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SB202190- and SB203580-Sensitive p38 Mitogen-Activated Protein Kinase Positively Regulates Heat Shock- and Amino Acid Analog-Induced Heat Shock Protein Expression

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When cells are exposed to proteotoxic stresses such as heat shock, amino acid analogs, and heavy metals, they increase the synthesis of the heat shock proteins (HSPs) by activating the heat shock transcription factor 1 (HSF1), whose activity is controlled via multiple steps including homotrimerization, nuclear translocation, DNA binding, and hyperphosphorylation. Under unstressed conditions, the HSF1 activity is repressed through its constitutive phosphorylation by glycogen synthase kinase 3β (GSK3β), extracellular regulated kinase 1/2 (ERK1/2), and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK). However, the protein kinase (s) responsible for HSF1 hyperphosphorylation and activation is not yet identified. In the present study, we observed that profile of p38 mitogen-activated protein kinase (p38MAPK) activation in response to heat shock was very similar to those of HSF1 hyperphosphorylation and nuclear translocation. Therefore, we investigated whether p38MAPK is involved in the heat shock-induced HSF1 activation and HSP expression. Here we show that the p38MAPK inhibitors, SB202190 and SB203580, but not other inhibitors including the MEK1/2 inhibitor PD98059 and the PI3-K inhibitor LY294002 and wortmannin, suppress HSF1 hyperphosphorylation in response to heat shock and L-azetidine 2-carboxylic acid (Azc), but not to heavy metals. Furthermore, heat shock-induced HSF1-DNA binding and HSP72 expression was specifically prevented by the p38MAPK inhibitors, but not by the MEK1/2 inhibitor and the PI3-K inhibitors. These results suggest that SB202190- and SB203580-sensitive p38MAPK may positively regulate HSP gene regulation in response to heat shock and amino acid analogs.

Key Words: Heat shock protein, Stress, p38 mitogen-activated protein kinase

INTRODUCTION

The heat shock response is an evolutionary conserved mechanism to protect against various environmental stresses. When cells are exposed to stressful conditions such as heat shock, heavy metals, and amino acid analogs, they increase the expression of a specific set of proteins referred to as the heat shock proteins (HSPs)[7,9,15,17]. The HSPs serve mainly as chaperones by facilitating the correct folding, assembly, and intracellular transport of other cellular proteins Increased expression of the HSPs in response to stresses is controlled by the activation of trans-regulatory proteins, the heat shock factor (HSF)[7,9,15~17,21]. HSF1, one of the HSF family (HSF1-4), is known as a major mediator for the heat shock response. Activated HSF1 binds as a homotrimer to a conserved cognate promoter site, the heat shock element (HSE), composed of inverted repeats of the 5-base pair sequence 5'-nGAAn-3' located in the promoters of HSP genes[16,21]. In normal cells, HSF1 exists in both cytoplasm and nucleus, in complex with regulatory proteins including

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*Footnotes: The abbreviations used are: HSP, heat shock protein; HSF, heat shock factor; MAPK, mitogen-activated protein kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EMSA, electromobility shift assay; PKC, protein kinase C; Azc, L-azetidine 2-carboxylic acid

HSP70 and HSP90, which repress the DNA binding activity of HSF1[1,25]. Exposure of cells to proteotoxic stresses results in the appearance of non-native polypeptides and the requirement for molecular chaperones. As a consequence of the release of interacting chaperones, HSF1 monomer homotrimerizes, is hyperphosphorylated, and becomes transcriptionally competent, resulting in an increased synthesis of the HSPs[2,6,11,18-20].

In the HSF1 sequence, there are several putative phosphorylation sites for protein kinases including proline-directed kinases such as extracellular regulated kinase 1/2 (ERK), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and p38 mitogen-activated protein kinase (p38MAPK)[13,14]. Among these protein kinases, glycogen synthase kinase 3β (GSK3β), ERK1/2, and SAPK/JNK/protein kinase C (PKC) have been demonstrated to repress HSF1 by constitutive phosphorylation on serine residues of 303, 307, and 363 in the regulatory domain, respectively[4,5,8,14,22,23]. Upon exposure to proteotoxic stresses, HSF is hyperphosphorylated through an unidentified protein kinase (s). It was suggested that hyperphosphorylation does not play a role in the regulation of HSF1 trimerization and DNA binding activity but involves in a subsequent step, the acquisition of transactivation function, in the activation of the factor[24]. In this study, we tried to identify the protein kinase (s) that is involved in the HSF1 activation by stresses. Here we provide evidences that p38 MAPK may play a positive role (s) in the HSF1 activation in response to heat shock and amino acid analogs.

MATERIALS AND METHODS

1. Cell culture and heat shock treatment

NIH3T3 mouse embryo fibroblast cells and HeLa human adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC, CRL-1658 and CRL-2, respectively) and grown in Dulbecco Modified Eagle's Medium (Life Technologies, Inc.) supplemented with 1% penicillin-streptomycin (Life Technologies, Inc.) and 10% (v/v) fetal bovine serum (Life Technologies, Inc.) at 37℃ in a humidified incubator with 5% CO_2. For heat shock treatment, the cells were plated and stabilized for 24 hours at 37℃ and then exposed to heat shock at 39~45℃ for various times and recovered at 37℃ for several hours as it was required.

2. Gel electrophoresis and Western blotting

Cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed in RIPA (+) (0.1% SDS, 1.0% Triton, 1.0% Deoxycholate, 0.1% NaN_3 in PBS) containing 5 mM DTT, 1% protease inhibitor cocktail (Sigma), and 1 mM PMSF at 4℃ for 15 min. Protein concentrations were determined using the Bio-Rad protein assay method (Bio-Rad). Equal amount of proteins was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resulting gels were either stained with Coomassie Blue or transferred to nitrocellulose membrane (Sigma) or PVDF (Schleicher & Schuell). For Western blotting, the membrane was blocked with 5% skim milk in PBS-T (0.1% tween-20 in PBS) for 1 hour at room temperature on a shaker. After washing the membrane with PBS-T, it was incubated with antibodies to HSF1, HSP72/73 (N27, StressGen), phospho-38MAPK, p38MAPK, phospho-SAPK/JNK, and SAPK/JNK for overnight at 4℃. After washing the membrane with PBS-T three times, it was incubated with 1 : 2000 diluted HRP-conjugated secondary antibody and the antibody-specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Pharmacia Biotech.).

3. Confocal microscopy

NIH3T3 cells were grown on coverslips in multiwell culture plates and exposed to heat shock for indicated times. For immunostaining, the cells were fixed with 3.7% (wt/vol) formaldehyde in PBS for 30 min on ice, and permeabilized with 0.2% Triton X-100 in PBS for 20 min on ice. After blocking with 3% bovine serum albumin containing several drops of horse serum in TBS for 1 hour at room temperature, then the cells were incubated with anti-HSF1 (Neo Markers) antibodies diluted in blocking buffer, overnight at 4℃ or for 4 hours at room temperature, respectively. After washing three times with TBS plus 0.1% Triton X-100 (TBST), the cells were stained with FITC-conjugated secondary antibody for 1 hour and washed five times in TBST and mounted on slide glasses with Crystal Mount. Then the cells were observed under a confocal microscope (LSM510, Carl Zeiss).

4. Electromobility shift assay (EMSA)

Electromobility shift assay was performed according to

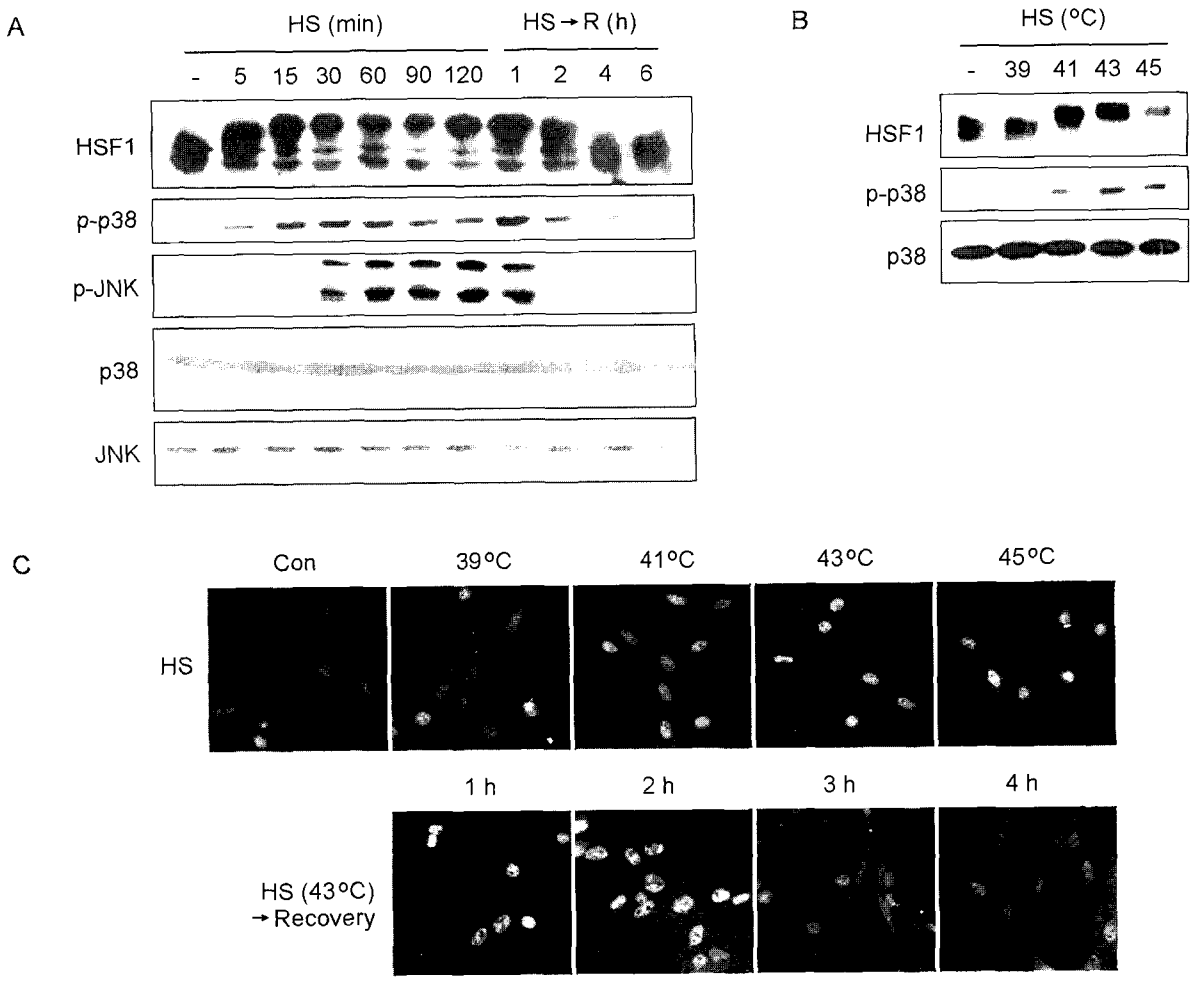

Fig. 1. p38MAPK and SAPK/JNK activation and HSF1 hyperphosphorylation and nuclear translocation by heat shock. **(A)** NIH3T3 cells were exposed to 43℃ for 5, 15, 30, 60, 90 and 120 min or recovered at 37℃ for 1, 2, 4, and 6 h after heat shock of 43℃ for 30 min. The cellular polypeptides were then analyzed by SDS-PAGE and Western blotting with antibodies to HSF1, phosphor-p38MAPK, p38MAPK, phospho-SAPK/JNK, and SAPK/JNK. **(B)** NIH3T3 cells were treated with heat shock of 39, 41, 43 and 45℃ for 30 min and the cellular proteins were subjected to SDS-PAGE and Western blot analysis with antibodies to HSF1, phospho-p38MAPK, and p38MAPK. **(C)** NIH3T3 cells plated on cover-glasses were treated with heat shock of 39, 41, 43 and 45℃ for 30 min or recovered at 37℃ for 1, 2, 3 and 4 h after heat shock of 43℃ for 30 min. The cells were fixed and then permeabilized, and stained with anti-HSF1 antibody and FITC-conjugated secondary antibody. The cells were observed with a confocal microscope (400X, Carl Zeiss, LSM510).

the method previously described[10]. Double stranded oligonucleotides containing the HSE consensus sequence (5'-GATCCTCGAATGTTCGCGAAAAG-3') were labeled using T4 oligonucleotide kinase (Promega) and (α-^{32}P)-ATP (Amersham Pharmacia Biotech., 3000 Ci/mmol, 10 mCi/ml). 15 μg of nuclear proteins was preincubated for 15 min at 4℃ in 19 μl of mixture containing binding buffer (20 mM Tris-HCl pH 7.5, 5% glycerol, 40 mM NaCl, 4 mM $MgSO_4$, 1 mM EDTA, 1 mM DTT, 50 μg/ml BSA), 1 μg of poly dI-dC and 1 μl of protease inhibitor cocktail (Sigma). Thereafter, binding reaction was performed for 40 min at room temperature with 1 μl (>50,000 cpm) of radio-labeled oligonucleotide in a final volume of 20 μl. Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis on a 4% acrylamide (acrylamide/bis-acrylamide, 29 : 1) gel at 30 mA for 1 h. Prior to sample loading, the gel was run for 30 min at 20 mA. After electrophoresis, the gel was dried and exposed to an X-ray film for 12~24 hours at -80℃. Supershift assay was performed in a similar fashion to EMSA. 2 μl of anti-HSF1 antibody was added to nuclear extracts before pre-incubation.

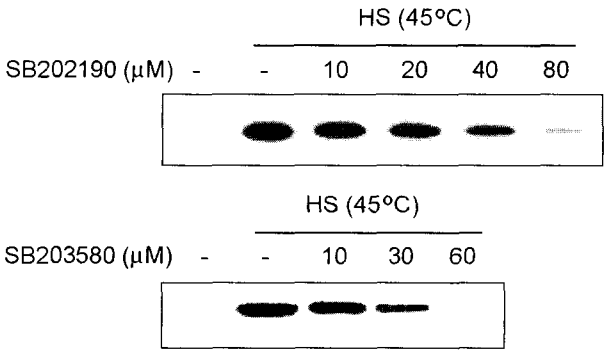

Fig. 2. Inhibition of p38MAPK by SB202190 and SB203580. NIH3T3 cells were pretreated with the p38MAPK inhibitors, SB 202190 and SB203580, for 1 h at various concentrations and then exposed to 45℃ for 30 min. The cellular polypeptides were then analyzed by SDS-PAGE and Western blotting with antibody to phospho-p38MAPK.

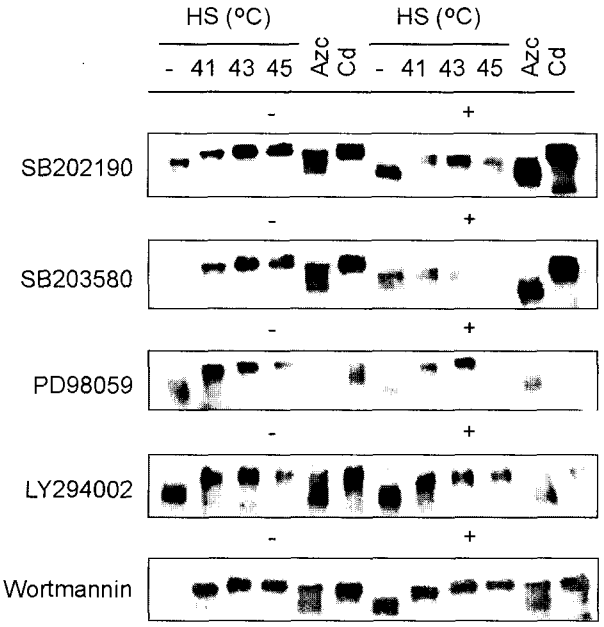

Fig. 3. HSF1 hyperphosphorylation by heat shock and amino acid analogs is partially but significantly inhibited by the p38 MAPK inhibitors, SB202190 and SB203580. NIH3T3 cells were pre-treated with either SB202190 (60 μM), SB203580 (60 μM), PD98059 (30 μM), LY294002 (20 μM), or wortmannin (100 nM) and then exposed to either heat shock (41, 43, 45℃ for 20 min), 5 mM L-azetidine-2-carboxylic acid (Azc, for 6 h) or 100 μM cadmium chloride (Cd, for 2 h). The cellular proteins were analyzed by SDS-PAGE and Western blotting with anti-HSF1 antibody.

RESULTS AND DISCUSSION

1. Profile of p38MAPK activation is similar to those of hyperphosphorylation and nuclear translocation of HSF1 in response to heat shock

As demonstrated previously[1,2,7,16,18,19,21,25], HSF1 was hyperphosphorylated upon exposure to heat shock (Fig. 1A). In addition, treatment of cells with heat shock caused nuclear translocation of HSF1 (Fig. 1C). Xia and Voellmy (1997) suggested that hyperphosphorylation does not play a role in the regulation of HSF1 trimerization and DNA binding activity but involves in a subsequent step, the acquisition of transactivation function, in the activation of the factor. We tried to identify the protein kinases responsible for stress-induced HSF1 hyperphosphorylation and activation. First, we examined alterations in the activities of SAPK/JNK and p38MAPK that are stress-inducible mitogen-activated protein kinase. As shown in Fig. 1, p38 MAPK and SAPK/JNK were activated in response to heat shock. p38MAPK was activated more rapidly than SAPK/JNK and the activation was prolonged. SAPK/JNK was inactivated more rapidly than p38MAPK. Interestingly, profile of p38MAPK activation was very similar to those of hyperphosphorylation and nuclear translocation of HSF1 (Fig. 1).

2. Hyperphosphorylation of HSF1 is inhibited by the p38MAPK inhibitors, SB202190 and SB203580

Since HSF1 possesses the phosphorylation sites for p38 MAPK[13,14] and p38MAPK activation profile is similar to that of hyperphosphorylation of HSF1, we postulated that p38MAPK may play a role (s) in the heat shock-induced HSF1 phosphorylation and activation. To test this, we used the p38MAPK inhibitors, SB202190 and SB203580. First, we examined concentrations of the p38MAPK inhibitors that are able to inhibit heat shock-induced p38MAPK phosphorylation and activation. As shown in Fig. 2, SB202190 and SB203580 inhibited heat shock induced p38MAPK phosphorylation and activation in a dose-dependent manner. Maximal inhibitory effects of SB202190 and SB 203580 were obtained at concentration of 80 μM and 60 μM, respectively. Then, we examined the effects of the p38 MAPK inhibitors on heat shockinduced HSF1 hyperphosphorylation. As shown in Fig. 3, HSF1 hyperphosphorylation in response to heat shock (41~45℃) was partially but significantly inhibited by the p38MAPK inhibitors, SB 202190 and SB203580. In contrast, other protein kinase inhibitors including PD98059 (the MEK1/2 inhibitor), LY 294002 and wortmannin (the PI3-K inhibitors) did not

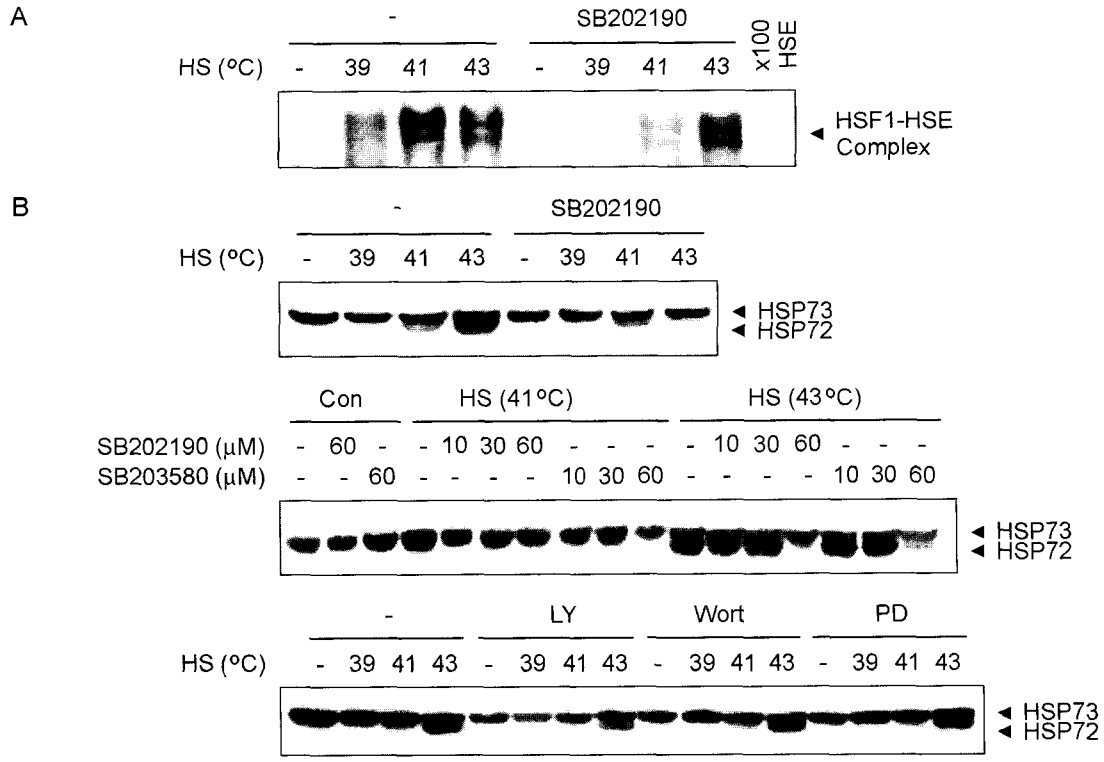

Fig. 4. HSF1 DNA-binding and HSP72 expression in response to heat shock are prevented by the p38MAPK inhibitors, SB202190 and SB203580. **(A)** NIH3T3 cells pre-treated with SB202190 (60 μM for 1 h) and then exposed to heat shock of 39, 41, 43℃ for 20 min. The nuclear extracts were prepared and analyzed by EMSA. The DNA-protein complex was disappeared by 100-fold excess of cold competitor. **(B)** NIH3T3 cells were pre-treated with either SB202190 (10~60 μM), SB203580 (10~60 μM), PD98059 (30 μM), LY294002 (20 μM), or wortmannin (100 nM) for 1 h and then exposed to heat shock of 39, 41, 43℃ for 20 min and recovered at 37℃ for 6 h. The cellular proteins were analyzed by SDS-PAGE and Western blotting with anti-HSP72/73 antibody.

exert inhibitory effects on heat shockinduced HSF1 hyperphosphorylation. Similarly, slight hyperphosphorylation of HSF1 by a proline analog, Azc, was also prevented by the p38MAPK inhibitors, but not by the MEK1/2 inhibitor and the PI3-K inhibitors. Interestingly, cadmium chloride-induced HSF1 hyperphosphorylation was not prevented by the p38MAPK inhibitors, MEK1/2 inhibitor, and PI3-K inhibitors. These results suggest that p38MAPK may be one of the protein kinases that are responsible for HSF1 hyperphosphorylation in response to heat shock and amino acid analogs, but not to heavy metals.

3. Heat shock-induced HSF1-DNA binding and HSP 72 expression is prevented by the p38MAPK inhibitors

To investigate whether phosphorylation of HSF1 by p38 MAPK is linked to the transcriptional activity of HSF1, we examined the effects of SB202190 on the HSF1-DNA binding and HSP expression by heat shock. The HSF1-DNA binding was significantly decreased by pretreatment of SB 202190 (Fig. 4A). The inhibitory effect of SB202190 was more prominent in cells exposed to heat shock of 39℃ and 41℃ than 43℃. Thus, p38 kinase-mediated phosphorylation of HSF1 is likely to contribute to more efficient DNA-binding capacity. In addition, heat shock-induced HSP72 synthesis was also prevented by the p38MAPK inhibitors, SB202190 and 203580, but not by the MEK1/2 inhibitor PD98059 and the PI3-K inhibitors, LY294002 and wortmannin (Fig. 4B). These results suggest that p38 MAPK-mediated phosphorylation of HSF1 may potentiate a cognate DNA-binding and subsequently transactivation capacity. p38MAPK has been shown to be implicated in the expression of BiP/GRP78, an endoplasmic reticulum resident molecular chaperone and which is related to cytosolic HSP72/73[3]. In addition, p38MAPK is known to confer cadmium-induced HSP70 expression in 9L rat brain tumor cells[12]. Since heat shock-induced HSF1 hyperphosphorylation is partially prevented by the p38MAPK inhibitors, other protein kinase (s) is likely to also participate

in HSF1 hyperphosphorylation. In addition, cadmium chloride-induced HSF1 hyperphosphorylation was not prevented by the p38MAPK inhibitors. Therefore, it will of interest to characterize other protein kinase (s) involved in the stress-induced HSF1 hyperphosphorylation/activation and HSP expression.

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