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Isolation of a cDNA clone specifying rat chaperonin 10, a stress-inducible mitochondrial matrix protein synthesised without a cleavable presequence

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Abstract

We have isolated a cDNA clone encoding chaperonin 10 from rat liver. The cDNA specifies a protein of 102 amino acids which, when transcribed and translated in vitro, yields a single basic product (pI > 9) that co-migrates exactly with the heat shock inducible cpn10 of rat hepatoma cells during 2D gel-electrophoresis. It is concluded that cpn10, unlike the majority of nuclear-encoded proteins of the mitochondrial matrix, is synthesised without a cleavable targeting signal and that, following removal of the initiating methionine, it becomes acetylated prior to mitochondrial import. Incubation of ^{3}H- or ^{35}S-labelled cpn10 with mitochondria confirms these conclusions and shows that cpn10 is imported into mitochondria in an energy-dependent process which is inhibited by the presence of 2,4-dinitrophenol.

Key words: Protein import; Acetylation; Amphiphilic helix; Heat-shock protein

1. Introduction

Recent reports have provided extensive evidence that folding of many if not most polypeptides depends on accessory proteins called molecular chaperones (see [1,2] for reviews). Chaperonins constitute a class of chaperones and the most studied members are the GroEL and GroES proteins of *Escherichia coli*, both of which are essential for full chaperonin function in vivo [3]. Eukaryotic homologues of GroEL have been identified in a large number of organisms, including fungi [4], insects [5], humans [6], plants [7] and rodents [8], while eukaryotic GroES homologues have been reported in mammals [9,10] and plants [11,12]. Important functional differences exist between the rodent and prokaryotic chaperonin 10 (cpn10) homologues [13] but the difficulty of obtaining significant quantities of these proteins from eukaryotic sources [9,10] has excluded a detailed analysis of their role in protein folding. Extensive characterisation of these proteins in established assays [10,14] is therefore best achieved via cDNA cloning and expression in an heterologous host. Furthermore, the finding that cpn10 isolated from rat liver mitochondria was acetylated [15], an event associated with the cytosol [16], raises questions both about the mechanism of import of cpn10 into mitochondria and the role of N-terminal acetylation in this process.

We report here the isolation and analysis of a full-length cDNA clone specifying the nuclear encoded cpn10 homologue from rat mitochondria and further demonstrate that this stress-inducible protein, unlike the majority of other imported mitochondrial matrix proteins, is synthesised without a cleavable targeting presequence.

2. Materials and methods

2.1 Oligonucleotides

Based on a partial cpn10 amino acid sequence [10], degenerate oligonucleotide mixtures deduced from the amino acid sequences, TKGGIM (5′-ACNAA(A/G)GGNGGNAT(A/T/C)ATG-3′, primer A) and DDKDYF (5′-(A/G)AA(A/G)TA(A/G)TT(C/T)TT(A/G)TC(A/G)TC-3′, primer B), were synthesised on an Applied Biosystems DNA Synthesiser 381.

2.2. Preparation of mRNA, cDNA synthesis, PCR and library screening

Rat hepatoma cells were grown in culture in the presence of the amino acid analogue L-azetidine 2-carboxylic acid, to induce the expression of cpn10 [10], and mRNA was isolated from 5×10^8 cells using a FastTrack mRNA isolation kit (Invitrogen). Partial first strand cDNA synthesis was performed at 37°C employing 1 μg mRNA, 10–30 pmol of primer B and 200 U of mouse Moloney leukemia virus reverse transcriptase (Gibco BRL) according to the manufacturer's protocol. For PCR reactions, cDNA derived from 0.6 μg mRNA was incubated with 8 pmol primer A, 8 pmol primer B, 1 mM dNTPs, 2.5 U *Taq* polymerase (Promega), 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$ and 0.1% Triton X-100, in a final volume of 40 μl with a 50 μl mineral oil overlay. Following 5 min heating at 94°C, 30 cycles of denaturation (94°C, 60 s), annealing (37°C, 120 s) and extension (74°C, 120 s) were performed. A PCR product of expected size (189 bp) was recovered and used to screen an adult rat liver cDNA library constructed in a λZAP II vector (Stratagene). Recombinants (400,000) were screened at high

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Abbreviations: cpn10, chaperonin 10; cpn60, chaperonin 60; OTC, rat ornithine transcarbamylase; p-OTC, the 39 kDa precursor of OTC.

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stringency essentially as described previously [17] and seven independent clones isolated. The cDNA insert of one clone was sequenced fully in both directions by the dideoxynucleotide chain termination procedure [18]. The nucleotide sequence data has been submitted to the EMBL Data Bank (accession number X71429).

2.3. In vitro synthesis and mitochondrial import

^{35}S-labelled rat p-OTC was synthesised as described previously [19]. For the synthesis of ^{35}S-Met- and ^{3}H-Leu-labelled cpn10, RNA was transcribed from the cDNA (cloned in pBluescript II SK−) using T_3 RNA polymerase and in vitro translated at 30°C for up to 30 min using nuclease-treated Flexi rabbit-reticulocyte lysate (Promega) devoid of additional Mg^{2+} or K^+. Mitochondrial import reactions and associated manipulations, such as dinitrophenol treatment, detergent solubilisation and proteinase K digestion, were carried out as previously described [20]. Analysis of imported p-OTC was performed using 12.5% SDS-PAGE in Tris-glycine buffer [21], while cpn10 was analysed using SDS-PAGE in Tris-Tricine buffer [22].

2.4. Additional analytical techniques

The growth of cultured rat hepatoma cells in the presence of amino acid analogues and the conditions for heat shock and isolation of ^{35}S-labelled mitochondrial matrix preparations were as described previously [10]. 2D-gel electrophoresis was performed using precast Immobiline Dry Strips (pH range 3–10.5; Pharmacia) in the first dimension and 16% (w/v) Tris-Tricine gels for the second dimension. Labelled proteins were detected using a Molecular Dynamics PhosphorImager or by fluorography, as indicated. Hydrophobic moments [23], isoelectric points and putative transport signals were predicted using the PC/Gene computer program (Intelligenetics Inc.).

3. Results

3.1. Cloning of cpn10 cDNA

mRNA was isolated from cultured cells grown in the presence of L-azetidine 2-carboxylic acid, a proline analogue known to induce rat cpn10 synthesis [10]. Following oligonucleotide-primed first-strand cDNA synthesis, a 189 bp PCR fragment was generated and used to select a full length cDNA clone of 368 bp containing a single open reading frame which encodes a 102 amino acid primary translation product (Fig. 1). The predicted amino acid sequence matches the amino acid sequence obtained directly on cpn10 purified from rat liver [15]

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1    CGCGGCGGCGTAAGTC ATG GCT GGA CAA GCT TTT AGG AAG TTT  43
1                     Met Ala Gly Gln Ala Phe Arg Lys Phe   9
44 CTT CCG CTA TTT GAC AGA GTA TTG GTT GAA AGG AGT GCC GCT  85
10 Leu Pro Leu Phe Asp Arg Val Leu Val Glu Arg Ser Ala Ala  23
86 GAA ACT GTG ACC AAA GGT GGC ATT ATG CTT CCA GAA AAG TCT 127
24 Glu Thr Val Thr Lys Gly Gly Ile Met Leu Pro Glu Lys Ser  37
128 CAA GGA AAA GTA TTG CAA GCA ACG GTC GTG GCT GTG GGA TCA 169
38 Gln Gly Lys Val Leu Gln Ala Thr Val Val Ala Val Gly Ser  51
170 GGC GGG AAA GGA AAG GGT GGA GAG ATT CAG CCT GTC AGT GTG 211
52 Gly Gly Lys Gly Lys Gly Gly Glu Ile Gln Pro Val Ser Val  65
212 AAA GTT GGA GAT AAA GTT CTT CTC CCA GAA TAT GGA GGC ACC 253
66 Lys Val Gly Asp Lys Val Leu Leu Pro Glu Tyr Gly Gly Thr  79
254 AAA GTA GTA CTA GAC GAC AAG GAT TAC TTC TTA TTT AGA GAT 295
80 Lys Val Val Leu Asp Asp Lys Asp Tyr Phe Leu Phe Arg Asp  93
296 GGC GAC ATT CTT GGA AAG TAT GTC GAC TGAAATCACTGTTGAAATG 341
94 Gly Asp Ile Leu Gly Lys Tyr Val Asp ***                102
342 GTGTCACATGAAGCTGCCCATTCCACT                           368
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Fig. 1. The cDNA and deduced amino acid sequence for rat chaperonin 10. The upstream in-frame stop codon and residues constituting a putative targeting signal are underlined.

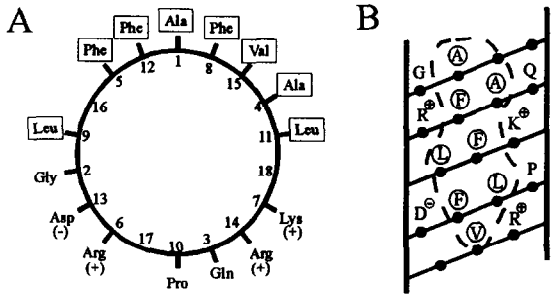

Fig. 2. α-Helical projections of the first 15 residues of rat cpn10. (A) Helical wheel axial projection with 3.6 residues per turn. Hydrophobic residues with a normalised consensus hydrophobicity > 0.5 [23] are boxed and charged residues indicated by + or − signs. (B) A cylindrical projection of the helix shown above. Hydrophobic residues with a normalised consensus hydrophobicity > 0.5 are circled and charged residues indicated by + or − signs. The segregation of the hydrophobic and hydrophilic amino acids is indicated with a broken line.

except for the presence of an initiating methionine which is removed followed by acetylation of Ala^2. The translation initiation codon preceded by an in frame stop-codon and a purine in position −3, as well as a G in position +4 [24], indicate that the isolated cDNA does specify the entire cpn10 coding region.

3.2. Cpn10 retains its targeting signal following mitochondrial import

The vast majority of mitochondrial matrix proteins are synthesised with highly degenerate and basic N-terminal targeting signals which are removed by specific matrix-located proteases upon import. The comparison between the sequence of the isolated mature protein and that predicted from the cpn10 cDNA, however, indicates that a transient targeting signal is absent from the primary cpn10 translation product. Since the targeting signals can often adopt amphiphilic α-helical conformations [25], we therefore looked for such information in cpn10 using a helical wheel projection (Fig. 2A). The projection shows that amino acids 1–15 have the potential to form an amphiphilic helix. The predicted mean hydrophobic moment of 0.73 is indicative of a high degree of amphiphilicity which is clearly illustrated in the cylindrical projection presented in Fig. 2B. To test whether this helix, or part thereof, constitutes a targeting signal, cpn10 produced in vitro was subjected to mitochondrial import assays (Fig. 3B,D) using p-OTC as a control (Fig. 3A,C). The inclusion of p-OTC as a control protein facilitates the assessment of the import efficiency of the mitochondrial preparation since this is directly reflected in the appearance of a processing product of higher electrophoretic mobility. The results show that cpn10 is translocated into a compartment which renders the protein inaccessible to externally added proteases and that this process is not accompanied by modification

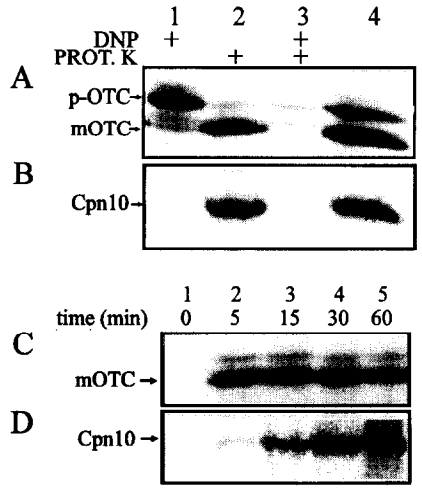

Fig. 3. Import of rat p-OTC and cpn10 into isolated mitochondria. (A,B) Mitochondria were incubated with ^{35}S-labelled p-OTC (panel A) or cpn10 (panel B) and analysed by SDS-PAGE and PhosphorImage analysis. Lane 4, standard import reaction; lanes 1 and 3, import reactions were performed in the presence of 2,4-dinitrophenol; lanes 2 and 3, the mitochondrial import mixtures were treated with proteinase K prior to electrophoresis. (C,D) Mitochondria were incubated with excess ^{35}S-labelled p-OTC (panel C) or cpn10 (panel D) for the indicated time periods and subsequently treated with proteinase K. Under these conditions the maximal amount of p-OTC imported represented 20% of the added precursor, while the minimal amount of cpn10 imported is about 5% of the total protein added.

of cpn10 itself. Thus in vitro translocated cpn10, like the matrix-located mature form of OTC, is not degraded by proteinase K (lane 2) unless prior detergent solubilisation of the mitochondria has been performed (data not shown). Furthermore, addition of dinitrophenol to mitochondria (lane 1) blocks import of cpn10 and p-OTC into the mitochondria since both forms are now accessible to protease treatment (lane 3), confirming that a transmembrane potential and/or ATP is required for import.

Although cpn10 behaved as a typical competent precursor during the import assays, we noticed that the overall rate of translocation was poor relative to that obtained for p-OTC (Fig. 3C,D). One potential explanation for this is that the protein may not have acquired its N-terminal acetylation during the in vitro translation in the rabbit reticulocyte lysate. The lack of this acetylation is expected to alter the amphiphilicity of the putative α-helix since a protonated N-terminus would be situated at the phase of the predicted α-helix containing the bulk of the hydrophobic residues (see Fig. 2) leading to a decrease in the affinity for membranes [26]. To address this problem, 2D gel analysis of the in vitro translation product was performed and compared to that obtained from ^{35}S-labelled mitochondrial extracts isolated from control and heat shocked rat hepatoma cells (Fig. 4). The ^{3}H-labelled in vitro translated and the ^{35}S-labelled stress-induced cpn10 from rat hepatoma cells migrated to an identical position in the 2D gels (Fig. 4, compare panels A, B and C). Furthermore, when the in vitro translated ^{3}H-labelled cpn10 was mixed with authentic cpn10 purified from rat liver, superimposable Coomassie stained and ^{3}H-labelled spots were obtained (data not shown). The protein appeared very basic, focussing at a pI > 9. A prediction of the pI for the acetylated and non-acetylated protein gives pIs of 9.65 and 9.44, respectively. These differences should be distinguished by the 2D electrophoretic procedure and the co-migration of the two forms therefore leads us to conclude that cpn10, like human fetal hemoglobin [27], is acetylated during in vitro translation in the rabbit reticulocyte lysate and therefore most probably also before import into mitochondria in vivo. The reason for the relatively poor in vitro import of cpn10 is therefore unresolved.

3.3. *Eukaryotic homologues of rat cpn10*

Chaperonin 60 homologues are ubiquitous and have

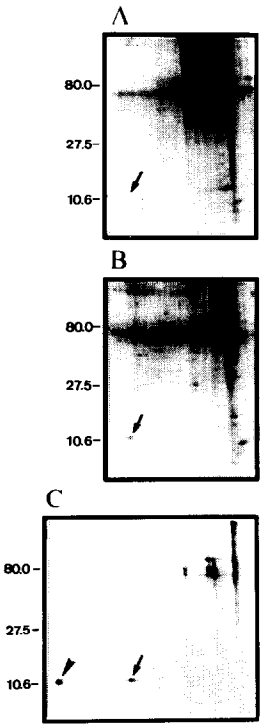

Fig. 4. In vitro translated and stress-induced cpn10 co-migrate during two-dimensional gel electrophoresis. Clonal rat hepatoma cells were metabolically labelled with ^{35}S-methionine and ^{35}S-cysteine. Matrix proteins from control cells (panel A) or cells heat shocked for 15 min (panel B) were separated by 2D gel electrophoresis and labelled proteins visualised by PhosphorImage analysis. (C) ^{35}S-Labelled matrix proteins were fortified with in vitro translated ^{3}H-labelled cpn10 and following electrophoresis subjected to both PhosphorImage analysis (detection of ^{35}S only, not shown) and fluorography (detection of both ^{35}S- and ^{3}H-labelled cpn10). The filled arrow points to the single cpn10 spot detected following both PhosphorImage analysis (detection of stress-induced cpn10 only) and fluorography (detection of both stress induced and in vitro translated cpn10), while the arrow-head shows the position of in vitro translated ^{3}H-labelled cpn10 subjected to SDS-PAGE and detectable only by fluorography. The gels are shown with the basic proteins on the left and the positions of molecular mass standards corresponding to 80, 27.5 and 10.6 kDa are indicated.

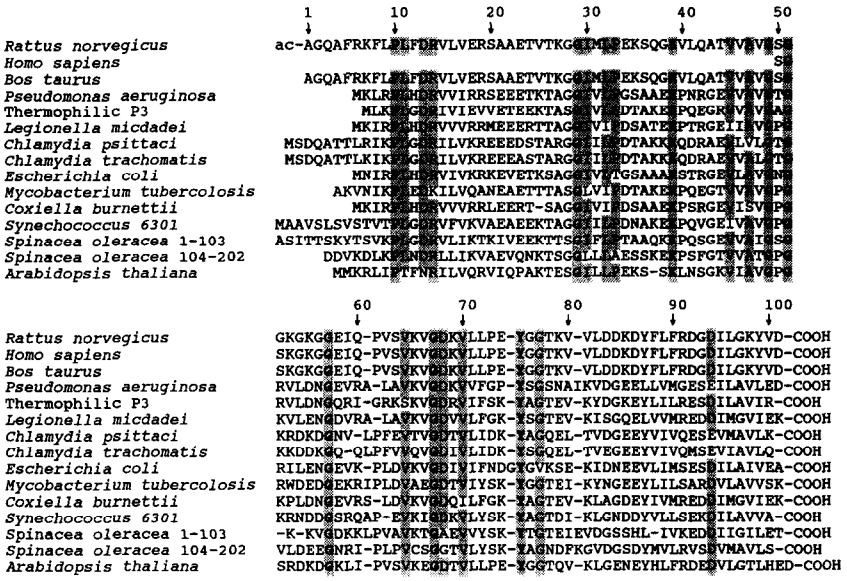

Fig. 5. Identification of highly conserved amino acid residues in cpn10 homologues. The primary structures of cpn10 homologues from the following species: *Rattus norvegicus* (this study), *Bos taurus* (EMBL accession no. X69556), *Homo sapiens* (residues 51–101, Genbank accession nos. HUM00TB07 and HUM00TBO25), *Pseudomonas aeruginosa* [37], Thermophilic strain PS3 [38], *Legionella micdadei* [39], *Chlamydia psittaci* [40], *Chlamydia trachomatis* [41], *Escherichia coli* [7], *Mycobacterium tubercolosis* [42], *Coxiella burnettii* [42], *Synechococcus* strain 6301 [43], *Spinachea oleracea* (amino acids 1–103) [11], *Spinachea oleracea* (amino acids 105–202) [11] and *Arabidopsis thaliana* [12] were compared. Residues appearing in at least twelve of the fifteen sequences are shaded grey and invariable residues shown in bold.

been shown to be essential for viability in bacteria [3] and in *Saccharomyces cerevisiae*, even when oxidative phosphorylation is not required (reviewed in [28]). Since cpn60 function relies on cpn10, the latter chaperone is also expected to be of ubiquitous appearance. A database search employing the cDNA sequence determined herein revealed that, in addition to a large number of prokaryotic homologues, highly homologous sequences have been obtained from *Spinacea oleracea* [11], *Arabidopsis thaliana* [12] and cattle (EMBL accession no. X69556) (Fig. 5). Two hitherto unidentified open reading frames obtained during a human cDNA library sequencing project also showed extensive homology with the rat cpn10 cDNA since only one amino acid residue change is predicted in a stretch of 50 amino acids (GenBank accession numbers HUM00TB037 and HUM00TB025). Recently, a human cpn10 was purified and characterised: it is identical to the rat cpn10 except for a substitution of a Ser residue for a Gly residue at position 52 (A. Cavanagh and H. Morton, personal communication). Twenty-one residues are highly conserved amongst all the listed cpn10 homologues. One of the eight very highly conserved residues (corresponding to Gly^{29} in rat cpn10) has been mapped to a mobile and functionally important domain in *E. coli* GroES [29].

4. Discussion

The vast majority of proteins imported into the mitochondrial matrix contain a basic and highly degenerate presequence of 15–40 amino acids located at the N-terminus of the primary translation product. Here we report the isolation of a cDNA clone encoding rat cpn10. Comparison of the translated cDNA sequence with that of the mature protein showed that the nuclear encoded cpn10 lacks a cleavable N-terminal presequence. However, a helical wheel projection demonstrated that the first 15 N-terminal amino acids may form an almost ideal amphiphilic helix with a large dipole moment characteristic of many mitochondrial targeting sequences [25,30]. Indeed mitochondrial import experiments (Fig. 3) demonstrated that cpn10 displayed import characteristics similar to those of p-OTC and the targeting information therefore resides within the mature protein. Only a small number of other mitochondrial matrix proteins which are not proteolytically processed have been identified conclusively from comparative protein and nucleotide sequence data. These are rhodanese [32], 3-oxoacyl-CoA thiolase [33], the β-subunit of the human electron- transfer flavoprotein [34], and very recently, a mitochondrial cpn10 from *S. cerevisiae* (S. Rospert and G. Schatz, pers. commun.).

The question arises as to why cpn10 does not possess a cleavable presequence. Several possibilities, between which we cannot discriminate at present, exist. Firstly, 25% of peptides containing a stretch of nine random amino acids and the last five residues of an authentic presequence constitute functional matrix-targeting signals [30]. A large number of ancestral bacterial proteins

could therefore have possessed functional targeting sequences by chance and bypassed the need for the addition of presequences during evolution of the eukaryotic cell. A less trivial reason why cpn10 is synthesised without a cleavable presequence may be that such a presequence is not compatible with mitochondrial cpn10 function due to the requirement for N-terminal acetylation in the cytosol. Although the intracellular location of cpn10 acetylation is not known, the cytosol is the likely compartment in which this event occurs [16], a conclusion which is supported by the identical mobility of in vitro and in vivo synthesised cpn10 during 2D gel-electrophoresis (Fig. 4). The importance of cpn10 acetylation may be inferred from protein folding studies using mammalian cpn60 in which it was demonstrated that *E. coli* cpn10 could not replace rat cpn10 [13] whereas rat cpn10 can function with *E. coli* cpn60 [10]. Although the two cpn10s exhibit extensive homology, one notable difference, however, is that rat cpn10 is N-terminally acetylated [15], and this may be important in this interaction since amino-terminal acetylation has been implicated in other protein–protein interactions [35].

We are presently addressing the functional significance of the cpn10 acetylation, if any, by expression of the cpn10 cDNA in *E. coli*, a host which has been shown not to perform N-terminal acetylation of some proteins which undergo this modification in their natural host [36].

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