Evidence for the Existence of Distinct Mammalian Cytosolic, Microsomal, and Two Mitochondrial GrpE-like Proteins, the Co-chaperones of Specific Hsp70 Members*

(Received for publication, January 27, 1998, and in revised form, May 18, 1998)

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We previously reported the cDNA cloning and characterization of a mammalian mitochondrial GrpE protein (~21 kDa, mt-GrpE1) and now provide evidence for the presence of distinct cytosolic (~40 kDa), microsomal (~50 kDa), and additional mitochondrial (~22 kDa, mt-GrpE2) GrpE-like members. While a cytosolic GrpE-like protein has recently been identified, the demonstration of both a microsomal and a second mitochondrial GrpE-like member represents the first in any biological system. Investigation of the microsomal and two mitochondrial GrpE-like proteins revealed that they bound specifically to Escherichia coli DnaK, and the complexes formed were not disrupted in the presence of 0.5 M salt but were readily dissociated in the presence of 5 mM ATP. The functional integrity of mt-GrpE1 and #2 was verified by their ability to specifically interact with and stimulate the ATPase activity of mammalian mitochondrial Hsp70 (mt-Hsp70). Analysis of the cDNA sequences encoding the two mammalian mitochondrial GrpE-like proteins revealed ~47% positional identity at the amino acid level, the presence of a highly conserved mitochondrial leader sequence, and putative destabilization elements within the 3′-untranslated region of the mt-GrpE2 transcript which are not present in the mt-GrpE1 transcript. A constitutive expression of both mitochondrial GrpE-like transcripts in 22 distinct mouse tissues was observed but possible different post-transcriptional regulation of the mt-GrpE1 and #2 transcripts may confer a different expression pattern of the encoded proteins.

Molecular chaperones of the 70-kDa heat shock protein (Hsp70) family function in a diverse number of vital cellular processes including protein folding, translocation of proteins across membranes, proteolysis, regulation of the stress response, and activation of signal transduction molecules (reviewed in Refs. 1–6). Members of the Hsp70 family have been found in all organisms examined, and representatives from distantly related organisms often display a high degree of sequence similarity. For example, at the amino acid level the prokaryotic Escherichia coli Hsp70 member (DnaK) exhibits ~51% positional identity with its higher eukaryotic mitochondrial homologue (mt-Hsp70) (7). Central to the function of Hsp70 members as molecular chaperones is a weak ATPase activity which, at least in the case of E. coli DnaK, is synergistically stimulated up to 50-fold by the heat shock proteins DnaJ and GrpE (8). DnaJ and GrpE appear to regulate the ability of DnaK to bind and stabilize unfolded proteins before their release in a reaction requiring ATP hydrolysis (9). It has been shown that DnaJ stimulates the hydrolysis of DnaK-bound ATP which allows DnaK (in the ADP-bound state) to interact more strongly with unfolded proteins. GrpE then acts as a “nucleotide exchange factor” to recycle DnaK into an ATP-bound state, thereby permitting the efficient release of substrate. Recently, the crystal structure of E. coli GrpE bound to the ATPase domain of DnaK was determined (10). This confirmed earlier studies that a dimer of GrpE binds asymmetrically to a single DnaK molecule in the absence of ATP (11–13). GrpE is proposed to facilitate release of DnaK-bound ADP by essentially wedging apart the DnaK ATPase domain, thus weakening the grasp of DnaK on ADP. Furthermore, a long α-helical region of GrpE is believed to extend to the C-terminal substrate binding domain of DnaK where it could possibly mediate an interdomain communication necessary for substrate release. Thus, DnaK/DnaJ/GrpE appear to function in a mechanistic fashion as a molecular “chaperone team” which, in one form or another, may operate in all compartments of eukaryotic cells.

Homologues of DnaK and DnaJ have been identified in several major compartments of the eukaryotic cell, including the cytosol, nucleus, endoplasmic reticulum (ER), mitochondria, and chloroplasts, and in many cases these chaperones exist in multiple isoforms (reviewed in Refs. 1–6). In comparison, eukaryotic homologues of GrpE have been detected only in mitochondria (14–18) and possibly chloroplasts (19), but in no case have isoforms been detected. While a chloroplast GrpE-like protein has been inferred through cross-reactivity with antibodies to E. coli GrpE (19), mitochondrial GrpE homologues from Saccharomyces cerevisiae (Mge1p, Yge1p, GrpEp, and GrpE2p), Drosophila melanogaster (Droe1p), and mammals (mt-GrpE1) were identified by their abilities to bind specifically to Hsp70 members, and the complexes formed are not disrupted in the presence of 1 M salt but readily dissociate in the presence of 5 mM ATP (15, 17, 18). Such a specific interac-

* This work was supported by grants from the National Health and Medical Research Council and from the Australian Research Council (to N. J. H. and P. B. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: Hsp70, 70 kDa heat shock protein; EB, equilibration buffer; ER, endoplasmic reticulum; Hap, Hsp70/Hsc70-associating protein; Hsc70, the constitutive isof orm of Hsp70; IMAC, immobilized metal affinity chromatography; mt, mitochondrial; EST, expressed sequence tag; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; bp, base pairs.

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tion was first observed between the E. coli members of GrpE and DnaK (20).

It is somewhat surprising then that the role of a GrpE-like protein may not be required for the proper functioning of Hsp70 homologues outside mitochondria and chloroplasts while DnaJ homologues exist in all additional compartments that contain Hsp70 members. Indeed, it is perhaps more likely that the three-component chaperone team of Hsp70, DnaJ, and GrpE is conserved in numerous compartments of eukaryotic cells, but that sequence similarities have been too low to allow facile identification through comprehensive searches of data bases (21). Thus, as opposed to members of the Hsp70 (DnaK), Hsp60 (GroEL), and Hsp10 (GroES) families from rat mitochondria and E. coli which exhibit 51, 49, and 45% positional identity at the amino acid level, respectively, the GrpE family exhibits only 21% positional identity, and comparison of 20 GrpE sequences identified only six invariant residues (21). Another likely reason for the paucity of identified GrpE-like proteins is their very low abundance in most systems (i.e. Mge1p and mt-GrpE#1 comprise ~0.03% of the total soluble mitochondrial proteins) (21, 22). Taken together, the low degree of sequence similarities and the low abundance of at least some GrpE members calls for both a sequence based and a functional approach to search for new GrpE-like chaperones. Indeed, very recently a cytosolic/nuclear located Hsp70/Hsc70-associating protein (Hap) (alternatively named BAG-1 or RAP46) with GrpE-like activity but a seemingly unrelated primary structure has been reported (23–25). Alternative translation initiation from a single Hap transcript in both human and mouse cells is believed to generate either a cytosolic ~36- and ~32-kDa Hap protein, respectively, or a ~50-kDa Hap protein with an N-terminal extension thought to be important in nuclear targeting (26). The finding of a cytosolic/nuclear-located protein with GrpE-like activity lends further support to the supposition that Hsp70 members operate as components of DnaK/DnaJ/GrpE-like chaperone teams irrespective of the organism or cellular location. Given the presence in the endoplasmic reticulum of both DnaJ-like members (Sec63 and Sec1p) and Hsp70-like homologues (BiP and Lhs1p), it is therefore not unlikely that microsomal proteins with GrpE-like activity exist (reviewed in Refs. 1–6).

In this study we have now further explored the complexity of the mammalian GrpE complement. We provide evidence that mammalian cells, in addition to their previously identified mt-GrpE#1 and cytosolic/nuclear located Hap members, contain a second distinct mitochondrial GrpE member (mt-GrpE#2) which functions as a co-chaperone for mt-Hsp70, plus a microsomal ~50-kDa protein which may serve as a partner for ER BiP or an as yet unidentified Hsp70 member.

MATERIALS AND METHODS

DnaK-affinity Chromatography and Immunological Techniques—Preparation of a DnaK-affinity column and subsequent chromatography was described previously (17). Isolation of bovine liver organelles was performed on sucrose density gradients by removing a broad region of the gradient where mitochondria predominantly reside with significant amounts of endoplasmic reticulum and peroxisomes being present. Soluble protein extracts were then purified from the preformed organelles. Following DnaK affinity chromatography, 100 µg of the 5 mM ATP eluate (predominantly comprised of bovine mt-GrpE#1) was mixed with an equal volume of Freund’s complete adjuvant as described by Harlow and Lane (28). Ear bleeds were performed 10 days after the third boost, and serum containing polyclonal antibodies to mt-GrpE#1 was collected and processed (28). The serum was supplemented with 0.02% (v/v) sodium azide and stored at −70 °C. Dilutions of 1 in 10,000 were used in immunostaining analysis.

For Western blotting, cellular protein fractions were resolved by SDS-PAGE in a 16% (w/v) Tris-Tricine gel (29) and transferred to nitrocellulose using a semidry transfer unit (28). The blot was probed with the rabbit antisera and HRP-labeled goat anti-rabbit IgG, followed by incubation with enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech) and subsequent exposure to Hyperfilm-MP (Amersham Pharmacia Biotech). Mitochondria (bovine liver) were prepared according to Hartman et al. (30), microsomes (porcine liver) were pelleted by centrifugation of the post-mitochondrial supernatant at 100,000 × g for 1 h, and the final supernatant was defined as the cytosolic (rat liver) fraction.

Stress Treatments, Metabolic Labeling, and Fractionation of Tissue Culture Cells—The growth of rat hepatoma (H4) cells in the presence of the amino acid analogue 1-azetidine-2-carboxylic acid, the conditions for heat shock, and the isolation of 35S-labeled cytosolic and mitochondrial proteins were as described previously (21). A crude cellular lysate was prepared by hypotonic lysis and Dounce homogenization. Cellular membranes and nuclei were removed as a pellet following a 754 × g centrifugation, and a crude mitochondrial pellet was obtained by recentrifugation of the supernatant at 10,000 × g. The remaining supernatant contained the cytosolic and microsomal fraction. Mitochondria were purified further on sucrose gradients and following incubation with 0.5% (w/v) Triton X-100 reduced (Sigma) at 4 °C for 1 h, a soluble mitochondrial lysate was obtained after centrifugation at 80,000 × g for 1 h.

Nucleotide Sequence Analysis—The verified nucleotide sequence encoding mouse mt-GrpE#2 was obtained by sequence analysis of two independently isolated expressed sequence tags (ESTs) from the I.M. A.G. Consortium Lawrence Livermore National Laboratory cDNA clones collection (I.M.A.G. Consortium CloneIDs 482996 and 478162 corresponding to GenBank™ accession nos. AA060861 and AA049605, respectively; see “Results” and “Discussion”) (31). Both strands of the two clones were sequenced using the dideoxynucleotide chain termination procedure of Sanger et al. (32).

Southern Blot Analysis—Rat genomic DNA (10 µg) was digested with the appropriate restriction enzyme (40 units at 37 °C for 8 h) and electrophoresed in a Tris-aceate-EDTA (TAE)-buffered 0.8% (w/v) agarose gel. The digested DNA was transferred onto a Hybond™-N+ nylon membrane (Amersham Pharmacia Biotech), and the blot was prehybridized 6 h at 65 °C in 5 × SSC, 0.5% (w/v) SDS, 100 µg/ml sheared salmon sperm DNA, and 5% Denhardt’s solution (0.1% (w/v) Ficoll™ 400, 0.1% (w/v) polyvinylpyrrolidone, and 0.1% (w/v) bovine serum albumin). The membrane was probed with a [32P]-labeled mouse mt-GrpE#2 cDNA fragment (nucleotides 65–690, Fig. 1), then stripped by incubation in 0.5% (w/v) SDS at ~90 °C for 10 min and reprobed with a [32P]-labeled rat mt-GrpE#1 cDNA fragment (GenBank™ accession no. U55340, nucleotides 96–729). The denatured probes were added to the prehybridization solution and hybridizations were carried out for 12 h at 65 °C. Final washes were performed for 10 min at 65 °C in 0.1× SSC supplemented with 0.1% (w/v) SDS, and the blots were analyzed using a Storm PhosphorImager and Image Quant software (Molecular Dynamics).

Expression of Mouse mt-GrpE#2, Rat mt-GrpE#1, and Rat mt-Hsp70 in E. coli—The cDNA coding regions specifying the mature translation products of mouse mt-GrpE#2 (579 bp), rat mt-GrpE#1 (570 bp), and rat mt-Hsp70 (1899 bp, GenBank™ accession no. S75280) were amplified with Vent™DNA polymerase (New England BioLabs) utilizing specific primer pairs in a 30-cycle polymerase chain reaction protocol (94 °C for 1 min at 60 °C, 55 °C for 90 s, and 72 °C for 90 s). The primer pairs used were: for mouse mt-GrpE#2 (5′-CTGGATCATATGAGCACTGCCACCCAAAGAA–3′ and 5′-AGCCGGATCTTAAGTACTCTTCCTGAGACCTCTAC-3′), for rat mt-GrpE#1 (5′-CCATTGGGATCTTGATAGATTAGATGATGATGATGATGAGAACCCCGCGGAACTAA–3′ and 5′-TTGGCTTAAATGATGATGATGATGATGATGATGATGATGATGAGAACCCCGGAACTAA-3′). PCR products were digested with NdeI and BstHI, ligated individually into the same predigested sites of the pET-14b vector (for mt-GrpE#1 + p2) or pET-3a vector (for mt-Hsp70) as specified in the manufacturer’s manual (4th Ed., Novagen). Note that the mt-Hsp70 construct was designed to remove a preexisting N-terminal T7-Tag™ and introduce a C-terminal hexahistidine tag preceded by a thrombin cleavage site. Constructs under the selection of ampicillin were then

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separately transformed into BL21(DE3) cells co-harboring the plysS plasmid under the selection of chloramphenicol (Novagen). The authenticity of each construct was verified by sequence analysis of ~300 bp from either end of each cDNA insert within the pET-14b or pET-3a vectors.

For expression of the recombinant mt-GrpE#1 and #2 proteins bearing an N-terminal hexahistidine tag (removable with thrombin), typically 1.5 liters of Luria broth containing 100 μg/ml ampicillin and 40 μg/ml chloramphenicol were inoculated with the transformants and shaken at 30 °C until the A600 nm was equal to 0.5. Synthesis of the recombinant protein was initiated by addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM, and incubation was continued at 30 °C for 3 h. Cells were then harvested and lysed by the lysozyme method according to Sambrook et al. (33). After centrifugation (16,000 × g for 10 min) the supernatant was applied at 4 °C to a 2-ml Talon™ metal affinity column (CLONTECH) equilibrated in 25 mM Tris-Cl (pH 8.0), 200 mM NaCl, and 10 mM β-mercaptoethanol. Following extensive washing with equilibration buffer (EB), the column was washed successively with 0.3 × NaCl in EB (i.e. 0.5 × NaCl final concentration), 10 mM imidazole in EB, 5 mM ATP, 10 mM MgCl, and 50 mM KC1 in EB, 2.5 mM CaCl2 in EB. Finally an essentially pure preparation of recombinant protein (~40 mg for mt-GrpE#1 and ~14 mg for mt-GrpE#2) was recovered by recirculating through the column a 20 ml solution of EB fortified with 2.5 mM CaCl2 and 10 units of human thrombin (Boehringer Mannheim) for 5 h. uncleaved recombinant protein retained on the IMAC column was eluted with 100 mM imidazole in EB (~20 mg for mt-GrpE#1 and ~7 mg for mt-GrpE#2) and subjected to gel filtration on a Superdex-200 (26/60, Amersham Pharmacia Biotech) column equilibrated in 0.1 M NaP, (pH 7.3) and 100 mM NaCl at 4 °C. The mass of hexahistidine-tagged recombinant mt-GrpE#1 + #2 were determined on an electrospray ionization-triple quadrupole mass spectrometer (PE SCIREX API300) essentially as described by Hartman et al. (30). A proteolytic fragment of recombinant mouse mt-GrpE#2 was subjected to SDS-PAGE in a 12.5% Tris-glycine gel (34), recovered with a gel-spin protein recovery kit (ScimacT), then taken for direct N-terminal protein sequencing as described previously (30).

Expression and subsequent purification of the recombinant mt-Hsp70 was carried out as described for mt-GrpE#1 and #2 except expression was performed for 16 h at 16 °C (instead of 30 °C). Typically ~10 mg of soluble mt-Hsp70 were purified from 1 l of culture.

Native PAGE—Non-denaturing PAGE was performed in 6% Tris-glycine gels by omitting SDS from the PAGE system (34). The protein samples were prepared in 25 mM Tris-Cl (pH 8.0), 50 mM NaCl, 50 mM KCl, 10 mM MgCl, 10 mM β-mercaptoethanol, and 50 units/ml ATPase. The samples were run at 100 V for 1 h after the migration of 0.05 mg/ml of bovine serum albumin.

ATPase Assay—The ATPase activity of recombinant rat mt-Hsp70 was measured by the conversion of γ-32P-ATP (1 μM; 4000 Ci/mmol, Bresapect) to γ-32P, essentially as described previously (8). Assays were carried out for 30 min at 30 °C in 50 μl of reaction mixtures containing 50 mM Tris-Cl (pH 8.0), 50 mM NaCl, 50 mM KCl, 10 mM MgCl, 2 mM dithiothreitol, 40 μM ATP, and the appropriate proteins. Reactions were terminated by spotting 2 μl samples onto Polygram CEL 300 polyethylenimine cellulose plates (Macherey-Nagel) where ~0.832P-ATP was separated from γ-32P, by one-dimensional chromatography using a solution of 1 M formic acid and 1 l LiCl. Hydrolyzed ATP was then quantified by Storm PhosphorImager analysis (Molecular Dynamics). The E. coli DnaJ protein was purchased from StressGen Biotechnologies Corp. while the recombinant rat mt-Hsp70, rat mt-GrpE#1 and mouse mt-GrpE#2 proteins were prepared without their hexahistidine tags as described earlier.

DNA Dot Blot Analysis—For identification of the tissue distribution of the mt-GrpE transcripts, an RNA dot blot with mRNA from 22 different mouse tissues (100–500 ng each) was purchased from CLONTECH (catalog no. 7771-1) and prehybridized, hybridized, and analyzed as described for the Southern blot analysis above.

RESULTS AND DISCUSSION

Identification of Distinct Cytosolic, Microsomal, and Mitochondrial GrpE-like Proteins—It has previously been established that E. coli GrpE binds strongly to immobilized DnaK in the absence of ATP (20). Utilizing this specific interaction we previously reported the DnaK-affinity purification of a single mitochondrial GrpE homologue (mt-GrpE#1) from rat, porcine, and bovine liver mitochondrial extracts (17). In the present work we again employed DnaK-affinity chromatography, combined with an antiserum to mt-GrpE#1, to identify additional GrpE-like members from a bovine liver organelar extract. Although SDS-PAGE analysis of the protein constituents, that were specifically retained on immobilized DnaK in presence of 1 mM KCl but were readily dissociated from DnaK in the presence of 5 mM ATP, revealed only the mt-GrpE#1 protein following staining with Coomassie Brilliant Blue, at least one other related protein appears to be contained in this eluate. Fig. 1A shows the Western blotting pattern observed when the mt-GrpE#1 antiserum was used to probe the protein constituents from the 5 mM ATP eluate (of the DnaK column) and several purified organellar preparations. As suspected, the antiserum detected predominantly mt-GrpE#1 in the 5 mM ATP eluate (Fig. 1A, lane 2) which co-migrated with a single species in the mitochondrial fraction (Fig. 1A, lane 1). The detection of a small amount of a slightly lower molecular weight species was previously determined to be a proteolytic breakdown product of the mt-GrpE#1 protein (Fig. 1A, lane 2) (17). The antiserum which was prepared by initial immunization with greater than 95% pure mt-GrpE#1 and three subsequent boosts with gel-purified mt-GrpE#1 also detected a minor amount of a ~50-kDa protein in the 5 mM eluate of the DnaK column (Fig. 1A, lane 2) which co-migrated with a single species in the microsomal fraction (Fig. 1A, lane 4). This observation represents the first evidence for a GrpE-like protein in the endoplasmic reticulum. Although there is a formal possibility that the antibodies which recognize the ~50-kDa protein were in fact produced against it and therefore do not identify mt-GrpE#1, it should be noted that the ~50-kDa protein forms a stable interaction with immobilized DnaK that cannot be disrupted in the presence of 1 mM KCl but is readily dissociated in the presence of 5 mM ATP, reminiscent of mammalian mt-GrpE#1, E. coli GrpE, S. cerevisiae Mge1p, and D. melanogaster Droep1 (15, 17, 18, 20). Furthermore, as will be discussed later, we have identified a second distinct mitochondrial GrpE member (mt-GrpE#2), and antibodies raised against the gel-purified mt-GrpE#2 alone cross-react with mt-GrpE#1 and a ~50-kDa microsomal protein (data not shown). This observation further underscores the probability that the ~50-kDa microsomal protein co-purifying with mt-GrpE#1, during DnaK-affinity chromatography, contains common epitopes and therefore possibly fulfills a similar function. To provide additional evidence that the ~50-kDa protein is a GrpE-like member, we tested if the ~50-kDa protein was a bona fide stress (inducible) protein. Rat hepatoma cells were grown in the presence of the amino acid analogue l-azetidine-2-carboxylic acid and following metabolic labeling, the cytosolic and microsomal fractions were analyzed by SDS-PAGE and phosphorimaging to detect de novo synthesized proteins (Fig. 1B). A strong induction of Hsp70 (Hsp72), and to a lesser extent Hsp60 (Hsp73), was observed following the stress treatment, but it was not possible to detect the metabolically labeled ~50-kDa microsomal protein, let alone observe an increase in intensity. However, upon Western blot analysis a clear increase in the total amount of the ~50-kDa protein was evident following l-azetidine-2-carboxylic acid treatment (Fig. 1C, lanes 2) but not following heat shock (data not shown), reminiscent of the induction pattern of mt-GrpE#1 and mt-Hsp70 during stress (21). In this regard the induction pattern of this protein also resembles that of BiP (reviewed in Ref. 35), a candidate partner for this protein. Taken together, we conclude that this stress-inducible ~50-kDa microsomal protein, which interacts

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Fig. 1. Mammalian GrpE homologues are found in several cellular locations. A, a bovine mitochondrial extract (50 μg; lane 1), bovine organellar proteins eluted from a DnaK column by 5 mM ATP (1.6 μg; lane 2), rat cytosolic extract (70 μg; lane 3), and a porcine microsomal extract (55 μg; lane 4) were electrophoresed in a Tris-Tricine gel, then transferred to nitrocellulose. The filter was probed with antisera raised against the bovine organellar proteins eluted from a DnaK column by 5 mM ATP followed by detection of bound antibodies with a secondary horseradish peroxidase-conjugated antibody. Synthesis of the microsomal 50-kDa GrpE-like protein is induced by L-azetidine-2-carboxylic acid (Aze). B, cytosolic and microsomal proteins (post 10,000 × g supernatant) metabolically labeled with [35S]methionine and [35S]cysteine were recovered either from untreated cells (Con, 18.0 μg; lanes 1), or cells grown in the presence of L-azetidine-2-carboxylic acid (18.4 μg; lane 2). Equal amounts of trichloroacetic acid-insoluble radioactive material (2 × 10^6 cpm) were loaded in each sample well, separated in a Tris-Tricine gel, and analyzed with a Storm PhosphorImager (Molecular Dynamics). The positions of Hsc70 (Hsp73) and Hsp70 (Hsp72) are indicated. C, an identical Tris-Tricine gel was loaded as described for panel B except the equal amounts of trichloroacetic acid-insoluble radioactive material were doubled (4 × 10^6 cpm/lane). Following blotting, the filter was probed with the antisem employed in panel A. D, an amino acid segment within the mammalian Hap protein is conserved in several members of the GrpE family. Comparison of an amino acid segment from Homo sapiens (GenBankTM accession no. U62940), Mus musculus (Swiss-Prot accession no. Q99933) and Mus musculus (Swiss-Prot accession no. Q60739) Hap (BAG-1, RAP46) with an analogous region in Rattus norvegicus mt-GrpE#1 (GenBank™ accession no. U62940), M. musculus mt-GrpE#2 (GenBank™ accession no. AF041060), and E. coli GrpE (GenBank™ accession no. X07863). Residues identical to those of human (h) and mouse (m) Hap sequences are highlighted in black while conserved residues (defined as A/G, I/L, R/K/H, and D/E/N/Q) are shaded gray. The numbering of the C-terminal residue (on the right-hand side) of each segment indicates its position within the entire protein sequence.

Specifically with DnaK in a nucleotide-dependent manner, represents an authentic member of the GrpE family, and it should be designated er-GrpE#1. We expect that er-GrpE#1 functions with BiP and an ER DnaJ homologue as part of a complete chaperone team that has already been defined in the mitochondrial, cytosolic, and perhaps chloroplastic compartments of eukaryotic cells. Screening of expression libraries using the antibodies prepared herein now opens the way for cloning of cDNAs and genes encoding this hitherto unknown co-chaperone-like protein.

Surprisingly, despite being undetected in the immunizing antigen, a single ~40-kDa protein was recognized by the antisem in the cytosolic fraction (Fig. 1A, compare lanes 2 and 3). We suspect that this protein corresponds to a recently characterized human ~36-kDa protein from humans and mice with GrpE-like activity (Hap, Bag-1, and Rap46) that resides in the cytosol (23–26). Despite the former report that the primary sequence of Hap does not display regions of significant similarity to previously identified members of the GrpE family (24), the cross-reactivity displayed here does indicate a structural similarity to mt-GrpE#1, and accordingly a small segment within the Hap protein that is conserved in several members of the GrpE family can readily be identified (Fig. 1D). The presence of such a conserved segment supports the likelihood that Hap cross-reacts with antibodies raised against mt-GrpE#1; however, we cannot dismiss the possibility that the ~40-kDa cytosolic protein represents an as yet uncharacterised polypeptide with GrpE-like structural and biochemical characteristics. A second ~50-kDa isoform of the human and mouse Hap protein has recently been shown to be generated by alternative translation initiation from a single Hap transcript. While this isoform has the same apparent molecular weight as the putative er-GrpE#1, Hap is incapable of binding both DnaK and BiP (23). Preliminary studies have shown that the ~50-kDa Hap isoform is localized to the nucleus (26).

Identification of a Second Mammalian mt-GrpE cDNA—Based on a consensus sequence for the GrpE family (21) a search of data bases revealed a previously uncharacterised mouse EST, from both 13.5–14.5 (GenBank™ accession no. AA049605) and 19.5 (GenBank™ accession no. AA060861) days post conception embryo cDNA libraries. These ESTs exhibit significant homology to our earlier reported rat mt-GrpE#1 cDNA sequence (21) but represent a distinct entity. Complete sequencing of the two EST clones revealed a 1445-bp cDNA (Fig. 2), where GenBank™ accession no. AA049605 (Fig. 2, nucleotides 4–1448) and GenBank™ accession no. AA060861 (Fig. 2, nucleotides 27–1448) were identical in a 1422-bp overlap. By comparison of the predicted amino acid sequence (Fig. 2) with several GrpE homologues (Fig. 3 and Table 1), it is concluded that the cDNA encodes a second mouse mitochondrial GrpE protein (named mt-GrpE#2) and in all likelihood only the initiation ATG codon is missing from the coding region of the cDNA clone. In support of this prediction, the presence of an ATG codon on the 5’-end of the cDNA would be complemented by the two adjacent 3’-nucleotides (Fig. 2, numbered 4 and 5) to initiate the translation of a 224-residue putative mitochondrial precursor protein (36). Furthermore, we have previously observed that the initiation ATG codon was absent from a multitude of mt-GrpE#1 cDNAs and corresponding ESTs, and could be recovered only following a 5’-rapid amplification of cDNA ends approach (21). We therefore conclude that some structural feature(s) of the mt-GrpE transcripts may hamper the synthesis of full-length mt-GrpE encoding cDNAs.
Southern analysis on restriction endonuclease digested rat genomic DNA (Fig. 4) clearly showed that mt-GrpE#1 and #2 are encoded by distinct genes which probably exist as single copies within the rat genome. Comparison of the N-terminal amino acid sequence of purified bovine mt-GrpE#1 with the deduced amino acid sequences of mouse mt-GrpE#2 and several mt-GrpE#1 members predicts the presence of a conserved cleavable mitochondrial targeting sequence, defined as residues 1–31 (where residue 1 is the predicted initiation methionine) for mouse mt-GrpE#2 and residues 1–27 for rat, mouse, and human mt-GrpE#1 (Fig. 3). Characteristic of mitochondrial targeting sequences, residues 8–19 of mouse mt-GrpE#2 and 4–21 of rat, mouse, and human mt-GrpE#1 have a strong potential to form amphiphilic α-helices with a high content of serine and basic residues but seldom acidic residues (37). As evident for bovine mt-GrpE#1, upon mitochondrial import, these N-terminal targeting sequences would presumably be proteolytically removed from the 224-residue mouse mt-GrpE#2 (25.0 kDa) and 217-residue rat mt-GrpE#1 (24.3 kDa) precursors to generate the mature proteins of 193 (21.5 kDa) and 190 (21.3 kDa), respectively. The predicted isoelectric points of the mature mouse mt-GrpE#2 protein is 6.8 and the mature rat mt-GrpE#1 protein is 6.5, while the precursors, due to their basic mitochondrial targeting sequences, have predicted pI values of 7.7 and 8.5, respectively. The specific detection of mt-GrpE#1 in mitochondria (Fig. 1A, lane 1) correlates well with these predictions. Surprisingly, expression of mt-GrpE#2 has thus far not been observed in any of the major cellular compartments within adult mammalian liver cells (discussed later) which has led us to believe that differential expression of the two mt-GrpEs may exist.

Properties and Interaction of mt-GrpE#1 and #2 with DnaK—In order to evaluate whether mouse mt-GrpE#2 might function as a co-chaperone for an Hsp70 member, we investigated if mt-GrpE#2 could form a stable complex with DnaK in the absence of ATP, a characteristic of several other GrpE family members (15, 17, 18, 20, 22, 38). The mature portions of both the rat mt-GrpE#1 and mouse mt-GrpE#2 proteins (predicted in Fig. 3) with N-terminal hexahistidine tags were thus synthesized in E. coli and then purified by immobilized metal affinity chromatography (IMAC) (Fig. 5A, lanes 3 and 5, respectively). As seen in Fig. 5, both mt-GrpE#1 and #2 form a specific complex with E. coli DnaK which is stable in the presence of 0.5 M NaCl but readily dissociated in the presence of 5 mM ATP (Fig. 5A, lanes 2 and 4, respectively). Both N-terminal protein sequencing (of the first 10 residues) and mass spectrometric analysis unequivocally revealed the identity of the interacting protein recovered in the 5 mM ATP eluate as E. coli DnaK. Specifically, the predicted mass of mature E. coli DnaK is 68,983.20 Da, while those obtained by mass spectrometry were 68,976 ± 6 Da (Fig. 5A, lane 2) and 68,983 ± 4 Da (Fig. 5A, lane 4). To dismiss the possibility that DnaK might bind directly to the IMAC resin rather than to mt-GrpEs, IMAC purification was also performed on lysates prepared from E. coli not subjected to prior induction of mt-GrpE#1 and #2 synthesis. In this case, DnaK was not detected in the 5 mM ATP eluates (data not shown).

The unequivocal demonstration of E. coli DnaK interaction with the recombinant mt-GrpEs suggested a functional integrity. To further characterize the properties of the two mammalian mt-GrpEs, they were subjected to gel filtration, and their elution times were compared with those of known standards. The cDNA and deduced amino acid sequence of mouse mt-GrpE#2. The numbering of nucleotides is shown on the left-hand side and the first base of the predicted initiator codon is numbered +1 (see text for details). Amino acids are given by their single-letter code, and they are numbered on the right-hand side. The predicted first residue of the mature protein is numbered +1 and the cleavable presequence (underlined) is thus defined as amino acids –27 to –1. A putative polyadenylation site is boxed and possible RNA destabilization sequences are double underlined. This sequence has been submitted to GenBank and given accession no. AF041060.
has previously been determined that, alone, both *E. coli* GrpE and *S. cerevisiae* Mge1p predominantly exist as dimers and to a lesser extent as higher oligomers (11–13, 39, 40). Likewise, both mt-GrpE#1 and #2 were observed to exist mostly in a dimeric form (with a native molecular mass of ~57 kDa) but higher oligomeric forms were also evident (Fig. 5B). In further support of a dimeric (or higher oligomeric) state of mt-GrpE#, we have observed that a proteolytic fragment of recombinant mt-GrpE#2 (lacking the hexahistidine tag and beginning with the N-terminal sequence PDGLGPSLAE... ) copurifies with the intact protein (containing the hexahistidine tag) through IMAC purification and subsequent gel filtration (Fig. 5A, lane 5). The fragment is not a result of nonspecific thrombin cleavage for it persists in the eluate when the thrombin cleavage step is omitted and alternatively the hexahistidine-tagged protein is eluted with 100 mM imidazole (data not shown).

### TABLE I

|          | *M. musculus* mt-GrpE#2 | *M. musculus* mt-GrpE#1 | *H. sapiens* mt-GrpE#1 |
|----------|-------------------------|-------------------------|------------------------|
| *M. musculus* mt-GrpE#2 | 46.1 (57.6) | 46.6 (57.9) |
| *M. musculus* mt-GrpE#1 | 46.1 (57.6) | 46.6 (57.9) |
| *R. norvegicus* mt-GrpE#2 | 46.6 (57.9) | 88.1 (83.9) |
| *H. sapiens* mt-GrpE#1 | 46.6 (57.9) | 88.1 (83.9) |
| *D. melanogaster* Droep | 36.8 (51.2) | 43.7 (56.9) | 49.7 (56.2) |
| *C. elegans* mt-GrpE | 35.8 (48.5) | 43.5 (49.9) | 40.1 (50.4) |
| *S. cerevisiae* Mge1p | 25.9 (46.0) | 30.6 (46.3) | 30.6 (45.4) |
| *E. coli* GrpE | 25.9 (43.8) | 26.9 (43.8) | 25.9 (44.5) |
| *M. musculus* Hap | 22.5 (39.9) | 20.5 (38.4) | 21.6 (40.4) |
| *H. sapiens* Hap | 16.7 (38.1) | 20.2 (37.6) | 20.2 (41.9) |

![Figure 3](#) Multiple amino acid sequence alignment showing the similarity between mouse mt-GrpE#2 and selected GrpE homologues. *Bos taurus* (N-terminal peptide, Ref. 17), *M. musculus* (compiled and edited from GenBank accessing nos. AA269811 and W70459), *R. norvegicus* (GenBank accessing no. U62940), *H. sapiens* (compiled and edited from GenBank accessing nos. AA252446 and N28348), *D. melanogaster* (GenBank accessing no. U34900), *Caenorhabditis elegans* (EMBL accession no. Z46996), *S. cerevisiae* (GenBank accessing no. D28059), and *E. coli* (GenBank accessing no. X07863). The alignment was made using the PILEUP program (Genetic Computer Group, Madison, WI), and the suspected initiating methionine was numbered +1 to facilitate comparison, since the processing sites are not known for most of the primary translation products. The N-terminal mitochondrial signal sequence from yeast Mge1p is underlined, and based on the mature N-terminal sequence of bovine mt-GrpE, the suspected site of signal sequence cleavage is indicated with an arrow for the other mt-GrpE precursors. Residues that are identical to the mouse mt-GrpE#2 sequence are highlighted in black while conserved residues (defined as A/G, Y/F, S/T, I/V/L, R/K/H, and D/E/N/Q) are shaded gray. The asterisks under the consensus sequence (generated by alignment of 20 GrpE sequences) indicate the positions of six strictly conserved residues (21). Note, in our previous study (21) 12 amino acids (. . . **KETM EKYKRALADTEN-LRQR** . . . ) from the rat mt-GrpE#1 protein were deduced incorrectly due to the presence of two frameshift errors in the cdna sequence. The correct sequence of rat mt-GrpE#1 (and predicted human mt-GrpE#1) is shown above and the changes have been made to GenBank accessing no. U62940.

![Figure 4](#) Multiple GrpE-like Members in Mammals

The percentage positional identity for individual pairs of sequence is shown at both the amino acid and nucleotide level (in parentheses) for several GrpE family members. Calculations were based on the multiple sequence alignment shown in Fig. 3. For the human and mouse Hap (BAG-1, Rap46) members, calculations were generated for individual pairs of sequence utilizing the GAP program (Genetic Computer Group, Madison, WI).
parent DnaK oligomer dissociating activity has been ascribed to E. coli GrpE previously (12). In that case it is suggested dimeric GrpE serves to stabilize monomeric DnaK in a 2:1 complex rather than actively disrupting DnaK oligomers. In the ER, it has been suggested that BiP interconversion between the oligomeric (inactive) and monomeric (active) state allows the cell to maintain a constant level of functionally active BiP available (42). Thus GrpE molecules, in addition to their role as nucleotide exchange factors for the stimulation of Hsp70 ATPase activity, may also serve to maintain adequate cellular levels of their functionally active Hsp70 counterpart by modulating the interconversion of oligomeric (inactive) and monomeric (active) forms of these chaperones.

We further tested the functional integrities of mt-GrpE#1 and #2 by investigating whether they, in the presence of a DnaJ member, could stimulate the intrinsic ATPase activity of mt-Hsp70 synergistically. Previous studies have shown that together E. coli GrpE and DnaJ can stimulate the low intrinsic ATPase activity of DnaK up to 50-fold, while alone GrpE and DnaJ stimulate the activity 2- and 13-fold, respectively (8, 43). As a mammalian mitochondrial homologue of DnaJ has not been identified, we employed E. coli DnaJ in the following ATPase assays because it has been observed to be functionally equivalent to S. cerevisiae Mdj1p (mt-DnaJ) in the synergistic stimulation of the Ssc1p (mt-Hsp70) ATPase activity with Mge1p (mt-GrpE) (44). Fig. 6C shows that E. coli DnaJ alone can indeed stimulate the ATPase activity of mt-Hsp70 (~6.5-fold), while together DnaJ and either mt-GrpE#1 or #2 synergistically stimulate the ATPase activity of mt-Hsp70 ~15-fold. The mt-Hsp70 ATPase activity was stimulated only weakly in the presence of either mt-GrpE#1 or #2 alone (~1.5-fold, Fig. 6C). Neither the mt-GrpE#1 or #2 preparations, nor the DnaJ preparation, exhibited any apparent ATPase activity (data not shown). It is concluded that mt-GrpE#1 and #2 both constitute active co-chaperones and function in a similar manner to E. coli GrpE in both the stimulation of the ATPase activity of mt-Hsp70 and the specific dissociation of the oligomeric states of mt-Hsp70.

**Expression of mt-GrpE#2**—A ubiquitous but varying level of expression for the rat mt-GrpE#1 transcript (~1.2 kilobase pairs) was previously seen in eight different tissues (21). Using a more expansive dot blot of murine mRNAs obtained from 22 different sources (Fig. 7A) and normalized to the mRNA expressed by SDS-PAGE in a 13% Tris-glycine gel followed by staining with Coomassie Brilliant Blue. Lane 1 contained molecular mass markers. B, the retention times (increasing from left to right) of several standard proteins, recombinant rat mt-GrpE#1 (E#1) and recombinant mouse mt-GrpE#2 (E#2) proteins (predicted from Fig. 3) were synthesized in E. coli with N-terminal hexahistidine tags, and following cell lysis were retained on ~2 ml of IMAC columns pre-equilibrated in 25 mM Tris-Cl (pH 8.0), 300 mM NaCl, and 10 mM β-mercaptoethanol. After extensive washing in EB, the columns were successively washed with 100 ml of EB containing 0.5 M NaCl in total), 100 ml of EB containing 5 mM ATP, 10 mM MgCl₂, and 50 mM KCl (lanes 2 and 4), 100 ml of EB containing 10 mM CaCl₂, and 20 ml of EB containing 10 mM CaCl₂ and 10 units of thrombin (lanes 3 and 5). The polypeptide content of the indicated fractions were analyzed by SDS-PAGE in a 13% Tris-glycine gel followed by staining with Coomassie Brilliant Blue. Lane 1 contained molecular mass markers. B, the retention times (increasing from left to right) of several standard proteins, recombinant rat mt-GrpE#1 (E#1) and recombinant mouse mt-GrpE#2 (E#2) were measured on a Superdex-200 (26/60) gel filtration column equilibrated in 0.1 M NaH₂PO₄ (pH 7.3) containing 100 mM NaCl. Eluates were monitored at 280 nm. A vertical dotted line is provided to help compare the three chromatograms while arrows and asterisks indicate the position of dimeric and higher oligomeric states of mt-GrpE#1 and mt-GrpE#2, respectively. The standards used were: blue dextran (2,000 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).
pression levels of eight different housekeeping genes, the expression of both the mt-GrpE#2 and #1 transcripts appeared to be ubiquitous (Fig. 7, B and C, respectively). We rule out nonspecific hybridization as negative controls such as: yeast total RNA and tRNA (grid position 5 F1 and F2, respectively); E. coli rRNA and DNA (grid position 5 F3 and F4, respectively); synthetic poly(rA) (grid position 5 G1); and Cot1 DNA (representing the most abundant repetitive sequences, grid position 5 G2) did not give rise to any hybridization signal. Furthermore, since the stringency of the hybridization was identical to that seen for the Southern blot in Fig. 3, cross-hybridization of the mt-GrpE#2 probe with mt-GrpE#1 transcript is most unlikely. Despite this apparent ubiquitous expression of both the mt-GrpE#2 and #1 transcripts appeared to be ubiquitous (Fig. 7, B and C, respectively). We rule out nonspecific hybridization as negative controls such as: yeast total RNA and tRNA (grid position = F1 and F2, respectively); E. coli rRNA and DNA (grid position = F3 and F4, respectively); synthetic poly(rA) (grid position = G1); and Cot1 DNA (representing the most abundant repetitive sequences, grid position = G2) did not give rise to any hybridization signal. Furthermore, since the stringency of the hybridization was identical to that seen for the Southern blot in Fig. 3, cross-hybridization of the mt-GrpE#2 probe with mt-GrpE#1 transcript is most unlikely. Despite this apparent ubiquitous expression of both the mt-GrpE#2 and #1 transcripts, we never detected any proteins in the 5 mM ATP eluates (of the DnaK columns) with an N-terminal sequence related to mt-GrpE#2. Neither did antibodies raised against the 5 mM ATP eluates recognize recombinant mt-GrpE#2, while recombinant mt-GrpE#1 was readily detected. The reason for this apparent contradiction between the ubiquitous presence of mt-GrpE#2 transcript and the absence of the corresponding translation product is not clear, but a similar situation has been documented for an osmotin-like protein in plants (45).

A possible explanation for the lack of mt-GrpE#2 accumulation could be the presence of two copies of the nonameric sequence UUAUUUA(U/A)(U/A) within the 3′-untranslated region of the mouse mt-GrpE#2 transcript (Fig. 2). These AU-rich elements are known to play a crucial role in the turnover of certain mammalian mRNAs, thus enabling an effective level at which gene expression can be controlled (46, 47). Perhaps more importantly, at least in this context, removal of the 3′-untranslated region from the granulocyte/macrophage colony-stimulating factor mRNA, which harbors the very same elements, effectively increases the stability of the transcript but decreases the efficiency of translation, implicating a role for 3′-untranslated region of granulocyte/macrophage colony-stimulating in translational control (review in Ref. 27). Whether this form of post-transcriptional regulation is employed on mt-GrpE#2 mRNAs is not known, but it might be relevant that we have observed E. coli BL21(DE3) cells to accumulate mt-GrpE#2 at significantly lower levels than does mt-GrpE#1. Notably, it has already been documented that overexpression of yeast Mge1p in situ has proven to be toxic to the cell (16). Notwithstanding these observations, it is clear that mt-GrpE#2 has all the hallmarks of a true Hsp70 co-chaperone, and it shall be intriguing to elucidate its exact role in future studies.

In conclusion, the findings in this report are significant for two reasons. First, we have provided evidence that a DnaK

![Fig. 6.](https://example.com/fig6.png)

**Fig. 6.** mt-GrpE#1 and #2 interact specifically with and stimulate the intrinsic ATPase activity of mt-Hsp70. A, purified mammalian mt-Hsp70 (mt-70, 2 μg), Hsc70 (2 μg), and BiP (2 μg) were analyzed by SDS-PAGE in a 13% Tris-glycine gel followed by staining with Coomassie Brilliant Blue. B, mixtures of these Hsp70 proteins (30 μg each) and either mt-GrpE#1 (E#1, 20 μg) or mt-GrpE#2 (E#2, 30 μg) were depleted of ATP, and the complexes formed were analyzed by native PAGE in 6% Tris-glycine gels followed by staining with Coomassie Brilliant Blue. In panels A and B molecular mass (MW) standards are shown for comparison. C, evaluation of the synergistic stimulation of mammalian mt-Hsp70 (mt-70, 1 μM) ATPase activity by E. coli DnaJ (J, 1 μM) and mt-GrpE#1 (E#1, 2 μM) or mt-GrpE#2 (E#2, 2 μM) was performed at 30 °C with 40 μM ATP for 30 min as described under “Materials and Methods.”

![Fig. 7.](https://example.com/fig7.png)

**Fig. 7.** mRNA dot blot analysis indicates a ubiquitous but varying level of the mt-GrpE#1 and mt-GrpE#2 transcripts in mouse tissues. Each dot contained approximately 100–500 ng of mRNA isolated from the indicated tissues, and loadings were normalized to the mRNA expression levels of eight different housekeeping genes. A, the diagram shows the type and position of mRNA and controls dotted onto the membrane. B, the filter was probed with the 32P-labeled mouse mt-GrpE#2 cDNA, then stripped and C, reprobed with the 32P-labeled rat mt-GrpE#1 cDNA.
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(Hsp70)/DnaJ/GrpE chaperone team exists in a microsomal location. Second, we have demonstrated that isoforms of GrpE-like molecules, as seen for both DnaK and DnaJ-like chaperones, can co-exist in a single compartment of a higher eukaryotic cell. This has not previously been found for GrpE family members from any other species, and its significance is yet to be determined in terms of mitochondrial chaperone activities and functions. The tools generated during this study will assist in elucidating the importance or otherwise of these components for the biogenesis and maintenance of proteins in higher eukaryotic cells.

Acknowledgments—We thank Joan Hoogenraad for assistance with the tissue culture work, Yoji Hayasaka for mass spectrometric analysis, and Helen Healey for help with the design of expression vectors.

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