HSP70 Deficiency Results in Activation of c-Jun N-terminal Kinase, Extracellular Signal-regulated Kinase, and Caspase-3 in Hyperosmolarity-induced Apoptosis*

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In this study we examined the function of heat shock protein 70 (HSP70) in the hyperosmolarity-induced apoptotic pathway using hsp70.1−/− mouse embryonic fibroblasts (MEFs). When the cells were exposed to hyperosmotic stress, an absence of HSP70 negatively affected cell viability. Caspase-9 and caspase-3 were rapidly activated, and extensive cleavage occurred in focal adhesion and cytoskeletal molecules in the hsp70.1−/− MEFs. In contrast, hsp70.1+/+ MEFs exhibited no caspase-9 or caspase-3 activation and finally recovered intact cell morphology when cells were shifted back to an isosmotic state. Because HSP70 might be involved in the regulation of mitogen-activated protein kinase (MAPK) activities with regard to various cellular activities, we also monitored MAPK phosphorylation. The absence of HSP70 affected c-Jun N-terminal kinase phosphorylation. However, it had no effect on p38. Sustained phosphorylation of extracellular signal-regulated kinase (ERK) was observed during the hyperosmolarity-induced apoptosis of hsp70.1−/− MEFs. Inhibition of ERK activity by the treatment of PD98059 accelerated the apoptotic pathway. ERK phosphorylation was precisely correlated with shift of mitogen-activated protein kinase phosphatase-3 from the soluble to insoluble fraction. Our results demonstrate that the inhibitory effect of HSP70 on caspase-3 activation is sufficient to inhibit apoptosis and that HSP70 exhibits regulatory functions to c-Jun N-terminal kinase and ERK phosphorylation in hyperosmolarity-induced apoptosis.

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Apoptosis can be induced by a variety of different signals, including the activation of the Fas or tumor necrosis factor receptors, deprivation of growth factors, excessive DNA damage, treatment with chemotherapeutic drugs, or stresses such as heat shock, hyperosmotic shock, or UV irradiation (1, 2). Apoptotic stimuli trigger mitogen-activated protein kinase (MAPK)1 cascades and the proteolytic cleavage of procaspases. Many apoptotic stimuli induce the release of cytochrome c from the mitochondria, and this released cytochrome c then binds to apoptotic protease-activating factor 1 with dATP in the cytosol. This apoptosome complex in turn activates caspase-9, and the activated caspase-9 results in the cleaving of downstream caspase-3 (3, 4). The apoptotic process is also mediated via mitochondria-independent pathways, which converge on the proteolytic activation of the executive caspase, caspase-3. Caspase-3 cleaves various cellular proteins, finally resulting in the morphological alteration of apoptotic cells. Apoptosis, which is characterized by cell shrinkage, membrane blebbing, nuclear breakdown, and DNA fragmentation, is an indispensable process in embryological development, tissue homeostasis, and regulation of the immune system (5, 6). Apoptotic malfunctions have been implicated in a myriad of human diseases, including cancer, neurodegenerative disorders, and ischemic stroke (7–9).

Heat shock protein 70 (HSP70) can be induced by various stresses, including heat shock, hyperosmotic stress, oxidative stress, UV irradiation, amino acid analogues, infection, inhibitors of energy metabolism, and heavy metals (10). In addition to its chaperone functions in protein folding and assembly, HSP70 protects cells from a number of apoptotic stimuli (11–15). Most living cells are sensitive to sudden exposures to apoptotic stimuli. A transition from a mild to a harsh stimulus or repeated exposures to stimuli, i.e. preconditioning, can render cells tolerant to cellular damage (16). HSP70, which is highly induced during preconditioning, performs a pivotal role in the process of apoptotic tolerance. HSP70 binds to specific target proteins, mediating their cellular activities. HSP70 can modulate stress-activated signaling through direct binding to c-Jun N-terminal kinase (JNK) (17). HSP70 inhibits apoptosis via its direct association with apoptotic protease-activating factor 1 (18).

Despite recent advances in our knowledge regarding the anti-apoptotic effects of HSP70, this function has not yet been confirmed precisely using HSP70-deficient cells. Therefore, we utilized mouse embryonic fibroblasts (MEFs) generated from hsp70.1−/− mice to directly observe the changes occurring to apoptotic molecules under HSP70-deficient conditions. Our results illustrate that HSP70 exerts an apoptosis-regulatory effect at multiple steps in the hyperosmolarity-induced apoptotic pathway. HSP70 deficiency leads to the activation of JNK, ERK, and caspase-3 in the apoptotic pathway. The HSP70-induced prevention of caspase-3 activation was critical in inhibiting the apoptotic process. In contrast to the pro-apoptotic functions of JNK and caspase-3, sustained activation of ERK played an inhibitory role with regard to apoptosis.

EXPERIMENTAL PROCEDURES

Materials—Antibodies against JNK, ERK, p38, phospho-JNK (Thr-183/Tyr-185), phospho-ERK (Thr-202/Tyr-204), and phospho-p38 (Thr-
180/Tyr-182) were from Cell Signaling (Beverly, MA). Monoclonal anti-
vinculin clone VIN-11-5 and anti-α-tubulin clone B-5-1-2 were purchased from Sigma. Paxillin and caspase-9 antibodies were ob-
tained from Transduction Laboratories (Lexington, KY) and Pharmingen, respectively (San Diego, CA). Anti-glycereraldehyde-3-phosphate dehy-
drogenase was from Abcam (Cambridge, UK), and anti-FAK was from Upstate Cell Signaling Solutions (Waltham, MA). Anti-HSP70, caspase-3 (H-277), mitogen-activated protein kinase phosphatase (MKP) 1, MKP-3, actin (1–19), and the secondary antibodies, goat anti-rabbit, goat anti-mouse, donkey anti-goat IgG conjugated to horse-
radish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The molecular weight standards for SDS-polyacylamide gel electrophoresis were obtained from Bio-Rad. The ECL detection kit was from Amersham Biosciences.

Cell Culture and Treatment—Mouse embryonic fibroblasts (MEFs) were prepared from hsp70.1+/+ and hsp70.1−/− mice embryos by the C57BL/6 at 3.5 postconception as described in Shim et al. (19). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Spontaneous immortalized MEFs were obtained with successive subculture, and they were used for transfection experiments. For the specific induction of hsp70.1, i.e. pre-conditioning with NaCl, culture media were replaced with Dulbecco’s modified Eagle’s medium supplemented with 100 mM NaCl and incubated for 6 h. For the lethal hyperosmotic condition, cells were exposed to the Dulbecco’s modified Eagle’s medium (DMEM) medium supplemented with 400 or 500 mM NaCl for 2 h and then allowed to recover in the isosmotic DMEM media for the indicated time. For heat preconditioning, culture plates containing cells were tightly wrapped with Parafilm and im-
mersed in a water bath at 43 °C for 30 min, then kept in a CO2 incubator at 37 °C for 6 or 16 h.

Cell Morphology, Immunofluorescence, and Viability—Morphological characteristics of MEFs were evaluated with an inverted phase contrast microscope (Olympus). Photographs were taken with a 35-mm camera (Olympus). For immunofluorescence, fixed MEFs were incubated with anti-α-tubulin monoclonal antibody, and primary antibody was visualized with an anti-mouse IgG fluorescein isothiocyanate conjugate (Santa Cruz Biotechnology). Texas Red X phallidin (Molecular Probes) was used to stain actin fibers. Cell viability was calculated from trypan blue exclusion assay.

Western Blotting—After washing with phosphate-buffered saline, the cells were scraped and collected in extraction buffer (1% Triton X-100, 0.5% sodium deoxycholate, 20 mM Tris-Cl, pH 7.5, 12 mM β-glycer-
phosphate, 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 3 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin). The collected cells were incubated on ice for 30 min. The lysate was centrifuged, and the protein amount in cleared lysates was quantitated with BCA protein assay reagents (Pierce). The equal protein concentrations were separated on a 10% SDS-PAGE gel. After transfer of protein to nitrocellulose membrane, the membrane was blocked with 5% (w/v) nonfat dry milk in 1x phosphate-buffered saline overnight at 4 °C. Each of the proteins was detected with its specific antibody. The antibody-antigen complex was detected using horseradish peroxidase-conjugated secondary antibody and visualized by a standard chemiluminesence method performed according to the manufacturer’s instruction. Soluble and insoluble fractions were pre-
pared as described in Taglom et al. (25).

Stable Transfection—MFG.hsp70.puro and MFG.puro plasmids (20) were transfected into hsp70.1−/− MEFS using Lipofectamine Plus reagent (Invitrogen) with the manufacturer’s protocol. After 48 h of incubation, puromycin (3 μg/ml) was added to the culture medium to select the transfected cells. The selection was continued for 10 days, after which the cells were maintained as a population, and the selected cells were used for the experiments.

RESULTS

hsp70.1−/− MEFs Exhibit No Basal HSP70 Expression and No HSP70 Induction by Hyperosmolarity—We previously gen-
erated hsp70.1−/− mice and demonstrated the differential expression of two inducible hsp70 genes, hsp70.1 and hsp70.3, depending on stress type (19, 20). Because heat shock induced the expression of both genes, i.e. hsp70.1 and hsp70.3, hsp70.1−/− and hsp70.1−/− MEFs exhibited drastic increases in HSP70 levels when exposed to heat shock (Fig. 1A). In contrast, mild hyperosmotic conditions (addition of 100 mM NaCl to culture media) resulted in HSP70 expression only in the hsp70.1+/+ MEFs due to the selective induction of hsp70.1

(Fig. 1B). This selective hyperosmotic stress-mediated induction of hsp70.1 was also confirmed by Northern blot analysis (data not shown). We detected no basal expression of HSP70 in the hsp70.1−/− MEFs under normal growth conditions (Fig. 1, A and B). This indicates that basal HSP70 level is attributed to the hsp70.1 gene expression. Thus, the HSP70 level in hsp70.1+/+ MEFs was far higher than in hsp70.1−/− MEFs under both normal and hyperosmotic conditions (Fig. 1B). The induced level of HSP70 was stably maintained for up to 24 h. With the other HSPs, HSP90 and HSP27, which are involved in apoptosis signaling, we detected no significant alterations oc-
curring in the hsp70.1−/− MEFs (Fig. 1C).

HSP70 Deficiency Renders Cells Vulnerable to Apoptotic Stress—When MEFs were exposed to lethal concentrations of NaCl (addition of 400 or 500 mM NaCl), hsp70.1−/− MEFs were found to be more resistant to cell death than the hsp70.1−/− MEFs due to their basal HSP70 expression levels (Fig. 2A, left panel). If the MEFs were preconditioned with 100 mM NaCl, in order to induce HSP70 expression only in the hsp70.1−/− MEFs, cell viability was observed to increase enor-
mously in the hsp70.1+/+ MEFs compared with the hsp70.1−/− MEFs. Fig. 2A depicts cell viability in the hsp70.1−/− and hsp70.1−/− MEFs either with or without pre-
conditioning. Cellular morphologies and HSP70 levels in the hsp70.1+/+ and hsp70.1−/− MEFs after preconditioning are
Multiple Functions of HSP70 in Hyperosmolarity-induced Apoptosis

Fig. 2. HSP70 level is critical for cytoprotection against hyperosmotically induced cell death. A, viabilities of hsp70.1+/+ and hsp70.1−/− MEFs. The percentage of surviving cells was estimated by trypan blue exclusion analysis. Cells were incubated in media containing either 400 or 500 mM NaCl for 2 h and then recovered for 17 h in isosmotic media with or without preconditioning with 100 mM NaCl for 6 h. C, control. B, morphological changes of MEFs under hyperosmotic conditions. After preconditioning with 100 mM NaCl for 6 h, hsp70.1+/+ and hsp70.1−/− MEFs were exposed to 500 mM NaCl for 2 h. The cells were then recovered in isosmotic media for 7 h (R7h) or 17 h (R17h). The preconditioned hsp70.1+/+ MEFs had large amounts of HSP70 compared with hsp70.1−/− MEFs.

shown in Fig. 2B. These results indicate that the loss of either HSP70 basal expression or inducibility impairs the cell tolerance to cell death signaling.

Cleavage of Caspase-3 and Caspase-9 Is Inhibited by HSP70—It has been reported that HSP70 plays an inhibitory role in caspase-3 activation via prevention of the active apoptosisomes (18, 21). However, because there is no available information regarding caspase-3 activity in hsp70-deficient cells, we compared caspase-3 cleavage between hsp70.1+/+ and hsp70.1−/− MEFs under hyperosmolarity-induced apoptotic conditions. Cleavage of caspase-3 was unambiguously detected in time- and dose-dependent manners in the hsp70.1−/− MEFs (Fig. 3A). No effect of preconditioning on caspase-3 cleavage was consistent with no induction of HSP70 in the hsp70.1−/− MEFs (Fig. 3B). In contrast to the hsp70.1−/− MEFs, caspase-3 was somewhat resistant to apoptotic cleavage in the hsp70.1+/+ MEFs, which is probably attributable to basal HSP70 levels (Fig. 3A). When the hsp70.1+/+ MEFs were preconditioned, caspase-3 cleavage was clearly inhibited (Fig. 3B). This indicates that the amount of HSP70 is a critical factor for the prevention of caspase-3 activation. Cleavage of caspase-9, which is an upstream molecule of caspase-3, was also inhibited in the hsp70.1+/+ MEFs after preconditioning (Fig. 3C). However, caspase-9 was cleaved, in a manner similar to that of caspase-3 cleavage in the hsp70.1−/− MEFs. To confirm whether or not HSP70 per se blocks caspase-3 cleavage and cellular damage, the hsp70 gene was transfected into hsp70.1−/− MEFs. Empty vector transfectants exhibited caspase-3 cleavage when exposed to hyperosmotic stress conditions, just as hsp70.1−/− MEFs. However, hsp70 transfectants did not exhibit caspase-3 cleavage under the same stressful conditions (Fig. 3D). Inhibition of cellular damage was also observed in the hsp70-transfected hsp70.1−/− MEFs (Fig. 3D).

HSP70 Is Essential for the Maintenance of Recovery Potential via Protection of Caspase-3 and Its Substrates—When MEFs were exposed to hyperosmotic stress, structural integrity of cytoskeleton was rapidly destroyed within 30 min. With shrinkage of cell volume, both hsp70.1+/+ and hsp70.1−/− MEFs were severely damaged in terms of cellular morphology and cytoskeletal integrity during the exposure to hyperosmotic stress (Fig. 4A). When cells were returned to an isosmotic state, i.e., recovery state, hsp70.1+/+ MEFs gradually recovered, and ultimately exhibited normal cellular morphology and cytoskeletal structure. However, hsp70.1−/− MEFs showed no recovery after transition from hyperosmotic to isosmotic media. Cellular damage proceeded, even after returning to isosmotic conditions, culminating in cell death. Because cells had shrunken and become deformed, we evaluated both focal adhesion status and the state of the cytoskeletal molecules. Despite cellular deformation, focal adhesion molecules (FAK, vinculin, paxillin) and cytoskeletal molecules (tubulin, actin) were found to have remained intact in the hsp70.1+/+ MEFs (Fig. 4B). By way of
Multiple Functions of HSP70 in Hyperosmolarity-induced Apoptosis

Comparison, FAK, paxillin, tubulin, and actin were all degraded with caspase-3 activation in the hsp70.1/−/− MEFs, even after cells had been shifted back to an isosmotic state. Vinculin was a protein implicated in resistance to apoptotic degradation. In the case of the hsp70.1/−/− MEFs, cellular damage was totally reversed during the recovery period, possibly due to the inhibition of caspase-3 activation by HSP70. When the caspase-3-specific inhibitor, DEVD-CHO, was applied, hsp70.1/−/− MEFs were rescued from the apoptotic cell death (data not shown). In this study we demonstrated that the inhibitory role of HSP70 on caspase-3 activation is indispensable, keeping the maintenance of recovery potential and, thus, cell viability.

HSP70 Regulates Phosphorylation of c-Jun N-Terminal Kinase and Extracellular Signal-regulated Kinase under Hyperosmotic Conditions—MAPKs are integral to the mechanisms by which cells respond to physiological stimuli and also to a wide variety of environmental stresses. Therefore, we assessed MAPK activities in MEFs exposed to lethal hyperosmotic conditions (Fig. 5A). Earlier and more severe phosphorylation of JNK was detected in the hsp70.1/−/− MEFs than in the hsp70.1/+/+ MEFs. JNK phosphorylation was detected at 30 min, peaking 2 h after exposure to hyperosmotic stress, and it decreased to basal levels when cells were returned to an isosmotic state, i.e. in recovery condition. Whereas the hsp70.1/+/+ MEFs exhibited no ERK phosphorylation, the hsp70.1/−/− MEFs exhibited prominent ERK phosphorylation. ERK phosphorylation was sustained, and the degree of phosphorylation during the apoptotic process was not observed to undergo any reduction (Fig. 5B). No detectable differences were observed between hsp70.1/+/+ and hsp70.1/−/− in terms of p38 MAPK phosphorylation (Fig. 5A).

It is generally accepted that JNK activation triggers apoptosis and ERK activation prevents apoptosis. However, it has been recently suggested that JNK acts as an anti-apoptotic molecule, and ERK could be pro-apoptotic on cell type and stimulus (22, 23). To clarify the roles of phospho-JNK and phospho-ERK in hyperosmolarity-induced apoptosis, we treated the cells with inhibitors before applying hyperosmotic stress. Treatment with ERK inhibitor (PD98059) accelerated cellular damage and reduced the viability of the hsp70.1/−/− MEFs (Fig. 5C). When JNK inhibitor (SP600125) was applied, cellular morphology recovered, and viability increased (Fig. 5C). This indicates that, whereas phospho-JNK acts as a pro-apoptotic molecule, phospho-ERK acts as an anti-apoptotic molecule in hyperosmolarity-induced apoptosis. To verify the anti-apoptotic function of ERK in the hyperosmolarity-induced apoptotic pathway, caspase-3 activity was analyzed in PD98059-treated hsp70.1/−/− MEFs. Caspase-3 activation and the cleavage of its substrate were also accelerated by treatment with PD98059 (Fig. 5D). hsp70.1/+/+ MEFs exhibited no significant differences in cellular morphology by the treatment of PD98059 or SP600125 (Fig. 5C).

Increase of ERK Phosphorylation Is Attributed to Decrease of Mitogen-activated Protein Kinase Phosphatase-3 in Soluble Fraction—Either activation of MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase) or inactivation of ERK phosphatases contributes to ERK phosphorylation. It has been recently reported that MKP-3 and MKP-1 could be effective phosphatases for the phosphorylation of ERK
Immunoblot assays were performed to detect each protein. Cstaining. F-actin and tubulin were stained using rhodamine-labeled phalloidin and anti-tubulin antibody labeled with fluorescein isothiocyanate, respectively. C, control. R means cells incubated in isosmotic media during the indicated time periods. cytoskeletal structures in the C, glyceraldehyde-3-phosphate dehydrogenase.

**DISCUSSION**

HSP70 is one of the most conserved molecular chaperones and is essential for proper folding and assembly of proteins. HSP70 also plays a key role in cell survival under various apoptotic stress conditions. HSP70 seems to act as an anti-apoptotic molecule at multiple points. HSP70 prevents apoptosis by inhibiting the JNK-signaling cascade (33, 34). HSP70 has been established as a negative regulator of apoptosis signal-regulating kinase 1 (27). We previously reported that HSP70 inhibits apoptosis by preventing apoptosis signal-regulating kinase 1 (27). We previously reported that HSP70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase-3 activation (15). Concurrently, two different groups reported that HSP70 inhibits apoptosis by preventing apoptosome formation (18, 21). Recently, the in vivo functions of HSP70 were studied with either hsp70.1- or hsp70.3-disrupted mice. Huang et al. (28) reported a marked increase of sensitivity to heat stress-induced apoptosis occurring in hsp70.1- or hsp70.3-disrupted mice. Our hsp70.1-deficient mice exhibited sensitivity of the renal medulla upon the application of osmotic stress (19). The hsp70.1-deficient mice appeared to have no protection against tumor necrosis factor-induced lethality (29). Genomic instability and enhanced radiosensitivity were also reported in hsp70.1- and hsp70.3-deficient mice (30). However, to our knowledge there has been no report yet demonstrating anti-apoptotic function at the molec-
ular level in hsp70-deficient cells. Therefore, in this study we endeavored to assess the differences in apoptotic molecular changes in the presence or absence of HSP70.

The hsp70.3 gene is not induced by hyperosmotic stress. hsp70.1−/− MEFs are completely HSP70-deficient in terms of basal expression and remain so even under hyperosmotic stress conditions. Therefore, hsp70.1−/− MEFs are useful for the study of HSP70 function in hyperosmolarity-induced apoptosis. The reason why hsp70.1 responds to hyperosmotic stress but hsp70.3 does not can be explained by the osmotic response element/tonicity response element. The osmotic response element/tonicity response element is found only in the hsp70.1 gene promoter (31). When we performed reporter gene analyses of both the hsp70.1 and hsp70.3 promoters, only the hsp70.1

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**Fig. 5. HSP70 regulates stress-induced MAPK phosphorylation.** A, MAPK status in hsp70.1−/− MEFs. After preconditioning with 100 mM NaCl MEFs were exposed to hyperosmotic conditions (500 mM NaCl) for the indicated time periods. Cell lysates were analyzed by immunoblot assay. Phosphorylated (p-) MAPKs were detected by phospho-specific MAPK antibodies. Total amounts of each MAPK were detected by anti-MAPK antibodies. C, control. R, recovered cells. B, sustained phosphorylation of ERK. ERK phosphorylation was detected at various time points during the hyperosmolarity-induced apoptotic process. C, changes of viability as a result of treatment with PD98059 or SP600125. Inhibitor was added 1 h before hyperosmotic stress. Graphs show the percentage of viable cells in PD98059- and SP600125-treated hsp70.1−/− MEFs at R7h and R17h, respectively. Viable cells were counted by trypan blue exclusion assay. D, effect of ERK inhibitor on the cleavage of caspase-3 and its substrates. Cell lysates were prepared from PD98059-treated hsp70.1−/− MEFs for Western blot analysis. PC means positive control for Western blotting with anti-HSP70 antibody.
Multiple Functions of HSP70 in Hyperosmolarity-induced Apoptosis

promoter exhibited reporter gene activity under hyperosmotic stress conditions (data not shown).

We addressed two major effects of HSP70 in the hyperosmolarity-induced apoptotic pathway. One was the inhibitory effect of HSP70 on caspase-3 activation. Caspase-3 activation is a critical step in the determination of cell fate under stressful conditions. If caspase-3 activation had already occurred, cells make no return from the apoptotic pathway. Otherwise, cellular damages could be cured when stressful insults are alleviated. Focal adhesion and cytoskeletal molecules were cleaved by caspase-3 in the absence of HSP70. Cells detached from the bottom and finally died with anoikis. We clearly demonstrated the blockage of caspase-3 activation by the natural caspase-3 inhibitor molecule, HSP70.

The other effect was the regulatory effect of HSP70 on MAPK activation, i.e. the suppression of JNK and ERK activity. It has been reported that HSP70 regulates JNK phosphorylation via two different mechanisms. HSP70 could inhibit the repression of JNK dephosphorylation under stressful conditions (26). HSP70-induced suppression of JNK activation was attributed to physical interactions occurring between HSP70 and JNK (17). HSP70 appears to be quite specific in its negative regulation of MAPK activation as a stress response. After stress, HSP70-Bag1 binding negatively affected ERK phosphorylation, finally affecting cell growth signals (32). HSP70 deficiency resulted in the phosphorylation of JNK in cells exposed to hyperosmotic stress. Sustained ERK phosphorylation occurred in the absence of HSP70 in the hyperosmolarity-induced apoptosis pathway.

HSP70 inhibits apoptosis through either its chaperoning function or its binding activities to specific target molecules. HSP70 can restore proteins that have been damaged or unfolded by stresses via its chaperoning activity (33). However, HSP70 can suppress the activation of apoptosis by non-protein damaging stimuli, implying that HSP70 might inhibit apoptosis through its effects on specific targets. HSP70 suppresses apoptosis via direct association with apoptotic protease-activating factor 1 and the blockage of the assembly of a functional apoptosome (18, 21). HSP70 is able to inhibit the apoptogenic effects of apoptosis-inducing factor (AIF) via specific interactions with AIF (35). HSP70 also binds directly to apoptosis signal-regulating kinase 1 and modulates stress-activated apoptosis signaling (17, 27).

MAPKs are typical signaling mediators that transmit intracellular signals initiated by extracellular stimuli to the nucleus. MAPK signaling regulates a variety of cellular activities, including cell growth, differentiation, survival, and cell death (17). The phosphorylation status of MAPK profoundly affects both cell fate and cellular function. Recent studies have revealed the direct coupling of the inactivation of MAPK to the activation of an MKP. This coupling might provide signal transduction fidelity, and MKPs function to tightly regulate substrate specificity and enzymatic activities (25, 36). MKP-3 specifically binds to and inactivates both ERK1 and ERK2 and may also play a key role in the spatio-temporal regulation of ERK activity in mammalian cells (37). Yaglom et al. (25) reported that the inactivation of MKP was involved in the regulation of ERK by heat shock. It has also been reported that the ERK-specific phosphatases MKP-1 and MKP-3 all the precise control of nuclear and cytoplasmic ERK activities (38). The constitutive induction of ERK phosphorylation was accompanied by reduced activity of MKP during cellular senescence (39).

In this study sustained ERK phosphorylation occurred in HSP70-deficient cells undergoing apoptosis. To elucidate whether MKP is involved in sustained ERK phosphorylation in
the hyperosmolarity-induced apoptosis pathway, we tried to detect MKP in HSP70-deficient cells. Because MKP was not detected in hsp70I/−− MEFs, probably due to its very low level of expression, we used SK-N-SH cells. Under hyperosmotic conditions, we observed that the MKP-3 level had decreased dramatically in soluble fraction, and its loss correlated neatly with ERK phosphorylation levels. HSP70 played a pivotal role to prevent MKP-3 aggregation under the hyperosmotic condition. Protein damaging treatments such as heat shock, oxidative stress, and ethanol caused significant damage of JNK phosphatases and ERK phosphatases (25, 26). It has been reported that HSP70 counteracts inactivation of phosphatases via its chaperoning activity (25, 26). Our results suggest that nonprotein-damaging treatment, e.g. hyperosmotic stress, also results in MKP-3 shift into the insoluble fraction. Inactivation of MKP-3 contributes to ERK phosphorylation, which exerts a cellular protection in the apoptotic pathway.

HSP70 exhibits crucial functions in the maintenance of cell homeostasis. HSP70 is generally considered to be an antiapoptotic molecule. However, HSP70 deficiency results in ERK activation due to MKP-3 inactivation in the apoptotic pathway. HSP70 can be used as a target molecule for pharmacological and therapeutic interventions, both, in apoptosis. Preconditioning, which induces HSP70 expression, inhibits cell death, thus resulting in myocardial and renal protection. The diverse roles of HSP70 suggest that HSP70 might be exploited in the development of novel therapeutic approaches.

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Multiple Functions of HSP70 in Hyperosmolarity-induced Apoptosis

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