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A role for a eukaryotic GrpE-related protein, Mge1p, in protein translocation

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ABSTRACT **The 70-kDa heat shock proteins (hsp70s) function as molecular chaperones in a wide variety of cellular processes through cycles of binding and release from substrate proteins coupled to cycles of ATP hydrolysis. In the prokaryote *Escherichia coli*, the hsp70 DnaK functions with two other proteins, DnaJ and GrpE, which modulate the activity of DnaK. While numerous hsp70s and DnaJ-related proteins have been identified in eukaryotes, to our knowledge no GrpE-related proteins have been reported. We report the isolation and characterization of a eukaryotic *grpE*-related gene, *MGE1*. *MGE1*, an essential nuclear gene of the yeast *Saccharomyces cerevisiae*, encodes a soluble protein of the mitochondrial matrix. Cells with reduced expression of Mge1p accumulate the precursor form of a mitochondrial protein. Since mitochondrial hsp70 is required for translocation of precursors of mitochondrial proteins from the cytosol into the matrix of mitochondria, these data suggest that Mge1p acts in concert with mitochondrial hsp70 in protein translocation.**

By binding to short regions of polypeptides, the 70-kDa heat shock proteins (hsp70s) act as molecular chaperones by preventing aggregation and improper folding (for reviews see refs. 1 and 2). The cycles of binding and release of hsp70s from these substrate proteins are coupled to cycles of ATP binding and hydrolysis (3, 4). In *Escherichia coli* the hsp70 DnaK cooperates with two cohort proteins, DnaJ and GrpE. DnaJ stimulates the hydrolysis of ATP, while GrpE promotes the release of bound nucleotide (5). Mutants of *dnaK*, *dnaJ*, and *grpE* have similar effects on a variety of cellular processes, including proteolysis, expression of *hsp* genes, and protein translocation, indicating cooperation among these proteins *in vivo* (6–8).

Eukaryotes contain numerous hsp70s and DnaJ-related proteins. For example, the matrix of mitochondria of *Saccharomyces cerevisiae* contains the hsp70 Ssc1p (9) and a recently identified DnaJ-like protein, Mdj1p (10). *SSC1* is an essential gene known to be critical for the translocation of proteins from the cytosol into the matrix (11). Consistent with its role as a molecular chaperone, Ssc1p binds to polypeptide chains in transit across the mitochondrial membranes (12, 13). Thus strains with a defective Ssc1p accumulate the precursor form of mitochondrial proteins destined for the matrix (11). Since it is known that two components of the hsp70–DnaJ–GrpE chaperone team are conserved, we anticipated that GrpE-related proteins would be found in eukaryotes as well. Here we report the characterization of a *grpE*-related gene from yeast which encodes a protein of the mitochondrial matrix.§ Because reduction of the amount of this protein, Mge1p, results in the accumulation of the precursor of a nuclear-encoded matrix protein, we propose that Mge1p acts with Ssc1p in protein translocation.

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MATERIALS AND METHODS

Cloning and Sequence Analysis of *MGE1*. A 352-bp region corresponding to the partial open reading frame of the putative yeast *grpE*-like gene was amplified by PCR, labeled by the random priming method, and used for Southern blot analysis of yeast genomic DNA using standard laboratory techniques (14). The complete gene was isolated by constructing a doubly size-selected genomic DNA library containing ≈4.2-kb *Eco*RI–*Xho* I genomic DNA fragments cloned in pRS306 (15). The library was screened by using the 352-bp PCR fragment corresponding to the C terminus of the putative open reading frame, using standard methods (14). The DNA sequence was determined on both strands by the dideoxynucleotide chain-termination method using Sequenase (United States Biochemical). The multiple sequence alignment was performed by using the PILE-UP program of the UW GCG-software package (version 8) after setting a gap-weight value of 6 and a gap length weight of 0.1. The sequence identity and similarity values were determined by the GAP program of the GCG package, using default parameters.

Strains and Plasmids. Yeast strains and plasmids used in this study are given in Table 1. A 1-kb *Ssp*I fragment containing the entire coding region of *MGE1* (nucleotides −8 through +1021, with +1 being the A of the initiating ATG) was inserted into the *Hin*cII site of pGEM-3Zf(+) (Promega) to generate plasmid pT7-MGE1, which was used for *in vitro* transcription and translation experiments. For overexpression experiments, the GAL1-MGE1 fusion plasmid was constructed by cloning a *Bam*HI–*Sph* I fragment from pT7-MGE1 in the GAL1/10 vector pBM272. The GAL1-MGE1 fusion was then inserted as a 1.6-kb *Eco*RI–*Sca* I fragment into the centromeric plasmid pRS313 to generate pSL32.

Western Analysis. A 1-kb *Bgl* II–*Eco*RI fragment containing codons 91–228 of Mge1p was inserted into the glutathione *S*-transferase fusion vector pGEX2T (Pharmacia) cut with *Bam*HI and *Eco*RI. The resulting 44-kDa GST-MGE1 fusion protein was isolated by affinity purification using glutathione-agarose beads (17). Antisera were raised by immunizing rabbits with the purified fusion protein. For Western blotting, protein samples were fractionated by SDS/PAGE, electrotransferred, and immunodetected by enhanced chemiluminescence (ECL) according to the supplier's instructions (Amersham).

Analysis of Mge1p Precursor Import. For *in vivo* analysis of precursor accumulation, total cell extracts were prepared as described previously (16) from a wild-type and a *mif1*^{ts} (Y164) mutant strain (generously provided by A. Horwich, Yale University) grown in liquid YPD medium (16) at 23°C or grown at 23°C and shifted to 37°C for 30 min. Samples containing 20 μg of total protein were analyzed by SDS/PAGE as described above.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U09565).

Table 1. Strains and plasmids

Strain or plasmid	Genotype	Ref. or source
Yeast		
PK81	*MATα ade2-101 lys2 ura3-52 leu2-3,112 Δtrp1 ssc1-2::LEU2*	16
PK82	*MATa his4-713 lys2 ura3-52 Δtrp1 leu2-3,112*	16
Y164	*MATα arg3 his4-519 leu2-3,112 mif1*	A. Horwich
PJ53	*MATa/α 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/can1-100 GAL2⁺/GAL2⁺ met2-Δ1/met2-1 lys2-Δ2/lys2-Δ2*	P. James
YM2271	*MATα ura3-52 his3-200 ade2-101 lys2-801 trp1-901 met⁻ gal4-542 gal80-538 LEU2::GAL1-lacZ*	M. Johnston
YM2263	*MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 met⁻ gal4-542 gal80-538 LEU2::GAL1-lacZ*	M. Johnston
DG407*	*MATα ura3-52 his3-200 ade2-101 lys2-801 trp1-901 met⁻ gal4-542 gal80-538 LEU2::GAL1-lacZ mge1-1::URA3*	This study
Plasmid		
pSL32	Amp^r *CEN6 HIS3 ARS1 GAL1-MGE1*	This study
pBM5391	Amp^r *CEN3 TRP1 ARS1 gal4^{ts}*	M. Johnston

*DG407 is a haploid derivative from the homozygous diploid obtained in a cross between YM2263 and YM2271. *MGE1* was disrupted as described in *Materials and Methods*. The viability of the *mge1-1* haploid was maintained by a centromeric plasmid encoding *MGE1*.

Mitochondria were prepared from strain PK82 grown in 1% yeast extract/2% peptone/3% glycerol/2% ethanol as described previously (16). [^{35}S]Methionine-labeled precursor proteins were synthesized *in vitro* by using rabbit reticulocyte lysate programmed with RNA transcribed by T7 or SP6 RNA polymerase. All methods for the translocation experiments have been described previously (16).

For submitochondrial fractionation, mitoplasts were prepared after import of radiolabeled precursor proteins by diluting intact mitochondria into 25 mM Hepes, pH 7.4 (swelling). After reisolation of the mitoplasts by centrifugation, mitoplasts were suspended in SEM (250 mM sucrose/1 mM EDTA/10 mM Mops, pH 7.2). Aliquots were treated with proteinase K at 150 mg/ml for 15 min at 0°C with or without disruption in 0.1% deoxycholate. Proteins were separated by SDS/PAGE and detected by autoradiography. Precursors used were those of cytochrome b_2 [Cyt b_2; a marker for intermembrane space (18)], Su9-DHFR [a marker for mitochondrial matrix (19)], and Mge1p.

Subcellular Localization of Mge1p. Mitochondria were prepared from PK82 as described for *in vitro* import (16, 20). After Dounce homogenization, aliquots were taken for total, cytosolic, and mitochondrial fractions. For separation of membrane and soluble components, mitochondria were suspended in 25 mM Hepes, pH 7.4, and sonicated for 50 s, followed by centrifugation for 20 min at 48,000 rpm in a Beckman TLA100.3 rotor. Mitoplasts were prepared as described (20) for *in vitro* import. After reisolation of the mitoplasts by centrifugation, the supernatant was precipitated with 10% trichloroacetic acid to recover intermembrane space proteins. Samples were separated by SDS/PAGE and immunodetected as described above.

Gene Disruption. The *URA3* gene carried on a 1.1-kb *Hin*dIII fragment was inserted into the *Hin*dIII site at codon 81 to construct *mge1-1*. The *mge1-1* fragment was liberated from plasmid sequences by digestion with *Pst* I and *Xho* I and used to transform the diploid strain PJ53. The resulting heterozygotes were placed on sporulation medium and the resulting asci were dissected.

***gal4^{ts}*-Regulated Expression of Mge1p.** For analyzing the effects of reduced Mge1p levels, total cell lysates for Western blots were prepared from cells grown in liquid YPD medium at the temperatures indicated and harvested at OD_{600} between 0.1 and 0.3. Cell pellets were suspended in PBS (150 mM NaCl/16 mM Na_2HPO_4/4 mM NaH_2PO_4) with 1 mM phenylmethylsulfonyl fluoride (PMSF) and disrupted with 1% SDS/0.1% Triton X-100, then 2× Laemmli buffer was added and the mixture was boiled for 7 min. Portions containing 0.125-OD equivalents of total cell lysates from each sample were resolved by SDS/PAGE and analyzed as described above.

RESULTS

Identification of a *grpE*-Related Gene, *MGE1*. The presence of the partial C-terminus-encoding sequence of a putative yeast *grpE* homologue upstream of the *KIN4* gene (21) was revealed by a data base search using the FASTA program (22) in which the DNA sequence upstream of the *KIN4* gene was used as a query (B. Baum, personal communication). The complete gene was isolated and sequenced as described in the *Materials and Methods*. The predicted protein of 228 amino acids has 34% identity and 57% similarity with GrpE of *E. coli* (23). When compared with GrpE, Mge1p has an N-terminal extension of 43 amino acids (Fig. 1). This extension has features characteristic of a typical mitochondrial leader sequence (24), being rich in hydroxylated (serine and threonine) and positively charged (lysine and arginine) residues and lacking negatively charged (glutamic and aspartic) residues. Thus we have named this gene *MGE1* (mitochondrial *grpE*).

Most nuclear-encoded mitochondrial proteins are synthesized as precursors in the cytosol and upon translocation into mitochondria they are processed to a smaller size by cleavage of their N-terminal leader sequence (24). While Mge1p normally migrates as a 21-kDa protein, it accumulates as a 28-kDa protein in a *mif1^{ts}* mutant strain at 37°C, where translocation of precursors of matrix proteins synthesized in the cytosol into the mitochondria is inhibited (Fig. 2*A*). To determine whether the *MGE1*-encoded protein could be translocated into mitochondria *in vitro*, radiolabeled Mge1p was synthesized in a reticulocyte lysate and added to energized mitochondria. Mge1p was translocated into a protease-resistant location in a reaction dependent on an electrochemical potential difference across the inner membrane (Fig. 2*B*), undergoing cleavage in the process.

Mge1p Is a Soluble Mitochondrial Matrix Protein. While the above experiments demonstrate the capacity of Mge1p to be translocated into mitochondria, they do not establish its cellular location *in vivo*. By using Mge1p-specific antibodies, Mge1p was localized to the purified mitochondrial fraction, while none was detected in the cytosolic fraction (Fig. 3*A*). On the other hand, a cytosolic hsp70, Ssa1p, was found in the cytosolic fraction but was barely detectable in the mitochondrial fraction.

Mitochondrial hsp70 (Ssc1p) is a soluble matrix protein (9, 11). To determine if Mge1p colocalized with Ssc1p we analyzed the distribution of Mge1p in mitochondrial fractions. The presence of Mge1p in the supernatant after sonication of mitochondria and centrifugation to pellet the membranes indicated that it is a soluble protein (Fig. 3*B*). To distinguish between an intermembrane space or matrix location, mitochondria were treated with a hypotonic solution

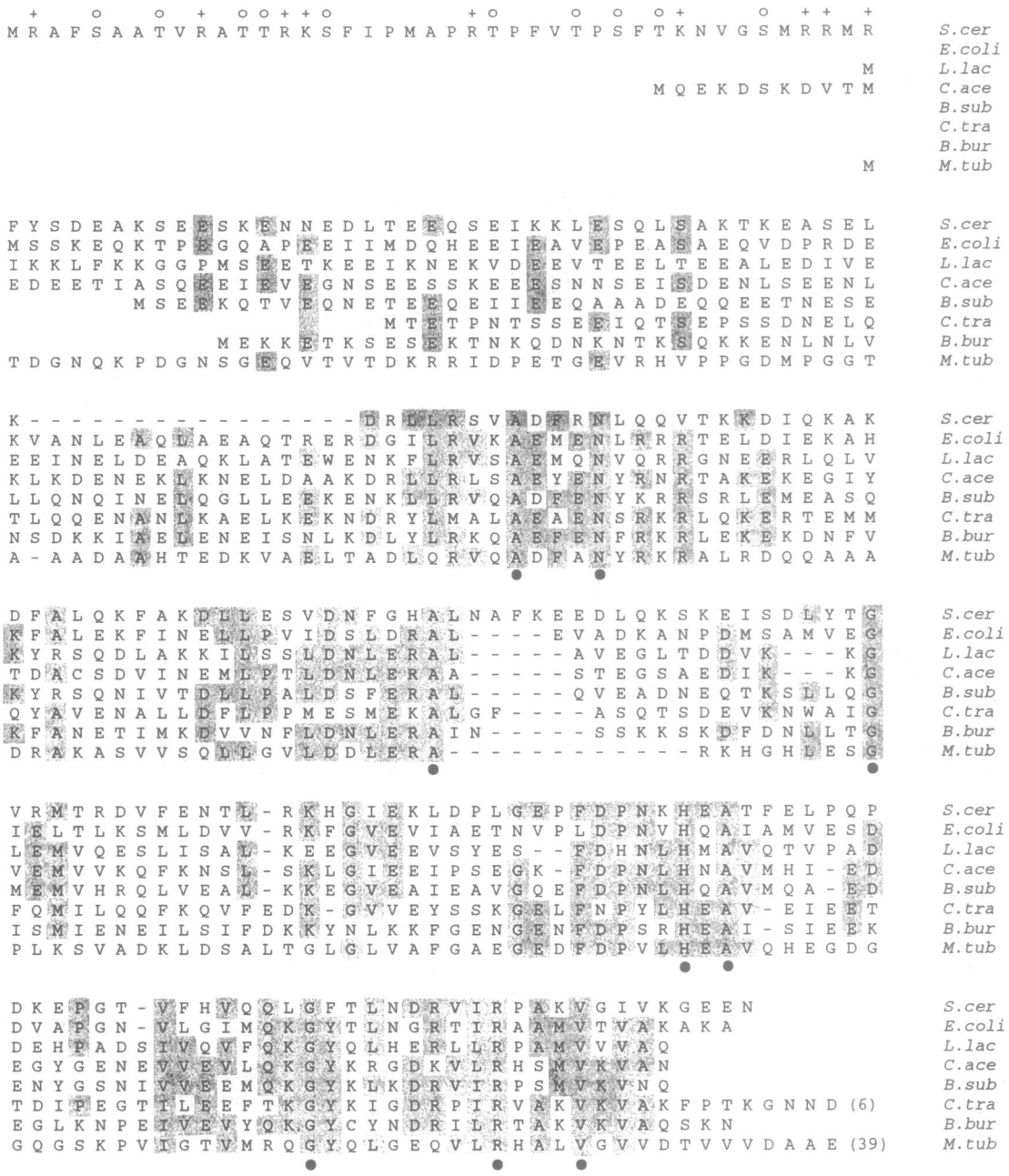

FIG. 1. Alignment of the predicted amino acid sequence of Mge1p (*S. cer*) with the amino acid sequences of various prokaryotic GrpE homologues: *E. coli*, *Lactococcus lactis* (*L. lac*), *Clostridium acetobutylicum* (*C. ace*), *Bacillus subtilis* (*B. sub*), *Chlamydia trachomatis* (*C. tra*), *Borrelia burgdorferi* (*B. bur*), and *Mycobacterium tuberculosis* (*M. tub*). The putative leader sequence of Mge1p is shown on the top row. Hydroxylated residues in the leader are indicated by open circles and positively charged residues by a plus above the sequence. Identical residues in regions sharing 50% or greater identity are shaded. Residues that are absolutely conserved in all known GrpE homologues are indicated with a filled circle below the sequence. Gaps that were inserted during the alignment are denoted by dashes. The numbers in parentheses at the end of some sequences correspond to the number of C-terminal amino acids that are not included in the figure. Sequence identity and similarity values (%) of various GrpE-like proteins as compared with *E. coli* GrpE are as follows: *S. cer* (33.7, 56.9), *L. lac* (29.4, 53.5), *C. ace* (30.7, 55.0), *B. sub* (31.6, 62.0), *C. tra* (29.1, 52.5), *B. bur* (28.0, 55.4), and *M. tub* (23.8, 47.1). Sequences of the prokaryotic GrpE-like proteins were obtained from GenBank (accession nos.: X07863, *E. coli*; M84964, *B. sub*; M96847, *B. bur*; M62819, *C. tra*; M74569, *C. ace*; Z19148, *L. lac*; and X58406, *M. tub*).

which ruptures the outer, but not the inner, membrane. Proteins of the intermembrane space such as cytochrome b_2 became susceptible to digestion with exogenously added protease, while matrix proteins such as Ssc1p did not. Mge1p became susceptible to added protease only after the inner membrane was disrupted by detergent (Figs. 2*C* and 3*C*), demonstrating that Mge1p is a soluble protein present in the matrix of mitochondria.

Genetic Analysis of MGE1. To determine if MGE1 is an essential gene we constructed a heterozygous diploid containing an insertion of the selectable marker *URA3* in *MGE1* (Fig. 4). Sporulation of the heterozygous diploid (*MGE1/mge1*::*URA3*) showed 2:2 segregation of viable versus inviable spores. The viable spores were Ura$^-$, indicating that the *URA3*-tagged disruption caused the inviability. The viability of Ura$^+$ spores could be rescued by a wild-type copy of *MGE1* carried on a centromeric vector. We conclude that *MGE1* is an essential gene.

Precursors of hsp60 Accumulate in Cells Limited for Mge1p Expression. Since in *E. coli* hsp70 and GrpE interact

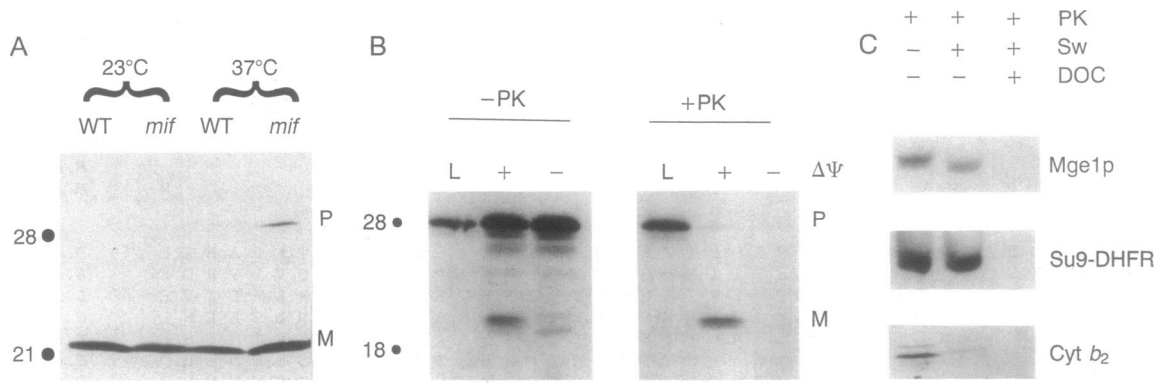

FIG. 2. Analysis of Mge1p import. (*A*) Accumulation of pre-Mge1p in a *mif1*^{ts} mutant strain grown under nonpermissive conditions (37°C). Positions of molecular mass markers in kDa are given on the left. WT, wild type; P, precursor; M, mature. (*B*) Import of pre-Mge1p, synthesized *in vitro*, into purified mitochondria is dependent on the membrane potential, ΔΨ. L, pre-Mge1 lysate; PK, proteinase K; P, precursor; M, mature. (*C*) Mge1p is imported into a protease-protected position in the mitochondrial matrix. PK, proteinase K; Sw, swelling; DOC, deoxycholate.

functionally (25) we wanted to determine if mitochondrial hsp70 and Mge1p, both of which are soluble matrix proteins, are involved in the same cellular processes. Towards this end we placed *MGE1* under control of the *GAL1* promoter. However, we found that strains containing such constructs were inviable under conditions where the *GAL1* promoter is active (i.e., galactose-based medium) even in the presence of the wild-type *MGE1* gene under the control of its own promoter. When shifted from glucose-to galactose-based medium, strains carrying this *GAL1-MGE1* construct overproduced Mge1p, leading us to conclude that excess Mge1p is toxic (data not shown).

To reduce expression of Mge1p, we took advantage of a system developed in the laboratory of Mark Johnston (Washington University, St. Louis) which allows for the regulation of *MGE1* under control of the *GAL1* promoter by temperature rather than by changing the carbon source. Strain DG407 contains disruptions in *MGE1* and *GAL4* which encodes the positive activator of galactose-responsive genes, and two plasmids, one having *MGE1* under the control of the *GAL1* promoter (pSL32) and the second carrying the *gal4*^{ts} allele (pBM5391). In this strain, transcriptional activation of galactose-responsive genes is no longer subject to complete repression by glucose due to the deletion of *GAL80*, a major effector of glucose repression (26); thus, genes under control of the *GAL1* promoter are activated constitutively by the *gal4*^{ts} protein in glucose-based medium in a temperature-dependent manner. The *gal4*^{ts} allele has 50% activity at 25°C, approximately 25% activity at 30°C, and only 1% of wild-type activity at 37°C (J. Dover and M. Johnston, personal communication).

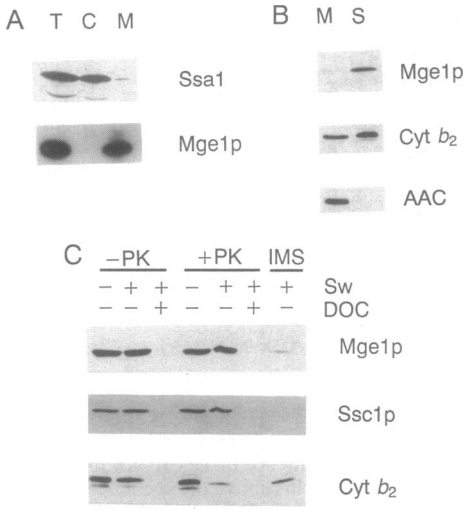

FIG. 3. Mge1p is a soluble mitochondrial matrix protein. (*A*) Separation of mitochondrial from cytosolic proteins. Twenty micrograms of mitochondria (M) and fractional equivalents of total cell lysate (T) and soluble cytosolic proteins (C) were analyzed by Western blotting. (*B*) Separation of membrane and soluble components of mitochondria. Twenty micrograms of purified mitochondria was subjected to sonication to separate membrane (M) and soluble (S) mitochondrial proteins. (*C*) Submitochondrial fractionation. Mitochondria (25 μg) were subjected to mitoplast formation (by swelling, Sw) and disruption with deoxycholate detergent (DOC) in combination with digestion with proteinase K (PK) to determine the submitochondrial localization of Mge1. Proteins of the intermembrane space (IMS; Cyt b_2) are released upon swelling, while matrix-localized proteins remain protease resistant after swelling. Antisera used were against Ssa1p (cytosolic), ATP/ADP carrier (AAC; mitochondrial inner membrane), cytochrome b_2 (Cyt b_2; soluble protein of the mitochondrial intermembrane space), Ssc1p (soluble protein of the mitochondrial matrix), and Mge1p.

Cells containing *MGE1* under the control of the *GAL1* promoter and the *gal4*^{ts} allele (DG407) had Mge1p levels comparable to control cells having *MGE1* under the control of its own promoter (Y2271 with pBM5391) at 26°C. Cells dependent upon *GAL1*-driven *MGE1* expression could grow at 30°C and 34°C even though the level of Mge1p was lower than in the control cells (Fig. 5*A*). However, these cells were inviable at 37°C, confirming that Mge1p is essential for growth. To determine the effect of reduced levels of Mge1p on protein translocation into mitochondria we tested for the accumulation of the precursor form of the mitochondrial matrix protein, hsp60 (Fig. 5*B*). The precursor form of hsp60 was not detected in extracts of cells grown at 21°C. At 30°C and 34°C, temperatures where the *gal4*^{ts} protein is less active and cells had reduced amounts of Mge1p, cells dependent on the *gal4*^{ts}-driven expression of Mge1p accumulated the precursor form of hsp60. These results suggest that Mge1p is required for import of proteins from the cytosol into the matrix, most likely acting with the mitochondrial hsp70, Ssc1p.

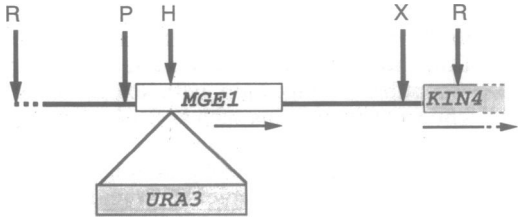

FIG. 4. Disruption analysis of *MGE1*. Restriction sites are indicated as R, *Eco*RI; H, *Hin*dIII; P, *Pst* I; and X, *Xho* I.

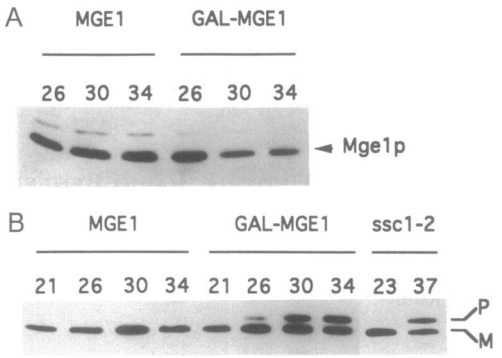

FIG. 5. Functional analysis of *MGE1*. (*A*) Effect of *gal4*^{ts} on expression of Mge1p; 0.125-OD equivalents of total cell lysates from cells grown at 26°C, 30°C, and 34°C were analyzed by immunoblotting with antisera against Mge1p. (*B*) Accumulation of hsp60 precursor under conditions of Mge1p depletion; 0.125-OD equivalents of total cell lysates from cells grown at 21°C, 26°C, 30°C, and 34°C were analyzed by immunoblotting with antisera against hsp60. P, precursor; M, mature. Representative data are shown from three separate experiments. Strains are MGE1 (*MGE1* under control of its own promoter), YM2271(pBM5391); GAL-MGE1 (*MGE1* under control of the *GAL1* promoter and *gal4*^{ts} activator), DG407(pSL32); and ssc1-2, PK81.

DISCUSSION

The parallels between GrpE of *E. coli* and Mge1p of *S. cerevisiae* mitochondria are striking. Both are essential (27) and interact functionally with hsp70s. GrpE and DnaK are involved in the same cellular processes such as proteolysis and regulation of the heat shock response; Mge1p and Ssc1p both appear to be involved in the translocation of proteins into mitochondria. DnaK and GrpE physically interact (28, 29); Mge1p-specific antibodies can coimmunoprecipitate Ssc1p imported into isolated mitochondria (data not shown), suggesting a physical interaction between the two mitochondrial proteins.

The action of hsp70s in cellular processes such as protein translocation requires cycles of binding and release from substrate polypeptides. An essential component of the cycle is an exchange of ATP for ADP (11). GrpE appears to act as a nucleotide exchange factor for DnaK (30). The results reported here are consistent with such an essential role for Mge1p, particularly in light of the fact that Mge1p colocalizes with and is involved in the same cellular process, protein translocation, as mitochondrial hsp70. Recently a mitochondrial DnaJ-related protein has been identified (10). Together these data provide strong evidence for the conservation of the three-component chaperone machinery of hsp70, GrpE, and DnaJ in diverse organisms. We predict that GrpE homologues will be found in other compartments of the eukaryotic cell and that hsp70, DnaJ, and GrpE homologues universally act as "chaperone teams."

We gratefully acknowledge Bobby Baum, who pointed out the similarity between GrpE and the predicted product of the partial open reading frame upstream of the *KIN4* gene; Mark Johnston, who made available to us the *gal4*^{ts} allele and the *gal4/gal80* disruption strain; Nikolaus Pfanner, who provided cytochrome b_2 and AAC antisera; and Richard Hallberg, who provided hsp60 antisera. We thank Liza Krainer for expert technical assistance. This work was supported by National Institutes of Health Grant 5 R01 GM31107 to E.A.C.

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