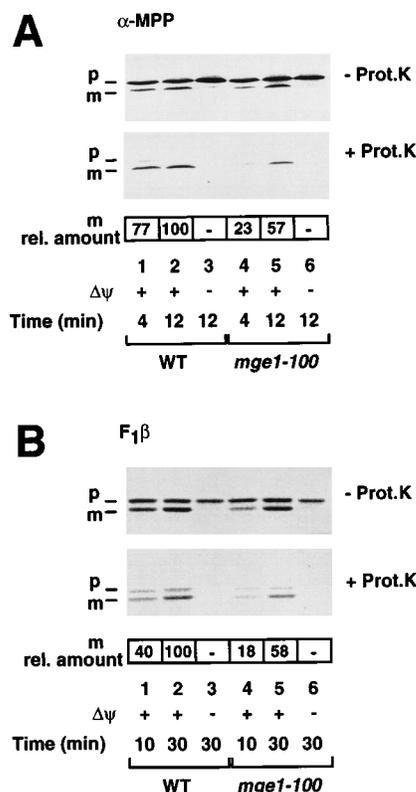


FIG. 4. Translocation of precursor proteins to a protease-protected location is retarded in *mge1-100* mitochondria. In vitro-synthesized precursors of the  $\alpha$  subunit of matrix processing peptidase ( $\alpha$ -MPP) (A) and the  $\beta$  subunit of  $F_1$ -ATPase ( $F_1\beta$ ) (B) were imported for the indicated periods at 25°C into mitochondria isolated from the *mge1-100* strain or the isogenic wild-type strain (WT). Prior to the import reactions, mitochondria were preincubated for 15 min at 37°C to induce the mutant phenotype. Incubations were carried out in the presence (+) or absence (-) of a membrane potential,  $\Delta\psi$ . For the lower panels, the samples were treated with proteinase K (Prot.K) after the import reaction. For quantification, the amount of mature protease-protected protein in wild-type mitochondria after the longer import time was set at 100. p, precursor form; m, mature form; rel., relative.

of preproteins was not caused by a reduction of the membrane potential  $\Delta\psi$  for the following reasons. Only translocation of the N-terminal presequence across the inner membrane requires  $\Delta\psi$ , while the mature part of preproteins is efficiently transported in the absence of  $\Delta\psi$  (23, 31, 37). Since the *mge1-100* mutation preferentially impaired translocation of the mature protein part, the translocation defect was not caused by a reduction of  $\Delta\psi$ . Therefore, we conclude that the mutant Mge1p leads to a retardation of membrane translocation of the mature part of preproteins.

**Maturation of proteins in the mitochondrial matrix is impaired in *mge1-100* mitochondria.** A hybrid protein composed of an N-terminal portion of the precursor of cytochrome  $b_2$  and the entire DHFR is of particular use for analyzing the function of mt-Hsp70 in maturation of imported proteins. The protein  $b_2\Delta$ -DHFR contains the matrix targeting signal of the cytochrome  $b_2$  precursor but lacks the intermembrane space sorting signal, and it is thus translocated into the mitochondrial matrix (15). In the matrix it is proteolytically processed in two steps, first by the mitochondrial processing peptidase to the i form and subsequently to the  $i^*$  form, possibly by the mitochondrial intermediate peptidase (40, 45, 46). Mitochondria with a temperature-sensitive mutation in mt-Hsp70 (*ssc1-2*) were preferentially impaired in the formation of the  $i^*$  form,

indicating an involvement of mt-Hsp70 in this maturation process (45).

We thus studied the question of whether the mutation in *MGE1* affected the maturation of  $b_2\Delta$ -DHFR. The hybrid protein was added to mitochondria in the presence of methotrexate, a specific ligand of DHFR, and therefore accumulated as a membrane-spanning intermediate (45). The N terminus was translocated into the matrix and processed twice in wild-type mitochondria (Fig. 5, upper panel, lane 1), whereas the folded DHFR part was retained on the cytosolic side and thus the protein remained accessible to externally added protease (Fig. 5, lower panel, lane 1). In the mutant mitochondria, the second processing to the  $i^*$  form was strongly inhibited (Fig. 5, lane 2). We carried out import of the hybrid protein in the absence of methotrexate to obtain complete translocation into the matrix (Fig. 5, lane 3). Also under this condition, formation of the  $i^*$  form was strongly inhibited in *mge1-100* mitochondria (Fig. 5, lane 4). This reduction in  $i^*$  formation indicates that the *mge1-100* mutation leads to retardation of preprotein maturation both with the membrane-spanning preprotein and with the fully imported preprotein.

**The interaction of mt-Hsp70 with preproteins is differentially affected by the mutation in *MGE1*.**  $b_2\Delta$ -DHFR interacts with mt-Hsp70 during import, as shown by specific coprecipitation of the preprotein with anti-mt-Hsp70 antibodies in the membrane-spanning form and in the imported form (44, 45). We analyzed the interaction of  $b_2\Delta$ -DHFR with mt-Hsp70 at the two import stages: membrane-spanning intermediate (Fig. 6, samples 1 to 4) and fully imported protein (Fig. 6, samples 5 to 8). The mitochondria were lysed with Triton X-100, and immunoprecipitations under nondenaturing conditions were performed with antibodies directed against mt-Hsp70 and pre-immune antibodies. The yield of coprecipitation of the membrane-spanning i form with mt-Hsp70 was moderately reduced for *mge1-100* mitochondria compared with that for wild-type mitochondria (Fig. 6; compare column 4 with column 2). In contrast, the yield of coprecipitation of  $b_2\Delta$ -DHFR fully imported into the matrix was significantly increased with *mge1-100* mitochondria (Fig. 6; compare columns 8 and 6). No coprecipitation was observed with preimmune serum (Fig. 6, columns 1, 3, 5, and 7). Moreover, no coprecipitation of i-form  $b_2\Delta$ -DHFR with mt-Hsp70 was observed when the preprotein

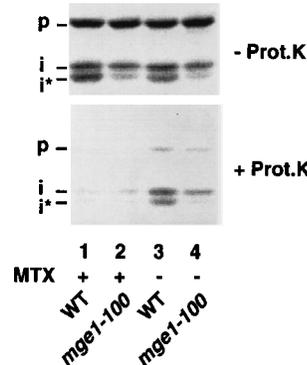


FIG. 5. Maturation of  $b_2\Delta$ -DHFR is impaired in *mge1-100* mitochondria. The in vitro-synthesized precursor protein  $b_2\Delta$ -DHFR was imported for 30 min at 25°C into mitochondria isolated from the *mge1-100* strain or the isogenic wild-type strain (WT). Prior to the import reactions, mitochondria were preincubated for 15 min at 37°C. Membrane-spanning intermediates were accumulated by incubation of the precursor protein with methotrexate (MTX) for 10 min on ice before the start of the import reaction. For the lower panel, samples were treated with proteinase K (Prot.K) after the import reaction. p, precursor form; i, and  $i^*$ , processed forms of  $b_2\Delta$ -DHFR.

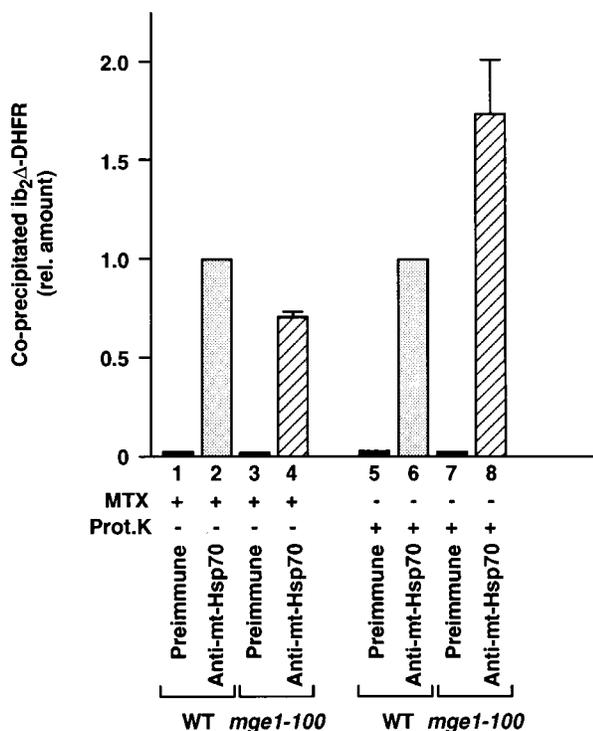


FIG. 6. Differential effect of the *mge1-100* mutation on coprecipitation of membrane-spanning and imported proteins with mt-Hsp70. The in vitro-synthesized precursor protein  $b_2\Delta$ -DHFR was accumulated in mitochondrial import sites in the presence of methotrexate (MTX) (samples 1 to 4) or completely imported into the matrix, followed by treatment with proteinase K (Prot.K) (samples 5 to 8) as described in the legend to Fig. 5. The mitochondria were reisolated and lysed, and coimmunoprecipitations with antiserum against mt-Hsp70 or preimmune serum were performed as described in Materials and Methods. Analysis was performed by SDS-PAGE and digital autoradiography. The amount of i-form  $b_2\Delta$ -DHFR ( $ib_2\Delta$ -DHFR) coprecipitated from wild-type mitochondria (43, 45) under each group of conditions was set at 1.0. Error bars indicate the standard errors of the means for four individual experiments. rel., relative.

was incubated with mitochondria in the absence of a membrane potential, excluding the possibility that the association occurred after lysis of the mitochondria (44, 45). As pointed out below (see Discussion), these results suggest that the mutation in Mge1p may favor an accumulation of mt-Hsp70 in the slow-binding/slow-release state described for DnaK.

**Reduced aggregation of imported proteins in *mge1-100* mitochondria.** Finally, we investigated folding and aggregation of proteins imported into the matrix. As a model substrate we used a fusion protein composed of the presequence of *N. crassa*  $F_0$ -ATPase subunit 9 and DHFR (Su9-DHFR) that is typically employed for this type of investigation (14, 27, 35).

After a preincubation of the isolated mitochondria at 37°C, Su9-DHFR was imported into wild-type and mutant mitochondria at 25 or 37°C. The import times were chosen such that they were within the kinetically linear range for the folding reaction (27, 34). The mitochondria were then treated with trypsin to remove nonimported protein and lysed with a non-ionic detergent. After addition of proteinase K, the degree of folding of the imported proteins was determined by the amount of protease-resistant DHFR present, since DHFR that is not completely folded is digested by protease under the conditions used (27, 34). Surprisingly, we did not observe a significant influence of the *mge1-100* mutation on the folding state of Su9-DHFR (Fig. 7A) despite the enhanced interaction

of imported proteins with mt-Hsp70 reported above.

The process of protein folding is a dynamic process, a competition between productive interactions which lead to the final active conformation and off-pathway reactions which lead to misfolding and aggregation. Aggregation is assessed by measuring the fraction of imported Su9-DHFR that is found in the Triton X-100-insoluble material. The aggregation of imported Su9-DHFR was decreased in the mutant mitochondria, particularly at 37°C, at which temperature the level of aggregation was about one-third of that found in wild-type mitochondria (Fig. 7B). This decrease suggests that an enhanced binding of mt-Hsp70 to fully imported proteins reduces the misfolding and therefore the aggregation of imported proteins in mutant mitochondria. Whether the *mge1-100* mutation has a direct effect on folding of imported proteins cannot be ascertained from this experiment, although it is conceivable that the reduction in misfolding and aggregation masks a partial folding defect.

## DISCUSSION

The analysis of an *MGE1* conditional allele, *mge1-100*, described in this report demonstrates that Mge1p functions in the

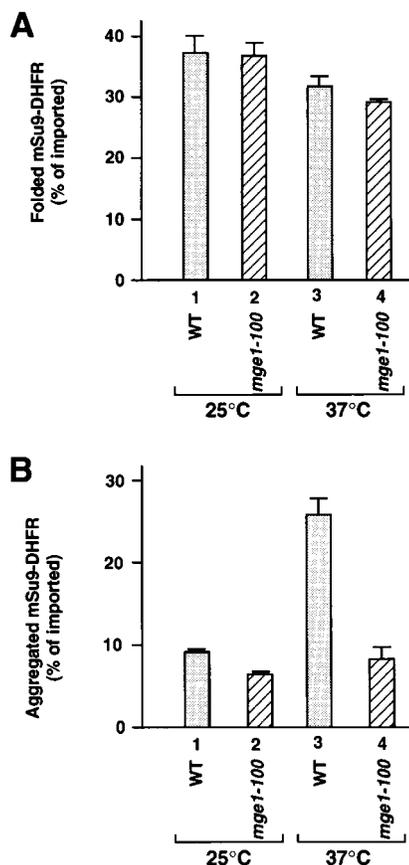


FIG. 7. Decreased formation of aggregated imported protein in *mge1-100* mitochondria. Import of  $^{35}\text{S}$ -labeled Su9-DHFR into wild-type and *mge1-100* mutant mitochondria was performed either for 1 min at 25°C after urea denaturation of the precursor protein or for 10 min at 37°C. After removal of nonimported proteins with trypsin, the reisolated mitochondria were lysed by Triton X-100 and the samples were split. Unfolded mature Su9-DHFR was digested with proteinase K. Aggregated material was pelleted (no treatment with proteinase K). Values for folding (A) and aggregation (B) are normalized to the total amount of imported mature Su9-DHFR in each experiment. Error bars indicate the standard errors of the means for three individual experiments.

translocation and maturation of mitochondrial preproteins. Mutant mitochondria showed a retardation of import of precursor proteins into the mitochondrial matrix. In addition, analysis of the hybrid protein  $b_2\Delta$ -DHFR, which undergoes a two-step maturation process involving two cleavage events in the matrix, revealed that the second processing step was significantly reduced in the *mge1-100* mitochondria. The control experiments described in the Results section indicate that these effects are a direct result of decreased Mge1p function rather than altered expression or stability of Mge1p or indirect effects on the integrity of the mitochondria.

Mge1p has been shown to form a complex with matrix Hsp70 (Ssc1p) which interacts directly with the translocating polypeptide chain. Therefore, a comparison of the effects of mutations in *SSC1* and *MGE1* on translocation and maturation of preproteins is instructive as to the mechanism of action of this chaperone complex. The defect in *mge1-100* mitochondria bears similarity to the defect in *ssc1-2* mitochondria. *ssc1-2* mitochondria accumulate membrane-spanning intermediates of preproteins because of retarded translocation of the mature part of the protein, whereas the transfer of the presequence is only slightly affected (7, 14). *ssc1-2* mutant Hsp70 is still able to interact with preproteins, but the release of polypeptides from Hsp70 is impaired, leading to a retardation of the reaction cycle of Hsp70 (7, 45). The association of mutant Mge1-100p with mt-Hsp70 is defective. In wild-type cells, all of the Mge1p can be found associated with mt-Hsp70, while only 30% of the Mge1-100p is associated, raising the possibility that the impaired binding of Mge1p to mt-Hsp70 may affect the reaction cycle of mt-Hsp70. In fact, the second processing step of the hybrid protein  $b_2\Delta$ -DHFR, which is particularly reduced in *ssc1-2* mitochondria (45), is also strongly inhibited in *mge1-100* mitochondria.

In addition to binding to Mge1p, mt-Hsp70 interacts with the inner membrane protein Mim44 as well as translocating polypeptides. The ATP-dependent binding of mt-Hsp70 to Mim44, an essential component of the import site, was not affected in the *mge1-100* mitochondria. The binding of mt-Hsp70 to membrane-spanning polypeptides was moderately, but significantly, reduced. However, the binding of mt-Hsp70 to fully imported polypeptides was clearly enhanced.

The extensive studies of DnaK and GrpE function in *E. coli* (20, 21, 24, 28, 38, 42) provide a basis for understanding the implications of the altered interaction of mt-Hsp70 with precursor proteins in the mitochondrial matrix. GrpE acts as a nucleotide release factor that stimulates the release of ADP from DnaK. Since the cellular concentration of ATP is normally higher than that of ADP, the subsequent binding of ATP is favored. Assuming that Mge1p acts analogously to GrpE, a decrease in the interaction between Mge1p and mt-Hsp70 would favor the ADP-bound form of mt-Hsp70. The ATP-bound form of Hsp70 would be converted to the ADP-bound form through action of the intrinsic ATPase activity of mt-Hsp70, and the decrease in Mge1p function would result in stabilization of this form because nucleotide exchange would not occur at the normal rate. Since the ADP-bound form of Hsp70 appears to cause the stabilization of the interaction with substrate proteins by decreasing the off-rate of Hsp70, a decrease in Mge1p function would be expected to stabilize the Hsp70-substrate protein interaction. Stabilization of the Hsp70-substrate interaction is in fact observed in *mge1-100* mitochondria, as indicated by the increase in the yield of fully imported preprotein that can be coprecipitated by mt-Hsp70-specific antibody for *mge1-100* mitochondria compared with that for wild-type mitochondria. A prolonged interaction of imported proteins with mt-Hsp70 is also indicated by the re-

duction in the formation of protein aggregates in *mge1-100* mitochondria. Binding to mt-Hsp70 apparently prevents the aggregation of imported proteins.

Why do we observe a reduced coprecipitation efficiency of membrane-spanning polypeptides with mt-Hsp70 in contrast to the increased efficiency with fully imported polypeptides? Perhaps the answer lies in the differences between the membrane-spanning preproteins and those that are fully imported and the effect of the *mge1-100* mutation on the Hsp70-substrate protein binding cycle. Complete import strictly requires binding of mt-Hsp70 molecules to the preprotein (7, 18, 26, 41, 44). The ADP-bound form of DnaK is suggested to be the slow-binding/slow-release form (24, 38, 42). If the defect in Mge1p in the mutant mitochondria stabilizes the ADP-bound form by preventing release of nucleotide from mt-Hsp70, then one would expect a reduction in the number of mt-Hsp70s interacting with membrane-spanning intermediates, since the binding step is slowed, leading to a reduced yield of coprecipitation of membrane-spanning preproteins. On the other hand, since the fully imported proteins must have already interacted with Hsp70 and their release from mt-Hsp70 would be slowed in the *mge1-100* mutant, the increase in coimmunoprecipitation that we observed in this case would be expected. This interpretation is supported by the observation (4, 43) that a depletion of matrix ATP decreased the yield of coprecipitation of membrane-spanning preproteins with mt-Hsp70 but increased the yield of coprecipitation of fully imported preproteins.

Mt-Hsp70 functions with a DnaJ homolog, Mdj1p, as well as with Mge1p. Unlike the *mge1-100* mutation, deletion of *MDJ1* produced a disturbance of folding of imported proteins but had no measurable effect on protein translocation (35). A deletion of *MDJ1* led quantitatively to the formation of petite colonies, while *mge1-100* cells can be maintained as [*rho*<sup>+</sup>]. In summary, the function of mitochondrial GrpE is required for both membrane translocation and maturation of preproteins, whereas mitochondrial DnaJ appears to have a more specialized role, i.e., involvement in the folding of imported polypeptides. The broad function of Mge1p fits with the observation that Mge1p is essential for viability of yeast cells under all growth conditions (1, 13, 19) while Mdj1p is not (35).

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