Targeted disruption of *hsp70.1* sensitizes to osmotic stress

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The 70 kDa heat shock protein (Hsp70) plays a critical role in cell survival and thermotolerance in response to various stress stimuli. Two nearly identical genes, *hsp70.1* and *hsp70.3*, in response to environmental stress, rapidly induce Hsp70. However, it remains unclear whether these two genes are differentially regulated by various stresses. To address the physiological role of the *hsp70.1* and *hsp70.3* genes in the stress response, we generated mice that specifically lack *hsp70.1*. In contrast to heat shock, which rapidly induced both *hsp70.1* and *hsp70.3* mRNA, osmotic stress selectively induced transcription of *hsp70.1*. In *hsp70.1*-deficient embryonic fibroblasts, osmotic stress markedly reduced cell viability. Furthermore, when osmotic stress was applied *in vivo*, *hsp70.1*-deficient mice exhibited increased apoptosis in the renal medulla. Taken together, our results demonstrate that differential expression of *hsp70* genes contributes to the stress response and that the *hsp70.1* gene plays a critical role in osmotolerance.

INTRODUCTION

Mammalian cells respond to environmental changes by immediate induction of heat shock proteins (HSPs). The 70 kDa heat shock protein (Hsp70) is one of the most highly conserved members of the HSP family (Lindquist and Craig, 1988). Hsp70 can act as a molecular chaperone and protects cells against subsequent exposures to lethal heat shock, which is capable of denaturing proteins (Li and Werb, 1982). Other workers and we have previously demonstrated that overexpression of HSP70 protects

cells from apoptosis both *in vitro* and *in vivo* (Seo *et al.*, 1996; Buzzard *et al.*, 1998; Ravagnan *et al.*, 2001). Mice deficient in *hsp70.2*, a testis-specific *hsp70* gene, are sterile, due to an increase in apoptotic cells in the testis (Dix *et al.*, 1996). Moreover, it has been reported that Hsp70 prevents activation of procaspase-9 (Beere *et al.*, 2000).

To date, seven members of the *hsp70* family have been identified in the murine genome, and three of these are clustered closely together on the same chromosome (Hunt and Calderwood, 1990; Walter *et al.*, 1994). Two genes in the cluster, *hsp70.1* and *hsp70.3*, encode identical proteins but have completely different sequences in the 3' untranslated region (UTR) (Milner and Campbell, 1990). It has been shown that the *hsp70.1* and *hsp70.3* transcripts are differentially expressed in cultured rat cells in response to heat shock (Angeletti *et al.*, 1996). *hsp70* genes were differentially expressed in response to various stresses, including heat shock, glucose starvation and osmotic stress (Tanaka *et al.*, 1988), and this differential regulation can also occur in a cell type-specific manner (Leppa *et al.*, 2001).

To understand the functional significance and cytoprotective role of differential expression of two *hsp70* mRNAs in response to stress *in vivo*, we generated *hsp70.1*-deficient mice by homologous recombination. Our results demonstrate that the *hsp70.1* and *hsp70.3* genes are induced to different degrees and by different regulatory mechanisms, depending on the specific stress conditions. Furthermore, loss of *hsp70.1* increases the susceptibility to osmotic stress-induced apoptosis, both *in vivo* and *in vitro*.

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RESULTS

hsp70.1 gene targeting

To understand the mechanisms of regulation of *hsp70* gene expression and the differential protective effects of Hsp70 against various stresses *in vivo*, we disrupted the *hsp70.1* gene in mice in which the promoter and part of the coding sequence of the *hsp70.1* gene had been replaced with the neomycin resistance gene (*neo*) (Figure 1A). Three independent embryonic stem (ES) clones containing the targeted *hsp70.1* allele were isolated and injected into C57BL/6 blastocysts. Genotyping of the offspring was determined by PCR (Figure 1B) and confirmed by Southern blot analysis with a flanking 3' UTR probe (Figure 1C). Most *hsp70.1*^{-/-} null mice were viable and exhibited no obvious phenotypic abnormalities during development.

hsp70.1 and *hsp70.3* genes differentially expressed under stress conditions

A previous report has shown that heat shock and osmotic stress induce differential expression of *hsp70* genes (Tanaka *et al.*, 1988), suggesting that *hsp70* genes may be regulated by different mechanisms. To examine the different transcriptional regulation of *hsp70* genes in response to heat shock or osmotic stress, we exposed mouse embryonic fibroblasts (MEFs) to heat shock (42°C for 30 min) or to osmotic stress (100 mM NaCl/DMEM for 6 h) and measured the level of *hsp70* mRNA expression (Figure 2). Two specific probes were designed to distinguish *hsp70.1* mRNA from *hsp70.3* mRNA, based on the divergent 3' UTR sequences of the two *hsp70* genes.

Using an *hsp70.1*-specific probe, northern blot analysis revealed a strong induction of a 3.1 kb transcript in MEFs derived from *hsp70.1*^{+/+} mice but none in MEFs derived from *hsp70.1*^{-/-} mice in response to heat shock (Figure 2). On the other hand, as expected, *hsp70.3* mRNA was induced by heat shock in both *hsp70.1*^{+/+} and *hsp70.1*^{-/-} MEFs (Figure 2). The induction of both *hsp70* genes rapidly reached a maximum within 1 h and remained elevated thereafter (data not shown).

In contrast, osmotic stress selectively increased *hsp70.1* mRNA but not *hsp70.3* mRNA (Figure 2). The level of *hsp70.1* mRNA was gradually increased in *hsp70.1*^{+/+} MEFs, beginning ~4 h after osmotic shock (data not shown). However, *hsp70.3* mRNA was not induced in either *hsp70.1*^{+/+} or *hsp70.1*^{-/-} MEFs (Figure 2). These results demonstrate directly that the two *hsp70* genes are differentially regulated by various stresses.

Western blot analysis following osmotic stress confirmed that Hsp70 was expressed exclusively in *hsp70.1*^{+/+} MEF cells. Hsp70 immunoreactivity was induced, beginning at ~4 h, and reached a maximum value at 8 h (Figure 3A and B). These results were confirmed by immunofluorescence staining analysis with anti-Hsp70 antibody (Figure 3C). Hsp70 was seen primarily in the nucleus and in the perinuclear cytoplasm in response to osmotic stress. This differs from the expression pattern of Hsp70 upon heat shock, which induces Hsp70 mainly in the nucleus.

The expression of various other HSPs, including heat shock cognate 70 (Hsc70), Hsp90s (Hsp84, Hsp86) and Hsp60, was not affected by osmotic stress in either *hsp70.1*^{+/+} or *hsp70.1*^{-/-} cells, whereas HSP27 was briefly induced and then decreased back to normal levels in both *hsp70.1*^{+/+} and *hsp70.1*^{-/-} cells

Fig. 1. Generation of *hsp70.1*-deficient mice. (A) Diagrams summarizing the genomic region at the *hsp70.1* locus, the *hsp70.1* targeting vector and the predicted structure of the mutated *hsp70.1* gene. PGK, promoter of phosphoglycerate kinase; Neo, neomycin resistance gene (black arrows); TK, thymidine kinase gene (gray arrow). The restriction enzymes were designated as follows: B, *Bam*HI; N, *Not*I; X, *Xho*I; E, *Eco*RI; H, *Hind*III. (B) PCR analysis of genomic DNA from wild-type mice (+/+), heterozygous mice (+/-) and homozygous null mice (-/-). PCR genotyping showed fragments of 0.5 and 1.2 kb for the wild-type and targeted *hsp70.1* alleles, respectively. (C) Southern blot analysis of genomic DNA purified from the *hsp70.1*^{+/+} and *hsp70.1*^{-/-} mice. The probe was derived from the 3' UTR of the *hsp70.1* gene and recognized a 12.5 kb *Eco*RI fragment for the targeted *hsp70.1* allele and a 10 kb *Eco*RI fragment for the wild-type allele.

Fig. 2. Differences in transcription of *hsp70.1* and *hsp70.3* mRNAs as induced by heat shock and osmotic stress. *hsp70.1*^{+/+} and *hsp70.1*^{-/-} MEF cells were left untreated (C) or exposed to heat shock (HS; 42°C for 30 min followed by recovery at 37°C for 1 h) or exposed to osmotic stress (NaCl; incubation in 100 mM NaCl/DMEM for 8 h). Total RNA was isolated and the samples (15 µg) were analyzed by northern blotting using specific *hsp70.1* and *hsp70.3* probes made from unique sequences of the 3' UTR of each *hsp70* gene. Equal sample loading was verified by 18S rRNA analysis of ethidium bromide-stained gels prior to transfer.

(Figure 3A and B). These results imply that no detectable increase in the expression of other HSPs compensated for the loss of *hsp70.1*.

Disruption of *hsp70.1* causes loss of tolerance for osmotic stress

To examine whether the loss of Hsp70 induction impaired resistance to subsequent exposure to extreme osmotic stress, we exposed MEF cells to osmotic stress (600 mM NaCl/DMEM) for 90 min, with or without pretreatment (100 mM NaCl/DMEM), and determined the viability of the cells (Figure 4). Both MEFs showed similar levels of viability under unstressed (control)

Fig. 3. Effect of osmotic stress on HSP expression. (A) Western blot analysis of Hsp70, Hsp27, Hsp60 and Hsp90s accumulation. *hsp70.1*^{+/+} and *hsp70.1*^{-/-} MEF cells were exposed to 100 mM NaCl/DMEM for the indicated time periods. (B) Quantification of HSP expression upon moderate osmotic stress. Autoradiogram form was quantified by densitometry. Data for the 'fold induction' are shown relative to control levels. Each time point represents an average of three independent experiments. (C) Subcellular localization of HSP70. Cells treated with 100 mM NaCl/DMEM for 8 h were subjected to indirect immunofluorescence analysis using specific antibody against inducible Hsp70 (StressGene) as primary antibody and FITC-conjugated secondary antibody. Actin fibers were shown using an antibody against F-actin conjugated to Texas Red.

conditions, and no viable cells were observed when the severe osmotic stress was applied without any pretreatment. However, pretreatment with 100 mM of NaCl/DMEM before a subsequent addition of 600 mM of NaCl/DMEM markedly increased the cell viability of *hsp70.1*^{+/+} MEFs. In contrast, none of the *hsp70.1*^{-/-} cells survived under the same conditions. Figure 4 also shows that the pretreatment itself led to reduced cell viability and morphological changes in *hsp70.1*^{+/+} (20%) and *hsp70.1*^{-/-} (40%) MEFs. These results are consistent with the hypothesis that the lack of Hsp70 through loss of the *hsp70.1* gene may decrease tolerance against subsequent severe osmotic stress. Despite a mild induction of Hsp27, as shown in Figure 3, MEFs lacking *hsp70.1* were highly sensitive to osmotic stress, suggesting that Hsp70 plays a critical role in osmotolerance.

Enhanced apoptosis in *hsp70.1*^{-/-} renal medulla after osmotic stress

Renal medulla is necessarily exposed to high concentrations of toxic substances as well as to fluctuating concentrations of NaCl and urea. To address whether loss of Hsp70 may impair renal function of *hsp70.1*^{-/-} mice, we exposed mice to long-term osmotic stress by providing 3% NaCl in their drinking water for 30 days. In the salt-fed group of *hsp70.1*^{-/-} mice, body weight was significantly increased by 5–10% during the early period of the study and some mice died, although no differences were observed in either urine osmolality or in histological examination of the kidneys of wild-type and *hsp70.1*^{-/-} mice (data not shown).

Fig. 4. Effect of osmotic stress on cell survival. (A) Morphology of MEF cells growing for 17 h in isotonic DMEM after hyperosmotic stress: untreated cells (cont), cells treated with 600 mM NaCl/DMEM for 90 min, cells treated with 100 mM NaCl/DMEM for 6 h and cells pretreated with 100 mM NaCl/DMEM for 6 h and then exposed to 600 mM NaCl/DMEM for 90 min. (B) Quantification of cell viability. Analyses were performed by counting the number of cells in a defined area from two independent experiments.

Figure 5 shows that only *hsp70.1* mRNA was induced in the kidneys of wild-type mice in response to osmotic stress, whereas induction of two *hsp70* mRNAs in the kidney was not different when the mice were exposed to a whole-body thermal stress, which is consistent with the results shown in Figure 2. We then used immunohistochemistry to localize osmotic stress-induced Hsp70 in the kidney. Hsp70 was concentrated largely in the renal medulla of wild-type mice but could not be detected in *hsp70.1*^{-/-} mice (data not shown).

We also performed TUNEL staining assays and compared the labeled nuclei between renal medulla of wild-type and *hsp70.1*^{-/-} mice after osmotic and thermal stress (Figure 6). When osmotic stress was applied to the mice, the renal medulla of *hsp70.1*^{-/-} mice exhibited significantly high numbers of

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Fig. 5. Northern blot analysis of *hsp70* mRNA expression *in vivo*. Mice were separated into three groups: the control group (C, $n = 3$); the thermal-stressed group (HS, $n = 3$) pretreated in a 42°C chamber for 30 min to induce Hsp70 and then placed in the 44°C chamber for 45 min after recovery for 8 h; and the salt-fed group (NaCl, $n = 3$), which received 3% NaCl (w/v) in their drinking water for 30 days. The blot was hybridized with the same probes as described in Figure 2. Data for the 'fold induction' are shown relative to control levels. Analyses were conducted using results from two independent experiments.

TUNEL-positive nuclei, while that of wild-type mice had only a few apoptotic cells. In contrast, there was no significant difference in apoptosis after thermal stress. These results support the hypothesis that the loss of Hsp70 expression might be a critical factor in the increased apoptosis in these cells.

DISCUSSION

It was revealed that two major inducible *hsp70* genes are located in close proximity on the same chromosome (Milner and Campbell, 1990). This type of gene duplication has been proposed to provide evolutionary possibilities such that the expression of these genes may be regulated at multiple levels and that their role may be additive or synergistic. Such regulation could produce large amounts of a single protein or could enable one gene to compensate for the loss of the other. A recent report showed that *hsp70.1* and *hsp70.3* were expressed in response to heat shock and the absence of either gene increased the cellular susceptibility to heat stress-induced apoptosis (Huang *et al.*, 2001), suggesting that *hsp70.1* and *hsp70.3* have additive roles in thermotolerance. Heat shock may represent a severe injury against which cells have developed a rapid protective mechanism. Two inducible *hsp70* genes might be necessary to generate a sufficiently rapid response to the challenge posed by this severe condition.

However, several studies have suggested that expression of *hsp70.1* and *hsp70.3* could be regulated differentially, depending on the nature of the stress or the specific type of cell involved. In addition, the turnover rates of *hsp70.1* and *hsp70.3* mRNAs could differ. Here, we have demonstrated that these two genes are differentially regulated by osmotic stress. *hsp70.1* played a major protective role against osmotic stress, and *hsp70.3* could not compensate for its absence. These findings clearly demonstrate that organisms have complex regulatory mechanisms that lead to the differential expression of the *hsp70* genes. Such mechanisms may provide multiple regulatory pathways for cells to respond rapidly and specifically to stress challenges.

The reason why *hsp70.3* is expressed in response to heat shock but not in osmotic stress remains unanswered. While we have no evidence to explain the selective regulatory mechanism of *hsp70* expression, it is possible that the *hsp70.1* gene may have an osmotic response element (ORE) in the divergent

Fig. 6. Increased apoptosis in osmotically stressed kidneys of *hsp70.1*^{-/-} mice. The mice were separated into three groups as described in Figure 5. Paraffin-embedded sections were stained using the Apoptag kit (Intergene) and counterstained with propidium iodide. Images were obtained using a confocal scanning microscope.

5' or 3' UTR sequences. A tonicity-sensitive element (TonE) has been reported to mediate increased transcription of several genes, including the sodium/*myo*-inositol cotransporter gene in response to hypertonic stress (Rim *et al.*, 1998). Recently, we have found that *hsp70.1* has two TonE consensus sequences (TGGAAANNYYN; Rim *et al.*, 1998) in reverse orientation, at -831 and -2088 in the promoter region, while *hsp70.3* does not have similar sequences within 5 kb of upstream sequences. We are currently investigating the role of the promoter region in the selective induction of *hsp70.1* after osmotic stress.

The maintenance of intracellular osmotic pressure is of fundamental importance for cell survival. Because cells in the renal medulla are routinely challenged with 10-fold variations in extracellular osmolarity, the adaptation of these cells to extreme conditions is critical to maintain normal kidney function. Although compatible organic osmolytes are known to play a major protective role against this stress (Santos *et al.*, 1998), this protective response occurs very slowly and Hsp70 provides a rapid protective measure until organic osmolytes are fully accumulated (Cohen *et al.*, 1991; Rauchman *et al.*, 1997; Neuhofer *et al.*, 1998).

Our data show that only *hsp70.1* is induced in response to osmotic stress (Figures 2 and 5). The loss of *hsp70.1* abolishes the induction of Hsp70 in response to osmotic stress, thereby rendering cells highly susceptible to osmotic stress-induced cell death, both *in vitro* and *in vivo* (Figures 4 and 6). We observed no differences in the expression of other HSPs, suggesting that *hsp70.1* plays a unique role. These data provide the first

evidence suggesting that each member of the highly homologous *hsp70* family may have a specific role in opposing certain stressors *in vivo*.

We believe that our study provides an important initial clue for elucidating mechanisms underlying the protective function of renal cells under hyperosmotic conditions and that the *hsp70.1*^{−/−} mice will serve as a useful model for research into various kidney disorders.

METHODS

Generation of *hsp70.1* knockout mice. A murine genomic clone of *hsp70.1* (Hunt and Calderwood, 1990) was fused to a 7.5 kb fragment of a 5′ promoter, a neomycin-resistant gene, a 0.8 kb fragment of *hsp70.1* exon and a thymidine kinase gene as a targeting vector. The *hsp70.1*-targeted ES cells were generated and injected into C57BL blastocysts by standard procedures. Genotypes were determined by four-primers PCR and confirmed by Southern blotting of tail genomic DNA. All mutant mice used in these experiments were bred by backcross with a C57BL/6 strain.

Cell culture and animal experiments. MEF cells were prepared from *hsp70.1*^{+/+} and *hsp70.1*^{−/−} E13.5 p.c. embryos and cultured in isotonic DMEM. For osmotic stress, culture media were replaced with DMEM supplemented with 100 mM NaCl (100 mM NaCl/DMEM) and incubated for 6 h. The pretreated cells were exposed to the DMEM medium supplemented with 600 mM NaCl (600 mM NaCl/DMEM) for 90 min and allowed to recover in the isotonic DMEM for 17 h. For thermal stress, cells were placed in a 42°C water bath for 30 min and returned to a 37°C/CO₂ incubator for 1 h. For *in vivo* stress experiments, mice were divided into three groups: the control group (*n* = 6), the thermal-stressed group and the salt-fed group (*n* = 7) that received 3% NaCl (w/v) in their drinking water for 30 days.

Northern blot analysis. Total RNA was prepared from cultured cells or renal tissue using RNeasy (Promega), according to the manufacturer's instructions. Samples containing 15 µg of total RNA were separated on formaldehyde agarose gels and blotted onto nylon membranes (Amersham). The blot was hybridized with a probe specific for either the *hsp70.1* or *hsp70.3* 3′ UTR.

Western blot analysis. Whole-cell extracts (40 µg of total protein) were subjected to SDS-PAGE and transferred to PVDF filters (Millipore). Western blotting was performed using anti-Hsp70 (Santa Cruz), anti-Hsp27 (StressGene) and anti-Hsp90s (Affinity BioReagent). The blots were developed using the enhanced chemiluminescence method (Amersham).

Immunofluorescence and TUNEL staining. For immunofluorescence, fixed MEF cells were incubated with anti-Hsp70 monoclonal antibody (StressGene) and antibodies were visualized with an anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate (Dako). Texas Red-conjugated anti-F actin (Sigma) was used to stain actin fibers. For TUNEL assays, staining was detected using the Apoptag kit (Intergen) and counterstained with propidium iodide. Cells were visualized in a confocal microscope (Bio-Rad) and images were exported to Adobe Photoshop version 6.0.

Cell morphology and survival. Morphological characteristics of MEF cells were evaluated with an inverted phase contrast microscope (Olympus). Photographs were taken with a 35-mm camera (Nikon) and cells were counted.

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