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A mitochondrial homolog of bacterial GrpE interacts with mitochondrial hsp70 and is essential for viability

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Mitochondrial hsp70 (mhsp70) is located in the matrix and an essential component of the mitochondrial protein import system. To study the function of mhsp70 and to identify possible partner proteins we constructed a yeast strain in which all mhsp70 molecules carry a C-terminal hexa-histidine tag. The tagged mhsp70 appears to be functional *in vivo*. When an ATP depleted mitochondrial extract was incubated with a nickel-derivatized affinity resin, the resin bound not only mhsp70, but also a 23 kDa protein. This protein was dissociated from mhsp70 by ATP. ADP and GTP were much less effective in promoting dissociation whereas CTP and TTP were inactive. We cloned the gene encoding the 23 kDa protein. This gene, termed GRPE, encodes a 228 residue protein, whose sequence closely resembles that of the bacterial GrpE protein. Microsequencing the purified 23 kDa protein established it as the product of the yeast *GRPE* gene. Yeast GrpEp is made as a precursor that is cleaved upon import into isolated mitochondria. GrpEp is essential for viability. We suggest that this protein interacts with mhsp70 in a manner analogous to that of GrpE with DnaK of *E.coli*.

Key words: gene disruption/histidine tag/mitochondrial hsp70/protein import/*Saccharomyces cerevisiae*

Introduction

Mitochondria contain a set of molecular chaperones that are essential for the import of proteins from the cytoplasm and for the folding of these proteins in the mitochondrial matrix (Hartl *et al.*, 1992). The three mitochondrial chaperones that have been described so far (hsp60, cpn10 and mhsp70) are each essential for cell viability (Craig *et al.*, 1989; Reading *et al.*, 1989; Rospert *et al.*, 1993b). Whereas the chaperonins hsp60 and cpn10 do not participate in translocation, mitochondrial hsp70 (mhsp70) is a key player in this process. It binds incoming precursor proteins and 'pulls' them into the matrix. Though the mechanism of this transport function is still unclear, it probably involves multiple cycles of ATP-dependent binding to the precursor, followed by release coupled to ATP hydrolysis (Neupert *et al.*, 1990; Scherer *et al.*, 1990; Beasley *et al.*, 1992). According to this model, mhsp70 acts as an ATP-driven 'import motor'. In addition, mhsp70 may mediate protein folding in the matrix, but such a function remains to be proven.

In order to understand the mechanism by which mhsp70 acts, it will be necessary to set up a functional *in vitro* system that duplicates some of the reactions mediated by mhsp70 *in vivo*. Such a system will probably require additional proteins that serve as functional partners of mhsp70. In *Escherichia coli* and other bacteria, the mhsp70 homolog DnaK functions with at least two partners, DnaJ and GrpE (Georgopoulos and Welch, 1993). The mitochondrial homologs of these proteins have not yet been identified, although a preliminary report has described a *Saccharomyces cerevisiae* gene encoding a mitochondrial DnaJ-like protein (Rowley *et al.*, 1993).

In an effort to characterize the function of mhsp70, and in particular to identify its partner proteins, we have constructed a yeast strain in which mhsp70 carries a C-terminal hexa-histidine tag. This tag has allowed us to purify mhsp70 by a rapid and gentle procedure (Hochuli *et al.*, 1988) and to identify a novel protein that is specifically bound to mhsp70 when mitochondria are depleted of ATP. We have shown that this protein is a mitochondrial homolog of the bacterial GrpE protein, and accordingly have termed it GrpEp. This protein is essential for viability of yeast cells. We propose that it interacts with mhsp70 in the import of precursor proteins from the cytoplasm.

Results

A yeast strain containing hexa-histidine tagged mhsp70

In order to study how mhsp70 functions and to determine whether this function involves interaction with partner proteins, we constructed a yeast strain in which all mhsp70 molecules carry a hexa-histidine tag. This tag should facilitate the rapid isolation of mhsp70 by binding to Ni-nitrilo-triacetic acid (Ni–NTA) beads (Hochuli *et al.*, 1988) under conditions that should not disrupt interactions with bound partner proteins.

We constructed mhsp70 variants carrying the hexa-histidine tag either at the N- or the C-terminus. As the N-terminally tagged mhsp70 did not bind efficiently to the Ni–NTA resin (data not shown), we performed all subsequent experiments with the C-terminally tagged mhsp70. Construction of this mhsp70 variant is illustrated in Figure 1A and B.

The tagged mhsp70 is functional in vivo

When the C-terminally tagged mhsp70 was expressed in a haploid yeast strain whose chromosomal gene encoding mhsp70 had been disrupted, the cells grew as rapidly as wild-type cells (Figure 1C). In this experiment, the tagged mhsp70 was expressed from its authentic promoter, the gene was

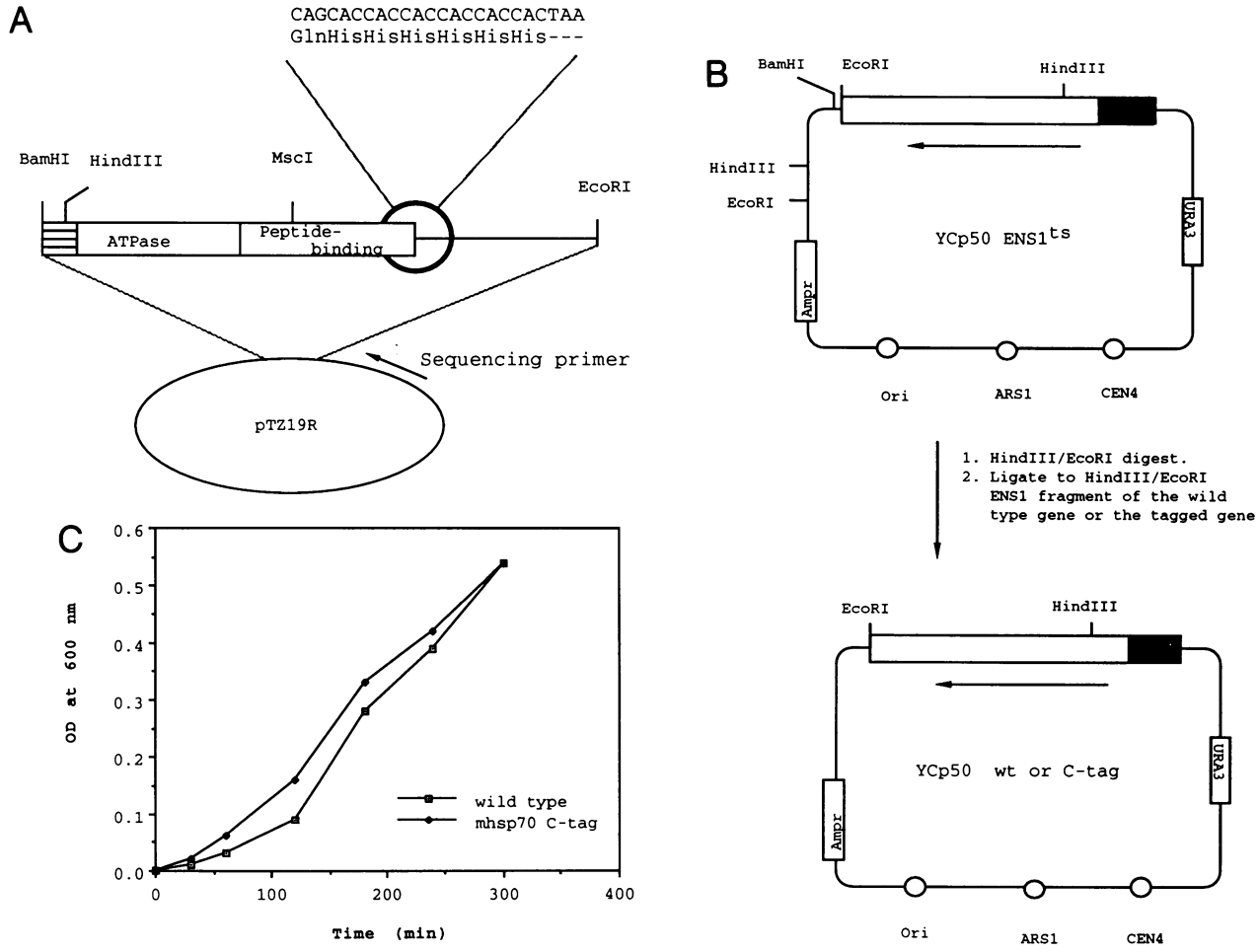

Fig. 1. Construction and *in vivo* test of mhsp70 carrying a C-terminal hexa-histidine tag. (**A**) A 2.3 kb *Bam*HI–*Eco*RI fragment containing the region encoding mhsp70 and the 3′-flanking sequence was cloned into the vector pTZ19R (New England Biolabs). The regions encoding the mitochondrial matrix targeting sequence (horizontal stripes), the ATPase domain and the peptide-binding domain of mhsp70 are marked. The 3′-terminal region where the hexa-histidine tag was added by PCR mutagenesis is enlarged. Thin lines represent plasmid DNA. The sequencing primer was a 17-mer distributed by New England Biolabs. (**B**) Shuttle vectors for expressing wild-type mhsp70 (YCp50-wt) or histidine tagged mhsp70 (YCp50-tag) under its own promoter (black box) in a haploid yeast strain whose chromosomal *ENS1/SSC1* gene had been disrupted. Both vectors were derived from YCp50-ENS1ts which encodes a temperature-sensitive mhsp70 protein (N.Morishima, unpublished). ARS, CEN4, replication sequences for yeast; ori, replication sequence for *E.coli*. (**C**) Growth of yeast strain JK9-dis (*MATa ens1::LEU2 trp1 his4 ura3*) expressing a plasmid-borne gene for wild-type or histidine tagged mhsp70. The plasmid (see B) carried the *URA3* gene. The transformants were grown at 30°C in minimal medium containing lactate as the main carbon source and tryptophan and histidine as nutritional supplements. Growth was monitored by absorbance at 600 nm.

carried by a centromere based vector present in only one to two copies per cell, and was able to grow on the non-fermentable carbon source lactate which requires optimal development of mitochondria (Pon and Schatz, 1991). The C-terminally tagged mhsp70 thus appeared to be functional *in vivo*.

Affinity purification of tagged mhsp70

The C-terminally tagged mhsp70 bound efficiently and selectively to the Ni–NTA resin and could be purified by sequential affinity chromatography on ATP–agarose (Scherer *et al.*, 1990) and the Ni–NTA resin. As the ATP-binding domain comprises the N-terminal part of mhsp70 (Flaherty *et al.*, 1990) whereas the histidine tag is C-terminal, this purification protocol selected against proteolytic fragments of mhsp70. A typical purification (Figure 2) yielded 0.7 mg of pure mhsp70 from 60 g of packed yeast cells. The overall recovery of mhsp70 was ~30%.

A 23 kDa mitochondrial protein copurifies with mhsp70

In order to identify proteins interacting with mhsp70, we incubated the Ni–NTA resin directly with an extract from mitochondria that had been depleted of ATP by preincubation with oligomycin, an inhibitor of mitochondrial ATP synthase. Proteins that had bound to the resin via histidine–Ni^{2+} interaction were then eluted by imidazole and analyzed by SDS–PAGE. As a further control for the specificity of this affinity step, we also performed the procedure with an extract from mitochondria containing only wild-type mhsp70. As expected, the resin bound the tagged mhsp70, but not the wild-type protein (Figure 3, compare lanes 3 and 5 with lanes 4 and 6). Interestingly, however, binding of the tagged mhsp70 was accompanied by binding of a 23 kDa protein. As judged by the staining intensity of the protein bands, the 23 kDa protein was recovered in substoichiometric amounts relative to mhsp70. The resin did not bind the 23 kDa protein upon incubation with extract

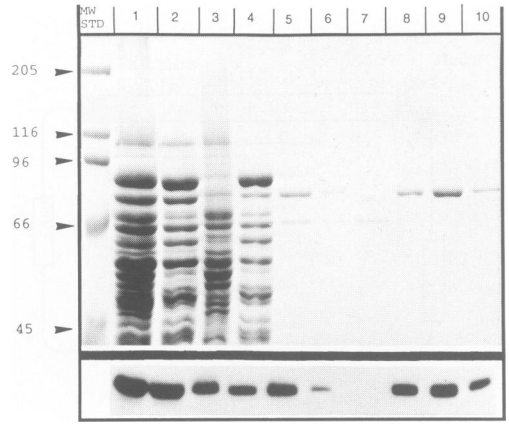

Fig. 2. Purification of histidine tagged mhsp70 from yeast mitochondria. (Upper panel) Samples from the various stages of the purification were analyzed by SDS−8% PAGE and staining with Coomassie Brillant Blue. Samples shown in lanes 1−8 represent equivalent amounts and can be directly compared with each other; the sample in lane 9 is 2.5-fold concentrated. Lanes: 1, Nycodenz-purified yeast mitochondria; 2, mitochondrial extract; 3, insoluble mitochondrial fraction; 4, proteins not adsorbed to ATP−agarose column; 5, proteins eluted with ATP from ATP−agarose; 6, proteins remaining on ATP−agarose after elution with ATP; 7, proteins not bound to Ni−NTA resin; 8, proteins eluted from Ni−NTA resin with imidazole; 9, 2.5 times the amount of sample shown in lane 8; 10, proteins not eluted from Ni−NTA resin with imidazole. MW STD, molecular weight standards. (Lower panel) Immune blot of the samples shown in the upper panel using an antiserum specific for mhsp70. Immune complexes were decorated with [^{125}I]protein A. A photograph of the autoradiogram is shown.

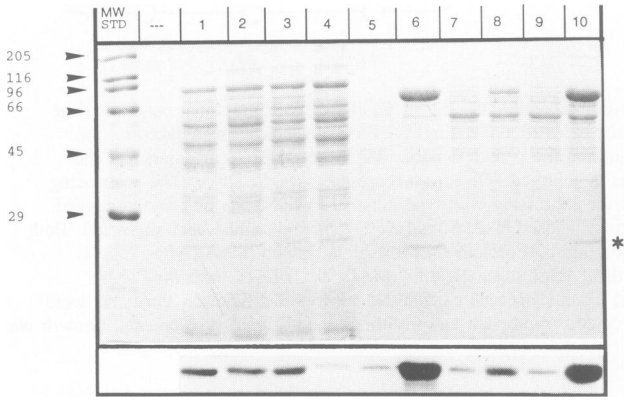

Fig. 3. A 23 kDa protein copurifies with the histidine tagged mhsp70. (Upper panel) Mitochondrial extracts (0.3 ml; 6 mg protein) from yeast strains expressing either wild-type or histidine tagged mhsp70 were incubated with 160 μl of equilibrated Ni−NTA resin. The resin was reisolated, the supernatant was saved, and mhsp70 was eluted from the resin with 0.9 ml of buffer B containing 300 mM imidazole. Each fraction (5% of the fraction for the samples in lanes 1−4, 100% of the fraction for samples in lanes 5−8) were analyzed by SDS−12% PAGE and staining with Coomassie Brillant Blue. Lanes: 1 and 2, mitochondrial extracts containing wild-type or histidine tagged mhsp70, respectively; 3 and 4, supernatant after incubating mitochondrial extract containing wild-type or histidine tagged mhsp70 with the Ni−NTA resin; 5 and 6, imidazole eluate of wild-type and histidine tagged extract from the Ni−NTA resin; 7 and 8, proteins from extract of wild-type or histidine tagged mitochondria not eluted from the Ni−NTA resin with imidazole; 9 and 10, total proteins from wild-type and histidine tagged extracts bound to Ni−NTA resin; MW STD, molecular weight standards. Asterisk, 23 kDa protein. (Lower panel) The samples shown in the upper panel were analyzed by SDS−12% PAGE and immunoblotting with an antiserum against mhsp70. Immune complexes were decorated with [^{125}I]protein A. A photograph of the autoradiogram is shown.

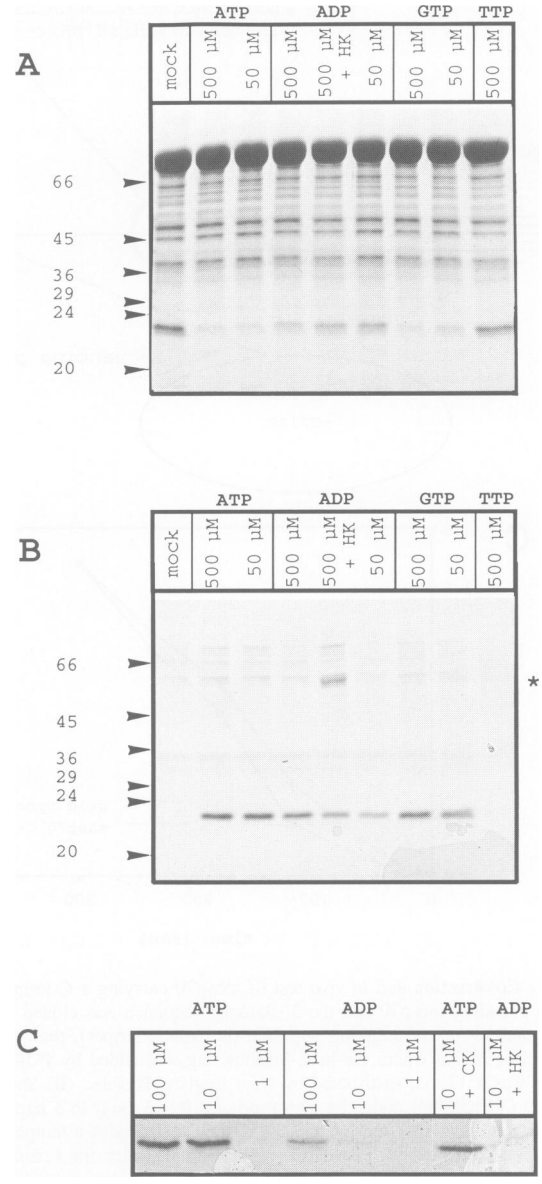

Fig. 4. Nucleotide-dependent release of GrpEp from mhsp70 bound to Ni−NTA resin. Mitochondrial extract from 20 μg of mitochondria containing histidine tagged mhsp70 was incubated with 120 ml of Ni−NTA resin. The resin was washed four times with 1 ml each of K^+-MOPS pH 7.4, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM imidazole, 0.25 M sucrose, 10% glycerol, 0.5 mM PMSF and resuspended in 2.7 ml of the same buffer. Aliquots (0.3 ml) of the suspension were incubated for 10 min at 30°C with the additions specified on top of each lane and in the following paragraphs to measure the nucleotide-dependent release of the 23 kDa protein. The aliquots were then centrifuged and pellets (**A**) and supernatants (**B** and **C**) were analyzed by SDS−12%PAGE and stained with Coomassie Brilliant Blue. (A) and (B) Lanes (counted from left): 1, no addition; 2, 500 μM ATP; 3, 50 μm ATP; 4, 500 μM ADP; 5, 500 μM ADP that had been preincubated with 0.15 U hexokinase and 20 mM 2-deoxyglucose; 6, 50 μM ADP; 7, 500 μM GTP; 8, 50 μM GTP; 9, 500 μM dTTP. Asterisk, hexokinase used for removing ATP. Arrowheads at left, molecular weight markers (sizes in kDa). (C) A similar, but separate release experiment was performed as described above. Nucleotide-dependent release of the 23 kDa protein was measured by adding the following: lanes (counted from left): 1, 100 μM ATP; 2, 10 μM ATP; 3, 1 μM ATP; 4, 100 μM ADP; 5, 10 μM ADP; 6, 1 μM ADP; 7, 10 μM ATP; 7, 10 μM ATP that had been preincubated with 0.1 U creatine kinase and 10 mM creatine phosphate; 8, 10 μM ADP that had been preincubated with 0.15 U hexokinase and 20 mM 2-deoxyglucose.

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-77   GC CAC GAA GGC CCC AAG TGC CGC TTT AGA TCC AAA GTA CAA

                                                    M   R
-36  GGA CAT ATT GTG GAA GAG TAA GTT GAA TAT TTG AAA ATG AGA

      A   F   S   A   A   T   V   R   A   T   T   R   K   S
 6   GCT TTT TCA GCA GCC ACC GTT AGG GCC ACA ACT AGG AAG TCG

      F   I   P   M   A   P   R   T   P   F   V   T   P   S
 48  TTC ATC CCA ATG GCA CCA AGA ACT CCT TTT GTG ACT CCA TCA

      F   T   K   N   V   G   S   M   R   R   M   R   F   Y
 90  TTT ACA AAG AAT GTA GGC TCA ATG AGA AGA ATG AGA TTT TAT
                        (S) (E) (E) (S) (K) (E)  N   N   E
      S   D   E   A   K   S   E   E   S   K   E   N   N   E
132  TCT GAT GAA GCC AAA AGT GAA GAA TCC AAA GAA AAC AAT GAA
     (D)  L   T   E   E   Q   ?   E
      D   L   T   E   E   Q   S   E   I   K   K   L   E   S
174  GAT TTG ACT GAA GAG CAA TCA GAA ATC AAG AAA TTA GAG AGC

      Q   L   S   A   K   T   K   E   A   S   E   L   K   D
216  CAG TTA AGC GCG AAG ACT AAA GAA GCT TCT GAA CTC AAG GAC
                    S   V   A   D   F   R
      R   L   L   R   S   V   A   D   F   R   N   L   Q   Q
258  AGA TTA TTA AGA TCT GTG GCA GAT TTC AGA AAT TTA CAA CAA
                                A   K   D   F   A   L
      V   T   K   K   D   I   Q   K   A   K   D   F   A   L
300  GTC ACA AAG AAG GAT ATT CAG AAA GCT AAG GAC TTT GCT TTA
      Q   K
      K   F   A   K   D   L   L   E   S   V   D   N   F
342  CAG AAG TTT GCA AAG GAT TTA TTG GAA TCT GTA GAT AAC TTT

      G   H   A   L   N   A   F   K   E   E   D   L   Q   K
384  GGT CAT GCT TTG AAT GCT TTT AAA GAG GAA GAC TTA CAA AAG

      S   K   E   I   S   D   L   Y   T   G   V   R   M   T
426  TCC AAG GAA ATT AGT GAT TTG TAT ACA GGG GTT AGA ATG ACA

      R   D   V   F   E   N   T   L   R   K   H   G   I   E
468  AGA GAT GTT TTT GAA AAC ACC CTA AGA AAG CAC GGT ATT GAA

      K   L   D   P   L   G   E   P   F   D   P   N   K   H
510  AAA TTA GAC CCA TTG GGA GAA CCA TTT GAT CCA AAT AAA CAC

      E   A   T   F   E   L   P   Q   P   D   K   E   P   G
552  GAA GCA ACG TTC GAG TTG CCA CAA CCT GAT AAG GAA CCG GGT

      P   V   F   H   V   Q   Q   L   G   F   T   L   N   D
594  ACT GTT TTC CAT GTA CAA CAA TTA GGT TTC ACC TTG AAT GAC

      R   V   I   R   P   A   K   V   G   I   V   K   G   E
636  AGA GTT ATC AGA CCA GCA AAA GTC GGA ATT GTT AAG GGC GAA

      E   N   -
678  GAG AAC TAA CCT TGA TGA AGA ATT ATT ACT ACC TAC GAC TTT

720  ATC TAT ATG TGC CTT TTT TCT TTC TCT GCC TGG TTT AGT TTT

762  ATT TAC TAC GTT TAC TTG TAT ATA TTT GTA AAC TAA TGA AAA

804  TAT AAA GAA ATA AAT CTC AAA ACA TGC TTT TAT ACT GTA AAA

846  ACT GGT GTT ATA TGG GTT AAT GAA AGG CAT ATA ATA TAC AAG

888  GGT GAC AAA TTT TTA GTA CTT TCG TAT ACA TTT TAA AGA AGA

930  CAC TTT TGT TTC TTG AGG ACT GGT CAA TTC CAT TTT TAT TTT

972  TTT GTA AAC AAT GAC GGC GAG TTT CAT TAG GAA CGT GAG CAG

1014 AAA AAT ATT ATC TGT TGT AAA CAA TCG CAT CGG TGG GAG AGC

1056 ATA AAC
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Fig. 5. Nucleotide sequence of the *GRPE* gene, deduced amino acid sequence of the protein product (GrpEp), and amino acid sequences of three tryptic peptides derived from the purified 23 kDa protein. Amino acids are given by their single letter code. The first base of the putative initiator codon is numbered as +1. The three underlined amino acid sequence regions are those determined by automated Edman degradation of three tryptic peptides derived from the purified 23 kDa protein. Residues in parentheses were one of two amino acids determined at that cycle. The sign '?' denotes that no amino acid was recovered at that cycle. EMBL database accession number X78350.

containing wild-type mhsp70; the 23 kDa protein thus bound to the resin as a result of being complexed with tagged mhsp70.

The 23 kDa protein is released from mhsp70 by ATP

The 23 kDa protein that had bound to the Ni−NTA resin via tagged mhsp70 could be released from the resin by incubation with ATP (Figure 4). ADP (in the presence of hexokinase and 2-deoxyglucose) also promoted release, although only at much higher concentrations than ATP. GTP, too, was partly effective whereas dTTP (Figure 4) and ATP-γ-S (not shown) as well as CTP (not shown) were inactive. These preliminary observations did not identify the rate at which ATP, ADP and GTP effect release; they merely provided an empirical basis for purifying the 23 kDa protein from a mitochondrial extract containing tagged mhsp70. Purification consisted of only three simple steps: binding of mhsp70 plus the 23 kDa protein to Ni−NTA resin in the absence of ATP, release of the 23 kDa protein from the resin by ATP and SDS−PAGE.

Cloning of the GRPE gene

The size of the 23 kDa protein and its ATP-sensitive interaction with mhsp70 suggested to us that it might be the mitochondrial homolog of bacterial GrpE (Zylicz *et al.*, 1987). Attempts to determine the N-terminal sequence of the 23 kDa protein were unsuccessful, perhaps because the N-terminus of the isolated protein was blocked. When we determined the sequence of three tryptic fragments one of the sequences was indeed nearly identical to a region of GrpE from *E.coli* (see below). The similarity was insufficient to prove homology of the 23 kDa protein to bacterial GrpE, but we obtained proof by cloning and sequencing the yeast gene encoding the 23 kDa protein (see below).

Cloning of the *S.cerevisiae GRPE* gene was facilitated by the report (Kambouris *et al.*, 1993) that a DNA sequence located upstream of the *KIN3* gene potentially encoded a protein resembling GrpE from *E.coli*. Based on this information, an oligonucleotide was designed and used to probe a *S.cerevisiae* cDNA library cloned in the bacteriophage lambda YES vector (Elledge *et al.*, 1991). Twelve recombinant plaques were detected that hybridized to this DNA probe. Since the DNA insert in all 12 lambda clones appeared to be of identical length, only two of them were subsequently subcloned and sequenced (Figure 5). The protein coding sequence most likely initiates at the ATG codon located at the nucleotide marked +1. This codon is the first ATG codon and, in addition, is preceded by a TAA termination codon, located in-frame upstream, at nucleotide −12. The open reading frame potentially encodes a 228 residue protein with a predicted M_r of 26 066. The deduced amino acid sequence between residues 47 and 207 exhibits an overall 35.4% identity to the corresponding segment of the GrpE protein of *E.coli* (Figure 6). The sequences of all three tryptic peptides that were derived from the 23 kDa protein could be precisely aligned with the predicted amino acid sequence of the GrpE-like protein. We conclude that the purified 23 kDa protein is the product of the cloned *GRPE*-like yeast gene.

Based on the nucleotide sequence of the cDNA clones, two DNA primers were designed in order to amplify the chromosomal copy of the gene. The amplified DNA product had the expected size and was subcloned and sequenced. Its nucleotide sequence was identical to that of the cDNA clones. This result showed that the chromosomal gene lacks introns.

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                                20        30        40        50        60
Yeast GrpE          MRAFSAATVRATTRKSFIPMAPRTPFVTPSFTKNVGSMRRMRFYSDEAKSEESKENNEDL
                                                                :| :: |:::::|::
E.coli GrpE                                 MSSKEQKTPEGQAPEEIIMDQHEEIEAVEPEASAEQV
                                                          10        20        30

                        70        80        90       100       110       120
Yeast GrpE          TEEQSEIKKLESQLSAKTKEASELKDRLLRSVADFRNLQQVTKKDIQKAKDFALQKFAKD
                    :  :::: :||:||   :::::  :| :||  |:: ||:: |: ||:||::|||:|| ::
E.coli GrpE         DPRDEKVANLEAQL---AEAQTRERDGILRVKAEMENLRRRTELDIEKAHKFALEKFINE
                         40        5O            60        70        80        90

                       130       140       150       160       170       180
Yeast GrpE          LLESVDNFGHALNAFKEEDLQKSKEISDLYTGVRMTRDVFENTLRKHGIEKLDPLGEPFD
                    ||  :|::::||    | : : : ::|:: :|: :| : : :::|| |:| ::  : |:|
E.coli GrpE         LLPVIDSLDRAL----EVADKANPDMSAMVEGIELTLKSMLDVVRKFGVEVIAETNVPLD
                       100           110       120       130       140       150

                             190       200       210       220
Yeast GrpE          PNKHEATFELPQPDKEPGTVFHVQQLGFTLNDRVIRPAKVGIVKGEEN
                    || |:|:  :   | :||:|: : | |:|||:|:||:|:|:::|:
E.coli GrpE         PNVHQAIAMVESDDVAPGNVLGIMQKGYTLNGRTIRAAMVTVAKAKA
                             160       170       180       190
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Fig. 6. Yeast GrpEp is similar to *E.coli* GrpE, but has an N-terminal extension. Identical and similar amino acids are connected by vertical bars and double dots, respectively. Horizontal bars denote gaps introduced to maximize the alignment.

In vitro synthesized GrpEp is imported into the matrix of isolated yeast mitochondria and cleaved to a smaller size

The sequence alignments shown in Figure 6 suggest that GrpEp is synthesized with an N-terminal extension. We suspected that this extension contained a matrix targeting signal because mhsp70, the partner of GrpEp, is located in the matrix. To test this assumption, we synthesized GrpEp *in vitro* and assayed import of the radiolabeled protein into isolated yeast mitochondria. As shown in Figure 7, GrpEp was indeed imported by mitochondria to a protease-inaccessible location (lanes 4 and 5). Import required an electrochemical potential across the inner membrane (compare lanes 2 and 3 with lanes 4 and 5). The imported protein was localized in the matrix, as it remained protease-inaccessible in mitoplasts (mitochondria with a disrupted outer membrane; lane 6). The imported protein was distinctly smaller than the *in vitro* translation product, suggesting removal of a matrix targeting signal by the matrix processing peptidase (compare lane 1 with lanes 4 and 5). The low overall efficiency of import and the sequence of the N-terminal extension (see Discussion) suggest that the matrix targeting signal is weak. Nevertheless, the properties of the GrpEp precursor are fully consistent with a role of the mature protein as a partner for mhsp70.

GrpEp is an essential protein

mhsp70 is essential for the viability of yeast cells (Craig *et al.*, 1989). In order to test whether the same is true for GrpEp, we disrupted one of the two chromosomal *GRPE* genes in a diploid yeast strain (Figure 8A), induced the cells to sporulate and tested the four-spore progeny from several asci for the presence of an intact *GRPE* gene and for viability on glucose-containing rich medium. Only spores containing an intact *GRPE* gene formed visible colonies. GrpEp is thus essential for viability of yeast cells.

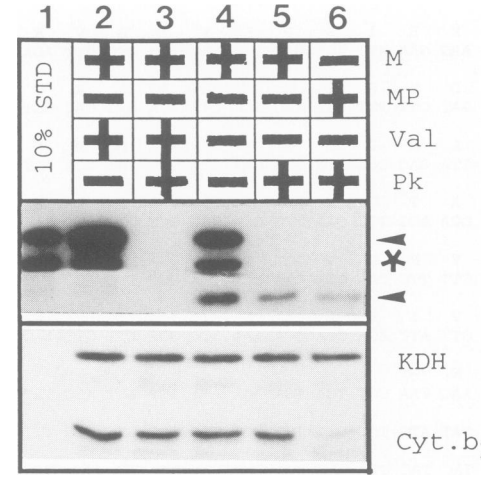

Fig. 7. The *in vitro* synthesized GrpEp precursor is imported into the matrix of isolated yeast mitochondria and processed to a smaller form. (Upper panel) The *GrpE* gene was placed under control of the SP6 promoter (plasmid pSP65, Promega), transcribed/translated *in vitro* in the presence of [^{35}S]methionine, and incubated for 10 min at 30°C in a final volume of 0.15 ml with 200 μg of yeast mitochondria under the conditions indicated. The mitochondria (M) were then either left untreated, treated with proteinase K, or converted to mitoplasts (MP) in the presence of proteinase K (Glick *et al.*, 1992) before being analyzed by SDS−12%PAGE and fluorography. Lanes: 1 (10% STD), 10% of the precursor that was added to each import assay. The lower band (asterisk) probably reflects aberrant initiation from the ATG codon at position +60; 2: mitochondria were uncoupled with 1 μg/ml valinomycin (Val) before adding precursor; 3, same as 2 except that mitochondria were treated with proteinase K (Pk; 50 μg/ml) after incubation with precursor; 4, mitochondria analyzed without subsequent treatment; 5, mitochondria treated with proteinase K (50 μg/ml) after incubation with precursor; 6, mitochondria converted to mitoplasts in the presence of proteinase K (50 μg/ml) after incubation with precursor. Upper and lower arrowheads at right, putative precursor and mature forms of GrpEp, respectively. (Lower panel) The samples shown in the upper panel were analyzed by SDS−12% PAGE and immunoblotting with antisera against α-ketoglutarate dehydrogenase (KDH, a soluble matrix marker) and cytochrome b_2 (Cyt. b_2, a soluble intermembrane space marker). Bound antibodies were visualized with [^{125}I]-protein A and fluorography.

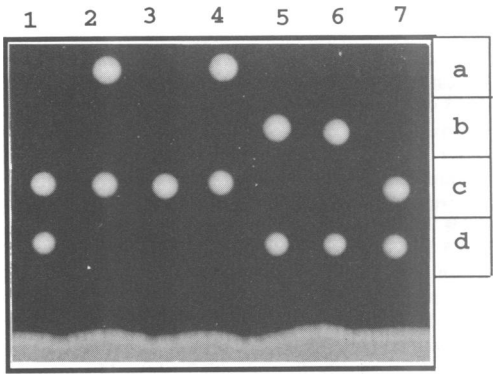

Fig. 8. *GRPE* is an essential gene. One of the two chromosomal *GRPE* genes in a diploid *S.cerevisiae* strain was disrupted by the *URA3* gene, the diploid was sporulated and seven different asci (numbered 1–7 on top of the figure) were dissected. The four spores (labeled a–d on the right margin of the figure) recovered from each of the asci were allowed to germinate and to grow for 3 days at 30°C on glucose-containing rich medium. The colonies were then photographed.

Discussion

The experimental system

In this work we have used a new approach for studying the mitochondrial chaperone system. Up to now, the interaction of chaperones with other proteins has been investigated by co-immunoprecipitation (Ostermann *et al.*, 1989; Prasad *et al.*, 1990; Manning-Krieg *et al.*, 1991; S.Rospert, S.Müller, G.Schatz and B.S.Glick, submitted), cofractionation on sizing columns (Koll *et al.*, 1992; Viitanen *et al.*, 1992; Rospert *et al.*, 1993a), coelectrophoresis on non-denaturing gels (Ostermann *et al.*, 1989; Prasad *et al.*, 1990; Koll *et al.*, 1992) and functional tests (Bertsch *et al.*, 1992; Rospert *et al.*, 1993a). Our present approach of using a yeast strain with histidine tagged mhsp70 permits the rapid and gentle isolation of mhsp70 as well as the identification of bound partner proteins. Since the tagged mhsp70 binds to the affinity resin via a C-terminal extension that is not present in the authentic protein, this binding would not be expected to disrupt interaction with a partner protein. As the tagged mhsp70 appears to be functional, the present system should be useful for identifying additional proteins that interact with mhsp70.

Properties of GrpEp

Although the overall sequence identity between yeast mitochondrial GrpEp and bacterial GrpE proteins is only ~30 %, identical and similar residues are present over the entire length of the sequences and optimal sequence alignment requires introduction of only a few gaps. mHsp70, too, is very similar to DnaK, its counterpart in bacteria (Georgopoulos, 1977; Craig *et al.*, 1989). In addition, GroES and GroEL homologs from *E.coli* and mitochondria can partially substitute for each other functionally *in vitro* (Rospert *et al.*, 1993a). We are currently testing whether yeast GrpEp or mhsp70 can functionally replace their bacterial homologs *in vitro* or *in vivo*. The similarities between the chaperone systems of mitochondria and bacteria are particularly compelling arguments for the prokaryotic origin of mitochondria.

Unlike bacterial GrpE proteins, yeast GrpEp is made as a larger precursor with an N-terminal extension that is cleaved upon import into mitochondria. We could not establish the precise length of this extension because the N-terminus of the isolated mature GrpEp was blocked. However, the sequence alignment shown in Figure 6 suggests a presequence length of 20–25 residues. This length is compatible with the size difference between the GrpEp precursor and the mature protein, and is typical of matrix targeting signals. The signal appears to be weak as its predicted helical amphiphilicity is low (data not shown; see Roise *et al.*, 1986; von Heijne, 1986; Roise and Schatz, 1988). Indeed, import of the GrpEp precursor into isolated mitochondria appeared to be less efficient than that of most other mitochondrial precursor proteins tested in our laboratory. Whether import of GrpEp is also inefficient *in vivo* remains to be tested.

Members of the hsp70 protein family also participate in protein translocation into the endoplasmic reticulum (Vogel *et al.*, 1990; Nguyen *et al.*, 1991; Sanders *et al.*, 1992) and probably also into chloroplasts (Yalovsky *et al.*, 1992; Tsugeki and Nishimura, 1993). It is thus conceivable that eukaryotic cells contain additional GrpEp isoforms which interact with hsp70 in the endoplasmic reticulum and in chloroplasts. As import of the GrpEp precursor into isolated yeast mitochondria is sluggish, some of the 'mitochondrial' GrpEp might even remain in the cytosol and function with one of the cytosolic members of the hsp70 protein family. We consider this last possibility unlikely because mhsp70 and cytosolic hsp70s differ appreciably in sequence, and because the cytosol lacks an enzyme that can specifically remove the matrix targeting sequence from mitochondrial precursor proteins.

Possible function of GrpEp

GrpEp is the major protein that copurifies with mhsp70 under conditions in which ATP levels have been depleted. In addition, GrpEp is released from mhsp70 by ATP, but very inefficiently or not at all by other nucleotides. This behavior parallels that of GrpE from *E.coli* in which GrpE and DnaJ participate in several reactions catalyzed by DnaK, the bacterial hsp70 homolog (Zylicz *et al.*, 1987). One of the major roles of GrpE is to accelerate the release of DnaK-bound nucleotide, thus allowing DnaK to recycle more efficiently (Liberek *et al.*, 1991). In principle, GrpEp could mediate the translocation of precursors across the inner membrane or their refolding in the matrix. As newly imported precursors are released from mhsp70 in an incompletely folded state (Manning-Krieg *et al.*, 1991), mhsp70 may not be directly involved in the refolding of imported precursors. Although we cannot exclude the possibility that the mhsp70–GrpEp system also mediates protein folding in the matrix, we propose that its main function is the the ATP-dependent transport of precursors across the mitochondrial inner membrane and the unfolding of mitochondrial precursors on the mitochondrial surface (Beasley *et al.*, 1992; Gambill *et al.*, 1993; Glick *et al.*, 1993). mHsp70–GrpEp may also be involved in the replication or transcription of mitochondrial DNA and the synthesis of mitochondrially encoded proteins (Georgopoulos, 1977). The availability of the *GRPE* gene and of the yeast strain expressing tagged mhsp70 should help us to purify these two proteins in sufficient amounts to study their functional interaction *in vitro*.

GrpEp is clearly a mitochondrial protein. As the eukaryotic

cytoplasm contains its own distinct set of hsp70 proteins (Craig, 1993) and DnaJ homologs (Caplan and Douglas, 1991; Atencio and Yaffe, 1992), it may also contain one or more GrpE homologs. The approach described here might be useful for detecting such proteins.

So far, every mitochondrial protein essential for cell viability has proved to be a component of the mitochondrial protein import system (Baker and Schatz, 1991). This protein family includes the two subunits of the general matrix processing peptidase (Yaffe and Schatz, 1984), hsp60 (Cheng *et al.*, 1989), mhsp70 (Craig *et al.*, 1989), cpn10 (Rospert *et al.*, 1993b), ISP42 (Baker *et al.*, 1990), ISP45/MIM44 (Maarse *et al.*, 1992) and Mas6p (Emtage and Jensen, 1993). GrpEp is now the ninth member of this selective group. This fact supports our suggestion that GrpEp cooperates with mhsp70 in the import of mitochondrial proteins from the cytoplasm.

Materials and methods

Yeast and E.coli strains

The *E.coli* strain DH5a was used for plasmid growth and genetic manipulations. The PCR experiments involving the *GRPE* gene (see below) were performed with genomic *S.cerevisiae* DNA prepared from strain GA24 (*MAT***a** *ura3 his3 pep4-3*). Disruption of the yeast *GRPE* gene was performed in the diploid *S.cerevisiae* strain JR182 (*MAT* **a**/α *his3/his3 his4/his4 ura3/ura3 leu2/leu2 trp1/trp1 ade8/ade8*). DNA sequencing was performed with plasmid pSK (Stratagen), and disruption of the *GRPE* gene with plasmid pBAD (kindly provided by L.M.Guzman-Verduzco and D.Belin).

The wild-type *S.cerevisiae* strain D273-10B (MATa; ATCC 25657) was used for the isolation of mitochondria for *in vitro* import studies. Cells were grown to late logarithmic phase in semisynthetic medium containing 2% lactate and 0.1% glucose (Daum *et al.*, 1982). Strains MH272-3fa (*MAT***a** *leu2 ura3 trp1 Δ-his3 ade2*) and MH272-3fα (*MAT*α *leu2 ura3 trp1 Δ-his3 ade2*) were used for determining the mating type, using the histidine markers *his3* and *his4* for auxotrophic selection of diploids. Other yeast strains are specified in a later section that describes the disruption of the gene encoding mhsp70.

Insertion of the hexa-histidine tag

The tag was introduced by three subsequent rounds of PCR amplification, performed in a final volume of 100 μl, containing 1 μg of template (pTZ19R-wt), 200 μM of each dNTP, 10 μl of 10-fold concentrated buffer (500 mM KCl, 100 mM Tris−HCl pH 8.8, 15 mM $MgCl_2$, 1% Triton X-100, 0.1% gelatin), 0.5 μM of each primer and 2.5 U Taq polymerase (AmpliTaq, Perkin Elmer). The reaction mixtures were incubated for 3 min at 94°C and submitted to 30 cycles (30 s at 94°C, 45 s at 55°C and 30 s at 72°C). The final cycle was an extension step of 7 min at 72°C. We used the following primers: *Msc*I-primer (5′-AAGGCTGACCAA**TTGG'CCA**-ACG-3′; the *Msc*I restriction site is bold), universal sequencing primer (5′-GTTTTCCCAGTCACGAC-3′), primer for the insertion of the hexa-histidine tag (5′-TCTGGTGAAACTAAGCAGCACCACCACCACCAC-CACTAAAAAGCAAATTCCTGT-3′) and antisense primer specific for the hexa-histidine tag. Two independent PCR amplifications were first performed: one with *Msc*I/hexa-histidine-antisense primers (350 bp amplified) and the other with the hexa-histidine-sense/universal sequencing primers (750 bp amplified). The two amplified fragments were gel-purified and then joined in a PCR amplification step with *Msc*I/universal sequencing primers. The resulting 1.1 kb amplification product was purified with the Prep-a-gen kit (Bio-Rad) and digested with *Eco*RI and *Msc*I. After an additional gel purification, the fragment was ligated into the vector pTZ19R-wt that had been cut with *Msc*I and *Eco*RI. To check for the presence of missense mutations ~350 bp of the amplified DNA fragment encoding the C-terminal part of mhsp70 and the hexa-histidine tag were sequenced (Sanger *et al.*, 1977), using as sequencing primers the *Msc*I primer and a primer specific only for the hexa-histidine tag (5′-GTGGTGGTGGTGGTGTG-3′).

Purification of C tagged mhsp70 from mitochondria

All steps were performed on ice or as close to 4°C as possible. Nycodenz-purified mitochondria (40 mg protein, Glick *et al.*, 1992) were resuspended to 10 mg protein/ml in breaking buffer (0.6 M sorbitol, 20 mM K^+−HEPES pH 7.4), diluted 9-fold with buffer A [20 mM K^+−HEPES pH 7.4, 100 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM phenylmethyl sulfonyl fluoride (PMSF), 1.25 μg/ml leupeptin, 0.75 μg/ml antipain, 0.25 μg/ml chymostatin, 0.25 μg/ml elastatinal, 5 μg/ml pepstatin], left for 20 min on ice and sonicated on ice for 5 min (15 s on/off pulsed periods, 80% duty cycle, W-375 sonicator with microtip, Heat Systems Ultrasonics) to release soluble matrix proteins. The sonicated suspension was centrifuged at 100 000 *g* for 30 min. The supernatant (10 ml) was applied to an ATP−agarose column (bed volume 1 ml; C8, Sigma) and recirculated through the column overnight. The column was washed successively with (i) 15 ml buffer A, 1% Triton X-100, (ii) 10 ml buffer A, 2 mM GTP, (iii) 10 ml buffer A, 0.5 M NaCl and (iv) 15 ml buffer A, and was then eluted for 6 h by a batch procedure with buffer A containing 7.5 mM MgATP. The ATP-eluate was adjusted to 5 mM imidazole pH 7.4 and incubated with 1 ml of Ni−NTA slurry (Qiagen) for 1 h. The slurry was washed successively with (a) buffer A, (b) buffer A, 1 mM ATP, 250 mM NaCl, (c) buffer A. Elution was performed with 2 ml 0.4 M imidazole pH 7.4, 20 mM K^+−HEPES pH 7.4, 25 mM KCl, 10% glycerol. Aliquots were quick frozen in liquid nitrogen and stored at −70°C. This procedure yielded about 700 μg of tagged mhsp70 from 40 mg of mitochondrial protein. Analysis of 8 μg of the purified protein by SDS−PAGE failed to reveal the presence of any impurities (see Figure 2).

Cloning of the S.cerevisiae GRPE gene

The *GRPE* cDNA was cloned from a *S.cerevisiae* cDNA library in the bacteriophage lambda YES vector (Elledge *et al.*, 1991; kindly provided by Dr S.J.Elledge), using a ^{32}P-labeled specific DNA primer (primer 1, 5′-AAGGATATTCAGAAAGCTAAGACTTTGCT-3′) defined by the upstream DNA sequence of the *S.cerevisiae KIN3* gene (Kambouris *et al.*, 1993). Nitrocellulose filter replicas were prepared from large Petri dishes containing $>10^4$ plaques of the *S.cerevisiae* cDNA library in lambda YES. The DNA was denatured by immersing the filter in a solution of 0.5 N NaOH, 1.5 M NaCl for 5 min, followed by an incubation for 5 min in 0.5 M Tris−Cl pH 7.4, 1.5 M NaCl. The filter was rinsed in 2×SSC solution and the DNA was fixed to the filter by baking for 30 min at 80°C in a vacuum oven. Prehybridization was done with 4×SSC and 5% skim milk at 55°C for 30 min, followed by hybridization with 5 pmol of γ-^{32}P-labeled DNA primer 1 (see above) for 12 h under the same conditions. The filter was washed twice with 2×SSC/ 0.1% SDS at room temperature for 20 min and once more with a 0.2×SSC/0.1% SDS solution at 55°C for 20 min. Positive plaques were subcloned into plasmid pSE936 (Elledge *et al.*, 1991).

Knowledge of the cDNA sequence of *S.cerevisiae GRPE* also permitted us to clone the gene directly from *S.cerevisiae* chromosomal DNA (extracted from strain GA24), using PCR and the following DNA primers: primer 2: 5′-CGGAATTCCATATGAGAAGCTTTTTCAGCAGCC-3′ and primer 3: 5′-CGGGGTACCATACAAGTAAACGTAGTAAATAAAAC-3′.

The PCR was performed in a final volume of 50 μl containing 0.5 μg of template DNA, 200 μM of each dNTP, 5 μl of a 10-fold concentrated buffer [100 mM KCl, 200 mM Tris−HCl pH 8.8, 20 mM $MgSO_4$, 1% Triton X-100, 100 mM $(NH_4)_2SO_4$], 2 μM of each DNA primer and 1 U Vent polymerase (New England Biolabs). The reaction mixtures were incubated for 1 min at 94°C and submitted to 30 PCR cycles (40 s at 92°C, 40 s at 50°C and 90 s at 75°C). The final cycle was an extension step (5 min at 75°C).

DNA sequencing and recombinant DNA techniques

The *GRPE* cDNA was subcloned into plasmid pKS at the *Xho*I site, whereas the chromosomal *GRPE* gene was subcloned into plasmid pKS at the *Sma*I site. Double-stranded plasmid DNA was sequenced (Sanger *et al.*, 1977) using the reverse primer 5′-AACAGCTATGACCATG-3′, the M13-20 primer 5′-GTAAAACGAGGCCAGT-3′, as well as the following primers: primer 1 (5′-AAGGATATTCAGAAAGCTAAGACTTTGCT-3′); primer 2 (5′-CGGAATTCCATATGAGAAGCTTTTTCAGCAGCC-3′); primer 3 (5′-CGGGGTACCATACAAGTAAACGTAGTAAATAAAAC-3′); primer 4 (5′-GGCTCAATGAGAAGAATG-3′); and primer 5 (5′-CCTTAGCTTTCTGAATATCC-3′). Buffers and reaction conditions for restriction enzymes and T4 DNA ligase were those listed by Boehringer Mannheim and New England Biolabs. Isolation of DNA fragments, preparation of plasmid DNA and other standard techniques were performed as described by Sambrook *et al.* (1989).

Disruption and replacement of the gene encoding mhsp70

One chromosomal copy of the gene encoding mhsp70 (termed *ENS1* by Morishima *et al.*, 1990, or *SSC1* by Craig *et al.*, 1987) was disrupted with the *LEU2* marker in the diploid strain JK9-3d (Morishima *et al.*, 1990). Leucine prototrophs were checked by Southern blotting, sporulation and

dissection of tetrads. The resulting strain JK9-dis (*MAT* **a**/α *ura3-52/ura3-52 his4/his4 trp1/trp1 leu2-3/leu2-3 ENS1/ens1::LEU2*) was transformed with plasmid YCp50-wt or with plasmid YCp50-C-tag (see Figure 1). Transformants were selected on media lacking leucine and uracil, and sporulated. The spores from individual asci were dissected on rich medium and spores containing the disrupted chromosomal copy of *ENS1/SCC1* and the rescuing single-copy plasmid were selected. The haploidy of the leucine prototrophs was ascertained by mating to yeast strains MH272-3fa or MH272-3fα.

Disruption of the S.cerevisiae GRPE gene

The *GRPE* gene was disrupted by subcloning it from pSKGRPE into vector pBAD, inserting the *URA3* gene into the unique *Hin*dIII site of *GRPE* (Figure 5), excising the interrupted *GRPE* gene at the flanking *Eco*RI and *Xba*I sites of the multiple cloning site of pBAD vector, and transforming the linear fragment into the diploid yeast strain JR182. Uracil-prototrophic transformants were checked for disruption of the *GRPE* gene by Southern blotting. To this end, genomic DNA was digested with *Ssp*I that cuts at nucleotide positions −8 and +1019 (Figure 5). The fragments were separated by electophoresis on a 0.8% agarose gel, blotted to a nitrocellulose membrane and probed with the ^{32}P-labeled, PCR-amplified entire *GRPE* gene.

Purification of the 23 kDa protein for microsequencing

Nycodenz-purified mitochondria (100 mg) was processed up to the 100 000 *g* supernatant as described in the purification procedure, except that the mitochondria were incubated with 1 μg/ml oligomycin for 5 min at 30°C before sonication. The supernatant (18 ml) was then mixed with 2.5 ml 1.8 M sucrose, 1 ml Ni−NTA resin, 400 μl 1 M imidazole pH 7.4 and 50 μl of 0.2 M PMSF (dissolved in dimethylsulfoxide) and the suspension was gently mixed at 4°C for 1.5 h. The resin was sedimented, washed three times with 10 ml buffer B (20 mM K^+−MOPS pH 7.4, 0.25 M sucrose, 200 mM KCl, 10% glycerol, 0.5 mM PMSF), and the 23 kDa protein was eluted with 5 ml buffer B, 10 mM MgATP for 30 min at 30°C. The collected supernatant was concentrated by precipitation with trichloroacetic acid and subjected to SDS−12% PAGE. Transfer of the protein onto PVDF membranes (Immobilon P) and enzymatic digestion of the membrane-bound protein with trypsin were done according to published procedures (Matsudaira, 1987; Fernandez *et al.*, 1992). The tryptic peptides were chromatographed on a C18 Vydac 218-TP51 reversed-phase HPLC column (250×1 mm, Vydac, Hesperia, CA) at a flow rate of 50 μl/min. Buffer I was 0.05% trifluoroacetic acid, buffer II was 0.05% trifluoroacetic acid in 80% acetonitrile. Peptides were eluted with the following program: 5 min, a mixture of 2% buffer II/98% buffer I; 90 min, a gradient from 2% buffer/98% buffer I to 75% buffer II/25% buffer I. The column effluent was monitored at 214 nm and selected peaks were sequenced on an Applied Biosystem 473A or 477A protein sequencer.

Miscellaneous

Published methods were used for transformation of yeast cells (Ito *et al.*, 1983), *in vitro* transcription/translation of precursor protein in the presence of [^{35}S]methionine (Hurt *et al.*, 1984), import into isolated mitochondria (Glick *et al.*, 1992) and immunoblotting (Haid and Suissa, 1983). Mitochondrial protein concentration was measured by absorption at 280 nm in the presence of 0.6% SDS. The concentration of purified mhsp70 was determined with the BCA procedure described in a company brochure of Pierce Chemical Co.

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