THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 25, pp. 17220–17227, June 23, 2006
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Heat Shock Protein 25 or Inducible Heat Shock Protein 70 Activates Heat Shock Factor 1

DEPHOSPHORYLATION ON SERINE 307 THROUGH INHIBITION OF ERK1/2 PHOSPHORYLATION[*][S]

Received for publication, January 4, 2006, and in revised form, April 18, 2006 Published, JBC Papers in Press, April 19, 2006, DOI 10.1074/jbc.M600062200

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The expression of heat shock proteins (HSPs) is known to be increased via activation of heat shock factor 1 (HSF1), and excess expression of HSPs exerts feedback inhibition of HSF1. However, the molecular mechanism to modulate such relationships between HSPs and HSF1 is not clear. In the present study, we show that stable transfection of either *Hsp25* or inducible *Hsp70* (*Hsp70i*) increased expression of endogenous HSPs such as HSP25 and HSP70i through HSF1 activation. However, these phenomena were abolished when the dominant negative *Hsf1* mutant was transfected to HSP25 or HSP70i overexpressed cells. Moreover, the increased HSF1 activity by either HSP25 or HSP70i was found to result from dephosphorylation of HSF1 on serine 307 that increased the stability of HSF1. Either HSP25 or HSP70i inhibited ERK1/2 phosphorylation because of increased MKP1 phosphorylation by direct interaction of these HSPs with MKP1. Treatment of HOS and NCI-H358 cells, which showed high expressions of endogenous HSF1, with small interfering RNA (*siRNA*) of either *HSP27* (*siHSP27*) or *HSP70i* (*siHSP70i*) inhibited both HSP27 and HSP70i proteins; this was because of increased ERK1/2 phosphorylation and serine phosphorylation of HSF1. The results, therefore, suggested that when the HSF1 protein level was high in cancer cells, excess expression of HSP27 or HSP70i strongly facilitates the expression of HSP proteins through HSF1 activation, resulting in severe radio- or chemoresistance.

The expressions of small HSPs, especially HSP25 (HSP27),[3] and the inducible HSP70 (HSP70i) have been shown to enhance the survival of mammalian cells exposed to various types of stimuli that induce stress and apoptosis (1, 2). Considering the key role of HSPs played in protection against stress-induced damage, it is of great importance to elucidate the regulatory mechanisms responsible for HSP expression.

The inducible HSP expression is regulated by the heat shock transcription factors (HSFs). In response to various inducers such as elevated temperatures, oxidants, heavy metals, and bacterial and viral infections, most HSFs acquire DNA binding activity to the heat shock element (HSE), thereby mediating transcription of the heat shock protein genes, which results in accumulation of HSPs (3, 4). Although several genes encoding multiple HSF isoforms have been identified in vertebrates (5, 6), only HSF1 is shown to be essential for the transcriptional activation of HSPs in mammalian organisms (4, 7). Under normal conditions, cellular HSF1 exists in a transcriptionally repressed state (8, 9), however, it is activated by stress in a multistep process involving trimerization, acquisition of HSE binding activity, novel phosphorylation, and trans-activation of HSP genes (3, 10, 11).

In recent years, a conceptual framework for stress-induced activation and feedback repression of HSF1 has emerged (3, 12, 13). However, our recent study indicated that, in RIF cells that have relatively low HSP25 and inducible HSP70 (HSP70i) expressions, HSP25 or HSP70i mutually coregulated each other and increased their own protein expression through HSF1 activation (14).

Recently, it was shown that ERK1/2 activation by mitogenic stimulation leads to phosphorylation of serines 303 and 307 of HSF1 and consequent association of HSF1 with 14-3-3. 14-3-3 binding inhibits both the transcriptional activity and nuclear accumulation of HSF1 (15). HSF1 seems to exert a reciprocal influence on cell proliferation in that HSP70, a major product of HSF1 transcriptional activity, inhibits the ERK pathway. It is intriguing to note that ERK1/2 is known to be a repressor of HSP synthesis (16), although HSP induction and ERK1/2 activation result in cytoprotection. HSF1 is subjected to additional layers of regulation, including up-regulation through binding the apoptosis modulator DAXX and modulation by ERK1/2 (17, 18). Hierarchical phosphorylation of HSF1 within its transcriptional regulatory domain by ERK1 (on serine 307) and glycogen synthase kinase-3 (GSK3) (on serine 303) was also reported (19, 20).

In this study, we attempted to further elucidate molecular mechanisms governing the relationship between HSPs and HSF1, and found that excess expression of HSP25/27 or HSP70i increased endogenous HSPs such as HSP25/27 or HSP70i through HSF1 activation, which resulted from its dephosphorylation of serine 307. Dephosphorylation of HSF1 on serine 307 was mediated by inhibition of ERK1/2 phosphorylation by direct interaction of MKP1 with HSP25 or HSP70i. Interac-

* This work was supported by Korea Institute of Science & Technology Evaluation and Planning and Ministry of Science & Technology (MOST) and the Korean government, through its National Nuclear Technology Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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[3] The abbreviations used are: HSP, heat shock protein; HSE, heat shock element; HSP70i, inducible heat shock protein 70; HSF, heat shock factor; ERK, extracellular signal-regulated kinase; RIF, radiation-induced fibrosarcoma; TR, thermoresistant clone of RIF; HOS, human osteosarcoma; siRNA, small interfering RNA; PBS, phosphate-buffered saline; HA, hemagglutinin.

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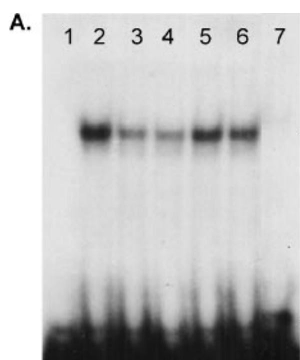
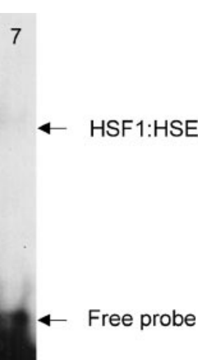
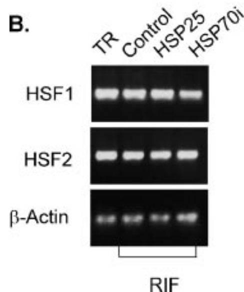
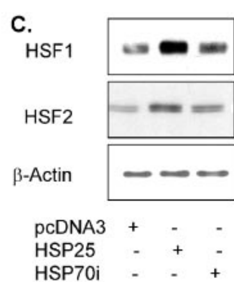
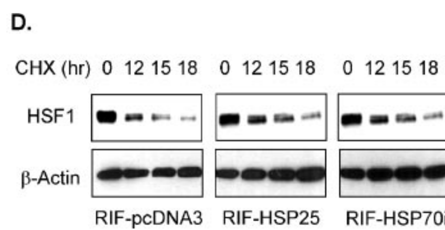
FIGURE 1. **Expressions of endogenous HSPs by overexpression of HSP25 and HSP70i.** *A*, representative Western blot analysis showing the relative levels of HSP25 and HSP70i in clones derived from RIF cells transfected with HSP25 or inducible HSP70 and TR cells, which are thermoresistance clones of RIF cells. *B*, activity of HSP25 or HSP70i promoter driving expression of the luciferase reporter gene in RIF cells, which were transfected with HSP25 or inducible HSP70 and grown in phenol red-less medium containing stripped serum. Luciferase activity was normalized to β-galactosidase transfection, which was included in all cotransfections to control for variation in transfection efficiency. Percentage of the empty pGL3 vector control is set at 100%. Mean $\pm$ S.D. of at least three independent experiments.











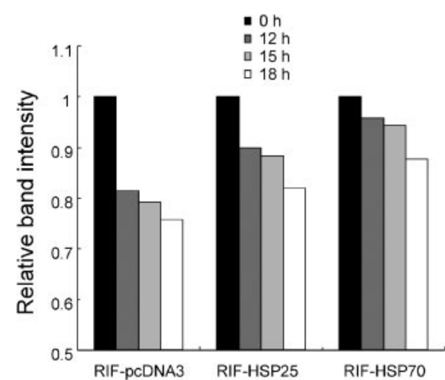


FIGURE 2. **HSF1 activation in HSP25 or HSP70i overexpressed cells.** *A*, gel mobility shift analysis of HSE binding activity in extracts of control and HSP25- or HSP70i-transfected RIF cells as well as TR cells. Nuclear extracts were prepared, and HSF1 DNA binding activity was measured by electrophoretic mobility shift assay using ^{32}P-labeled HSF1 DNA binding fragments, known as HSEs. The position of the HSF1-HSE complex and the free HSE DNA fragments are shown on the *right*. *Lane 1*, probe only; *lane 2*, TR; *lane 3*, RIF; *lane 4*, RIF-pcDNA3; *lane 5*, RIF-HSP25; *lane 6*, RIF-HSP70; *lane 7*, RIF-HSP70 + cold probe. *B*, mRNA expression of control and HSP25- or HSP70i-transfected RIF cells as well as TR cells. Total cellular RNA was extracted, reverse transcribed, and subjected to PCR. The products were electrophoresed on 1% agarose gel and stained with ethidium bromide. *C*, representative Western blot analysis showing the relative levels of HSF1 and HSF2 in control and HSP25- or HSP70i-transfected RIF cells. *D*, protein extracts were prepared at the indicated time points after cycloheximide treatment (20 μg/ml) in control, HSP25-, or HSP70i-overexpressed RIF cells, and Western blot analysis was performed using anti-HSF1 (*upper*). *Relative band intensity* means relative values that were calculated from densitometric scans of immunoblots, and values of control were based as 1 (*lower*). The results represent one of three independent experiments.

tion between MKP1 and HSP25 or HSP70i increased MKP1 phosphorylation, which resulted in ERK1/2 dephosphorylation.

EXPERIMENTAL PROCEDURES

Reagent—Anti-HSP25, anti-HSP70, anti-ERK, anti-phospho-ERK, anti-MKP1, anti-β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-HA was from Cell Signaling Technology (Beverly, MA), anti-HSF1 was from Neo Marker (Fremont, CA), and anti-phosphoserine was from Sigma.

Plasmids—Wild type mouse *Hsp25* and inducible *Hsp70* were cloned into pcDNA3. The mutant construct, *Mkp1* (C258S), was constructed by PCR by using the overlap extension primer. Cysteine was replaced by serine to generate a phosphatase activity-deficient mutant. The mutants *Hsf1*-S307A and *Hsf1*-S307D were constructed using the method as described above.

Cell Culture—RIF (radiation-induced fibrosarcoma), TR (thermoresistant clone of RIF), HOS (human osteosarcoma), and NCI-H358 (human non-small cell lung cancer) cells were cultured in Dulbecco's minimal essential medium and in RPMI 1640 (Invitrogen) supplemented with heat-inactivated 10% fetal bovine serum (Invitrogen) and antibiotics at 37°C in a humidified incubator with 5% CO_2.

Cell Transfection—Pre-designed siRNA for human *HSP25* and inducible *HSP70* (Ambion catalog numbers 19706, 51187, and 16706) and negative control siRNA were purchased from Ambion, Inc. (Austin, TX). The cells were transfected with the siRNAs for 48 h with the use of Lipofectamin™ 2000 (Invitrogen). Transient transfection of all cell types was carried out using Plus Lipofectamine™ reagent (Invitrogen) and Lipofectamine™ reagent.

Immunofluorescence Analysis—For immunofluorescence analysis, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and then washed three times with PBS. Cells were then incubated with anti-HSP25/HSP70 and anti-HA diluted 1:200 in PBS with 5% fetal bovine serum for 1 h at room temperature in a humidified chamber. Excess antibody was removed by washing coverslips three times with PBS. Cells were then incubated with fluorescein isothiocyanate-conjugated secondary antibody (Dako, Produktionsvej, Denmark) at 1:200 dilution in PBS with 5% fetal bovine serum for 4 h, and then incubated with 0.5 μg/ml propidium iodide (Molecular Probes, Inc., Eugene, OR) for 5 min at room temperature. After washing three times with PBS, coverslips were mounted onto microscope slides using ProLong antifade mounting reagent (Molecular Probes). The slides were examined by a confocal laser scanning microscope (Leica Microsystems).

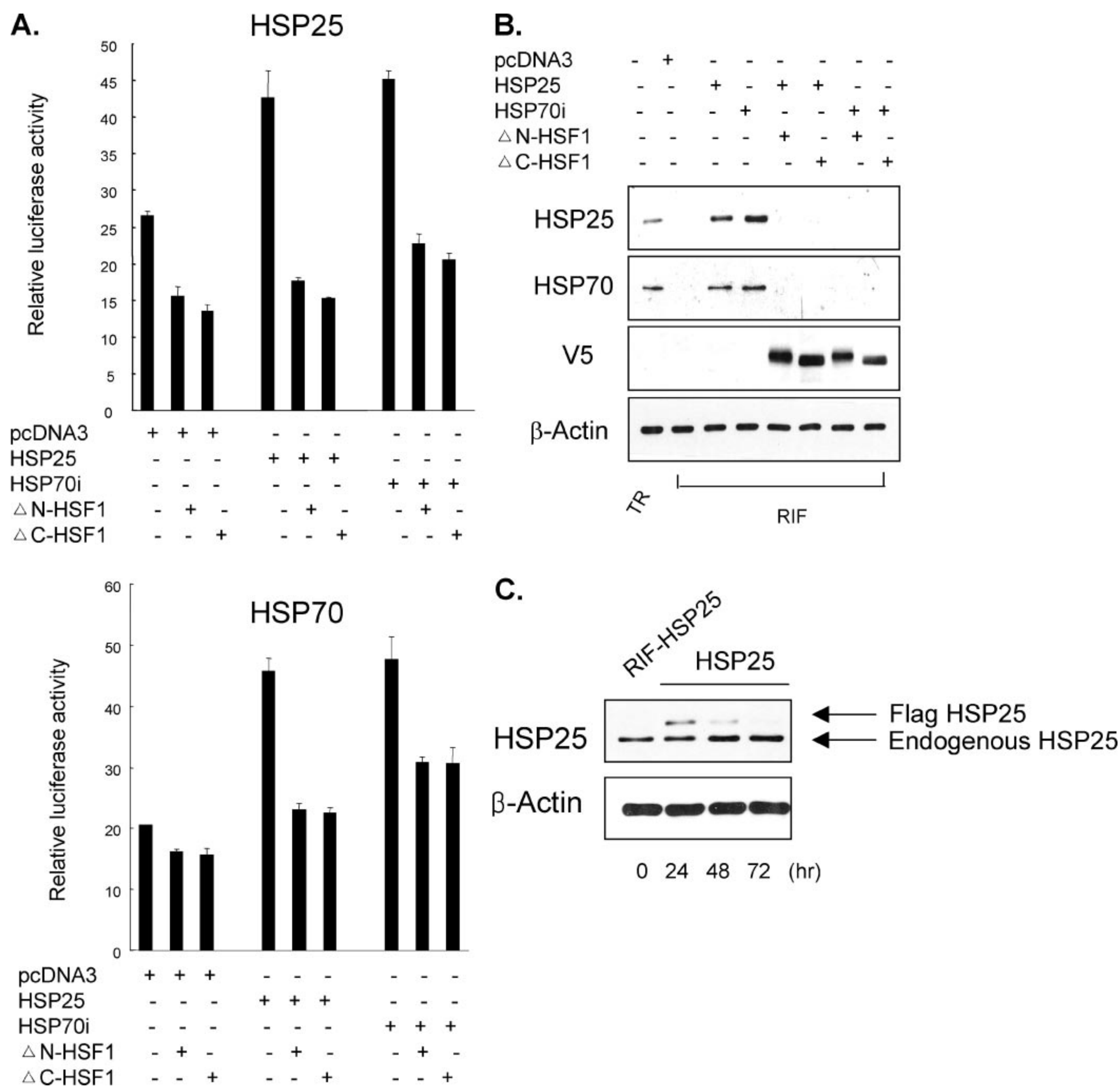


FIGURE 3. **Effects of mutant forms of HSF1 on endogenous expression of HSPs in HSP25 or HSP70i overexpressed cells.** *A*, activity of HSP25 (*upper*) or HSP70i (*lower*) promoter driving expression of the luciferase reporter gene in RIF cells transfected with HSP25 or inducible HSP70 with or without cotransfection of N-terminal deletion (Δ*N*) or C-terminal deletion (Δ*C*) mutants of HSF1, grown in phenol red-less medium containing stripped serum. Luciferase activity was normalized to the β-galactosidase transfection, which was included in all cotransfections to control for variation in transfection efficiency. Percentage of the empty pGL3 vector control is set at 100%. Mean ± S.D. of at least three independent experiments. *B*, representative Western blot analysis showing the relative levels of HSP25 and HSP70i in control and HSP25- or HSP70i-transfected RIF cells, with or without cotransfection of V5 tagged-N-terminal deletion (Δ*N*) or -C-terminal deletion (Δ*C*) mutants of HSF1. *C*, Western blot analysis of HSP25 protein level in the extracts at 24, 48, and 72 h of transient transfection of FLAG-tagged HSP25 vector.

Polyacrylamide Gel Electrophoresis and Western Blot—For PAGE and Western blots, cells were solubilized with lysis buffer (120 mM NaCl, 40 mM Tris (pH 8.0), 0.1% Nonidet P-40), the samples were boiled for 5 min, and an equal amount of protein (40 μg/well) was analyzed on 10% SDS-PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and processed for immunoblotting. Blots were further incubated with horseradish peroxidase-conjugated secondary antibody diluted at 1:5,000, and specific bands were visualized by chemiluminescence (ECL, Amersham Biosciences). Autoradiographs were recorded onto X-Omat AR films (Eastman Kodak Co.).

Immunoprecipitation—Cells (1×10^7) were lysed in immunoprecipitation buffer (50 mM HEPES (pH 7.6), 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40). After centrifugation (10 min at 15,000 × *g*) to remove particulate material, the supernatant was incubated at 4 °C with antibodies (1:100) against anti-HSF1 or MKP1 with constant agitation. The immunocomplexes were precipitated with protein A-Sepharose (Sigma) and analyzed by SDS-polyacrylamide gel electrophoresis using enhanced chemiluminescence detection (Amersham Biosciences).

Reporter Assay—Cells were plated at about 8×10^4 cells per 35-mm dish and cultured for 24 h before transfection. After incubation, cells

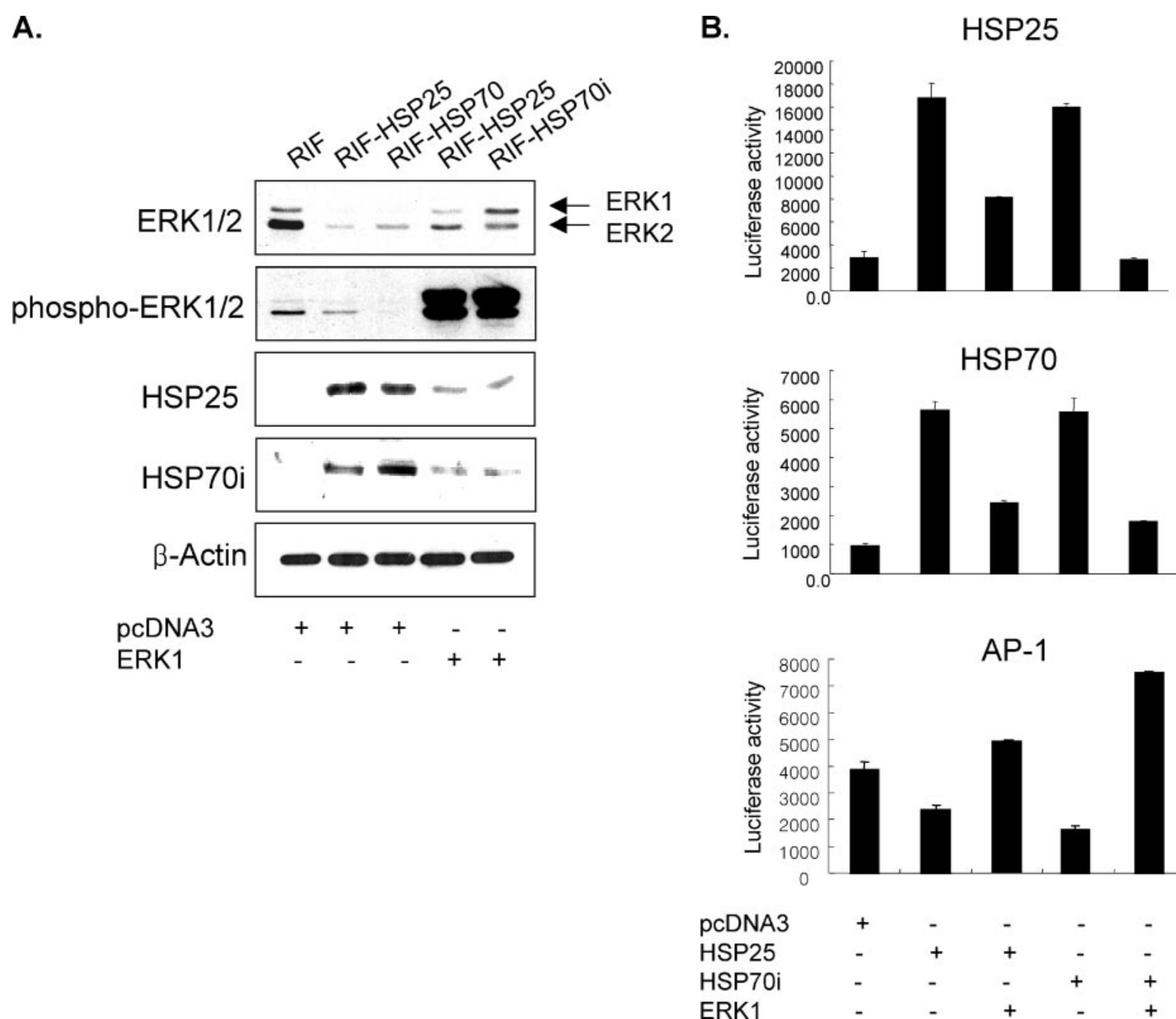


FIGURE 4. **Inhibition of ERK1/2 phosphorylation by HSP25 and HSP70i overexpression.** *A*, representative Western blot analysis showing the relative levels of phospho-ERK1/2 in control and HSP25- or HSP70i-transfected RIF cells, with or without cotransfection of ERK1 expression vectors. *B*, activity of HSP25 (*upper*), HSP70i (*middle*), or AP-1 promoter (*lower*) driving expression of the luciferase reporter gene in RIF cells, which were transfected with HSP25 or inducible HSP70 with or without cotransfection of ERK1, and grown in phenol red-less medium containing stripped serum. Luciferase activity was normalized to the β-galactosidase transfection, which was included in all cotransfections to control for variation in transfection efficiency. Percentage of the empty pGL3 vector control is set at 100%. Mean ± S.D. of at least three independent experiments.

were transfected with 200 ng of β-galactosidase expression vector pSV110 and 200 ng of reporter gene, along with HSP25, inducible HSP70, and AP-1 expression vector. Cells were harvested 48 h later, luciferase activity was assayed, and results were normalized to the β-galactosidase expression (Promega). All the results represent the mean of three independent experiments.

Electrophoretic Mobility Shift Assay—Two complementary single-strand DNA oligonucleotide, each 36 bases long and containing the HSE oligonucleotide, were annealed and used in the electrophoretic mobility shift DNA binding assay. The DAN sequences were 5′-GATCCTCGAAGGTTCGAGGATCCTCGAAGGTTCGAG-3′ and 3′-GAGCTTCCAAGCTCCTAGGAGCTTCCAAGCTCCTAG-5′. The HSE was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, and 5 μg of nuclear extract was incubated with the [^{32}P]HSE at room temperature for 30 min. Sequentially the HSF1-HSE complex was separated from unbound HSE on 6% Tris-borate-EDTA gel. The gel was dried, and the autoradiogram was taken.

RESULTS

Overexpression of HSP25 and HSP70i Facilitated Expressions of Endogenous HSPs Such as HSP25 and HSP70i in RIF Cells—When pcDNA3 vectors carrying *Hsp25* or *Hsp70i* were transfected to RIF cells and stable cell lines were obtained, either HSP25 or HSP70i overexpressed cells were found to facilitate mutual expressions of the corresponding HSPs as well as their own protein expressions comparable with that of TR cells that are thermoresistant RIF cells, and showed high expressions of HSP25 and HSP70i (14) (Fig. 1*A*). We examined 2 or 3 clones and similar phenomena were obtained (supplemental Fig. 1). The reporter assay for *Hsp25* and *Hsp70i* also indicated that the promoter activity for both *Hsp25* and *Hsp70i* were significantly activated by HSP25 or HSP70i overexpression (Fig. 1*B*), suggesting that excess expressions of HSP25 and HSP70i increased promoter activity of both *Hsp25* and *Hsp70i*.

HSF1 Activation Was Observed in HSP25 or HSP70i Overexpressed Cells—Because HSF1 is known to bind to HSE to promote HSP transcription (3), nuclear fractions were incubated with ^{32}P-labeled HSE, and the HSF1-HSE complex was subjected to polyacrylamide gel electrophoresis. As seen in Fig. 2*A*, the amount of HSF1-HSE complex was found to have significantly increased in both *Hsp25*- and *Hsp70i*-transfected cells. TR cells showed strong activation of HSF1, and stable transfection of *Hsp25* or *Hsp70i* also showed increased HSF1 activation. Moreover, binding of HSF1-HSP70i in the cytosol of RIF cells was abundant, however, no binding activity was shown in NIH3T3 cells (supplemental Fig. 2). Reverse transcriptase-PCR analysis for HSF1 and HSF2

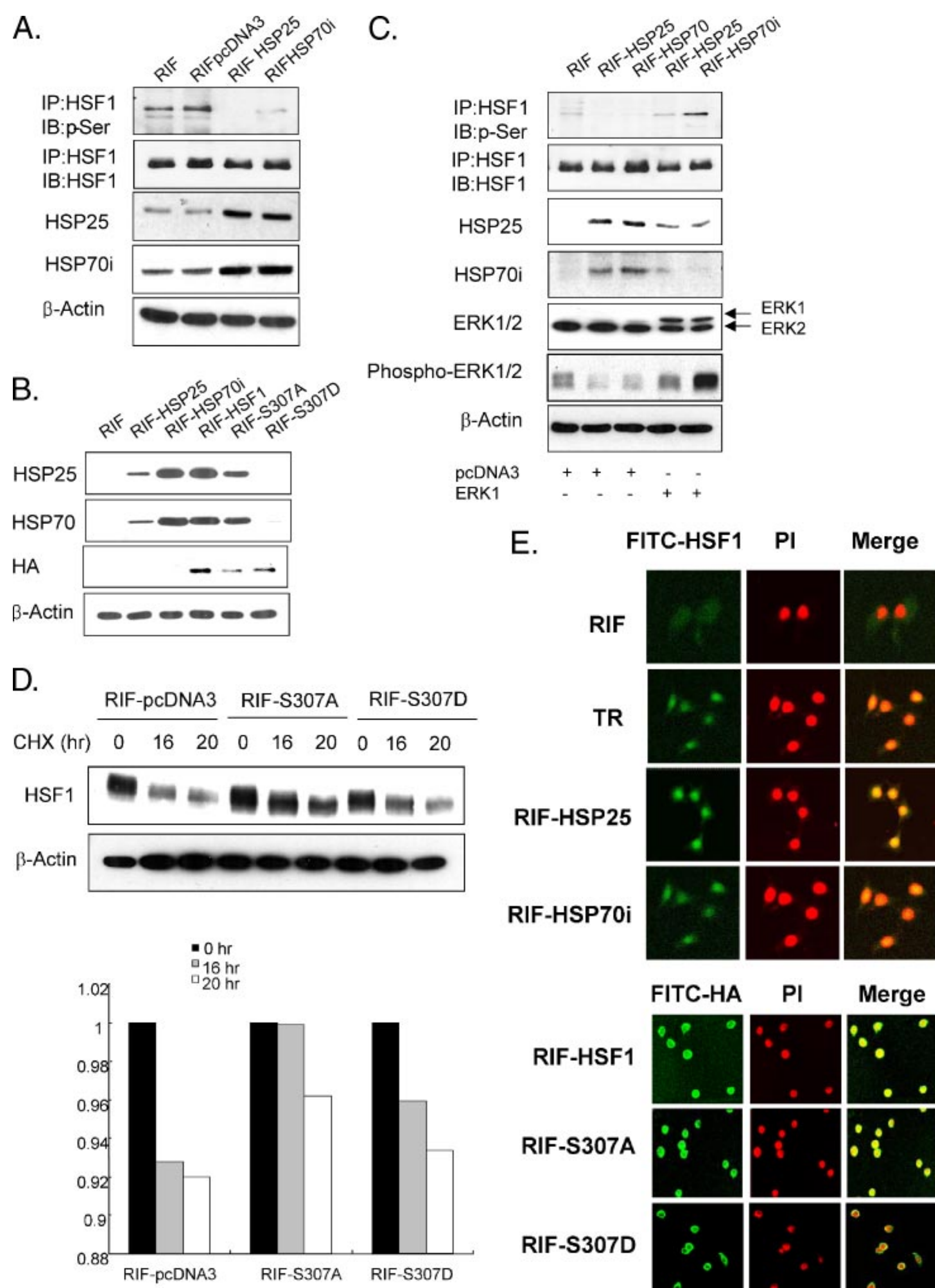


FIGURE 5. **Dephosphorylation of HSF1 on serine 307 by HSP25 or HSP70i overexpression.** *A*, representative immunodetection of phosphoserine (*p-Ser*) and HSF1 after immunoprecipitation with anti-HSF1 antibody in lysates of RIF cells transfected with control, HSP25, and HSP70i vectors (*A*). *B*, representative Western blot analysis showing the relative levels of HSP25 and HSP70i in control and HSP25- or HSP70i-transfected RIF cells, with or without cotransfection of HA-tagged wild type HSF1 and point mutants of HSF1 (S307A and S307D). *C*, immunodetection of phosphoserine and HSF1 after immunoprecipitation with anti-HSF1 antibody in lysates of RIF cells transfected with control, HSP25, and HSP70i vectors, with or without cotransfection of ERK1 expression vectors. *D*, protein extracts were prepared at the indicated time points after cycloheximide treatment (20 μg/ml) of RIF cells transfected with pcDNA3 and point mutants of HSF1 (S307A and S307D), and Western blot analysis was performed using anti-HSF1 (*upper*). Relative band density was calculated and presented as a histogram (*lower*). *E*, localization changes of HSF1 in control, HSP25- and HSP70i-overexpressed RIF cells (*upper*) or HA-tagged HSF1 in HSF1 wild type, HSF1-S307A, and HSF-S307D mutants transfected RIF cells. Cells were fixed with formaldehyde and immunostained with either anti-HSF1 or anti-HA antibodies. The results shown are representative of two independent experiments. *FITC*, fluorescein isothiocyanate; *CHX*, cycloheximide; *PI*, phosphoinositol; *IB*, immunoblot.

revealed that the amounts of these genes were not significantly different by HSP25 or HSP70i overexpression (Fig. 2*B*). However, a greater amount of HSF1 and HSF2 proteins was found in HSP25 or HSP70i overexpressed cells, suggesting post-translational regulation of HSF1 by HSP25 or HSP70i (Fig. 2*C*). Determination of the half-life of HSF1 also indicated that HSP25 or HSP70i overexpression increased HSF1 stability (Fig. 2*D*).

Mutant Forms of Hsf1 Abolished the Enhanced Expression of HSPs by HSP25 or HSP70i—To examine whether HSF1 activation by ectopic expression of HSP25 or HSP70i was responsible for the increased expression of endogenous HSPs, C and N terminus deletion mutants of *Hsf1* were transiently transfected to the stably transfected cells of *Hsp25* or *Hsp70i*. Fig. 3*A* shows that increased promoter activity of both *Hsp25* and *Hsp70i* was dramatically inhibited by additional transfection of deletion mutants of *Hsf1*. Western blot analysis also indicated that transient transfection of C terminus and N terminus deletion mutants of *Hsf1* to either HSP25 or HSP70i overexpressed cells did not induce any endogenous HSP25 and HSP70i at all. As shown in Fig. 3*B*, overexpression of HSP25 and HSP70i facilitated endogenous expression of HSPs through HSF1 activation. To elucidate whether ectopic expression of HSP25 or HSP70i can affect endogenous expression of these proteins, transient transfection of HA-tagged *Hsp25* was performed, and HSP25 protein expression was studied at 24, 48, and 72 h after transfection. As shown in Fig. 3*C*, ectopic expression of HA-tagged HSP25 was decreased at 48 h of transfection and could not be detected at 72 h, however, endogenous *Hsp25* expression was increased from 48 h of transfection, which coincided with the decrease of extrinsic HSP25. Therefore, the results indicate that HSP25 or HSP70i affect expression of the corresponding endogenous HSPs through HSF1 activation.

HSP25 and HSP70i Inhibited ERK1/2 Phosphorylation—Because ERK1/2 activity is involved in HSF1 phosphorylation and activation

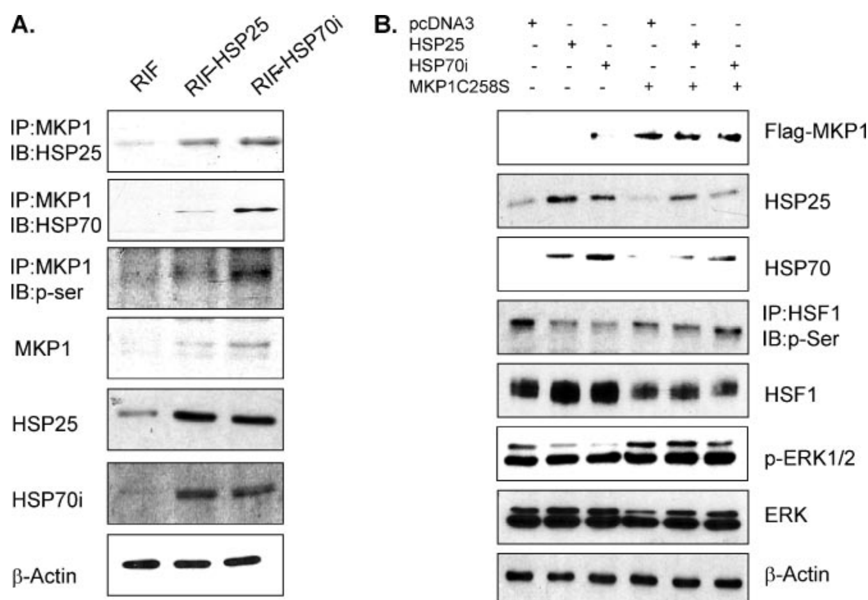


FIGURE 6. **Dephosphorylation of ERK1/2 in HSP25- or HSP70i-overexpressed cells.** Immunodetection of phosphoserine (*p-Ser*), HSP25, and HSP70i after immunoprecipitation with anti-MKP1 antibody in lysates of RIF cells transfected with control, HSP25, and HSP70i vectors (*A*), with or without contransfection with the point mutant form of MKP1 (C258S) (*B*). Western blot analysis was also performed using anti-FLAG, HSP25, HSP70i, HSF1, phospho-HSF1, MKP1, phospho-ERK1/2, and ERK1/2 antibodies. *PI*, phosphoinositol; *IB*, immunoblot.

(21), the status of ERK1/2 phosphorylation was examined. Stable transfection of *Hsp25* or *Hsp70i* inhibited ERK1/2 phosphorylation, and transient transfection of *Erk1* to HSP25- or HSP70i-overexpressed cells abolished this increased expression of endogenous HSPs (Fig. 4*A*). When the reporter assay for *Hsp25* or *Hsp70i* was performed, transient transfection of *Erk1* inhibited promoter activities of *Hsp25* or *Hsp70i* in HSP25- or HSP70i-overexpressed cells. However, inhibited transcriptional activity of AP-1 of one ERK1/2 downstream pathway in overexpressed cells of *Hsp25* or HSP70i was restored or more augmented by *Erk1* transfection (Fig. 4*B*). Therefore, the data strongly indicate that HSP25 or HSP70i overexpression induced dephosphorylation of ERK1/2, which might affect HSF1 phosphorylation.

Serine Phosphorylation of HSF1 Was Reduced by HSP25 or HSP70i Transfection—Because ERK1/2 activity affects serine phosphorylation of HSF1 (21), immunoprecipitation of HSF1 and immunoblotting of phosphoserine were performed. Serine phosphorylation of HSF1 disappeared in *Hsp25*- or *Hsp70i*-transfected RIF cells (Fig. 5*A*). Because ERK1/2 activation and consequent phosphorylation of serine 307 of HSF1 lead to inactivation of HSF1 (19), wild type or phosphorylation-deficient mutants of serine 307 (S307A) of *Hsf1* were transiently transfected to stably overexpressed RIF cells of HSP25 or HSP70i. Expressions of HSP25 and HSP70i also disappeared in the transfectant of the S307D mutant, whereas transfectant of wild type *Hsf1* and S307A expressed these proteins. Therefore, the results indicate that phosphorylation of HSF1 on serine 307 inactivated HSF1, and that HSP25 and HSP70i dephosphorylated HSF1 on serine 307 (Fig. 5*B*). To clarify whether down-regulation of ERK1/2 by HSP25 or HS70i is related to HSF1 phosphorylation, *Erk1* was transiently transfected to HSP25 or HSP70i overexpressed cells. Fig. 5*C* shows that HSF1 phosphorylation was restored and expression of HSP25 or HSP70i was reduced by *Erk1* transfection. In addition, S307A had a longer half-life than that of wild type *Hsf1* and S307D when detected using HA-tagged mutants (Fig. 5*D*). Confocal microscopy also revealed that nuclear expression of HSF1 was more abundant in HSP25 or HSP70i overexpressed cells than that of RIF control cells. Moreover, HA-tagged *Hsf1*-S307A mutant as well as wild type *Hsf1* were located in the nucleus, however, the majority of *Hsf1*-S307D mutant was not present in nucleus; *Hsf1*-S307D was present in the nuclear membrane and did not enter the nucleus (Fig. 5*E*). Therefore, these results indicate that HSP25 or HSP70i inhibited the nuclear translocation of HSF1 via decreased phosphorylation on serine 307, which in turn led to transcriptional activation of HSF1.

Interaction between HSP25 or HSP70i and MKP1 Dephosphorylated ERK1/2—Because heat shock increased expressions of the phosphorylated form of MKP1, which was found to be associated with ERK1/2 and JNK1/2 inactivation (22), we determined MKP1 phosphorylation in HSP25- or HSP70i-overexpressed cells. As seen in Fig. 6*A*, serine-phosphorylated MKP1 expression was increased in HSP25 or HSP70i overexpressed cells, and interaction between HSP25 or HSP70i and MKP1 was also increased. Transient transfection of site-directed mutants of *Mkp1* on cysteine 258 to serine (*Mkp1*-C258S), which abolished phosphatase activity of MKP1 (23), revealed that expressions of HSP25, HSP70i, and HSF1 were reduced by *Mkp1-C258S* transfection, and phosphorylations of both HSF1 and ERK1/2 by HSP25 or HSP70i were restored by this mutant (Fig. 6*B*), even though mutation of *Mkp1* on cysteine 258 did not affect the binding activity with HSP25 or HSP70i (data not shown). The data indicate that the site of cysteine 258 on MKP1 is important for phosphorylation of ERK1/2.

siRNA of HSP27 or HSP70i Treatment Inhibited HSF1 Expression—To unravel the physiological relevance of regulation existing between HSP27 or HSP70i and HSF1, HOS and NCI-H358 cells, which showed increased expressions of HSP27, HSP70i, as well as HSF1 (Fig. 7), were transfected with siRNA of *HSP27* (*siHSP27*) or *HSP70i* (*siHSP70i*). Endogenous expression of HSPs as well as HSF1 expression was found to be dramatically inhibited in *siHSP27* or *siHSP70i*-transfected HOS and NCI-H358 cells (Fig. 7). In addition, inhibited ERK1/2 phosphorylation was restored by these siRNAs, suggesting that HSP27 or HSP70i facilitated HSF1 activation by ERK1/2 dephosphorylation.

DISCUSSION

HSPs are known as the molecular chaperone because of its major role in protein folding (24–27). Because HSF1 induces *Hsp70i* and *Hsp25* gene expression in response to heat stress, it has been suggested that HSPs may function in a negative feedback mechanism to return HSF1 to its inactive monomeric state (12, 13). However, in the present study, ectopic transfection of HSP25 or HSP70i in RIF cells increased the expression of endogenous HSPs, such as HSP25 and HSP70i, through

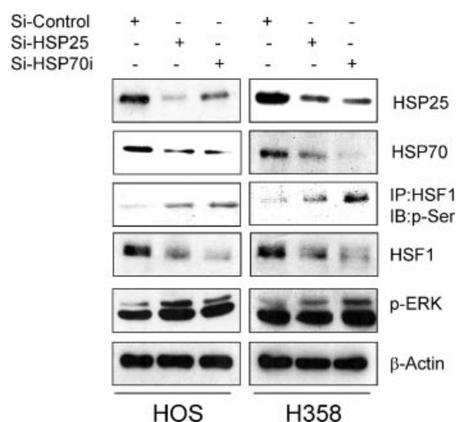


FIGURE 7. **Inhibition of HSF1 by siRNA of HSP27 or HSP70i.** HOS and NCI-H358 (human lung carcinoma cells) were transiently transfected with siRNAs of a control (*Si-Control*), HSP27 (*Si-HSP27*), and HSP70i (*Si-HSP70i*), and protein extracts were prepared and Western blot analysis was performed. *PI*, phosphoinositol; *IB*, immunoblot.

activation of HSF1. NIH3T3 cells or L929 cells, which showed relatively low HSF1 expression did not exhibit such phenomena (supplemental Fig. 3). Therefore, when HSF1 expression was high, such as in RIF, HOS, and NCI-H358 cells, overexpression of HSP25 or HSP70i facilitated more expression of endogenous HSPs, which may result in more severe radio- or chemoresistance.

Overexpression of HSP25 or HSP70i in RIF cells induced transcriptional activation of endogenous HSP25 and HSP70i, which was mediated by HSF1 activation (Figs. 1–3). Moreover, treatment of HOS and NCI-H358 cells, which showed high expression of HSF1 protein, with *Hsp27* or *Hsp70i* siRNA inhibited endogenous expression of HSPs and HSF1 (Fig. 7), suggesting that HSP25 or HSP70i overexpression activated HSF1. A recent study, which derived a similar conclusion to ours, also suggested that cognate HSP70 was required for regulation of HSF1 during heat stress and subsequent target gene expression, and also showed greatly decreased HSF1 activation when transfected with siRNAs targeted to cognate *Hsp70* (28).

Upon activation, HSF1 undergoes several modifications such as trimerization and localization to specific nuclear structures. Independent of monomer to trimer transition, phosphorylation of HSF1 is strongly induced during heat shock. Although the bulk phophorylation level of HSF1 correlates well with its transcriptional activity, phosphorylation of Ser-303, Ser-307, and Ser-363 has been shown to repress the transactivation capacity of HSF1 (19, 20, 29) and phosphorylation of Ser-307 is affected by ERK1/2 activation (21). The result in the present study showed that HSP25 or HSP70i overexpression dephosphorylated HSF1 on serine 307 of HSF1 (Fig. 4). The phosphorylation mimicked mutant form of serine 307 inhibited expression of endogenous HSPs in HSP25 or HSP70i overexpressed cells, suggesting that HSP25 or HSP70i affected the phosphorylation status of HSF1, especially on serine 307. Moreover, the increased half-life and nuclear translocation of the dephophorylated mimicked mutant of serine 307 (Fig. 4) indicated that dephosphorylated HSF1 on serine 307 was the transcriptionally active form of HSF1. ERK1/2 phosphorylation was inhibited in *Hsp25*- or *Hsp70i*-transfected cells (Fig. 5) and ERK1/2 phosphorylation has been reported to be involved in HSF1 phosphorylation on serine 307 (16, 21). ERK1/2 was found to be overexpressed in *Hsp25*- or *Hsp70i*-transfected cells, and transcriptional activation of endogenous HSP25 or HSP70i was abolished, suggesting that HSP25 or HSP70i-mediated ERK1/2 down-regulation may be involved in dephosphorylation of HSF1 on serine 307. Phosphatase involved in ERK1/2 signaling is MKP1. MKP1 is a phosphatase with dual specificity, induced by various stresses, and inactivates ERKs (30). ERK1/2 phosphorylates serine 307 of HSF1 (31). HSP70 has been shown to suppress ERK phosphorylation by direct interaction with MKP1 (32). In the present study, ectopic expression of HSP25 or HSP70i increased MKP1 phosphorylation on the serine residue, resulting in increased binding activity with MKP1 and ERK1/2 dephosphorylation. Our results (Fig. 6) that HSP25 also bound MKP1 is a new finding, suggesting that HSP25 also has a potentiality of MKP1 activation. In addition, the phosphatase activity defective mutant of *Mkp1* (*MKP1-C258S*) restored ERK1/2 phosphorylation and HSF1 expression, suggesting that interaction of MKP1 with HSP25 or HSP70i activated MKP1 phosphorylation, which was correlated with ERK1/2 dephosphorylation. Based on the data obtained, we suggest that overexpression of HSP25 or HSP70i activates HSF1, which is mediated by interaction with MKP1. Interaction of MKP1 with HSP25 or HSP70i activates MKP1 phosphorylation and then ERK1/2 inactivation, resulting in HSF1 dephosphorylation on serine 307. Because HSF1 dephosphorylation facilitated translocation of HSF1 to the nucleus (Fig. 4) (3, 12, 33), it is highly likely that this activated HSF1 enhanced more expression of endogenous HSPs such as HSP25 and HSP70i.

In conclusion, the present data suggest the possibility that overexpression of HSPs does not always show feedback inhibition of HSF1. In certain cancer cells that have HSF1 excessively expressed, overexpression of HSPs facilitated more endogenous HSPs production through HSF1 activation.

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