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Cloning and disruption of the gene encoding yeast mitochondrial chaperonin 10, the homolog of *E. coli* groES

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The mitochondrial chaperonin system consists of chaperonin 60 (also termed hsp60), which is homologous to *E. coli* groEL, and chaperonin 10, which is homologous to *E. coli* groES. In yeast, chaperonin 60 function has been shown to be essential for viability. We report here that the same is true for chaperonin 10. We have cloned, sequenced and disrupted the nuclear chaperonin 10 gene *CPN10* from *Saccharomyces cerevisiae*. This gene encodes a protein of 11,372 Da that is imported into the mitochondrial matrix without detectable cleavage. Haploid cells lacking a functional copy of *CPN10* fail to grow at temperatures between 23 and 37°C.

Protein import; Protein folding; hsp60; groEL

1. INTRODUCTION

Protein folding in bacteria, mitochondria and chloroplasts is assisted by a chaperonin system that consists of two components [1–5]. Chaperonin 60 (cpn60; termed groEL in *Escherichia coli*, Rubisco binding protein in chloroplasts, and hsp60 in mitochondria) is a homo-oligomer of 60 kDa subunits organized into two stacked heptameric rings. Chaperonin 10 (cpn10; termed groES in *E. coli*) is a homo-oligomer consisting of 10 kDa subunits in *E. coli* and mitochondria, and 24 kDa subunits in chloroplasts. The *E. coli* protein forms a single heptameric ring. In *E. coli*, cpn60 and cpn10 are required for growth at temperatures as low as 17°C [6].

The mechanism by which cpn60 and cpn10 mediate protein folding is still poorly understood. It is generally agreed that cpn60 binds incompletely folded polypeptide chains, thereby suppressing their aggregation. The bound chains can then be released in a folding-competent form by addition of cpn10, potassium ions and ATP. According to this model, cpn60 traps folding intermediates, and cpn10 mediates their release. However, this picture may be over-simplified, as release of some folding intermediates from cpn60 can occur in the absence of cpn10 [7,8]. Thus the function and general importance of cpn10 are still unclear.

The in vivo role of a protein can be readily tested in yeast, as nuclear genes can be specifically inactivated by disruption or deletion [9]. This approach has shown that hsp60 is essential for viability of yeast cells [10]. Recently we identified the cpn10 protein of yeast mitochondria, established its role in the in vitro release of an unfolded polypeptide from chaperonin 60, purified it as a complex together with chaperonin 60, and determined its partial amino acid sequence [11]. Here we have used this sequence information to clone the nuclear *CPN10* gene from yeast, and to show that haploid cells lacking a functional copy of this gene are inviable at temperatures between 23 and 37°C.

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Abbreviations: cpn60, chaperonin 60 (hsp60, Rubisco binding protein, groEL); cpn10, chaperonin 10.

2. MATERIALS AND METHODS

2.1. *Strains and plasmids*

The *E. coli* strain UT580 was used for plasmid constructions. Disruption of the *CPN10* gene was performed in the diploid *S. cerevisiae* strain JK9-3d (*ura3, trp1, leu2, his4*) [12]. Culture, transformation, and sporulation methods were as described [13–16]. *Saccharomyces cerevisiae* was grown on YPD medium or, where appropriate, SD minimal medium supplemented with the required amino acids.

2.2. *Cloning and disruption of* CPN10

The *CPN10* gene was cloned from yeast chromosomal DNA [17] by the polymerase chain reaction (PCR) [18], using two degenerate primers that were designed from the partial amino acid sequence of yeast cpn10 [11]. The size of the amplyfied PCR fragment corresponded to that predicted from comparisons with the sequences of homologous genes from other organisms. The purified PCR fragment was labeled with ^{32}P and used to screen the yeast genomic DNA library, R1S18A [19]. Several independent clones were identified and the region of the insert corresponding to *CPN10* was sequenced [20]. Disruption of *CPN10* was performed after subcloning the gene by PCR into the pCR II cloning vector (Invitrogen Corp.). The sequence of this PCR product was identical to that of the genomic clone. The plasmid used for disruption of *CPN10* was constructed by inserting the *LEU2* gene into the *Sma*I site of *CPN10* (Fig. 1). For transformation of *S. cerevisiae*, the modified *CPN10* gene was excised using the flanking *Xba*I and *Dra*III sites within the genomic DNA (Fig. 1). Diploid leu$^+$ transfor-

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mants were checked for disruption of the *CPN10* gene by Southern blotting [15].

2.3. *In vitro transcription–translation of CPN10*

A fragment containing the coding region of *CPN10* (Fig. 1) was ligated into the plasmid pSP65 (Promega). This construction placed the *CPN10* gene under control of the SP6 promoter. Import of in vitro-synthesized cpn10 into yeast mitochondria, protease treatment, and preparation of mitoplasts were as described [21,22]. Electrophoresis was performed by Tricine SDS-PAGE [23].

3. RESULTS

The yeast *CPN10* gene encodes a protein of 106 amino acids with a calculated mass of 11,372 Da (Fig. 1). Like mammalian cpn10 proteins [2,4], yeast cpn10 is basic, with a predicted pI of 9.8. In contrast, groES from *E. coli* is acidic, with a pI of 5.2 [24]. The amino acid sequence of yeast cpn10 shows significant identity with cpn10 proteins from bacteria and from mammalian mitochondria (Fig. 2).

cpn10 should be located in the mitochondrial matrix, as it interacts with the matrix-localized chaperonin 60 [11]. Indeed, the predicted N-terminal portion of yeast cpn10 has the potential to form a positively charged α-helix, and thus resembles a typical matrix-targeting signal. cpn10 proteins from rat and bovine mitochondria have N-terminal portions with similar structural properties [25] (Fig. 2), whereas bacterial cpn10 proteins

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-205        T TCG TGT GAA GGT CAT TAA CGG GGT AAT GCC CGT CAT CAA TGA TAG

              XbaI                                        Hind III
-159  AAA TTT CTA GAA TCT TGA AGC AAT GGG AAA AAG AAG CTT GAT TAT GTA TAA

                                                                    DraI
-108  AAT TAG TCA AAA CGC TTA TAC AGT ACA AAC TTA TTA TGT GCT AGG TTT AAA

 - 57  ATA ATT TCT GAT AGA AGC CAT TGA TCG AAA ATA TAT TGA ATC TAC AGA AAA

                M   S   T   L   L   K   S   A   K   S   I   V   P   L   M
 -  6  AAA AGA ATG TCC ACC CTT TTG AAG TCT GCT AAA TCT ATC GTT CCA TTG ATG

        D   R   V   L   V   Q   R   T   K   A   Q   A   K   T   A   S   G
   45  GAC CGT GTC CTT GTC CAA AGA ATC AAG GCA CAA GCA AAG ACA GCA TCC GGG

        L   Y   L   P   E   K   N   V   E   K   L   N   Q   A   E   V   V
   96  TTG TAT TTA CCT GAA AAG AAC GTG GAG AAG TTA AAC CAA GCT GAA GTT GTT
                    SmaI
        A   V   G   P   G   F   T   D   A   N   G   N   K   V   V   P   Q
  147  GCC GTA GGC CCG GGC TTT ACT GAT GCT AAT GGT AAT AAG GTT GTT CCT CAA

        V   K   V   G   D   Q   V   L   I   P   Q   F   G   G   S   T   I
  198  GTT AAA GTT GGT GAC CAA GTT TTG ATT CCA CAG TTT GGT GGT TCT ACC ATT

        K   L   G   N   D   D   E   V   I   L   F   R   D   A   E   I   L
  249  AAA TTG GGT AAC GAC GAT GAA GTT ATT CTT TTC AGG GAC GCT GAA ATC CTG

        A   K   I   A   K   D   *
  300  GCT AAG ATT GCC AAG GAC TAA GAA TGA TGT CTT CTT CAA CAG AGA TTT TAT

  351  ATA CAT ACT CTA TAT GTA TGT ACC TGT AAA TAG GCC ATT ATG TAT GAC AGG

  402  AAA AAA AAA TAA TTG AAC TCA AAT TTC TTC TGC TAT TCA GTT GCT GCC CCC

  453  AAT TGT CTG CTA ATA AAA TTG ATC TTA TTC ATC AAT AGG AAT TTT AGG GAC

       DraIII
  504  ACT ATG TGT A
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Fig. 1. Nucleotide sequence of the *CPN10* gene and deduced amino acid sequence of the encoded protein. Restriction sites used for cloning and disrupting the gene are indicated.

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S. cerevisiae   MSTLLKSAKSIVPLMDRVLVQRIKAQAKTASGLYLPEKNVEKLNQAEVVAVGPG

E. coli                 MNIRPLHDRVIVKRKEVETKSAGGIVLTGSAAAKSTRGEVLAVGNG
C. burnetii             MKIRPLHDRVVVRRLEERT-SAGGIVIPDSAAEKPSRGEVISVGPG
B. taurus       MAGQAFRKFLPLFDRVLVERSAAETVTKGGIMLPEKSQGKVLQATVVAVGSG
R.norvegicus    AGAQAFRKPLPLFDRVLVERSAAETVTKGGIMLPEKSEGKVLEATVVAVGSG

S. cerevisiae   FTDANGNKVVPQVKVGDQVLIPQFG-GSTIKLGNDDEVIL-FRDAEILAKIAKD

E. coli         RILENGEVKPLDVKVGDIVIFNDGY-GVSEKIDNEEVLIMS--RSDILAIVEA
C. burnetti     KPLDMDEVRSLDVKVGDQILFGK-YAGTEVKLAEDEYIVMR--EDDIMGVIEK
B. taurus       SKGKGGEIQPVSVKVGDKVLLPE-YGG---KVVLDDKDYFLFRDGDILGKYVD
R. norvegicus   GKGKGGEIEPVSVKVGDKVLLPE-YGGT--KVVLDDKDYFLFRDGDILGKYVD
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Fig. 2. Comparison of the amino acid sequences of cpn10 from yeast mitochondria, rat mitochondria (*Rattus norvegicus* [25]), bovine mitochondria (*Bos taurus*; S.J. Pilkington and J.E. Walker, GenBank accession no. X69556), and the procaryotes *E. coli* [28] and *Coxiella burnettii* [29]. Boldfaced residues are conserved or conservative in all five sequences.

lack this feature (Fig. 2). Moreover, in vitro-synthesized radiolabeled cpn10 is imported by isolated yeast mitochondria (Fig. 3). Import is inhibited by dissipating the electrochemical potential across the mitochondrial inner membrane (Fig. 3). Protease treatment of mitoplasts (mitochondria in which the outer membrane has been selectively disrupted) confirms that cpn10 is imported into the matrix (Fig. 3). Unlike most other matrix-targeted proteins, cpn10 does not undergo detectable proteolytic processing upon import.

To study the function of yeast cpn10, we disrupted one of the two chromosomal copies of the *CPN10* gene in a diploid strain. Southern blotting confirmed that the transformants contained both a wild-type copy and a disrupted copy of *CPN10* (Fig. 4A). The diploid cells were then sporulated, the resulting tetrads were dissected, and spores were allowed to germinate at 23, 30 and 37°C. At all of these temperatures, only two spores from each tetrad formed visible colonies (Fig. 4B). Spores carrying the disrupted *CPN10* gene germinated

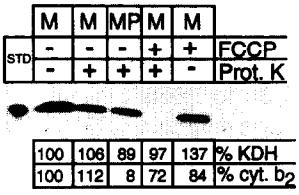

Fig. 3. In vitro-synthesized radiolabeled cpn10 is imported into the mitochondrial matrix. Import was for 10 min at 25°C. In two control samples, the membrane potential was dissipated by including 25 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) in the import mixture. Where indicated, the mitochondria (M) were treated after import with 0.1 mg/ml proteinase K for 30 min on ice. An additional sample was converted to mitoplasts (MP) in the presence of 0.1 mg/ml proteinase K. All samples were precipitated with 5% trichloroacetic acid and subjected to SDS-PAGE and fluorography. Efficiency of mitoplast generation and intactness of the inner membrane of mitoplasts were checked by immunoblotting of a portion of each sample, using antibodies against α-ketoglutarate dehydrogenase (KDH; matrix marker) and cytochrome b_2 (cyt. b_2; inter-membrane space marker). The amount of marker proteins present in mitochondria after import without proteinase K treatment was taken as 100%. STD: 20% of the amount of labeled cpn10 added to each sample.

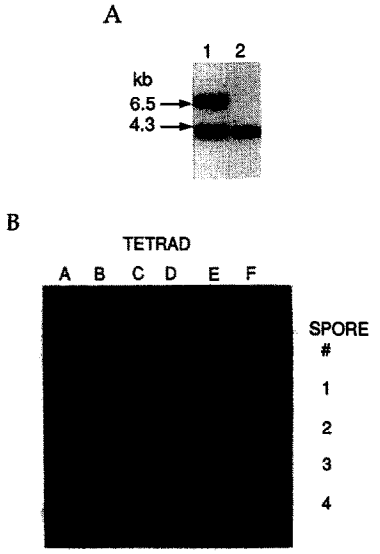

Fig. 4. (A) Southern blot of the diploid transformants (lane 1) and of the wild-type yeast strain (lane 2), using the coding region of *CPN10* as a probe. Cells transformed with the disrupted gene showed an additional band that was about 2 kb larger than the band from the undisrupted *CPN10* gene. (B) Sporulation of the heterozygous diploid strain and dissection of the spores yielded two viable and two non-viable spores; all viable spores were leucine-requiring and thus carried the wild-type *CPN10* gene. Letters on top indicate individual tetrads that were dissected.

and underwent about 4–7 cell divisions, but then stopped growing. The number of cell divisions decreased with increasing temperature, perhaps reflecting the involvement of cpn10 in a temperature-induced stress response [4]. Preliminary results suggest that yeast cells containing a disrupted *CPN10* gene may be viable at 18°C. At normal growth temperatures, however, cpn10 is essential for the viability of yeast cells.

4. DISCUSSION

Mitochondria contain hundreds of different proteins, most of which are dispensable for yeast growth. cpn10 is the latest addition to a small group of mitochondrial proteins the deletion or inactivation of which is lethal under conditions optimal for growth of wild-type cells. These proteins include ISP42/MOM38, mitochondrial hsp70, ISP45/MIM44, MAS6/MIM17, MIM23, hsp60, and the two subunits of the matrix processing protease [26,27]. All of these proteins are components of the machinery that mediates import, processing and folding of nuclear-encoded mitochondrial proteins. The availability of the *CPN10* gene should help to define the role of cpn10 in mitochondrial biogenesis.

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