Isolation and characterisation of a cDNA encoding rat mitochondrial GrpE, a stress-inducible nucleotide-exchange factor of ubiquitous appearance in mammalian organs

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Abstract In contrast to the *E. coli* chaperones DnaK, GroEL and GroES, cDNAs encoding mitochondrial homologues of DnaJ and GrpE from higher eukaryotes have yet to be reported. Based on peptide sequences, we have isolated a cDNA encoding a 217 residue nuclear encoded precursor of rat mitochondrial GrpE (mt-GrpE) including a typical mitochondrial presequence of 27 residues. Western blotting revealed that the 21 kDa GrpE homologue is present exclusively in the mitochondrial fraction where it comprises only ~0.03% of the total soluble protein, while Northern blotting showed that the mt-GrpE transcript is present in most if not all organs. By contrast to other mitochondrial chaperones, the levels of mt-GrpE and its transcript in cultured cells are only marginally increased in response to the proline analog L-azetidine 2-carboxylic acid but not by heat shock. Furthermore, members of the GrpE family exhibit a much lower degree of sequence identity than do the well studied members of the Hsp70, Hsp60 and Hsp10 families.

Key words: GrpE; Mitochondria; Stress induction; Eukaryote; cDNA

1. Introduction

Heat shock proteins have been found in all organisms examined and representatives of their individual families in distantly related organisms often exhibit very high degrees of sequence similarity (reviewed in [1]). For example, members of the ubiquitous Hsp70 family exhibit about 51% amino acid sequence identity between the prokaryotic *E. coli* DnaK homologue and its eukaryotic mitochondrial counterpart (mt-Hsp70) [2]. The high conservation of Hsp70 homologues throughout evolution is thought to help maintain their function as molecular chaperones in such diverse cellular reactions as protein folding, hormone receptor function, proteolysis and protein translocation. Central to the function of Hsp70 homologues as molecular chaperones is a weak ATPase activity which at least in the case of DnaK is accelerated about 50-fold by the concerted effort of the heat shock proteins DnaJ and GrpE [3]. Furthermore, DnaJ and GrpE appear to regulate the ability of DnaK to bind unfolded proteins and release them in an ATP-dependent manner and together they comprise a 'chaperone machine' which, in one form or the other, may operate in all compartments of eukaryotic cells.

In mitochondria of the unicellular organism *S. cerevisiae*, the Hsp70 homologue appears to function as part of a chaperone machine during the processes of mitochondrial protein import, folding and degradation. ATP hydrolysis is central to the import of proteins into mitochondria and has been linked to an indispensable function of the mt-Hsp70 ATPase activity in a 'Brownian ratchet' like mechanism (reviewed in [4,5]). The ratchet like mechanism of mt-Hsp70 in *S. cerevisiae* and its nucleotide-dependent binding to substrate proteins during protein import into mitochondria is strictly controlled by Ygelp (GrpEp, Mgelp) [6], a mitochondrial homologue of *E. coli* GrpE [7,8], and to some extent Tim44 [9], an inner membrane component with possible homology to the *E. coli* DnaJ protein [10]. Previous cloning and sequencing of the *S. cerevisiae* Ygelp gene revealed a sequence similarity to the bacterial counterpart which is much lower than that seen in other heat shock protein families such as Hsp70, Hsp60 and Hsp10. Whether this is a general trend for the GrpE family awaits identification and cloning of GrpE homologues from multicellular organisms, which surprisingly has not been reported despite the isolation and characterization of an incomplete 'chaperone team' of DnaK and DnaJ homologues in virtually every cellular compartment of higher eukaryotes.

Based on the peptide sequences of affinity purified bovine mt-GrpE obtained in our previous study [11], we now report the cloning of a cDNA specifying rat mt-GrpE and show that this homologue exhibits only 20% positional identity with its bacterial and fungal counterparts. We further conclude that the encoded GrpE homologue is of low abundance, only slightly stress inducible and confined to mitochondria but found in most, if not all, organs in accordance with its suspected vital cellular function.

2. Materials and methods

2.1. Synthesis of oligonucleotide primers

Based on peptide sequences obtained from bovine mt-GrpE [11], degenerate oligonucleotides representing all possible codon usages were synthesised on an Applied Biosystems DNA Synthesiser. The amino acid sequences and corresponding oligonucleotide sequences (in parenthesis) are: AKQKNDG [primer no. 1: 5′-GCNAA(A/G)-CA(A/G)AA(A/G)AA(C/T)GA(C/T)GG-3′] and FDPYEHEA [primer no. 2: 5′-A(A/G)CT(A/G)GGNAT(A/G)CT(C/T)GT(A/G)CT-(C/T)CG-3′].

The oligonucleotides employed in 5′-RACE analysis were those designed by Frohman et al. [12] combined with those designed from a partial cDNA clone (nucleotides 40–951, Fig. 1) of mt-GrpE, namely the antisense primer no. 3 [5′-CCCCTCCACAGGGGTGTG-GA-3′] and nested antisense primer no. 4 [5′-GCAACCTCCAG-CAAGTCCTT-3′].

The primer sets used in semi-quantitative RT-PCR of various chaperone transcripts were: for mt-Hsp70 RT-70a [5′-ATGGA-TCCATGGCGTCAGAAGCAATCAAGGGTGC-3′] and RT-70b

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Abbreviations: Azc, L-azetidine-2-carboxylic acid; Hsp, heat shock protein; mt, mitochondria; RACE, rapid amplification of cDNA ends

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[5′-TCACAGCGACAGGCCACTAAG-3′], for Hsp60 RT-60a [5′-CAAATGAAGAGGCTGGGGATGGCA-3′] and RT-60b [5′-GAGCAGGTACAATGGACTGAACAC-3′], for Hsp10 RT-10a [5′-ATGGCTGGACAAGCTTTT-3′] and RT-10b [5′-GCTTCATGTGACACCATTTCAA-3′] and for mt-GrpE RT-Ea [5′-TGCACAGCTACAAAACAAAAG-3′] and RT-Eb [5′-CAAGCCCGCCCACCTTTGA-3′].

2.2. Isolation of mRNA, RT-PCR, cDNA library screening and 5′-RACE analysis

mRNA was isolated from 5×10^7 clonal rat hepatoma cells (H4) [13] using a Fast Track mRNA isolation kit (Invitrogen) and partial first strand cDNA was generated with primer no. 2 and SUPERSCRIPT II RNase H$^-$ Reverse Transcriptase (Gibco BRL) according to the manufacturer's protocol. Using the partial first strand cDNA and primers no. 1+2, a 440 bp PCR product was produced using a 30 cycle protocol (94°C for 60 s, 50°C for 90 s and 72°C for 90 s) according to the manufacturer's specifications. The PCR product was recovered and used to screen an adult rat liver cDNA library constructed in the λZAP II vector (Stratagene). Recombinants (5×10^5) were screened essentially as described previously [14] and two independent clones were isolated. Both strands of the two clones were sequenced using the dideoxy method of Sanger et al. [15].

Cloning of nucleotides −14 to 39 of the final cDNA (see Fig. 1) was performed with the aid of primers no. 3 and 4 by 5′-RACE PCR according to Frohman et al. [12].

2.3. Immunological techniques

100 μg of both bovine mt-GrpE [11] purified by affinity chromatography and recombinant rat Hsp60 [16] were mixed with an equal volume of Freunds complete adjuvant (Gibco BRL) and injected subcutaneously into 7-month-old New Zealand white rabbits. The rabbits were then boosted, three times at 6-week intervals, with approx. 50 μg of the corresponding antigen excised from SDS-PAGE gels, developed into a slurry and mixed with an equal volume of Freunds incomplete adjuvant as described by Harlow and Lane [17]. Ear bleeds were performed 10 days after the third boost and sera containing polyclonal antibodies to mt-GrpE and Hsp60 were collected and processed [17]. The sera were supplemented with 0.02% (v/v) sodium azide and stored at −70°C. Dilutions of 1 in 10000 were used in immunostaining analysis.

For Western blotting, cellular protein fractions were resolved by SDS-PAGE in a 16% (w/v) Tris-Tricine gel [18] and transferred to nitrocellulose using a semi-dry transfer unit [17]. The blot was probed with the rabbit anti-mt-GrpE serum and HRP-labelled goat anti-rabbit IgG, followed by incubation with enhanced chemiluminescence (ECL) detection reagents (Amersham) and subsequent exposure to Hyperfilm-MP (Amersham). The filter was reprobed with serum raised against Hsp60 and bound antibodies were detected as described above.

Rat liver mitochondria were prepared according to Hartman et al. [13], microsomes were pelleted by centrifugation of the post-mitochondrial supernatant at $100000\times g$ for 1 h and the final supernatant was defined as the cytosolic fraction.

2.4. Northern analysis

For identification of the tissue distribution of mt-GrpE transcripts, a Northern blot with mRNA from eight different rat tissues (2 μg each) was purchased from Clontech and probed with a ^{32}P-labelled mt-GrpE cDNA probe (nucleotides 82–719, Fig. 1) in Express Hyb hybridisation solution (Clontech) for 1 h at 68°C. Final washes were performed twice for 20 min at 65°C in 0.1×SSC supplemented with 0.5% (w/v) SDS and filters were analysed using a Storm phosphorimager and Image QuaNT software (Molecular Dynamics).

2.5. Stress treatments, metabolic labelling and fractionation of tissue culture cells

The growth of H4 cells in the presence of the amino acid analog L-azetidine-2-carboxylic acid (Azc), the conditions for heat shock and the isolation of ^{35}S-labelled cytosolic and mitochondrial proteins were as described by Hartman et al. [13]. For Azc stress, H4 cells (5×10^7 H4 cells) at 70–80% confluence were grown for 10 h in the presence (stressed cells) or absence (control cells) of 5 mM Azc and then labelled for 2 h in methionine- and Azc-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% dialysed fetal calf serum, [^{35}S]methionine and [^{35}S]cysteine at 10 μCi/ml (Tran^{35}S-label, New England Nuclear, 1165 Ci/mmol). For heat shock treatments, cells were heat shocked at 45°C (stressed cells) for 17 min and then metabolically labelled for 12 h at 37°C in fresh, prewarmed methionine free DMEM supplemented with 10% dialysed fetal calf serum, [^{35}S]methionine and [^{35}S]cysteine at 10 μCi/ml. Control cells were labelled in an identical fashion for both treatments.

A crude cellular lysate was prepared by hypotonic lysis and Dounce homogenisation. Cellular membranes and nuclei were removed as a pellet following a $754\times g$ centrifugation and a crude mitochondrial pellet was obtained by recentrifugation of the supernatant at $10000\times g$. The remaining supernatant represented the cytosolic fraction. Mitochondria were further purified on sucrose gradients and following incubation with 0.5% (w/v) Triton X-100 Reduced (Sigma) at 4°C for 1 h, a mitochondrial supernatant was obtained after centrifugation at $80000\times g$.

2.6. Semi-quantitative RT-PCR

H4 cells (5×10^7) grown to 70–80% confluence at 37°C were either (i) grown a further 12 h at 37°C (control cells), (ii) supplemented with 5 mM Azc and grown an additional 12 h (Azc stress) at 37°C or (iii) grown an additional 7 h at 37°C followed by 20 min at 45°C and then at 37°C for 5 h (heat shock). Cells were harvested at the same time point and mRNA isolated as above.

First strand cDNA was generated using 1 μg of mRNA and oligo(dT)$_{15}$. 10% of each cDNA batch was used in separate PCRs with the primer pairs for mt-Hsp70 [RT-70a and RT-70b], for Hsp60 [RT-60a and RT-60b], for Hsp10 [RT10a and RT10b] and for mt-GrpE [RT-Ea and RT-Eb] in a 30 cycle PCR protocol as described above.

3. Results and discussion

3.1. Cloning of rat mt-GrpE cDNA

Based on peptide sequences we previously obtained for bovine mt-GrpE [11], degenerate oligonucleotides were designed and employed in RT-PCR to generate a cDNA fragment of expected size (440 bp). This PCR product was used to probe a rat liver cDNA library and a mt-GrpE cDNA clone (nucleotides 40–951, Fig. 1) lacking an ATG initiation codon was thus obtained. As several attempts to isolate a full length cDNA from two different libraries failed, 5′-RACE PCR was employed to define 53 bp corresponding to the 5′-end of the rat mt-GrpE transcript (nucleotides −14 to 39, Fig. 1). An ATG codon (nucleotides 1–3) that fulfills the Kozak requirements for initiation of translation in eukaryotes [19] was evident in three independently cloned 5′-RACE PCR products. The nucleotide sequences of these products were all identical in a 305 bp overlap with the sequence of the cDNA clone initially isolated from the library. We are therefore confident that the composite cDNA represented in Fig. 1 corresponds to a single GrpE transcript.

Search of databases revealed a rat (GenBank accession number H32411), three mouse (GenBank accession numbers W08216, W55413, W30513) and 16 human expressed sequence tags (ESTs) with a high degree of positional identity to parts of the rat mt-GrpE cDNA. None of these ESTs encode a full-length mt-GrpE and most if not all contain numerous obvious sequencing mistakes, such as stop codons within the open reading frame. Of the 20 ESTs, only three human sequences (TIGR HCD assembly no. THC94455) contain an ATG start codon and none of the mt-GrpE transcripts contain a poly(A) tail despite the presence of a putative polyadenylation signal in the rat transcript sequenced in this report (Fig. 1). Together, these results indicate that the mt-GrpE transcript may be somewhat unstable or alternatively difficult to reverse

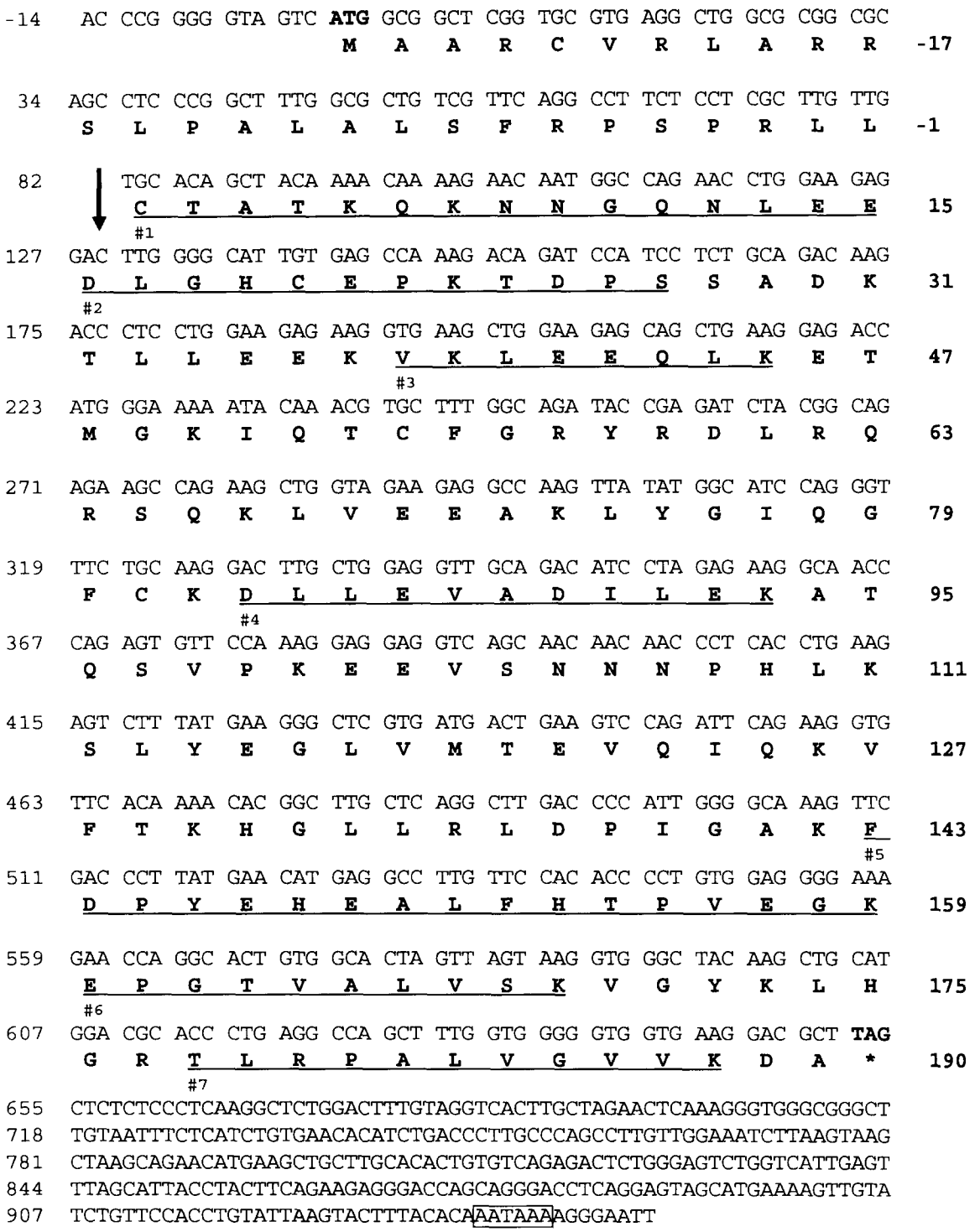

Fig. 1. The cDNA and deduced amino acid sequence of rat mt-GrpE. The numbering of nucleotides is shown on the left-hand side and the first base of the putative initiator codon is numbered +1. Amino acids are given by their single-letter code and they are numbered on the right-hand side. The first residue of the mature protein is numbered +1 and the cleavable presequence is defined as amino acids −27 to −1 with the point of cleavage indicated with an arrow. Regions of the deduced amino acid sequence that are almost identical to peptide sequences obtained from purified bovine mt-GrpE [11] are underlined and labelled #1 to #7. A putative polyadenylation site is boxed. This sequence has been submitted to GenBank and given accession number U62940.

transcribe. This may explain why screening of cDNA libraries failed to produce full length clones.

The combined information, obtained from sequencing a partial rat cDNA clone and 5′-RACE clones reveals a complete rat mt-GrpE cDNA comprising a coding region of 651 nucleotides and a minimal transcript size of 965 nucleotides (Fig. 1). Comparison of the deduced amino acid sequence with the N-terminal sequence of purified bovine mt-GrpE (Fig. 1) reveals a 27 residue N-terminal targeting sequence which, upon mitochondrial import, is proteolytically removed

Fig. 2. Multiple sequence alignment of the predicted amino acid sequences of GrpE proteins (or homologues) from: *Rattus norvegicus* (this study, GenBank accession number U62940), *Homo sapiens* (compiled and edited from GenBank accession number N28384 and TIGR HCD assembly no. THC94455), *Drosophila melanogaster* (GenBank accession number U34903), *Caenorhabditis elegans* (EMBL accession number Z46996), *Saccharomyces cerevisiae* (GenBank accession number D26059), *Mycobacterium tuberculosis* (GenBank accession number X58406), *Streptomyces coelicolor* (EMBL accession number X77458), *Borrelia burgdorferi* (GenBank accession number M96847), *Methanosarcina mazeii* (GenBank accession number X74353), *Escherichia coli* (GenBank accession number X07863), *Haemophilus influenzae* (GenBank accession number L44715), *Francisella tularensis* (GenBank accession number L43367), *Bacillus subtilis* (GenBank accession number M84964), *Staphylococcus aureus* (GenBank accession number D30690), *Lactococcus lactis* (GenBank accession number X76642), *Clostridium acetobutylicum* (GenBank accession number M74569), *Chlamydia trachomatis* (GenBank accession number M62819), *Synechococcus* sp. (partial sequence, EMBL accession number D28550), *Caulobacter crescentus* (EMBL accession number U33324) and *Mycoplasma genitalium* (EMBL accession number U39697). The alignment was made using the PILEUP program (Genetic Computer Group, Madison, WI) and the initiating methionine residue was numbered 1 as the processing sites are not known for most of the primary translation products. Residues that are conserved in at least 14 of the 20 sequences are highlighted. Conserved residues are defined as A/G, Y/F, S/T, I/V/L, R/K/H and D/E/N/Q. The asterisks under the consensus sequence indicate the positions of five strictly conserved residues. The symbols (▼) and (+) above the sequence show the positions of amino acid changes in characterised mutants from *E. coli* [22] and *S. cerevisiae* [6], respectively. Since the N-terminal sequence has not yet been obtained from mt-GrpE homologues other than bovine mt-GrpE, the suspected initiating methionine has been numbered +1 to facilitate comparison.

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from the 217 residue primary translation product (~24.3 kDa) to generate the mature protein of 190 amino acids (~21.3 kDa). The predicted isoelectric point (p*I*) of the mature rat mt-GrpE protein is 7.0 whilst the precursor, due to its basic mitochondrial targeting sequence, has a predicted p*I* of 8.9.

3.2. *Members of the GrpE family exhibit a relatively low degree of sequence identity at the amino acid level*

The deduced amino acid sequences of rat and human mt-GrpE exhibit 88.9% positional identity, while positional identities with other GrpE family members are: 41.8% for mitochondrial Droe1p from *D. melanogaster* (GenBank accession number U34903), 33.3% for *C. elegans* GrpE (EMBL accession number Z46996), 25.9% for mitochondrial Yge1p from *S. cerevisiae* [7], 20.8% for *E.coli* GrpE [20] and 19.1% for archaebacterial *M. mazei* GrpE [21]. Comparison of the deduced amino acid sequences of 19 GrpE family members reveals that five residues are strictly conserved, four of which reside in the C-terminal third of GrpE (Fig. 2). A segment within the N-terminal third of four bacterial GrpE molecules has been predicted to form a coiled-coil secondary structure which has been postulated to facilitate protein-protein interaction [22].

Members of the GrpE family exhibit a much lower degree of sequence identity than do the well studied members of the Hsp70, Hsp60 and Hsp10 families. Thus, the 21% positional identity of rat mt-GrpE with *E. coli* GrpE can be compared with the 51% [2], 49% [16] and 45% [13] positional identities we have determined for the rat mitochondrial homologues of *E. coli* DnaK (mt-Hsp70), GroEL (Hsp60) and GroES (Hsp10), respectively.

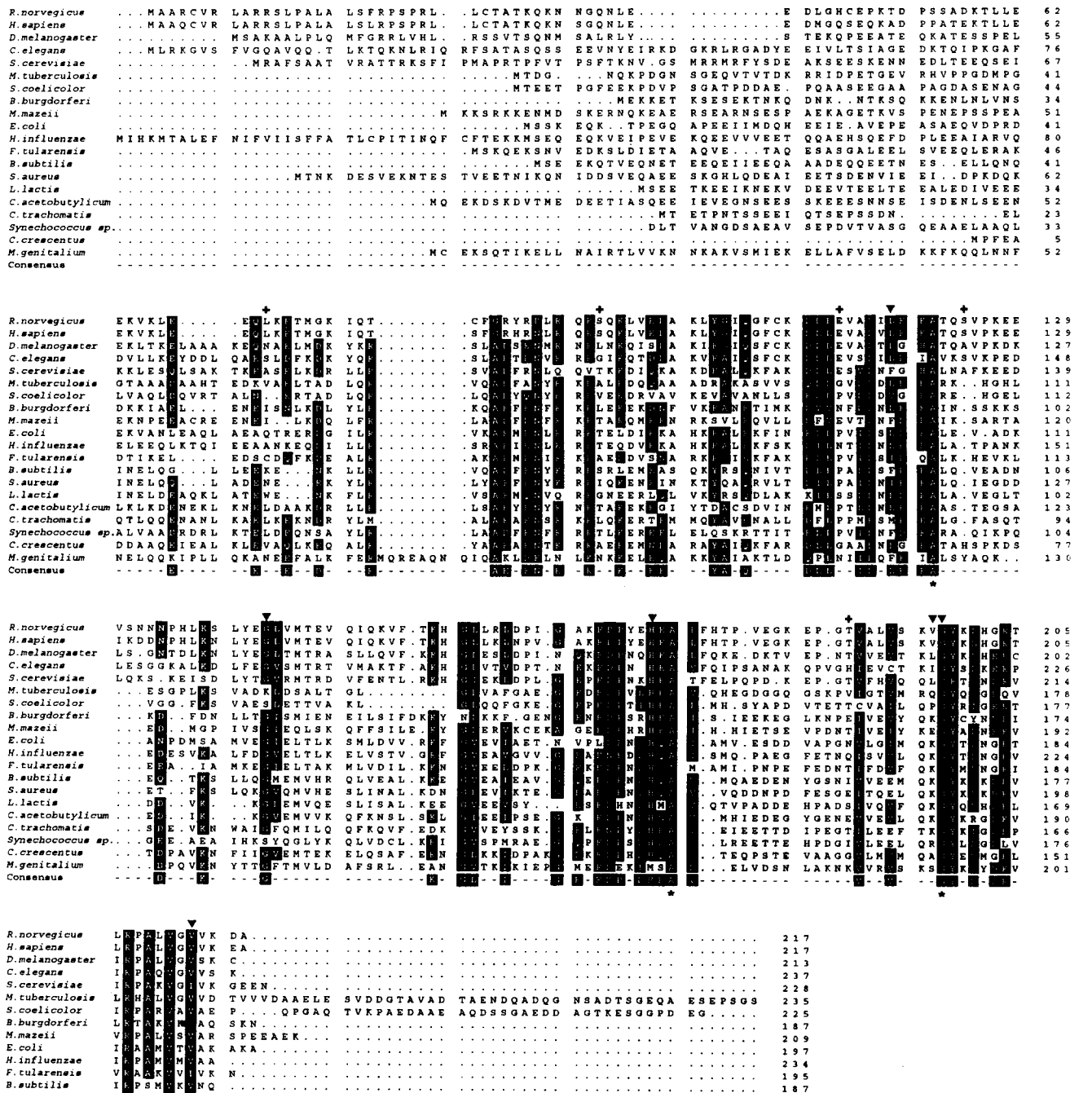
The low conservation of the primary structures between the eukaryotic and bacterial GrpE homologues appears not to have changed structural features to a significant extent. Thus, in keeping with the functional interchangeability of the rat and bacterial chaperonins [13,25], mammalian mt-GrpE like *E. coli* GrpE binds with high affinity to immobilised *E. coli* DnaK, the interaction being readily terminated in the presence of 5 mM ATP but persisting in the presence of 1 M KCl [11].

3.3. *mt-GrpE is a low abundance mitochondrial protein of ubiquitous appearance in mammalian organs*

As seen for yeast Yge1p [23], polyclonal antibodies raised against mammalian mt-GrpE revealed an exclusive mitochondrial location for the 21 kDa protein (Fig. 3). Thus, by contrast to the appearance of DnaK and DnaJ homologues in a number of compartments of both yeast and mammalian cells, GrpE homologues appear confined to mitochondria and have yet to be defined in plant organelles. It is somewhat surprising that the role of GrpE like molecules may not be required for the proper functioning of Hsp70 homologues in other compartments whilst recognisable homologues of DnaJ have been found in the cytosol, mitochondria and endoplasmic reticulum of yeast and mammalian cells (reviewed in [1]). Recently a novel rat cytosolic protein with molecular chaperone activity termed HiP was found to stabilise a labile interaction of cytosolic Hsp70, in the ADP-bound state, with Hsp40 and unfolded polypeptides [24]. This protein bears no obvious sequence relationship to GrpE and appears not to be functionally equivalent.

We have previously noted a relatively high concentration of the mitochondrial chaperones mt-Hsp70 [2], Hsp60 (unpublished results) and Hsp10 [13,25]. By contrast, our previous attempts at purifying mt-GrpE revealed a very modest concentration for this protein [11]. This inference is supported by the Western blot analysis shown in Fig. 5B from which it is concluded that mt-GrpE constitutes about 0.03% of the Triton-soluble mitochondrial protein fraction as also determined for *S. cerevisiae* Yge1p [23]. This value can be compared with a figure of about 1% for mt-Hsp70 [2,23] and 1.3% for Hsp60 (Fig. 5B). Assuming that GrpE functions as a monomer, mt-Hsp70 as a monomer and Hsp60 as a tetradecamer, mt-GrpE therefore appears about 10-times and 1–2-times less abundant than mt-Hsp70 and Hsp60, respectively. This is in accordance with the function of Yge1p as an ADP/ATP exchange catalyst during the cycles of protein binding and release by mt-Hsp70 which is an integral part of mitochondrial protein import and subsequent folding [6,23,26,27]. Consistent with this function of Yge1p we have further shown that a small fraction of rat mt-GrpE (but not Hsp60) resides in the membrane fraction of lysed mitochondria (data not shown). By analogy to fungal mitochondria [6,10,28–30], it is therefore tempting to speculate that the membrane association of mt-GrpE occurs through complex formation with mt-Hsp70 which in turn may bind to the mammalian homologue of Tim44.

By contrast to the restricted location of mt-GrpE within cells, Northern blot analysis revealed the presence of a ~1.2 kilobase mt-GrpE transcript mRNA in all organs stud-



ied (Fig. 4). At equal mRNA loading, the mt-GrpE transcript was particularly abundant in heart, kidney and liver tissues, perhaps because mitochondria are abundant in these organs. This widespread expression is expected since the *S. cerevisiae* YGE1 gene is essential for cell viability and is further underscored by the fact that the 20 ESTs related to mt-GrpE were obtained from sources as divergent as white blood cells, testis, brain, liver, spleen, melanocytes, ovary and pheochromocytoma (PC)-12 cells.

3.4. mt-GrpE synthesis is induced slightly by amino acid analogue treatment but not by heat shock

Consistent with their role as facilitators of protein folding, the synthesis of many chaperones have been noted to increase under conditions which are not conducive to protein folding (reviewed in [1]). In *E. coli* the GrpE gene, like the DnaK/DnaJ and GroEL/GroES operons, is part of the heat shock regulon and thus transcribed/translated at a higher rate in response to heat shock (reviewed in [31]). Whilst mammalian

Hsp60 and Hsp10 are clearly heat shock inducible [13,32], surprisingly, neither mammalian mt-Hsp70 nor *S. cerevisiae* mt-GrpE (Ygelp) appears to be inducible by heat shock [23]. It was therefore of interest to investigate whether rat mt-GrpE is synthesised at elevated rates in response to stress. Rat hepatoma cells were either heat shocked or grown in the presence of the proline analogue Azc and following metabolic labelling, cellular fractions were analysed by SDS-PAGE and phosphorimaging to detect de novo synthesised proteins (Fig. 5A). Inspection of the cytosolic fractions clearly indicated a sharp induction of Hsp72 by both types of stress and similarly a significant but less pronounced increase in mitochondrial Hsp60. Under no circumstances was it possible to detect metabolically labelled mt-GrpE, let alone observe an increase in its intensity. In contrast a 28 kDa protein, putatively identified as the cysteine containing rat Hsp28 (GenBank accession number S67755), was clearly accumulated at a higher rate following heat shock. When these observations were backed up by Western blot analysis (Fig. 5B) similar results were obtained, except now a slight increase in the total amount of both Hsp60 and mt-GrpE was noticeable upon close inspection of the corresponding filters.

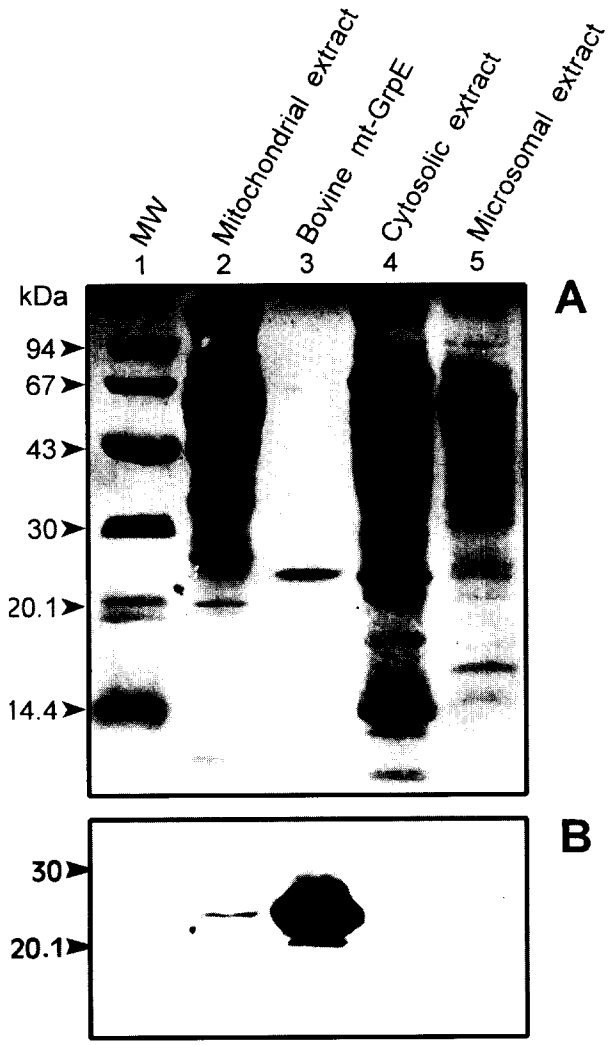


Fig. 3. Western blot analysis confirms a mitochondrial location of mt-GrpE. (A) Molecular weight markers (lane 1), mitochondrial extract (50 μg; lane 2), bovine mt-GrpE (1.6 μg; lane 3), cytosolic extract (70 μg; lane 4) and microsomal extract (55 μg; lane 5) were electrophoresed in a Tris-Tricine gel, transferred to nitrocellulose and stained with Ponceau S. (B) The filter was probed with rabbit anti-mt-GrpE antiserum followed by detection of bound antibodies with a secondary HRP-conjugated antibody.

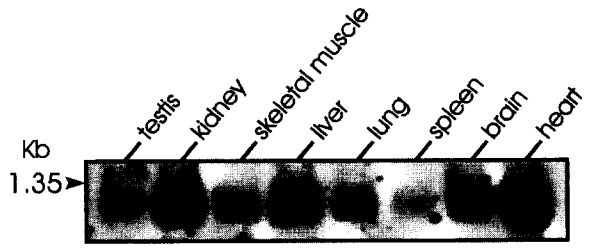


Fig. 4. Northern blot analysis indicates a ubiquitous but varying level of mt-GrpE mRNA in rat organs. Each lane contained approx. 2 μg of mRNA isolated from the indicated tissues and loadings were adjusted to contain equal amounts of β-actin mRNA.

In an attempt to further investigate whether such perceived increases in mt-GrpE levels could be correlated with an increase in mRNA steady-state levels, semi-quantitative RT-PCR was performed in triplicate and each time a similar relative intensity of the various chaperone transcripts was observed (Fig. 5C). As expected from previous metabolic labelling experiments [13,32], mt-Hsp70 mRNA levels increased in response to Azc treatment but not in response to heat shock whilst Hsp60 and Hsp10 mRNA levels increased in response to both treatments. These responses are in accord with nuclear run off experiments and Northern analysis we have performed in a parallel study [33] and indicates that the RT-PCR procedure, when performed carefully, will give a reliable indication of comparative transcript levels. The RT-PCR results obtained with the mt-GrpE specific primer set mirrored that obtained for mt-Hsp70 and thus indicated that the levels of mt-GrpE do not increase in response to heat shock but marginally in response to amino acid analogue treatment. Consistent with this observation, the yeast YGE1 gene is not induced by heat shock [7] and it appears that neither the *S. cerevisiae* nor the *C. elegans* grpE gene contains heat shock elements [7,34].

In conclusion, we have cloned a cDNA encoding a ubiquitously expressed mt-GrpE from a higher eukaryote. We have shown that the degree of conservation within the GrpE family is much lower than that seen for other chaperone members such as Hsp70, Hsp60 and Hsp10. However, it appears that this relatively low degree of sequence conservation has not been accompanied by a parallel diversification of function. The very specific interaction between mammalian mt-GrpE and DnaK combined with its stress inducibility signifies that the essential features of the bacterial DnaK/GrpE system have been maintained during the evolution of mitochondria in higher eukaryotes. A more rigorous test of this assumption will be facilitated with the availability of a mt-GrpE clone and corresponding antibodies.

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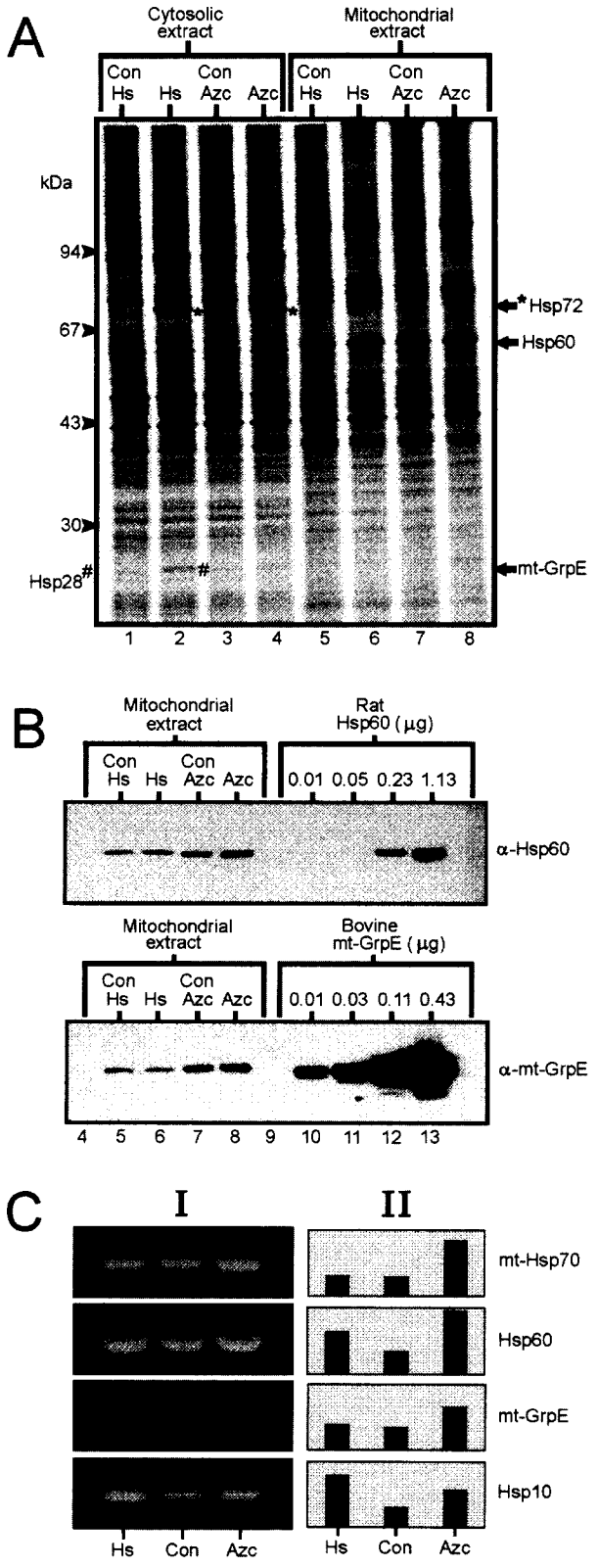

Fig. 5. mt-GrpE is a low abundance protein and its synthesis appears to be only slightly stress inducible. (A) Cytosolic (lanes 1–4) and mitochondrial (lanes 5,6) proteins metabolically labelled with [^{35}S]methionine and [^{35}S]cysteine were recovered either from untreated cells (Con; lanes 1,3,5,7), cells subjected to heat shock (Hs; lanes 2,6) or cells grown in the presence of Azc (lanes 4,8). Equal amounts of trichloroacetic acid-insoluble radioactive material (2×10^5 cpm) were loaded in each sample well, separated in a Tris-Tricine gel and analysed with a Storm phosphorimager (Molecular Dynamics). The indicated positions of Hsp70, Hsp60 and mt-GrpE were determined by electrophoresis of purified stress proteins in adjacent lanes followed by protein staining. A protein believed to represent Hsp28 is also indicated. (B) A Tris-Tricine gel was loaded in the order described above with equal amounts of trichloroacetic acid-insoluble radioactive material (4×10^5 cpm). Standard amounts of purified rat Hsp60 and bovine mt-GrpE were loaded in adjacent lanes 10–13 (as indicated). Following blotting to nitrocellulose, the filter was sequentially probed with antiserum against mt-GrpE and against Hsp60. The amount of mitochondrial supernatant proteins loaded were: 7.4 μg for control heat shock (Con Hs), 5.6 μg for heat shock (Hs), 15.6 μg for control Azc and 15.1 μg for Azc treatment. (C) Semi-quantitative RT-PCR analysis confirms a slight stress-inducibility of mt-GrpE by Azc but not by heat shock. (I) RT-PCR was performed on several chaperone mRNA transcripts isolated from untreated (Con), heat shocked (Hs) or Azc treated clonal rat hepatoma cells (as indicated). PCR products were separated on a 1% agarose gel supplemented with 0.02% (w/v) ethidium bromide. (II) The gel was scanned then quantitated with an ImageQuaNT program (Molecular Dynamics). The histograms indicate the relative amounts of pixels counted, for each transcript, in terms of the three cellular treatments indicated. The complementary primer sets were tested three times and each time similar relative intensities of the various chaperone transcripts were observed.
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