Identification and characterization of molecular interactions between mortalin/mtHsp70 and HSP60

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Mortalin/mtHsp70 (mitochondrial Hsp70) and HSP60 (heat-shock protein 60) are heat-shock proteins that reside in multiple subcellular compartments, with mitochondria being the predominant one. In the present study, we demonstrate that the two proteins interact both *in vivo* and *in vitro*, and that the N-terminal region of mortalin is involved in these interactions. Suppression of HSP60 expression by shRNA (short hairpin RNA) plasmids caused the growth arrest of cancer cells similar to that obtained by suppression of mortalin expression by ribozymes. An over-expression of mortalin, but not of HSP60, extended the *in vitro*

lifespan of normal fibroblasts (TIG-1). Taken together, this study for the first time delineates: (i) molecular interactions of HSP60 with mortalin; (ii) their co- and exclusive localizations *in vivo*; (iii) their involvement in tumorigenesis; and (iv) their functional distinction in pathways involved in senescence.

Key words: co-localization studies, heat shock protein 60 (HSP60), mortalin/mtHsp70 (mitochondrial Hsp70), p53 function, senescence.

INTRODUCTION

Mortalin/mtHsp70 (mitochondrial Hsp70) was first cloned as a novel member of the hsp70 family of proteins from the cytoplasmic fractions of normal fibroblasts that is non-inducible by heat. It is a 74 kDa protein, comprising 679 amino acids with a 46-amino-acid-long signal peptide, which undergoes Ca²⁺-dependent autophosphorylation and has a high degree of identity with other members of the hsp70 family, including *Escherichia coli* DnaK (51%), *Saccharomyces cerevisiae* SSC1p (65%), the constitutive cytosolic hsp70 from rat, hsc70 (46%), and endoplasmic-reticulum isoform BiP (49%). Although predominantly found in the mitochondria, mortalin/mtHsp70 exists in extramitochondrial sites, interacts with several proteins, and functions in pathways related to the control of cell proliferation, apoptosis, stress and tumorigenesis [1–8]. Mortalin is a ubiquitous protein [2,9], its expression level correlated with muscle activity, mitochondrial activity and biogenesis, and is induced by low levels of ionizing radiation, glucose deprivation, calcium ionophore, ozone and hyperthyroidism [5]. As a chaperone protein, mortalin confers cellular resistance on various stress conditions. At the same time, it functions in many constitutive cellular processes, including maintenance of mitochondrial function. Studies on the yeast homologue of mtHsp70/Ssc1 have shown that it is essential for the translocation of cytosolic precursor proteins across two mitochondrial membranes [10–12].

HSP60 (heat-shock protein 60), a second mitochondrial stress chaperone, has been shown to be involved in protein assembly and folding [11,13], cellular stress and apoptotic phenotypes [14,15]. Although predominantly detected in the mitochondria, both HSP60 and mtHsp70 were found in extramitochondrial subcellular sites by a variety of approaches, and in a variety of cells and tissues [16–24]. HSP60 expression is up-regulated in many kinds of tumours [25–29]. Some studies have shown co-regulation

and co-modification of HSP60 and mortalin during cellular stress conditions, including cellular toxicity, injury, mitochondrial activity and tumorigenesis [30–32]. These studies have suggested that the two mitochondrial chaperones are closely related, and might interact or work together. However, such interactions of these proteins and their functional relevance to cellular normal and cancer phenotypes have not been elucidated. The present study provides first evidence of the *in vitro* and *in vivo* interactions of mortalin and HSP60, and defines their functional involvement in the continued proliferation of tumour cells and their distinct roles in cellular senescence.

EXPERIMENTAL

Cells culture and transfections

Normal human fibroblasts (TIG-1), osteosarcoma (U2OS) and its derivative cells, NARF [stably transfected with IPTG (isopropyl β-D-thiogalactoside)-inducible p14^{ARF}; kindly given by Gordon Peters, Cancer Research UK, Lincoln's Inn Fields Laboratories, London, U.K.], were cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% (v/v) fetal bovine serum, penicillin, streptomycin and fungizone (Life Technologies, Inc.) at 37 °C in an atmosphere of 5% CO₂/95% air in a humidified incubator. NARF cells were induced for p14^{ARF} expression by addition of 0.1 mM IPTG for 24–48 h.

Transfections were performed using LipofectAMINE™ Plus (Life Technologies, Inc). Typically, 1 and 6 μg of plasmid DNA were used per 60% confluent cells in 12-well and 10 cm dishes respectively. Immunostaining of transfected cells was performed after 24–36 h of transfection. For stable transfections, cells were expanded by 1:4 splitting and then selected in G418 (100 μg/ml)-supplemented medium. G418-selected cells were passaged until they stopped dividing.

Abbreviations used: CCD, charge-coupled device; GST, glutathione S-transferase; HSP60, heat-shock protein 60; IPTG, isopropyl β-D-thiogalactoside; mtHsp70, mitochondrial Hsp70; NP40, Nonidet P40; PD, population doubling; shRNA, short hairpin RNA; siRNA, small interfering RNA.

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In vivo co-immunoprecipitation

NP40 (Nonidet P40) lysate (400 µg of protein) from COS7 cells was incubated with control, anti-HSP60 or anti-mortalin antibodies overnight at 4°C. Immunocomplexes were pelleted by incubation with Protein A-agarose (20 µl; 30 min at 4°C with slow agitation) and centrifugation (10000 rev./min at 4°C for 2 min). The pellet was washed with NP40 lysis buffer and examined for binding partners by Western blotting with anti-mortalin, anti-HSP60 or anti-GST (glutathione S-transferase) antibodies.

Recombinant GST-tagged mortalin/mtHsp70 deletion mutants and *in vitro* binding assays

Recombinant GST-tagged mortalin was prepared as described previously [33]. Purified recombinant GST-tagged mortalin proteins (0.5–1 µg) were mixed with COS7 cell lysate (400 µg of protein) in 400 µl of NP40 lysis buffer. The mixture was incubated with an anti-HSP60 antibody at 4°C for 1–2 h. Immunocomplexes were analysed for the presence of mortalin by Western blotting with monoclonal anti-GST antibody (B-14; Santa Cruz Biotechnology) [33].

Double immunostaining of mortalin/mtHsp70 and HSP60

For immunofluorescence studies, cells were plated on glass coverslips placed in a 12-well culture dish at densities ranging from 10^3 – 10^4 cells/coverslip. After 24 h, when cells had attached to the surface and spread well, they were washed with cold PBS and then fixed with pre-chilled methanol/acetone (1/1, v/v) mixture for 5 min on ice. Fixed cells were washed with PBS, permeabilized with 0.5% (v/v) Triton X-100 in PBS for 15 min and blocked with blocking reagent (Sigma) for 1 h. They were incubated with polyclonal anti-HSP60 antibody (N-20; Santa Cruz Biotechnology) and anti-mortalin antibodies (monoclonal anti-mtHsp70; Affinity Bioreagents) and polyclonal anti-mortalin antibody [4] for 1 h at room temperature, washed three times with 0.2% Triton X-100 in PBS, and then incubated with Alexa-488-conjugated rabbit anti-goat and Alexa-594-conjugated goat anti-mouse (Molecular Probes) secondary antibodies and mouse-, rabbit- and goat-biotin, followed by washings with 0.2% Triton X-100 in PBS.

For quantum dots staining, cells were incubated with QD (Qdot™ 525 Streptavidin Conjugate, Qdot™ 655 rabbit IgG and Qdot™ 655 mouse IgG; Quantum Dot Corporation, Hayward, CA, U.S.A.) in Qdot Incubation Buffer (1:2000). After three washings of 10 min each with 0.2% Triton X-100 in PBS and a final wash with PBS, the coverslips were mounted on glass slides with Fluoromount (Difco). The cells were examined on a Carl Zeiss microscope attached with either a Photometrics Synsys monochrome CCD (charge-coupled device) or an AxioCam HRC colour camera (Zeiss). Confocal images were taken with an LSM510 microscope (Zeiss) attached with a PMT (photo-multiplier) detection system. The extent to which the two proteins overlapped was assessed by combining the two images using either Metamorph or AxioVision software.

Constructions of siRNA (small interfering RNA) vectors

For construction of siRNA expression vectors, the tetracycline-inducible U6 promoter vector was used [34,35]. Target sites for siRNA were selected using an algorithm (<http://www.igene-therapeutics.co.jp>). Sequences of the siRNA target sites for HSP60 are shown in Table 1.

Table 1 Target site sequences used for shRNA expression vectors for HSP60

Name	Sequence
HSP60-102	5'-GTG AGA TGA GGA GCC AGT ACC-3'
HSP60-160	5'-TTC AAG CAT TAA GGC TCG GGC-3'
HSP60-335	5'-CAT CTT GAA CAA GTT TAG CTC-3'
HSP60-712	5'-TGG AGA AAT ATA GCC TCG ATC-3'

Colony-forming assays

Transfected cells were selected in puromycin (2 µg/ml)-supplemented medium for 48 h. Selected cells (1000) were plated in 6 cm dishes (in triplicate), induced for shRNA (short hairpin RNA) expression by addition of 5 µM doxycycline in the medium, and left to form colonies over the subsequent 2 weeks with a regular change of medium every third day. Colonies were fixed in methanol, stained with 0.1% Crystal Violet solution, photographed and counted.

Lifespan studies

Normal human fibroblasts (TIG-1) were stably transfected with expression plasmid encoding haemagglutinin-tagged human HSP60 protein at 30 PDs (population doublings). Cells were selected in 50 µg of G418/ml of medium, and were serially passaged until they stopped dividing. Split ratios were kept at 1:16 for rapidly dividing cells, and 1:4 for slowly dividing cells to allow them to undergo approx. 4 and 2 PDs per passage respectively. U2OS cells were induced to undergo senescence with exogenous expression of p14^{ARF}. Mortalin, HSP60 and p14^{ARF} (NeoMarkers Ab-3, NS-1115-PO) expression levels were examined by immunostaining and Western blotting, as described above.

RESULTS AND DISCUSSION

In vivo binding of mortalin/mtHsp70 with HSP60

In vivo binding of mortalin and HSP60 proteins was examined by co-immunoprecipitation assays. Immunocomplexes obtained with either anti-mortalin or anti-HSP60 antibody were examined for the presence of the other binding partner. As shown in Figure 1(A), mortalin was detected in HSP60 immunocomplexes (lane 1). Of note, repeated immunoprecipitation of HSP60 from the same lysate showed decreasing amounts of not only HSP60, but also its co-precipitating mortalin protein (Figure 1A, lanes 2–4). Interestingly, HSP60-immunodepleted sample showed the presence of HSP60 (Figure 1A, lane 14) although it was decreased as compared with the input (Figure 1A, lane 12). Furthermore, mortalin was also decreased in HSP60-immunodepleted lysate as compared with the pre-serum-immunodepleted lysate. These data implied that: (i) anti-HSP60 antibody did not immunoprecipitate all of HSP60 in the cells, probably due to its binding to other proteins; (ii) anti-HSP60 antibody could precipitate HSP60–mortalin complexes; and (iii) some, but not all, of HSP60 binds to mortalin. We also performed immunoprecipitation with anti-mortalin antibody (Figure 1A, lanes 5–8) and examined these for co-precipitation of HSP60. Whereas HSP60 was detected in the first mortalin immunoprecipitation (Figure 1A, lane 5), repeated precipitations from the same lysate did not precipitate HSP60 along with mortalin (Figure 1A, lanes 6–8). These data revealed that a small amount of mortalin and HSP60 forms complexes in cells.

Figure 1 Mortalin and HSP60 binding *in vivo*

(A) Mortalin and HSP60 were repeatedly precipitated with respective specific polyclonal antibodies, as indicated. Co-precipitated mortalin protein in HSP60 immunocomplexes and HSP60 in mortalin immunocomplexes were detected by Western blotting with specific monoclonal antibodies. Note that some mortalin co-precipitated with HSP60 (lanes 1–4), and similarly some HSP60 co-precipitated with mortalin (lanes 5–8). Rabbit IgG was used as a negative control (lanes 9–11). Detection of mortalin and HSP60 in immunodepleted lysates showed some decrease of mortalin in HSP60-immunodepleted lysates, and vice versa (lanes 12–15). (B) GST-tagged mortalin was mixed with cell lysates from which HSP60 was immunoprecipitated. Co-precipitated GST-tagged mortalin was detected by Western blotting with anti-GST antibody. Note that in lane 1, no GST-mortalin was added. Thus the control antibody pulled-down unspecific proteins (molecular masses close to GST-mortalin; lanes 1,

Figure 2 Double immunostaining of mortalin and HSP60

Co-localization of mortalin and HSP60 in human normal embryonic-lung fibroblasts (MRC-5) and osteosarcoma (U2OS) cells with Qdot bioconjugates. Note that the two proteins overlap by approx. 70% (shown as the yellow colour). Distinct red and green staining was visible both in normal and transformed cells.

Mortalin binds to HSP60 via its N-terminal region

To characterize the binding of mortalin and HSP60, we used GST-tagged recombinant mortalin proteins made in bacteria, as described previously [33]. The purified full-length and deletion mutants of GST-tagged mortalin proteins were mixed with cell lysates from COS7 cells. HSP60 was immunoprecipitated with anti-HSP60 antibody. Co-precipitation of GST-tagged mortalin was detected by Western blotting of the immunocomplexes with an anti-GST antibody. The full-length GST-mortalin protein was immunoprecipitated with HSP60 (Figure 1B). Consistent with the immunoprecipitation of GST-mortalin with HSP60, the HSP60-depleted lysates showed less GST-mortalin as compared with the control antibody-depleted lysates (Figure 1B, lanes 6 and 8). GST-N-terminal-mortalin fragments containing amino acid residues 105–435 (Figure 1C) and 105–252 (Figure 1D) were co-precipitated with HSP60. The C-terminal fragment containing amino acid residues 403–679 (Figure 1C) or 250–435 (Figure 1D) did not show co-precipitation with HSP60. On the basis of these data, the HSP60-binding region of mortalin is assigned to its N-terminal region from amino acid residues 105–252.

Exclusive 'niches' of mortalin and HSP60 in spite of their predominant co-existence

We next examined subcellular localization of mortalin and HSP60 by double immunofluorescence obtained by Qdot-conjugated antibodies. Qdot staining was chosen because of: (i) the narrow and symmetric emission spectra, and hence the absence of cross-talk between the multi-detection channels; (ii) broad absorption profiles that enabled the use of a single excitation or a long-pass emission filter; and (iii) simultaneous recording of colour images with a CCD camera. As shown in Figure 2, Qdot staining patterns of mortalin and HSP60 showed that, in addition to their co-localization (shown in yellow), each protein has a specific locale (Figure 2), implying that the two proteins exist together at some, but not all, subcellular localizations. We also confirmed the co-localization and specific localization of the two proteins by confocal laser microscopy (results not shown).

2, 5 and 6) that cross-reacted with anti-GST antibody. GST-mortalin (mot-2) co-precipitated with HSP60, but not with isotype matched control antibody. Supernatant after immunoprecipitation of HSP60 was also run on a gel to confirm the presence of GST-mortalin in control precipitation. (C and D) GST-tagged deletion mutants of mortalin were added to the cell lysates, following which HSP60 was immunoprecipitated. Whereas full-length mortalin (C) and its mutants containing amino acid residues 105–435 (C) or 105–252 (D) immunoprecipitated with HSP60, neither GST alone nor residues 250–435 (D) or 403–679 (C) showed binding.

Figure 3 HSP60 knock-down causes growth arrest of osteocarcinoma cells

(A) Expression levels of HSP60 subsequent to the stable transfections of indicated shRNA expression plasmid in U2OS cells as examined by Western blotting are shown. Target sites 102 and 160 were better than 335 and 712. The morphology (B) and colony-forming efficiency (C) of vector and HSP60 shRNA expression plasmid-transfected U2OS cells are shown.

Knock-down of HSP60 or mortalin in human osteosarcoma cells causes their growth arrest

We transfected tetracycline-inducible human osteosarcoma (U2OS) cells with shRNA plasmids for HSP60 (Figure 3 and Table 1). Transfected cells were selected in growth medium supplemented with puromycin (2 µg/ml) for 48–96 h. They were then induced to express shRNA for HSP60 by adding doxycycline (5 µM) in the medium. Cells were assayed for HSP60 expression by Western blotting. As shown in Figure 3(A), three of the four plasmids caused significant reduction in the expression of HSP60. Cells compromised for HSP60 expression were subjected to colony-forming assays. Equal numbers of cells were plated in 10 cm dishes. In two independent experiments, a significant reduction in the colony-forming efficiency occurred in cells that showed greater reduction in HSP60 expression. There were almost no colonies formed for cells transfected with shRNA plasmids 102 and 160 (Figures 3B and 3C). Cells transfected with shRNA plasmids 335 and 712 showed a decrease in the number of colonies as compared with the vector-transfected control, and this was proportional to the level of expression of HSP60. Similar results were obtained in the case of human breast carcinoma (MCF7) cells. From these data, we were able to conclude that HSP60 is required for the growth of tumour cells. This was similar to the effect of mortalin suppression obtained by helicase-coupled ribozymes in human transformed cells [36,37]. An increase in HSP60 expression has been reported in many tumours [25–29], and supports the role of HSP60 in tumorigenesis.

Overexpression of mortalin, but not of HSP60, extended the *in vitro* lifespan of normal human fibroblasts

We had earlier found that overexpression of mortalin extends the *in vitro* lifespan of normal human fibroblasts [38]. In the present study, we investigated whether HSP60 has a similar potential. Normal human fibroblasts (TIG-1; PD24) were transfected with expression plasmids encoding either human mortalin (hmt-2) or HSP60 proteins. Selected cells were passaged *in vitro* until

Figure 4 Lifespan extension of human fibroblasts

Overexpression of mortalin, but not HSP60, causes lifespan extension of human fibroblasts (A). Vector and HSP60-transfected cells phased out at 59 PDs, and did not show any increase in cell number over the next 4 weeks. Mortalin-transfected cells underwent 71 PDs in two independent experiments. (B) Induction of senescence in U2OS cells by exogenous expression of p14^{ARF} causes a decrease in mortalin, but not in HSP60. Actin was used as an internal loading control, and p53 was used as a downstream marker for p14^{ARF} activity.

they reached senescence and stopped dividing. Whereas mortalin-overexpressing cultures underwent approx. 71 PDs, the HSP60 and vector-transfected control cells senesced at 58 PDs (Figure 4A). Cells were maintained in the senescent stage for more than 4 weeks. Overexpression of mortalin, but not of HSP60, caused a temporary escape from senescence, resulting in an *in vitro* lifespan extension of normal cells. The data revealed functional divergence of mortalin and HSP60, and may involve a function of mortalin as a mitochondrial import motor in addition to its chaperone function, similar to HSP60 [10–13]. We next induced senescence in U2OS (lacking endogenous p14^{ARF}) cells by exogenous expression of p14^{ARF} [39], and examined the involvement of mortalin and HSP60 after senescence was induced as described. As shown in Figure 4(B), whereas the level of mortalin expression was reduced, the level of HSP60 expression showed no change.

Mortalin has a differential subcellular distribution in normal and transformed cells. Induction of senescence in transformed cells by introduction of a single chromosome [40], chromosome fragments and genes [41] or chemicals [42,43] was found to be accompanied by reversion of subcellular distribution of mortalin from the non-pancytoplasmic to the pancytoplasmic type. Two other studies have reported the change in distribution of mitochondria in response to heat-shock treatment in chicken embryo fibroblasts [44], and in response to viral infection in mammalian cells [45,46]. Interestingly, another study reported that functional inactivation of mtHsp70 (mortalin) causes mitochondrial aggregation in yeast [47]. This was shown to be independent of the defects in mitochondrial protein import or mitochondrial translocases [47]. It was proposed that mtHsp70 is essential for optimizing the functions of as-yet-unidentified heat-labile protein(s) in the mitochondrial matrix in controlling mitochondrial morphology [47], and this warranted the characterization of mtHsp70 complexes with other mitochondrial proteins. In the present study, we have characterized the interaction of mortalin and HSP60 and found that, although the two proteins are required for continued proliferation of cancer cells, they nevertheless have distinct functions in cellular senescence pathways. Although essential functions of mortalin, such as mitochondrial protein import, intracellular trafficking and chaperone activity, are likely to underlie such functional distinctions, further studies are warranted to resolve the

molecular details of their functions, which should also validate their future use as potential targets for cancer therapeutics.

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