Identification and characterization of molecular interactions between glucose-regulated proteins (GRPs) mortalin/GRP75/peptide-binding protein 74 (PBP74) and GRP94

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A heat-shock protein (hsp) 70 family member mortalin/glucose-regulated protein (GRP) 75/peptide-binding protein 74 (PBP74) has been localized to various cellular compartments including mitochondria, endoplasmic reticulum and cytoplasmic vesicles. Here we describe its interactions with an endoplasmic reticulum protein GRP94, a member of the hsp90 family of GRPs. Interactions were identified, confirmed and characterized by far-Western screening, *in vivo* reporter and co-immunoprecipitation assays. Interacting domains of the two proteins were also characterized by mutational analysis. Such interactions of these two GRPs may be important for function of either or both and therefore provide important information for further studies.

Key words: binding domain, far-Western screening, *in vivo* precipitation, mortalin–GRP94 interaction, two-hybrid assay.

INTRODUCTION

Mortalin is a member of the heat-shock protein (hsp) 70 family of proteins. The two mortalin cDNAs (mot-1 and mot-2), which encode two proteins that differ by two amino acids in the C-terminus, were cloned from normal and immortal mouse cell lines, respectively [1,2]. These proteins are differentially distributed in normal (pancytosolic) and immortal (perinuclear) cells [3,4]. Mortalin (mot-2) has also been identified as peptide-binding protein 74 (PBP74), mthsp70 (mitochondrial hsp70) and glucose-regulated protein (GRP) 75, and assigned roles in antigen processing, T-cell recognition, *in vivo* nephrotoxicity and radioresistance (for a review, see [5]). It has been localized to mitochondria, endoplasmic reticulum, plasma membrane and cytoplasmic vesicles [6–8].

The level of mortalin/mthsp70/GRP75 (referred to as mot-2 henceforth) correlates with muscle activity [9], mitochondrial activity and biogenesis [10] and is induced by low levels of ionizing radiation, glucose deprivation [11], calcium ionophore [12] and ozone [13]. mot-2 expression is upregulated in human transformed and tumour cell lines and tumours [14–16], and is decreased during induction of differentiation in HL-60 promyelocytic leukaemia cells [17]. mot-2-overexpressing derivatives of HL-60 cells were markedly impaired for induction of differentiation [17]. mot-2 binds to p53 [18–20]. Its overexpression in NIH 3T3 and MRC-5 (normal human diploid fibroblasts) cells led to their malignant transformation and life-span extension, respectively [14,21], and is shown to be mediated, at least in part, by inactivation of p53 function by mot-2 [18,21].

Mortalin cDNA isolates from normal and immortal human cells that show differential staining patterns showed identical sequences, implying that differential distribution of the protein in human cells ([4] and S. C. Kaul, T. Yaguchi and R. Wadhwa, unpublished work) is due to either protein modifications or cellular factors and does not originate from distinct cDNAs as in mouse cells. Human mortalin cDNA, when overexpressed in NIH 3T3 cells, resulted in their malignant transformation similarly to the mouse mot-2 cDNA. Based upon this activity assay human mortalin cDNA was called hmot-2. These studies have suggested that there are at least two mechanisms operating for differential distribution of the mortalin protein. One is by distinct cDNAs, mot-1 and mot-2, found in mouse, and the other by as-yet-undefined protein modifications/interactions or cellular factors found in mouse and human cells.

The present study was undertaken to isolate mortalin-binding proteins. GRP94 was isolated as a candidate mortalin-binding protein by far-Western screening of a cDNA library from normal human fibroblasts with His-tagged mot-1 recombinant protein. Mortalin–GRP94 interactions were characterized by yeast and mammalian two-hybrid assays and were also confirmed by *in vivo* co-immunoprecipitation of the proteins. Deletion mutants of mortalin and GRP94 showed that the N-terminal region of GRP94 and at least two regions of mortalin, one in each of the N- and C-termini, are engaged in their interactions.

EXPERIMENTAL

Cell culture and transfections

Human lung fibroblasts (MRC-5), carcinogen-transformed liver fibroblasts (SUSM-1), human glioblastoma cells (T98G), ECV 304 cells, mouse normal embryonic fibroblasts from CD1-ICR mice (CMEF), mouse immortal fibroblasts (NIH 3T3 and RS-4) and monkey kidney cells (COS7) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, streptomycin and fungizone (Life Technologies) at 37 °C in an atmosphere of 5% CO_2/95% air in a humidified incubator. Transient transfections were performed using LIPOFECTAMINE™ (Life Technologies). Typically, 3 μg of plasmid DNA was used for a 6 cm dish. After 48 h of transfections cells were harvested and analysed as indicated.

Abbreviations used: GRP, glucose-regulated protein; hsp, heat-shock protein; GST, glutathione S-transferase; GFP, green fluorescent protein.

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Far-Western screening

A cDNA library (10^7 p.f.u.) from human fibroblasts (W138) in λZapII vector was infected into bacteria, plated and incubated at 42 °C until minute plaques were seen. Nitrocellulose membranes were placed in the plates and were incubated for a further 3–4 h at 37 °C. Membranes were peeled off, air dried and incubated with His-tagged mot-1 protein overnight. These were then washed with 5% (v/v) skimmed milk in Tris-buffered saline (0.1 M Tris/HCl/0.15 M NaCl, pH 7.5) with 0.05% (v/v) Tween 20 and processed as a standard Western with anti-His-tag antibody as described below. Positive plaques were isolated from the plates, amplified and processed similarly until 100% of the plaques in the plates were positive. Four rounds of screening were performed and cDNA insert from plaques was obtained in pBluescript by co-infection of the helper phage R408 as described by manufacturer's instructions. Isolated clones were sequenced with T3 and T7 primers using an ABI automatic sequencer.

Cloning of GRP94

Total cellular RNA (1 μg) from human lung fibroblasts (MRC-5) was annealed with random hexanucleotides and reverse-transcribed for 75 min at 37 °C using Superscript II reverse transcriptase (Life Technologies). The reaction mixture was then heated at 95 °C for 5 min and chilled on ice. PCR was performed on 1/18th of the reverse-transcription mixture by using Ampli*Taq* DNA polymerase (Perkin-Elmer) and GRP94 sense (5′-CGCG-GATCCTGAGGGCCCTGTGGGTGCTG-3′) and antisense (5′-ATTTCACATTCCCTCTCC-3′) primers under the conditions of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 2 min, for a total of 30 cycles. The PCR product was purified (Geneclean II kit, BIO 101) and cloned into the pGEMT Easy vector (Promega). The GRP94 insert was excised, cloned into yeast and mammalian two-hybrid vectors and checked for integrity by sequencing.

Mammalian two-hybrid analysis

GRP94, mot-1 and mot-2 cDNAs were cloned into mammalian expression plasmids (mammalian two-hybrid system, Clontech, Palo Alto, CA, U.S.A.). These encoded fusion proteins of the Gal4 DNA-binding domain (Gal4DB) and GRP94, and of the VP16 activation domain (VP16AD) and either mot-1 or mot-2. Single-cell microinjections were performed with a mixture of DNA containing GRP94, mot-1 or mot-2, β-galactosidase reporter plasmid and pEGFPC1 (to detect microinjected cells) using an Eppendorf semi-automated microinjection system mounted on an inverted Zeiss microscope. Cells were fixed and stained for β-galactosidase expression after 48 h using the β-galactosidase staining kit (Boehringer Mannheim). Cells were visualized under a Zeiss microscope.

In vivo co-immunoprecipitation

Nonidet P-40 lysates (400 μg) from COS7 cells were precleared by incubating with 20 μl of Protein A–agarose (Gibco) for 1 h at 4 °C. The supernatant was incubated with slow agitation overnight at 4 °C with 20 μg of control (isotype-matched IgG) or anti-GRP94 antibody (C-19; Santa Cruz Biotechnology). Immunocomplexes were pelleted by incubation with Protein A–agarose (30 min at 4 °C with slow agitation) and centrifugation. Pellets were washed with Nonidet P-40 lysis buffer, electrophoresed on an SDS/polyacrylamide gel (7.5%) and transferred to PVDF membrane (Millipore, Bedford, MA, U.S.A.) by semi-dry transfer. The membrane was probed with monoclonal anti-GFP (green fluorescent protein) antibody (Clontech).

Immunofluorescence microscopy

Cells grown on glass coverslips placed in 35 mm plastic dishes were washed with cold PBS and fixed with a pre-chilled methanol/acetone (1:1, v/v) mixture for 5 min on ice. Fixed cells were washed with PBS, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked with 2% BSA in PBS for 20 min. Cells were double-stained with anti-GRP94 (C-19) and anti-mortalin antibodies and were visualized by secondary staining with donkey anti-goat IgG (Alexa-488-conjugated) and donkey anti-rabbit IgG (Alexa-594-conjugated; Molecular Probes). After six washes in PBS with 0.1% Triton X-100, cells were overlaid with a coverslip with Fluoromount (Difco). The cells were examined on an Olympus microscope with epifluorescence optics or a Fluoview confocal laser-scanning microscope (Olympus, Tokyo, Japan). Images were saved as TIFF files and imported into Adobe Illustrator for labelling. The extent to which the two proteins were similarly distributed was assessed by combining the two images using Fluoview software. With this program the individual mortalin and GRP94 images were seen as typical red and green fluorescence and the co-localized regions appeared as yellow.

In vitro binding assays

For preparation of recombinant mortalins, the open reading frame of mortalin cDNA and its various deletions were amplified by PCR with sense and antisense primers with *Bam*HI and *Sal*I sites, respectively. These were then cloned into pGEX-6P-1 (Amersham Pharmacia Biotech) to yield glutathione S-transferase (GST)-tagged proteins. The recombinant proteins were purified as described in [22] and the manufacturer's instructions (Amersham Pharmacia Biotech). The purity of preparations was examined by SDS/PAGE. Aliquots of the purified protein were stored at −20 °C in small volumes to avoid repeated freeze–thaw cycles.

Purified recombinant GST-tagged mortalin proteins (0.5–1 mg) were mixed with COS7 cell lysate (400 μg of protein) in 400 μl of Nonidet P-40 lysis buffer. The mixture was incubated with an anti-GRP94 antibody at 4 °C for 1–2 h. Immunocomplexes were separated by incubation with Protein A–agarose (20 μl) for 30 min followed by centrifugation. After heating at 95 °C in SDS sample buffer, proteins were resolved on SDS/PAGE, transferred to nitrocellulose membrane by semi-dry transfer, Western blotted with monoclonal anti-GST antibody (B-14; Santa Cruz Biotechnology) and detected by ECL chemiluminescence (Amersham Pharmacia Biotech).

Yeast two-hybrid assay

The matchmaker yeast two-hybrid system (Clontech) was used to determine the *in vivo* interaction between mortalin and GRP94. All of the procedures were performed according to the manufacturer's protocol. Briefly, full-length GRP94 cDNA was cloned into the pAct-2 yeast expression vector, thereby encoding a fusion protein with the Gal4 activation domain (Gal4AD; pAct-2/GRP94). Mortalin cDNA was similarly subcloned into the pODB8 vector to encode a fusion protein with the Gal4 DNA-binding domain (pODB8/mortalin). The pODB8 vector also encodes a gene that compensates for a deficiency in tryptophan biosynthesis in the yeast host cells, whereas the pAct-2 vector encodes a gene that compensates for a deficiency in leucine biosynthesis. The Y187 yeast reporter strain, which is unable to synthesize leucine, tryptophan or histidine, was co-transfected with pAct-2/GFP94 and pODB8/mortalin, or with other combinations of the vectors, and selected on tryptophan(−) and

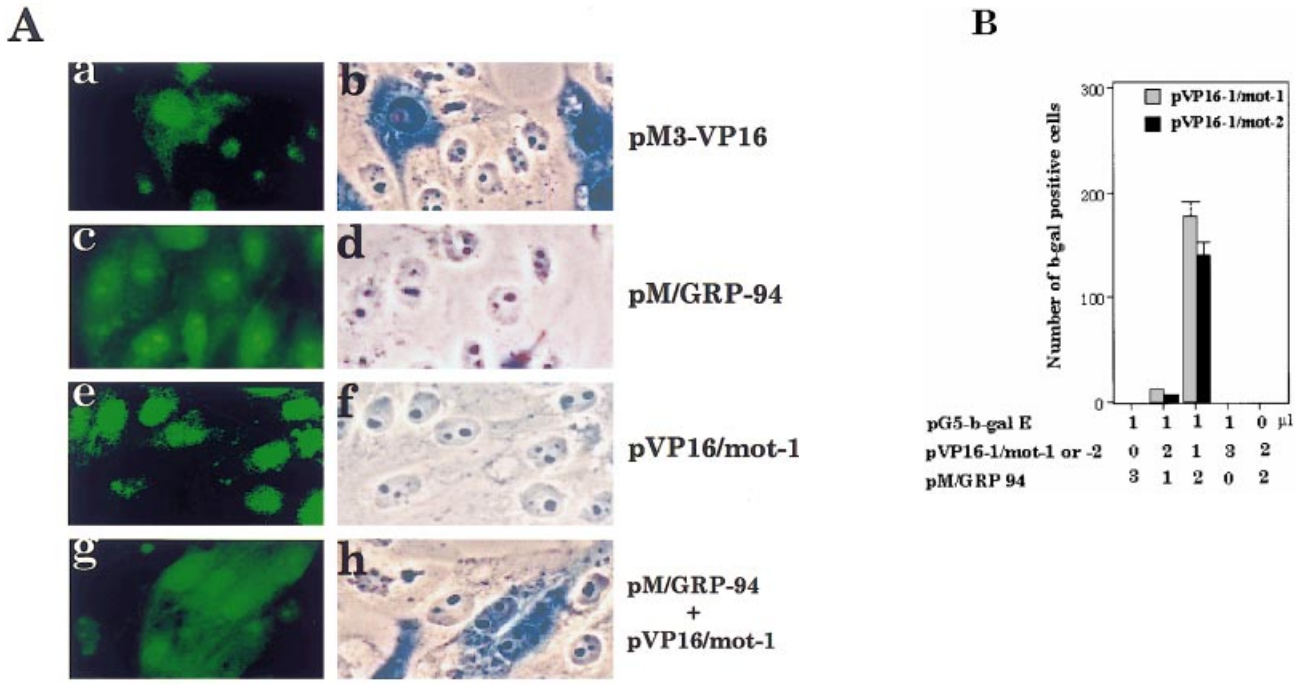

Figure 1 GRP94–mortalin interactions by mammalian two-hybrid system

(**A**) Indicated plasmids (0.1 μg/μl) were microinjected into COS7 cells. The pEGFPC1 plasmid encoding GFP protein was co-injected for the identification of the injected cells (left-hand panels). Cells were fixed at 48 h following the microinjection and were stained for β-galactosidase activity. Staining was positive (right-hand panels) for cells injected with both GRP94 and mot-1 plasmids. M3-VP16 was used as a positive control. (**B**) The number of cells with blue stain were counted from three experiments; in each experiment 250–300 cells were injected with the indicated plasmids. Both mot-1 and mot-2 interacted with GRP94. b-gal, β-galactosidase.

leucine(−) SD (synthetic dropout) agar plates. The interaction of GRP94 and mortalin was assayed in transformed cells as a function of either histidine compensation or β-galactosidase activity. For histidine-compensation assays, selected cells were streaked on to agar plates containing yeast medium deficient in leucine, tryptophan and histidine, and colony formation was monitored. β-Galactosidase activity was assayed in cells streaked on to agar plates containing yeast medium deficient in leucine and tryptophan, but containing histidine so that all transfectants could grow. After a period of cell growth, replicas of the cell colonies were produced on paper filters, then assayed for β-galactosidase activity using X-Gal as a substrate. Enzyme activity was indicated by the development of a blue colour.

Western-blot analysis

The protein sample (20–40 μg) separated by SDS/PAGE was electroblotted on to PVDF membrane using a semi-dry transfer blotter. Immunoassays were performed with anti-mortalin [1], anti-GRP94 (C-19), anti-GFP or anti-actin (Boehringer Mannheim) antibodies.

RESULTS AND DISCUSSION

In vivo interactions of GRP94 and mortalins

The His-tagged mot-1 protein was used to isolate its binding protein by far-Western screening as described in the Experimental section. The isolation of GRP94 as a mot-1-binding protein prompted us to characterize the interactions between these two proteins. To confirm that the two proteins interact we employed mammalian two-hybrid assay in which GRP94 and mouse mortalins (both mot-1 and mot-2) were cloned into mammalian

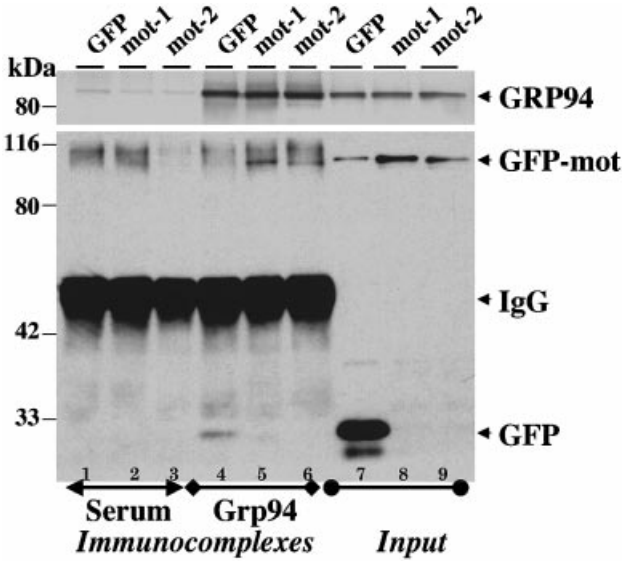

Figure 2 Co-immunoprecipitation of GRP94 and GFP-tagged mortalins

Lysates from cells expressing GFP, GFP-mot-1 and GFP-mot-2 (input lanes, 7–9) were immunoprecipitated with control or anti-GRP94 antibody. Immunocomplexes were probed with an anti-GFP monoclonal antibody. GFP-mortalins (both mot-1 and mot-2) co-precipitated with GRP94, seen as 110 kDa bands in lanes 5 and 6 corresponding to the proteins in input lanes 8 and 9. Expression of GFP protein was at least 10-fold higher (30 kDa band in input lane 7) compared with GFP-mortalins; only a negligible amount was seen in GRP94 immunocomplexes (lane 4). No precipitation of mortalins was obtained with isotype-matched IgG (lanes 1–3); the 115 kDa band is a non-specific reactant to IgG, recognized by GFP antibody (lanes 1–6). Bands corresponding to the IgG heavy chain are also visible in immunocomplexes. The input gel (lanes 7–9) shows 10% of the lysate used for immunoprecipitation.

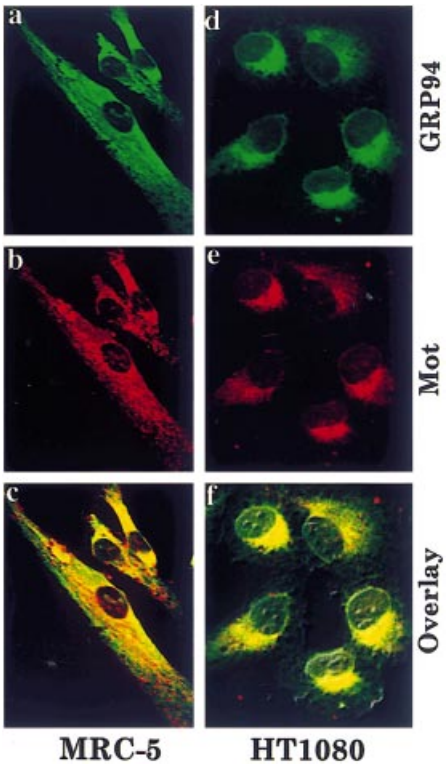

Figure 3 Immunolocalization studies for GRP94 and mortalin

Double immunolocalization of GRP94 (**a** and **d**) and mortalin (**b** and **e**) in human normal fibroblasts (MRC-5) and fibrosarcoma (HT1080). Co-localization of GRP94 and mortalin is visible as yellow staining (**c** and **f**).

expression vectors, encoded as fusion proteins with the Gal4 DNA-binding domain and VP16 activation domain, respectively. COS7 cells were microinjected with GRP94, mot-1 or mot-2 and reporter plasmids, and were monitored for activation of a β-galactosidase reporter gene that was dependent on the interaction of the two proteins by β-galactosidase staining of cells. Any traces of blue colour were counted as positive. As shown in Figure 1, microinjection of both (GRP94 and mot-1 or mot-2), but not of single, expression plasmids led to the activation of the reporter gene. The data, besides confirming the interactions, suggested that both mot-1 and mot-2 could interact with GRP94.

We next analysed the *in vivo* interactions between the two native proteins by co-immunoprecipitation assay (Figure 2). COS7 cells were transfected with pEGFPC1 and pEGFPC1/mortalin plasmids and the cell lysates were subjected to immunoprecipitation with anti-GRP94 antibody. GRP94 immunocomplexes when analysed by Western blotting with anti-GFP antibody. GFP-mot-1 and GFP-mot-2 proteins were detected in GRP94 immunocomplexes (Figure 2, approx. 110 kDa bands in lanes 5 and 6). Although the expression level of GFP was 10-fold higher compared with the GFP-tagged mot-1 and mot-2 proteins (Figure 2, input lanes 7–9), the amount of GFP detected in GRP94 immunocomplexes was negligible (Figure 2, lane 4). Control precipitations with isotype-matched IgG did not bring down mortalin protein; the approx. 115 kDa band seen in lanes 1–6 is a non-specific reactant to control, GRP94 and GFP antibodies. The data demonstrated that the GRP94 interacts with mortalin *in vivo*. Consistent with the mammalian two-hybrid data, both the mot-1 and mot-2 proteins were found to interact with GRP94.

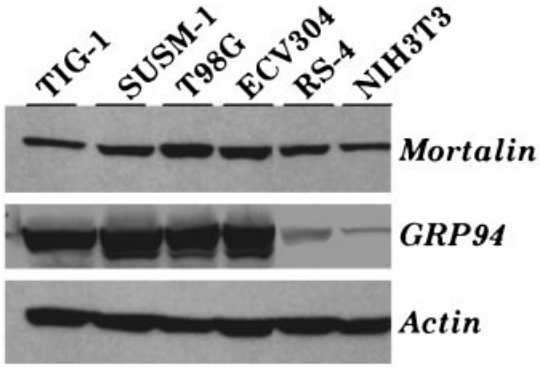

Figure 4 Western analysis of mortalin and GRP94 in mouse (NIH 3T3 and RS-4) and human (TIG-1, SUSM-1, T98G and ECV 304) cells

Normal and immortal mouse (results not shown) and human fibroblasts were double-stained for mortalin and GRP94. Negative controls, including the staining with secondary antibodies alone, confirmed the specificity of the immunostaining. The two proteins co-localized to a great extent in human normal and immortal cells (Figure 3). In mouse cells only a limited co-localization of two proteins was observed in normal or immortal cells (results not shown) and this was attributed to a lower level of expression of GRP94 in mouse cells (Figure 4).

GRP94/mortalin-binding domains

To define the binding domains of the two proteins we employed yeast two-hybrid analysis and *in vitro* pull-down assay. To define the binding domain of GRP94 to mortalin, yeast cells were transformed sequentially with expression plasmids encoding

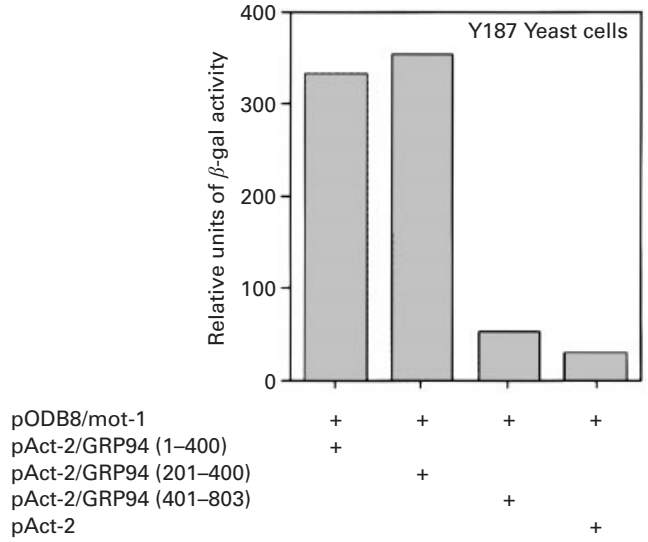

Figure 5 Mortalin-binding domain of GRP94

Yeast cells were transformed with plasmids encoding mot-1 and the indicated deletion mutants of GRP94. Activation of the β-galactosidase reporter gene was quantified by liquid β-galactosidase assay. GRP94 amino acid residues 1–400 and 201–400, but not 401–803, activated the reporter gene.

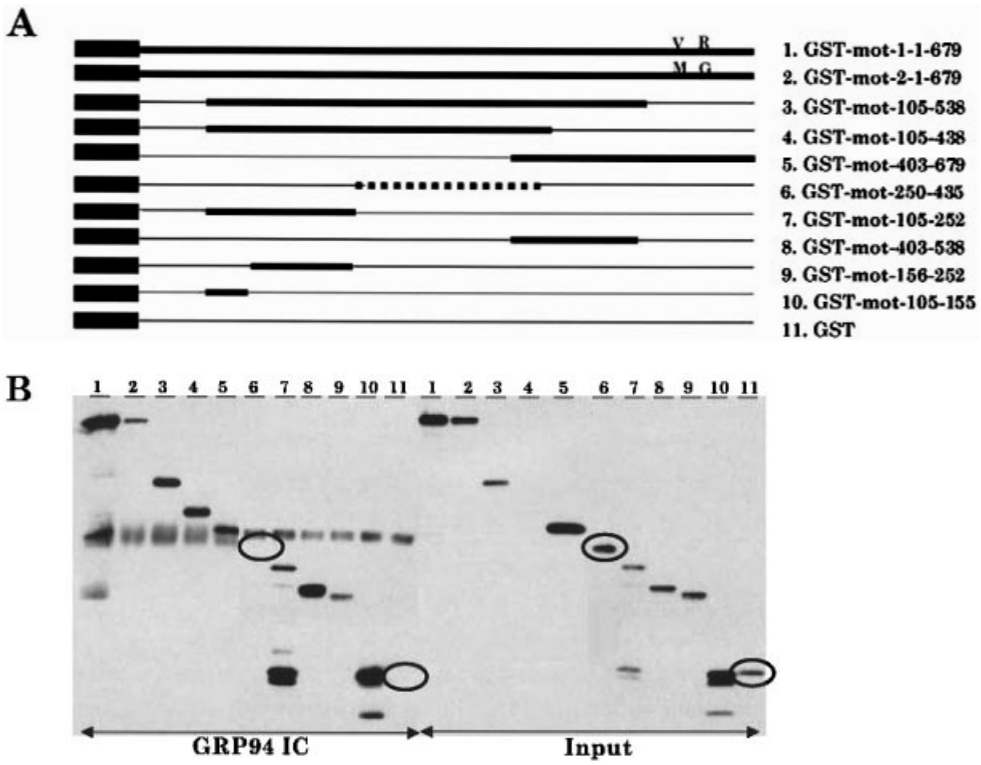

Figure 6 Mapping the GRP94-binding domain of the mortalins

(**A**) Schematic representation of mortalin deletion mutants. The two amino acids (residues 618 and 624) that differ between mot-1 and mot-2 are indicated. Thick and thin lines represent regions that were present and absent in the indicated deletion mutants, respectively; mutant names represent the inclusion of amino acid residues. The deletion mutant that did not bind to GRP94 is shown by a thick dotted line. (**B**) GST-tagged mortalin deletion mutants were mixed with COS7 cell lysates and were immunoprecipitated with anti-GRP94 antibody. GRP94 immunocomplexes (IC) were detected for the presence of various GST-tagged deletion mutants of mortalins [corresponding to the numbered constructs in (**A**)] by Western blotting with anti-GST tag antibody (left half of the panel). The input gel (right half of the panel) shows the GST–mortalin signal on the lysates that were immunodepleted with anti-GRP94 antibody, showing the absence of, or very little, GST–mortalin left in the supernatants in some cases (lanes 4 and 7). The bands corresponding to the deletion mutants that did not show binding are circled.

Gal4DB–mortalin and Gal4AD–GRP94 proteins. The interaction between the two proteins was monitored by measuring the β-galactosidase activity quantitatively by liquid β-galactosidase assay. Three different GRP94 constructs encoding GRP94 amino acid residues 1–400, 201–400 and 401–803 in the Gal4AD–GRP94 fusion protein were also employed. From the data most of the mortalin-binding region was assigned to amino acid residues 201–400 of GRP94 (Figure 5).

To identify the region of mortalin that interacts with GRP94, a GST pull-down assay was performed using the GST–mortalin fusion proteins; several deletion mutants of GST–fusion recombinant mortalin proteins were constructed (Figure 6A) and subjected to *in vitro* pull-down assay as described below. Various recombinant GST-tagged mortalin proteins were mixed with COS7 cell lysates and the mixture was incubated with anti-GRP94 antibody. GRP94 immunocomplexes were analysed for the presence of GST–mortalin constructs by Western blotting with anti-GST antibody. As seen in Figure 6(B), full-length mot-1, mot-2 or deletion mutants encoding the N-terminal or C-terminal regions interacted with GRP94 protein. Neither the mortalin amino acid residues 250–435 nor the GST protein by itself interacted with GRP94. These data suggested that there are at least two regions of mortalin that interact with GRP94, one in the N-terminus and the other in the C-terminus. Taken together with the above data, we concluded that mot-2 and GRP94 interact *in vitro* and *in vivo*. GRP94 has been shown to have autonomous oligomerization activity that is regulated by its C-terminus [23,24]. Binding of mortalin to its N-terminus therefore may not affect its dimerization. On the other hand at least two domains of mortalin, one in the ATP-binding region and the other in the peptide-binding domain [25], show binding to GRP94. This may modify activities of mortalin protein, including its chaperonin and mitochondrial-import functions [26,27].

The GRPs are a highly conserved family of stress proteins, and function as molecular chaperones that have translocase and foldase activities [24]. Although localized to more than one cellular compartment [28,29], GRP94 and GRP78 reside mainly in the endoplasmic reticulum, which was also identified as one of the two main sites of mot-2 residence [8]. Here we have shown that mot-2 binds to the endoplasmic-reticulum-resident protein GRP94. Increased synthesis of GRPs occurs in mammalian cells exposed to a variety of agents, including 2-mercaptoethanol, tunicamycin and A23187, which perturb calcium homoeostasis, and amino acid analogues, and in many tumours as a consequence of a hypoxic environment [16,30,31]. GRPs contribute to tumour proliferation by their multiple activities, many of which remain unidentified [32,33], and confer resistance to anti-cancer treatments. The mot-2–GRP94 interactions defined in the present study may provide important insights into these phenomena and therefore warrant further study.

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