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Molecules and Cells

Functional HSF1 Requires Aromatic-Participant Interactions in Protecting Mouse Embryonic Fibroblasts against Apoptosis Via G2 Cell Cycle Arrest

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The present study highlighted the aromatic-participant interactions in in vivo trimerization of HSF1 and got an insight into the process of HSF1 protecting against apoptosis. In mouse embryonic fibroblasts (MEFs), mutations of mouse HSF1 (W37A, Y60A and F104A) resulted in a loss of trimerization activity, impaired binding of the heat shock element (HSE) and lack of heat shock protein 70 (HSP70) expression after a heat shock. Under UV irradiation, wild-type mouse HSF1 protected the MEFs from UV-induced apoptosis, but none of the mutants offered protection. We found that normal expression of HSF1 was essential to the cell arrest in G2 phase, assisting with the cell cycle checkpoint. The cells that lack normal HSF1 failed to arrest in the G2 phase, resulting in the process of cell apoptosis. We conclude that the treatment with UV or heat shock stresses appears to induce the approach of HSF1 monomers directly *via* aromatic-participant interactions, followed by the formation of a HSF1 trimer. HSF1 protects the MEFs from the stresses through the expression of HSPs and a G2 cell cycle arrest.

INTRODUCTION

Heat shock proteins (HSPs) were induced immediately when the cells respond to strong, acute or toxic conditions to restore homeostasis and improve the survival of cells (Lindquist and Craig, 1988; Morimoto et al., 1994; 1997). The protective role of HSPs is sufficient to protect the cells and tissues from diverse environmental stresses including hydrogen peroxide and other oxidants, toxic chemicals, extreme temperatures and ethanol-induced toxicity. Heat shock proteins have been reported to regulate apoptosis and cell death, affecting both the extrinsic and intrinsic pathways of apoptosis (Joly et al., 2010). In addition, there is evidence suggesting that HSPs are involved in drug resistance, making cells more resistant to the cytotoxic effects of anticancer drugs that are capable of inducing apoptosis (Samali and Cotter, 1996).

Heat shock transcription factor 1 (HSF1) is a central regulator of the synthesis of inducible HSPs in mammals. In the normal state, HSF1 exists in the cytoplasm in an inert monomeric state. On the other hand, when cells are stressed, HSF1 forms a homo-trimeric complex and is translocated to the nucleus, where it specifically binds the heat shock element (HSE), a conserved regulatory DNA sequence upstream of the heat shock genes (Pirkkala et al., 2001; Voellmy, 2004). Previous studies have suggested that mammalian HSF1 trimer formation depends on intermolecular hydrophobic non-covalent interactions in its trimerization domain (Farkas et al., 1998; Rabindran et al., 1993). Importantly, aromatic amino acids are highly conserved in HSF1 and are believed to be essential for HSF1 trimerization. The DBD structure of Drosophila HSF suggests that an aromatic residue, Tyr92, had intramolecular interactions with the helix (H1) residues (Vuister et al., 1994). A previous study by Lu et al. (2009a) reported that in human HSF1, Tyr60 can form intramolecular interactions with the first α-helix (H1), which exists in the DNA-binding domain (DBD) of HSF1. Two cysteine residues (C36 and C103) in HSF1 (hHSF1) participate in the formation of an intermolecular disulfide bond (SS bond), which is required for DNA binding (Lu et al., 2009b). The aromatic residues, Trp37 and Phe104, are involved in the formation of intermolecular SS bonds. This mechanism of the interactions of aromatic residues (Trp37, Tyr60 and Phe104) in human HSF1 provided a model of HSF1 activation.

The present study examined three aromatic residues in mouse HSF1 as well as their roles in HSF1 trimerization activity and expression of HSP70. The experiments were performed using MEFs cell-lines after treatment such as heat shock and UV irradiation. Apoptosis and cell cycle were observed after the UV and cisplatin treatment.

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MATERIALS AND METHODS

Plasmid construction

The mouse HSF1 cDNA vector pCDNA3.1/hgy-mouse *hsf1* was kindly provided by Dr. Sang-Gun Ahn, Chosun University, Korea. The special amino acids (Trp37, Tyr60 and Phe104 to alanine) were changed by PCR-mediated, site-directed mutagenesis.

Cell lines, cell culture and transfection

Mouse embryonic fibroblast (MEF) cells, wild type or mutant *mhsf1*$^{-/-}$, were a generous gift from Dr. Benjamin (Dr. Benjamin's Cardiology Research Lab University of Utah). The cells were cultured at 37°C in a CO_2 incubator. The DMEM medium supplemented with 4.5 g/L glucose, 4 mM L-glutamine, penicillin, 100 μg/ml streptomycin and 10%FBS was used as the growth medium. The wild-type HSF1 (pCDNA3.1-HSF1) and HSF1 mutants were transfected into the MEF hsf1$^{-/-}$. The MEFs were cultured in a 6-well cell plate and transfection was performed when the cells reached 50-80% confluence. One μg of DNA in 100 μl of Opti-MEM I Reduced Serum Medium without serum was added to each well of the cells to be transfected. For each well of the cells, 4 μl of dilute Lipofectamine™ was added to the above diluted DNA solution, mixed gently and incubated for 25 min at room temperature to form the DNA-Lipofectamine™ complexes. The medium was removed and replaced with 200 μl of complete growth medium. 100 μl of the DNA-Lipofectamine™ complexes was added directly to each well. The cells were incubated for 18-24 h post-transfection before assaying for transgene expression. Twenty four hours after transfection, fresh medium containing 700 μg/ml G418 was added for selection. The antibiotic containing medium was replaced every 3-4 days.

HSF1 covalent trimerization assays

MEF cells were heat-treated at 42°C for 1 h and incubated for 24 h. The whole cell extracts were prepared by thawing the cell pellets in RIPA buffer at 4°C. The cell extracts were cleared by centrifugation at 12,000 × *g* for 15 min at 4°C, and the supernatants were used for the HSF1 trimerization studies. EGS [ethylene glycol bis(succinimidyl succinate)] cross-linking was carried out, as described previously (Ahn and Thiele, 2003). The samples were resolved by SDS/PAGE and analyzed by immunoblotting using the anti-HSF1 antibody.

Ultraviolet treatments

After removing the growth medium, the cell culture dishes were washed with PBS. The MEFs cells were irradiated for 30 s with an irradiance of 0.6 mW/cm^2. The UV irradiance was modulated by adjusting the UV lamp (Spectronics, USA) output, which was calibrated with a UV sensor. The UVB spectrum was between 280 nm and 320 nm with a peak irradiance at 312 nm. The total UVB exposure volume was 15 J/m^2.

Electrophoretic mobility shift assays

The nuclear extracts were prepared from the stress-treated MEF cell lines, as described by (Ahn and Thiele, 2003). The extract was incubated with 32^P-labeled HSE (5′-GATCCTCGA ATGTTCGCG AAAAG-3′) for 15min at room temperature in a binding buffer (20 mM Tris-HCl pH 7.5, 5% glycerol, 40 mM NaCl, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.05 mg/ml BSA) containing 1 μg of poly(dI-dC) and the protease inhibitors (Sigma). The nucleoprotein-oligonucleotide complexes were resolved by electrophoresis on a 4% polyacrylamide gel at 30 mA for 1 h. Before sample loading, the gel was run for 30 min at 20 mA.

Western blot analysis

For Western blot analysis, the cells were washed twice with PBS and collected with NP-40 lysis buffer. The cell extracts were quantified using a BCA protein assay kit (Bio-Rad). Equal aliquots of the cell extracts were separated on SDS-PAGE. The proteins were then transferred to PVDF membranes, blocked and probed with the antibody against HSP70. The primary antibody-bound proteins were detected using an enhanced chemiluminescence substrate system (Promega).

MTT assay

Twenty four hours after UV-irradiation, the cell viability was evaluated using a Cell Growth Determination Kit, MTT based (sigma). A MTT solution in amounts equal to 10% of the culture volume was added to the cultures. The dishes were returned to the incubator and incubated for 4 h. After incubation, the culture fluid was removed and MTT solvent was added in amounts equal to the original culture volume. The MTT assay was carried out using the standard protocol, and the optical density was measured at 570 nm using a spectrometer.

Cell cycle analysis and apoptosis detection

The cells were seeded on a 10 cm plate and treated with UV-irradiation or 2 mg/ml cisplatin (sigma). After further incubation for 24 h, the cells were trypsinized, pelleted and washed with PBS. The cells were fixed with 70% ethanol. After overnight incubation, the samples were washed with cold PBS and re-suspended in a DNA staining solution (20 μg/ml propidium iodide, and 0.5 mg/ml RNase in PBS). The cells in the different phases of the cell cycle were analyzed using a FACScan flow cytometer analysis system (Beckman Coulter, Cytomics FC500). The apoptosis was measured by using the Annexin V-FITC Apoptosis Detection Kit (Komabiotech, Korea).

RESULTS

Heat-induced HSF1 activation in mouse embryonic fibroblasts (MEFs) requires aromatic-participant interactions

Our previous studies have shown that in human HSF1, the loss of conserved aromatic amino acid residues (Trp37, Tyr60, and Phe104) could impair heat-induced trimerization and DNA-binding (Lu et al., 2009b). Moreover, an intricate intramolecular interaction between H1 and Tyr60 stabilizes the structure of the human HSF1 DNA-binding domain, and facilitates the formation of the intermolecular aromatic pairs between Trp37 and Phe104 (Ahn and Thiele, 2003; Lu et al., 2008).

To further identify the above interactions on the mammalian cell-line level, mouse HSF1 was cloned into the pCDNA3.1 vector, PCR-mediated point mutated, and transit transfected into HSF1-knock-out (*hsf1*$^{-/-}$) mouse embryonic fibroblasts (MEFs). The expressions of HSF1 in these cell lines were confirmed by Western blots in each treatment. After culturing the cells for 24 h, the MEF cells harboring the wild-type HSF1 (or W37A, Y60A, F104A mutants) were heat-treated at 42°C for 1 h, extracted with RIRA buffer, and examined by trimerization and DNA-binding activity assays. Similar to human HSF1, the wild-type mouse HSF1 showed full heat-induced activities, but none of the mutants (W37A, Y60A and F104A mHSF1) showed these activities (Figs. 1A and 1C). Furthermore, the heat stress-induced HSP70 protein expression assay showed similar results (Fig. 1B), which strongly suggests the importance of these residues on HSF1 activation.

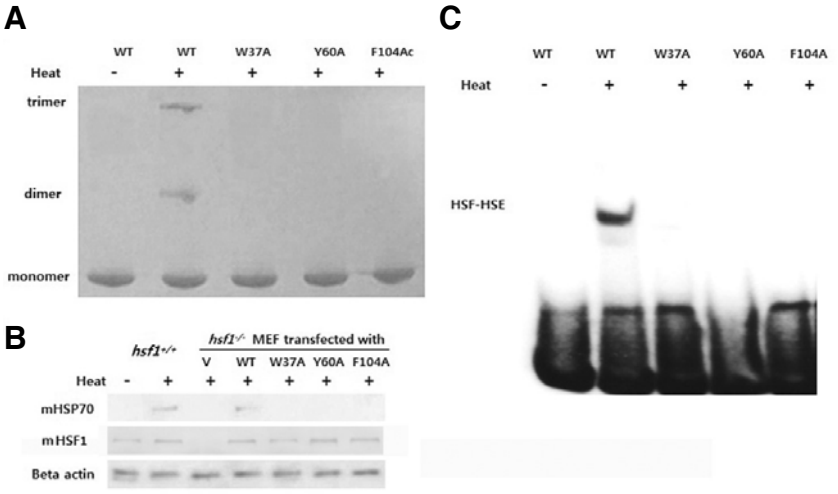

Fig. 1. Aromatic-participant interactions are essential for heat-induced HSF1 trimerization and HSP70 expression in mouse embryonic fibroblasts (MEFs). (A) Trimerization activity of HSF1 in the MEF hsf1$^{-/-}$ harboring wild-type (WT) vector or mutants of HSF1 (W37A, Y60A and F104A). The samples were heat activated (42°C for 1 h) and trimerization was analyzed by Western blot. (B) HSF1 and HSP70 expression was analyzed by Western blot in hsf1$^{+/+}$ and hsf1$^{-/-}$ harboring the empty vector (V) or wild-type (WT) or the mutants of HSF1. Beta-actin serves as the protein loading control. (C) The DNA-binding activities of HSF1 were measured by EMSA.

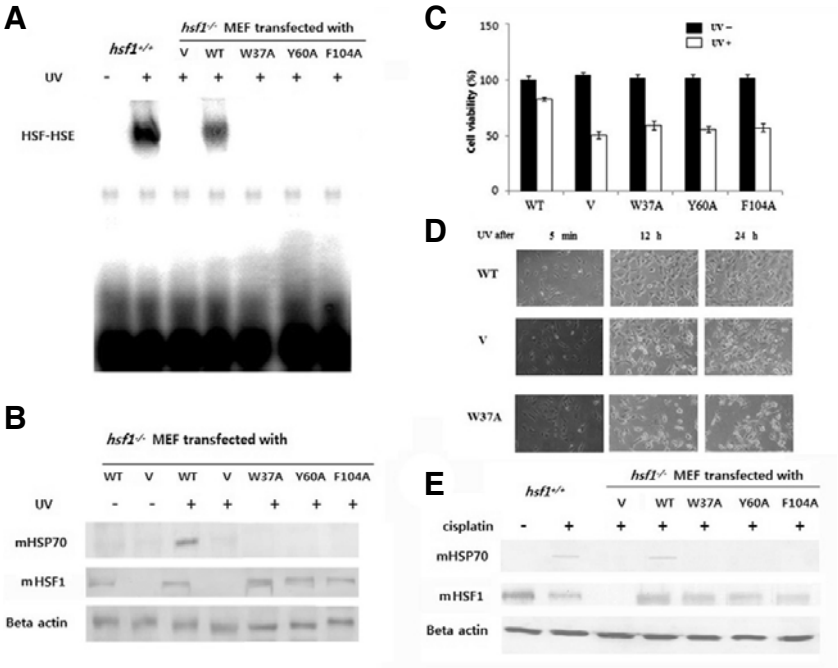

Fig. 2. HSF1 activation also requires aromatic amino acid residues in UV^{-} irradiated MEFs. (A) The UVinduced DNA-binding activities of HSF1 were measured by EMSA in hsf1$^{+/+}$ and hsf1$^{-/-}$ MEFs cell lines harboring the empty vector (V) or wild-type (WT) or mutants of HSF1 (W37A, Y60A and F104A). (B) UVirradiation induced HSF1 and HSP70 in the cell lines transfected with wild-type mHSF1 compared to that with an empty vector (V) and mutants. The cell lysates were prepared after UV-irradiation and subjected to Western blotting using HSP70 and anti-actin antibodies. Beta-actin serves as the protein loading control. (C) The cell viabilities of different cell lines were measured at 24 h after UV-irradiation using the MTT assay. (D) The MEFs cell lines were treated with 15 J/m^2 of UV light and the cell images were captured at various time points under a microscope. (E) Western blots of HSF1 and HSP70 after exposing the MEFs to 2 mg/ml cisplatin. Beta-actin was used as the protein loading control.

HSF1 activation also require aromatic amino acid residues in UV- irradiated MEF cells

The above results showed the heat-treated mouse HSF1 requires Trp37, Tyr60 and Phe104 residues. This study examined whether UV irradiation could also trigger mouse HSF1 activation without Trp37, Tyr60 or Phe104 in MEF cells. The ability of the cell lines for HSE-binding was analyzed. As shown in Fig. 2A, EMSA analysis of nuclear extracts showed that the *hsf1*$^{+/+}$ cell line with UV-irradiation gave rise to activated HSF1 that bound the HSE element. This HSE binding activity was also observed in the nuclear extracts from *hsf1*$^{-/-}$ MEF harboring the wild-type HSF1 (WT). On the other hand, neither the W37A, nor the Y60A or F104A HSF1 exhibited HSE binding activity. As the HSF1 mutants lost their trimerization activity, they were unable to bind HSE. The level of HSP70 expression was then measured in the *hsf1*$^{-/-}$ cell lines under UV stress (Fig. 2B). Similar to the heat treatment results, only the cells transfected with wild-type HSF1 showed HSP70 expression. The expression of HSP70 induced by UV stress was disabled by mutations of Trp37, Tyr60 or Phe104. This shows that the UV-stressed activation of HSF1 DNA-binding activity requires aromatic-participant interactions, in which the residues of Trp37, Tyr60 and Phe104 were most important.

Cell death and apoptosis in UV or cisplatin-treated MEFs

The cell viability was determined using an MTT assay. As shown in Fig. 2C, the cells transfected with the wild-type HSF1 exhibited high resistance to UV-treated cell death. The cell lines transfected with the empty vector or mutated HSF1 showed no such resistance. The cell images captured by a microscope expressed a similar result. In contrast to those transfected with the empty vector and mutants, the level of cell death was significantly lower in the wild-type cells stressed by UV after 12 and 24 h induction (Fig. 2D).

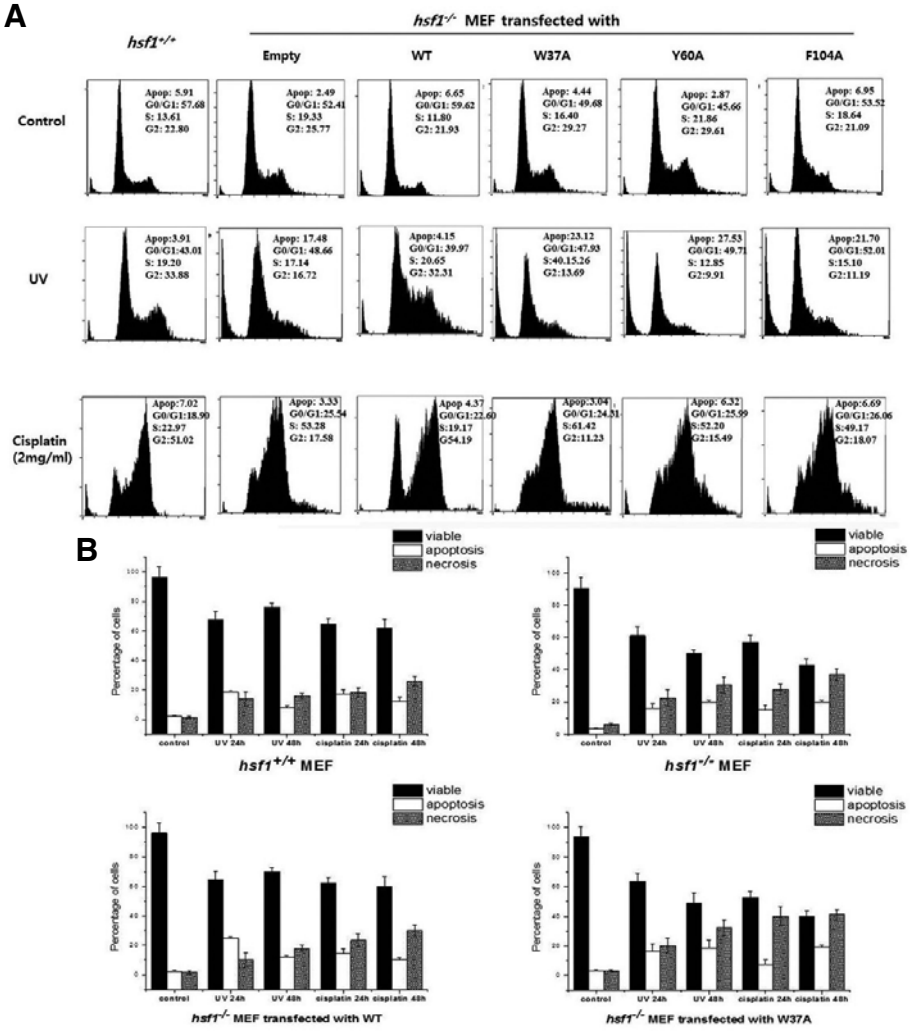

Fig. 3. HSF1 is required for G2 cell cycle arrest. (A) The cell cycle phase distribution of MEFs hsf1$^{+/+}$ and hsf1$^{-/-}$ harboring the empty vector (V) or wild-type (WT) or the mutants of HSF1 (W37A, Y60A and F104A) in response to UV and 2 mg/ml cisplatin. (B) Cell apoptosis was determined with annexin V-FITC and PI staining and analyzed by flow cytometer. Results are the means of 3 independent experiments: error bars indicate SD.

HSP70 can inhibit apoptosis of mouse embryonic fibroblasts, whereas the normal transcription of HSP70 requires the natural trimerization of HSF1.The cell lysates were also detected with the HSP70 antibody to determine if inducible HSP70 is expressed during a cisplatin treatment. Both *hsf1*$^{+/+}$ and *hsf1*$^{-/-}$ cells transfected with wild-type HSF1 revealed HSP70 induction when exposed to 2mg/ml cisplatin. No induction of HSP70 was observed in the *hsf1*$^{-/-}$ cells transfected with the HSF1 mutants (Fig. 2E). In addition, the amount of HSP70 expression was lower than that exposed to UV irradiation.

We extended our study to examine whether these cell lines undergoes apoptosis after UV and cisplatin treatment. Induction of apoptosis was determined with annexin V-FITC and PI staining (Fig. 3B). The data demonstrate that both the hsf1$^{+/+}$ and hsf1$^{-/-}$ become less viable during the experiment. However, the hsf1$^{+/+}$ or hsf1$^{-/-}$ MEFs with the wild-type HSF1 vector showed a recovery from apoptosis after the treatments, comparing with the HSF1-/- MEFs with the mutant vector or empty vector. These data indicate that loss of HSF1 interfere with cell growth and apoptosis.

Effects of Trp37, Tyr60 and Phe104-relied HSF1 on MEFs cell cycle under UV and cisplatin treatment conditions

To determine if the induced apoptosis is associated with cell cycle arrest, the cells were stained with PI and measured by flow cytometry. As shown in Fig. 3A, when treated with UV-irradiation, the percentage of *hsf1*$^{+/+}$ cells in the G2 phase increased significantly from 22.80% to 33.88%. Accumulation in the G2 phase was accompanied by decreases in the G1 phase. The *Hsf1*$^{-/-}$ cell line after UV-treatment showed a high sub-G1 peak corresponding to an increased in the population of apoptotic cells to 17.48% from a control value of 2.49%. The *hsf1*$^{-/-}$ cells transfected with the wild-type HSF1 vector showed a similar result to that of the *hsf1*$^{+/+}$ cells. The proportion of apoptotic cells transfected with the mutant vectors (Trp37, Tyr60 and Phe104) increased after being treated with UV stress. Results from 3 independent experiments analyzing cell cycle phase distribution are presented in Supplementary Table 1.

Cisplatin, a widely used chemotherapeutic agent, was tested for its ability in cell cycle arrest. Cisplatin was proved to be a DNA-damaging agent by forming intrastrand and interstrand crosslinks (Attardi et al., 2004). A concentration of 2 mg/ml was selected for the cisplatin treatment after prep-experiments. As expected, obvious G1/S accumulation was observed in the cells lacking normal HSF1 after treatment, either in *hsf1*$^{-/-}$ cells or *hsf1*$^{-/-}$ transfected with the mutant vectors (Fig. 3A). On the other hand, *hsf1*$^{+/+}$ cells or wild-type HSF1 vector-transfected *hsf1*$^{-/-}$ cells, which both express HSF1, showed G2 phase arrest.

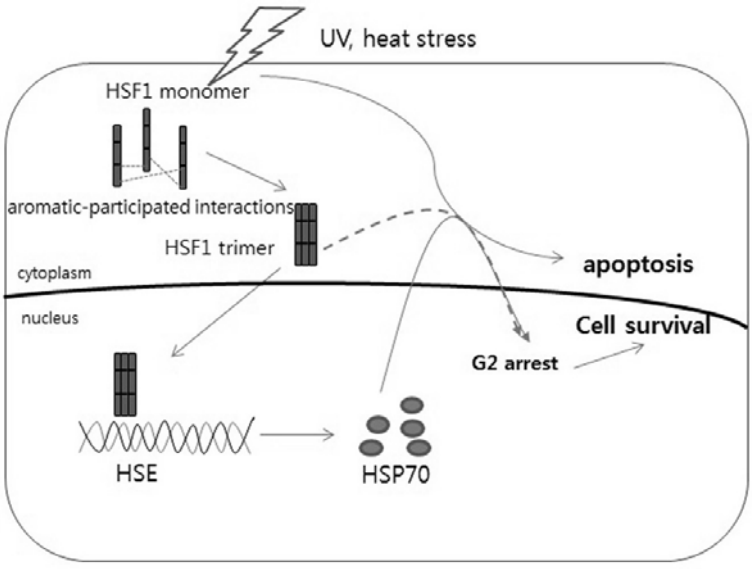

Fig. 4. Schematic diagram of functional HSF1 protects cells against apoptosis. The aromatic-participant interactions in HSF1 were induced by the stresses including UV, heat or other treatments, leading to the conformational changes in HSF1. The trimerized HSF1 translocates to the nucleus and induces the transcription of HSP genes. The stresses also induced apoptosis in the cells. In the absence of HSF1, the cells undergoing apoptosis will be died. HSF1 protects the cells from apoptosis via regulating the G2 cell cycle arrest and with the assistance of the HSF70.

This also suggests the ability of HSF1 to repair DNA breaks.

DISCUSSION

Cells under normal conditions and cell lines in culture undergo apoptosis when exposed to a range of cytotoxic agents (Takayama et al., 2003). Many studies reported that heat shock proteins play important roles in increasing the resistance to apoptosis. HSP70, as an anti-apoptotic protein, affects different pathways of apoptosis (Liu et al., 2010). Pro-apoptotic c-Jun N-terminal kinase (JNK) (Park et al., 2001), apoptosis protease activating factor-1 (Apaf-1) (Joly et al., 2010), apoptosis inducing factor (AIF) (Ravagnan et al., 2001), and the phosphatidylinositol 3-kinase pathway were also reported to help HSP70 block apoptosis. The present study found that the aromatic-participant interactions, in which Trp37, Tyr60 and Phe104 were most important, played an important role in the expression of HSP70 in MEFs, increasing the resistance to UV-stressed apoptosis. Because the normal expression of HSP70 requires the correct trimerization of HSF1, the interactions were found to be essential for the UV-stressed activation of HSF1 and the HSF1 DNA-binding activity.

UV irradiation was suggested to generate oxidative stress through the production of reactive oxygen species (ROS) in cells (Creagh et al., 2000). Cisplatin, an anticancer agent, induces DNA damage (Attardi et al., 2004). Although UV irradiation and cisplatin induces different changes in the cell cycle phase, both studies demonstrated the necessity of HSF1 for activation of the G2 cell cycle checkpoint (Fig. 3). Cells that lack HSF1 were unable to arrest in the G2 phase after exposure to cisplatin, resulting in defective G2 arrest and DNA repair, which explains the accumulation of cells in the G1/S phase. Under oxidative damage (UV irradiation), the loss of HSF1 results in apoptosis, whereas normal HSF1 leads to G2 phase arrest. These results were similar to those reported by Luft et al. in that HSF1-mediated thermotolerance prevents cell death and results in G2/M cell cycle arrest (Luft et al., 2001).

According to these results, the aromatic-participant interactions in HSF1 appear to be the direct target of stresses, such as UV and heat shock. As illustrated in Fig. 4, the stresses induce the approach of three HSF1 monomers via aromatic-participant interactions, which leads to the formation of a HSF1 trimer. The trimers then translocate to the nucleus and regulate the expression of HSP proteins (Vabulas et al., 2010). The present study provided evidence that HSF1 protects the cells from UV or heat stresses through the expression of HSPs and through G2 cell cycle arrest. Another point is that HSF1 is needed for the cells to accomplish the G2 checkpoint. There were no inducible HSP70 expression or G2 arrest in the absence of HSF1, and the cells undergo apoptosis. The precise roles of HSF1 or HSP70 in G2 cell cycle arrest are unclear, which will be a subject of future studies.

In conclusion, aromatic residues are important in the trimerization activity of HSF1. The aromatic-participant interactions can be induced by stresses that can lead to a conformational change in HSF1. *In vivo* DNA binding and stress-inducible trans-activation of the HSP70 gene protects the cells from stress-induced apoptosis *via* G2 cell cycle arrest. HSP70 was reported to cause oncogenesis and chemotherapy resistance. A recent study provides evidence that p27 exerts its tumor-suppressive function by suppressing HSP27 and HSP70 expression at the transcriptional level (Liu et al., 2010). Shunmei E. et al. also suggested a role of HSF1 in the augments of Fas-mediated apoptosis in cancer cells (Shunmei et al., 2010). Therefore, it is important to develop drugs that can block the aromatic-aromatic interactions, thereby inhibiting HSF1 activation for the suppression of oncogenesis.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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