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ARTICLES

JNK Phosphorylates the HSF1 Transcriptional Activation Domain: Role of JNK in the Regulation of the Heat Shock Response

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Abstract The role of c-Jun NH_2-terminal kinase (JNK) signaling cascade in the stress-inducible phosphorylation of heat shock factor 1 (HSF1) was investigated using known agonists and antagonists of JNK. We showed that treatment of HeLa cells with MG132, a proteasome inhibitor and known JNK activator, caused the transcriptional activation domain of HSF1 to be targeted and phosphorylated by JNK2 in vivo. Dose-response and time course studies of the effects of heat shock and anisomycin treatment showed a close correlation of the activation of JNK and hyperphosphorylation of HSF1. SB203580 inhibited JNK at the 100 μM concentration and significantly reduced the amount of hyperphosphorylated HSF1 upon heat shock or anisomycin treatment. SB203580 and dominant-negative JNK suppress hsp70 promoter-driven reporter gene expression selectively at 45°C but not at 42°C heat stress, suggesting that JNK would be preferentially associated with the protective heat shock response against severe heat stress. The possibility that JNK-mediated phosphorylation of HSF1 may selectively stabilize the HSF1 protein and confers protection to cells under conditions of severe stress is discussed. J. Cell. Biochem. 82: 326–338, 2001.

Key words: SB203580; dominant-negative; MG132; proteasome inhibitor; hsp70

The activation of heat shock gene transcription during the stress response is mediated by a family of heat shock transcription factors (HSF) which binds to heat shock elements in the promoters of heat shock genes. HSF1 is one of three HSF family genes in humans (HSF1, 2, and 4) and it mediates the major cellular stress response. Activation of HSF1 is accomplished by a stress-induced conversion of monomers to trimers, translocation of HSF1 from the cytosol to the nucleus, additional post-translational modifications including phosphorylation of serine residues, and transcriptional competence with enhanced heat shock gene expression [Wu, 1995; Morimoto, 1998; Morimoto and Santoro, 1998]. DNA binding activity and transcriptional competence of HSF1 are regulated by distinct mechanisms and can be uncoupled in certain cell lines and conditions [Jurivich et al., 1992; Bruce et al., 1993; Davidson et al., 1995].

A functional role for constitutive phosphorylation of HSF1 has been reported in which a mitogen-activated protein (MAP) kinase signaling cascade of the ERK family was implicated in the phosphorylation of HSF1 in unstressed cells, which results in repression of hsp70 promoter activation in vivo [Knauf et al., 1996]. In addition, constitutive phosphorylation by glycogen synthase kinase 3 and certain protein kinase C isoforms are also implicated in HSF1 repression in unstressed cells [Chu et al., 1998]. On the other hand, the function of stress-induced HSF1 hyperphosphorylation has been elusive so far because the activation of heat shock gene transcription by mammalian HSF1 can occur in the absence of stress-inducible phosphorylation [Sarge et al., 1993].

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Moreover, the time course of HSF1 activation suggests that an inducible phosphorylation would be a later event that is unlikely to affect other preceding activation processes such as oligomerization, DNA binding, and transcriptional activation [Cotto et al., 1996]. Therefore, the upstream kinase that mediates the inducible phosphorylation of HSF1 and its function in the cellular stress response remains to be identified.

In addition to the HSF1-mediated heat shock response, the JNK-mediated activation of transcription factors such as c-Jun, ATF-2, and Elk-1 serves as an independent stress response in the cell [Minden and Karin, 1997; Ip and Davis, 1998; Davis, 2000]. The signaling cascades for JNK and HSF1 share some common features. They are activated primarily by broad environmental stresses such as heat shock, osmotic shock, amino acid analogues, and toxic chemicals. As a common regulator, Hsp70 and a cellular reducing agent such as glutathione negatively regulate both signaling pathways, suggesting a potential inter-regulation between two stress responses. They also function as a protective response upon stress even though JNK is implicated in apoptosis in certain environmental and cellular conditions.

Previously, we investigated the possible interaction between the JNK and HSF1 stress responses and observed that JNK activation was a consistent indicator for the inducible phosphorylation of HSF1 [Lu et al., 2000; Park and Liu, 2000]. The link between JNK and stress-inducible phosphorylation of HSF1 is further strengthened by the suggestions that anticancer drugs and proteasome inhibitors induce hyperphosphorylation of HSF1 through the JNK pathway [Kim et al., 1999; Pirkkala et al., 2000]. Here, we provide evidence that human HSF1 is a direct in vivo downstream target of a JNK2 isoform which binds and phosphorylates the HSF1 transcriptional activation domain. We also discuss the possible functions of JNK-mediated HSF1 phosphorylation in the heat shock response.

MATERIALS AND METHODS

Materials

The polyclonal rabbit anti-hHSF1 antibody was prepared by immunizing a rabbit with histidine-tagged hHSF1 produced in *E. coli* and purified by affinity chromatography using Ni-NTA resin from Qiagen. ATF2, Protein G-Agarose, and antibodies for ERK1/2 [ERK1 (K-23)-G], JNK1/2 [JNK2 (FL)], phospho-specific JNK1/2 [P-JNK (G-7)], and p38 MAP kinase [p38 (C-20)-G] were purchased from Santa Cruz Biotech. Antibodies for phospho-specific ERK1/2 and p38 MAP kinases were from New England BioLabs. M2 anti-FLAG monoclonal antibody was from Sigma (St. Louis, MO). Histidine-tagged intact HSF1 and truncated histidine-tagged HSF1 mutant, TAD, were produced in *E. coli* and purified through Ni-NTA resin. The plasmids expressing FLAG-tagged dominant-negative JNK, JNK1-APF, and JNK2-APF were of kind gift from Dr. Roger Davis (University of Massachusetts, Worcester, Massachusetts). The use of pHB-CAT/pCMV-CAT and pCMV-βGAL in the chloramphenicol acetyltransferase assay was as described previously [Huang et al., 1994]. All other biochemical and chemicals were of molecular biology or reagent grade.

Expression of HSF1 and the HSF1 Transcriptional Activation Domain in HeLa

A DNA fragment encoding intact HSF1 was inserted into the NdeI and XhoI sites of the pPROEx1 procaryotic expression vector (Life Technology). A mammalian expression vector expressing histidine-tagged HSF1 was constructed by inserting an end-filled RsrII-XhoI fragment from pPROEx1-HSF1 into the pCEP4 mammalian episomal vector. The N-terminal region of intact HSF1 in pPROEx-HSF1 and pCEP4-HSF1, respectively, was deleted by restriction enzyme digestion with EheI and FseI, followed by blunting with T4 DNA polymerase. The DNA fragment containing the C-terminal transactivation domain (TAD) of HSF1 was generated by self-ligation with T4 DNA ligase. The in-frame fusion of the HSF1 C-terminal fragment to a histidine tag was confirmed by DNA sequencing and the affinity of anti-HSF1 antibody to the expressed protein. To express histidine-tagged intact HSF1 and TAD, HeLa cells were transfected at 50–80% confluence in 35 mm tissue culture plates. For each transfection, 2 μg of DNA which has been diluted with 100 μl of antibiotic- and serum-free DMEM were mixed with 6 μl of lipofectamine reagent in an equal volume of DMEM. After incubating the lipofectamine-DNA mixture with cells for 6 h, the medium was replaced with fresh growth medium and further incubated for 24 h.

Cell Culture, Preparation of Whole Cell and S-100 Cytoplasmic Cell Extracts, and In Vitro Activation of HSF1

Human HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum at 37°C. Unless indicated otherwise, the condition of heat shock was 30 min at 45°C. Cells were harvested by removal of the incubation medium, rinsing the cells twice with phosphate-buffered saline (100 mM NaCl, 100 mM sodium phosphate, pH 7.5), scraping the cells off the substratum with a rubber policeman, and pelleting the cells by centrifugation at 2,000*g* for 2 min at room temperature. For preparation of whole cell extract, the cell pellet was rapidly frozen in liquid nitrogen, thawed at room temperature for 5 min, and then resuspended in 100 μl of buffer C containing 20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM EDTA, and a cocktail of protease and phosphatase inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μM each of leupeptin, pepstatin, and aprotinin, 20 nM okadaic acid, 1 mM sodium vanadate, and 40 mM NaF]. The cell suspension was incubated on ice for 20 min, centrifuged at 10,000*g* for 10 min at 4°C, and the supernatant fraction was used as the whole cell extract. For preparation of the cytoplasmic S-100 extracts, the cell pellet was rinsed once with 1 ml of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF), pelleted by centrifugation, and resuspended in five packed cell volumes (PCV) of buffer A. After a 10 min incubation on ice, cells were re-pelleted by centrifugation, resuspended in 2X PCV of buffer A and then lysed by homogenization (10 strokes) in an all-glass Dounce homogenizer (B type pestle). The cell homogenate was centrifuged at 3,500*g* for 10 min at 4°C, and the supernatant was removed and used as the cytosol extract. The cytosol extract was mixed with 0.11 volumes of buffer B (0.3 mM HEPES (pH 7.9 at 4°C), 1.4 mM KCl, and 30 mM $MgCl_2$), and centrifuged for 60 min at 200,000*g* at 4°C. The supernatant was dialyzed for 5–8 h against 50 volumes of buffer D (20 mM HEPES (pH 7.9 at 4°C), 25% (v/v) glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT), and centrifuged at 12,000*g* for 20 min at 4°C. In vitro activation of HSF1 was done essentially as described previously [Mosser et al., 1990]. Briefly, S-100 cell extracts were incubated at 43°C for 1 h or at 37°C with 2% Nonidet P-40.

Analysis of HSF DNA-Binding Activity by the Electrophoretic Mobility Shift Assay

The sequence of the HSE oligonucleotide and the electrophoretic mobility shift assay procedure used to determine HSF binding to the HSE were as described previously [Choi et al., 1990]. Briefly, a reaction was carried out using 30 μg of cell extract protein, 0.5 μg of poly (dI-dC)-poly(dI-dC), and 250 pg of ^{32}P-labeled HSE in a total reaction volume of 20 μl. After a 25 min incubation at room temperature, the reaction mixtures were subjected to analysis in a low ionic strength 4% polyacrylamide gel. HSE binding activity was quantified either by scanning the autoradiogram with a densitometer or by excising an appropriate portion of the gel and counting by liquid scintillation spectrometry.

Immune Complex Protein Kinase Assay

Cells were incubated with 1 ml of ice cold gentle lysis buffer containing protease and phosphatase inhibitors (10 mM NaCl, 20 mM PIPES (pH 7.0), 0.5% NP-40, 0.05% 2-mercaptoethanol, 5 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 20 μg/ml of leupeptin and aprotinin, and 40 mM NaF) for 5 min on ice and cell homogenate was prepared by three cycles of repeated freezing and thawing. Aliquots containing 0.5 mg protein were immunoprecipitated with phospho-specific anti-JNK1/2 or p38 kinase antibodies for 3 h at 4°C. Immunoprecipitates were pooled by adding 20 μl of Protein G-Agarose for 1 h at 4°C, and collected by centrifugation at 1,000*g* for 5 min at 4°C. Pellets were washed four times with gentle lysis buffer and one time with kinase buffer (50 mM HEPES (pH 7.5), 0.1 mM EDTA, 0.1 mg/ml BSA, 0.1% 2-mercaptoethanol, and 0.15 M NaCl). After suspending pellet in 20 μl kinase buffer, 1 μg of ATF2, intact HSF1 or TAD-HSF1 was added and the kinase reaction was performed by adding 10 μl of an ATP mix (9.3 μl kinase buffer, 0.06 μl 50 mM ATP, pH 7.0, 0.2 μl 2.0 M $MgCl_2$, and 0.44 μl γ-^{32}P ATP (10 mCi/ml)) for 20 min at 30°C. The reaction was terminated by adding SDS-PAGE sample buffer and analyzed by a SDS-PAGE. Gels were fixed, dried and autoradiographed at −70°C.

DNA Transfection and Chloramphenicol Acetyltransferase Assay

HeLa cells were transfected with Lipofectamine-DNA mixtures (0.25 μg of each pHB-CAT/pCMV-CAT and pCMV-βGAL) according to the manufacturer's recommendation (Gibco BRL). After 24 h of transfection, heat-induced expression of pHBCAT/pCMV-CAT was tested by shifting cells from a 37 to 42°C incubator for 1 h or 45°C incubator for 30 min, and then further incubated for 6 h at 37°C. Cells were washed twice with phosphate-buffered saline, detached and pelleted by centrifugation at 3,000*g*. Extracts were prepared by lysing the cell pellet in 150 μl of 250 mM Tris, pH 8.0, and cleared by centrifugation at 16,000*g* for 30 min. Aliquots of the 16,000*g* supernatant fraction containing 10 μg protein were assayed for a β-galactosidase activity. Then, aliquots of cell extracts normalized by β-galactosidase activities were used to assay for chloramphenicol acetyltransferase activity using 0.2 μCi ^{14}C-chloramphenicol at 37°C for 30 min. The relative percent of ^{14}C-chloramphenicol acetylated was determined by densitometric scanning of the autoradiogram.

RESULTS

JNK Binds to and Phosphorylates the HSF1 Transcriptional Activation Domain

Because JNK phosphorylates the transcriptional activation domain of its downstream transcription factors including c-Jun, ATF-2, and Elk-1 [Minden and Karin, 1997; Ip and Davis, 1998], we investigated if potential phosphorylation sites of JNK exist in the transcriptional activation domain of human HSF1. We constructed a truncated HSF1 mutant, TAD, consisting of the trimerization suppressor domain and two consecutive transcriptional activation domains (Fig. 1A). Notably, TAD does not retain the constitutive HSF1 phosphorylation sites, serine 303 and 307 [Kline and Morimoto, 1997]. The wildtype histidine-tagged HSF1 and truncated histidine-tagged TAD were purified through Ni-NTA column and subjected to an immune complex kinase assay with activated JNK induced by anisomycin (Fig. 1B). JNK can efficiently phosphorylate the truncated mutant of HSF1 in vitro, suggesting that TAD serves as an efficient JNK substrate, comparable to the genuine JNK substrate ATF2.

Transfection of the TAD construct into HeLa cells revealed a low expression level, suggesting that the expressed protein might not be stable in the cell. The proteasome inhibitor MG132 is known to activate JNK as well as induce HSF1 activation, including inducible phosphorylation and transcriptional activation [Pirkkala et al., 2000]. To investigate the involvement of JNK in MG132-induced hyperphosphorylation of HSF1, TAD was transfected into HeLa cells with or without MG132 and various JNK constructs (Fig. 1C). MG132 caused a mobility shift of TAD into a slower migrating band, suggesting that TAD is inducibly phosphorylated upon JNK activation and that this is further enhanced by heat shock or anisomycin treatment (Fig. 1C, lanes 4 and 5). The involvement of JNK in the inducible phosphorylation of HSF1 is clearly demonstrated by the fact that a JNK dominant negative mutant, JNK2-APF, completely blocked the MG132-induced TAD mobility shift (Fig. 1C, lane 7). In contrast, co-transfection of the dominant negative JNK1-APF and TAD did not show any significant difference compared with the control, suggesting that JNK2 is more likely to play a role in HSF1 phosphorylation. However, JNK1-APF is expressed at a lower level than JNK2-APF (see Fig. 1D), so it remains to be determined if JNK2 has an independent role from JNK1 in HSF1-inducible phosphorylation.

Because most JNK substrates have demonstrated a stable association with JNK regardless of its kinase activity, we assayed for co-immunoprecipitation of JNK-APF and HSF1 in HeLa cells. We were unable to detect a stable complex between intact wildtype HSF1 and JNK in an in vivo co-immunoprecipitation assay or using an in vitro binding assay with purified HSF1 (data not shown). However, co-expression of TAD and JNK2-APF results in coprecipitation of the two proteins and the inhibition of inducible TAD phosphorylation, suggesting that the open exposure of the HSF1 transcriptional activation domain without its targeted JNK phosphorylation facilitates JNK2 binding (Fig. 1D, lane 4). Because of the lower expression of JNK1-APF and a non-specific signal around the position of JNK1-APF on the fluorogram, it is not clear if JNK1 could bind to TAD to the same extent as JNK2.

The Activation Profile of JNK Correlates With Inducible HSF1 Phosphorylation

Activation of the three known MAP kinases by increasing concentrations of anisomycin was

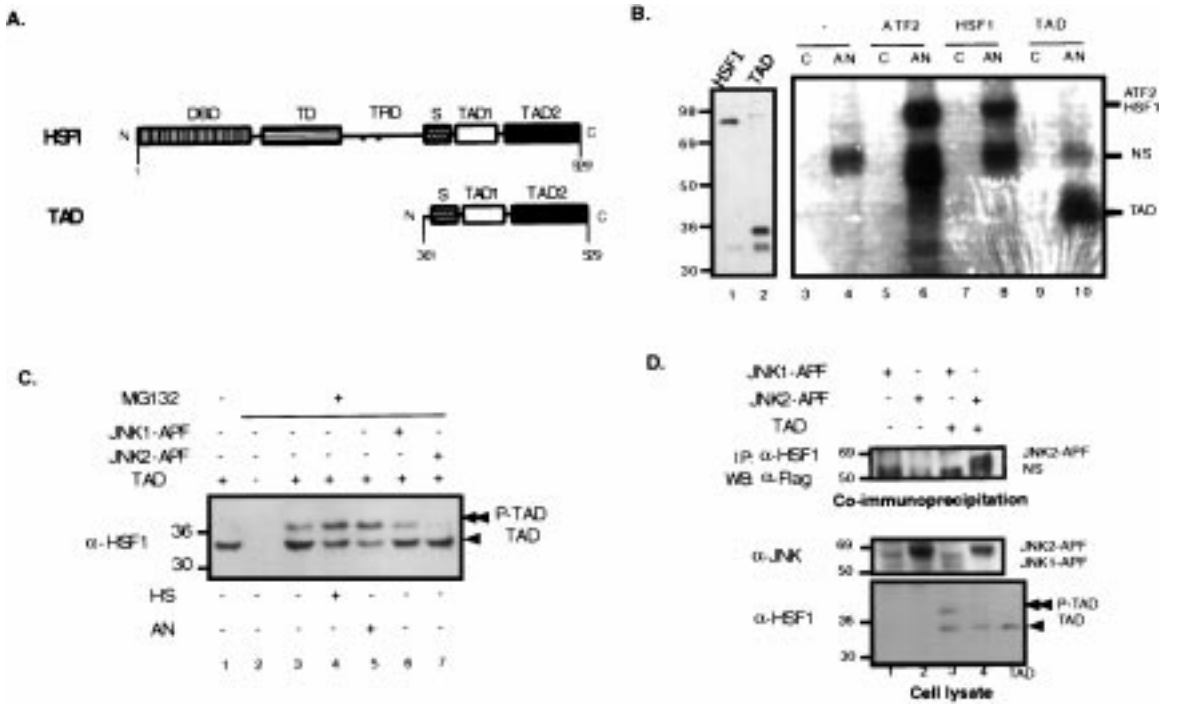

Fig. 1. JNK binds to and phosphorylates the transcriptional activation domain of HSF1. **A:** Schematic presentation of wild-type histidine-tagged- HSF1 (1–529) and the truncated histidine-tagged HSF1 mutant, TAD (361–529). Constitutive phosphorylation sites serine 303 and 307 are indicated by *. The conserved domains of HSF1 are indicated as follows: DBD, DNA-binding domain; TD, trimerization domain; TRD, transcriptional regulatory domain; S, suppressor of trimerization; TDA, Transactivation domain [TAD1; TAD 1; TAD2, TAD 2.] **B:** JNK phosphorylates the HSF1 transcriptional activation domain in vitro. HeLa cells were serum-starved for 12 h (C, Control) or further treated with anisomycin (1 μg/ml) for 1 h at 37°C at the end of starvation (AN, Anisomycin). Cell extracts were prepared and subjected to immunoprecipitation using phospho-specific JNK antibody as described in Methods. One microgram of ATF2, wild-type HSF1 or TAD was added to the in vitro kinase reaction at 30°C for 30 min, and analyzed by SDS-PAGE and autoradiography **(lane 3–10)**. In parallel, 100 ng of purified HSF1 and TAD that have been used in the immune complex kinase reaction were analyzed by Western blotting and probing with anti-HSF1 antibody **(lanes 1 and 2)**. NS, Nonspecific signal. **C:** Dominant negative JNK inhibits MG132-induced phosphorylation of TAD. HeLa cells were transiently transfected with either vector alone or vector expressing TAD, along with dominant negative mutants, JNK1-APF or JNK2-APF. After 24 h of transfection, MG132 was added at 10 μM for 15 h or cells were further treated with heat shock for 30 min **(lane 4)** or anisomycin for 1 h **(lane 5)**. The antigenic signals for TAD and its phosphorylated form are indicated on the right of the fluorogram. **D:** Co-expression of JNK and TAD results in co-precipitation. HeLa cells were transfected with dominant-negative JNK alone (lanes 1 and 2) or co-transfected with dominant-negative JNK and TAD (lanes 3 and 4). After 24 h of transfection, MG132 was added at 10 μM for 15 h and then cell lysates were subject to immunoprecipitation with anti-HSF1 antibody and then analyzed by Western blotting for FLAG-tagged JNK1/2 APF for its coprecipitation **(top panel)**. Coprecipitated JNK2-APF and a non-specific signal (NS) are indicated at the right of fluorogram. Aliquots of cell lysates containing 30 μg protein were analyzed by Western blotting for recombinant JNK and TAD expression **(middle and bottom panel** respectively). Recombinant TAD expressed in *E. coli* was included in the last lane of the anti-HSF1 Western blot for the unphosphorylated control.

compared with inducible HSF1 phosphorylation in terms of its mobility shift on a SDS-PAGE (Fig. 2A). Mobility shift of HSF1 induced by anisomycin is due to the inducible phosphorylation which probably occurs on the transcriptional activation domain of HSF1 based on the mobility shift of TAD as shown in Figure 1C, but phosphorylations on other domains may contribute to the observed mobility retardation of HSF1 as shown in Figure 2A. Treatment of cells with as little as 50 ng/ml of anisomycin leads to phosphorylation of both HSF1 and JNK, whereas activation of ERK1/2 or p38 kinase requires at least a 10-fold higher concentration. Therefore, among three MAP kinases JNK seems to exclusively contribute to the mobility retardation and inducible phosphorylation of HSF1 after treatment with anisomycin.

We next examined the down regulation of HSF1 hyperphosphorylation in the presence or absence of anisomycin during post recovery (37°C) after heat shock treatment. The time

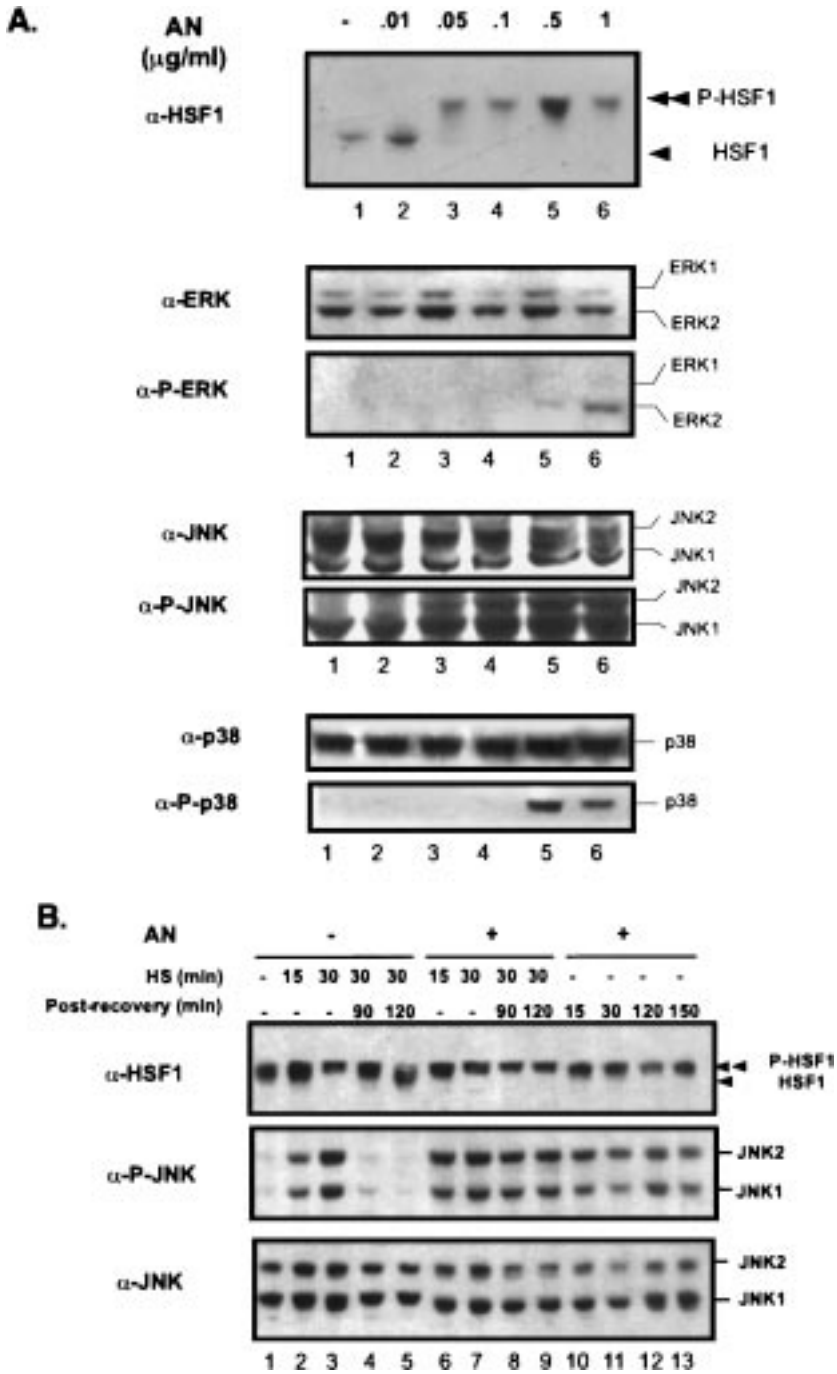

Fig. 2. Activation profile of JNK is correlated with inducible HSF1 phosphorylation. **A:** Effects of increasing concentration of anisomycin on HSF1 hyperphosphorylation and activation of the ERK1/2, JNK1/2, and p38 kinases. Confluent HeLa cells were incubated with concentrations of anisomycin from 10 ng/ml–1 μg/ml at 37°C for 1 h. Aliquots of whole cell extracts containing 30 μg protein were used for Western blot analysis by probing for HSF1 and for the total or activated forms of the ERK1/2, JNK1/2, and p38 kinases according to Methods. The position on the fluorogram of the hyperphosphorylated- (P-HSF1, double arrow heads) and control-form (HSF1, single arrow head) of HSF1 and of the antigenic signals for ERK1/2, JNK1/2, and p38 kinases are indicated. **B:** Time course of the down regulation of hyperphosphorylated HSF1 upon heat shock. Plates of HeLa cells were divided into three groups: heat shock without anisomycin treatment **(lanes 1–5)**, heat shock in the presence of 500 ng/ml of anisomycin **(lanes 6–9)**, and anisomycin (500 ng/ml) treatment alone **(lanes 10–13)**. After heat shock at 45°C for the time period as indicated, plates of HeLa cells were recovered at 37°C for the specified post-recovery time on the fluorogram. Whole cell extracts containing 30 μg protein were analyzed by Western blotting for HSF1, activated JNK, and total JNK.

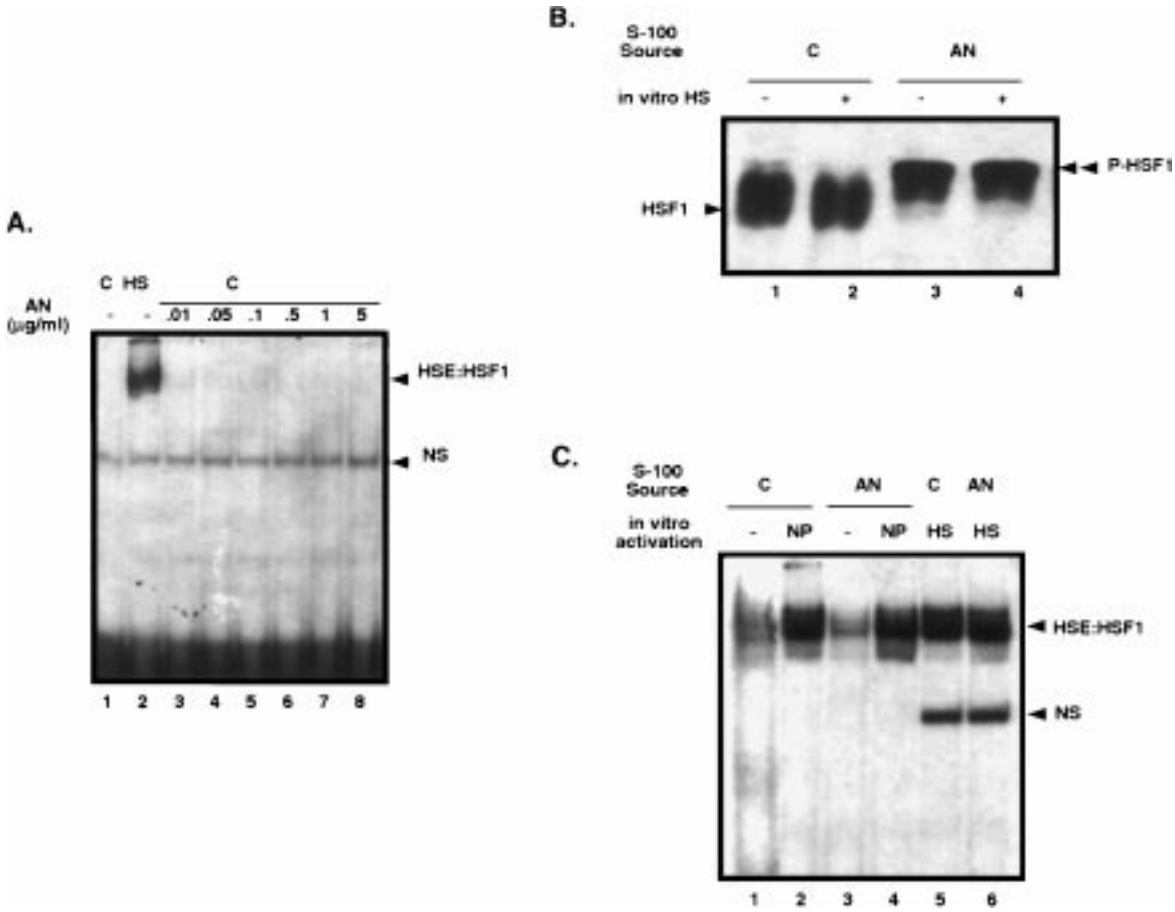

Fig. 3. Anisomycin induces the hyperphosphorylation but not the activation of HSF1. **A:** Effects of anisomycin on HSF1 DNA binding activity. HeLa cells were incubated at 37°C for 1 h in the absence or the presence of increasing concentrations of anisomycin as indicated. As a heat shock control, a plate of cells was placed in a 45°C incubator for 30 min. Whole cell extracts were prepared and aliquots of the cell extracts containing 10 μg of protein were used in an electrophoretic mobility shift assay for protein binding to ^{32}P-labeled HSE. **B–C:** Comparison of DNA binding activity between hyperphosphorylated HSF1 and its counterpart control. S-100 cytoplasmic cell extracts were prepared from unstressed control and anisomycin-treated HeLa cells respectively as described in Methods. 50 μg of S-100 cell extracts from control or anisomycin-treated HeLa cells were subject to in vitro activation by either incubating at 43°C for 1 h (in vitro HS) or at 37°C with 2% Nonidet P-40 (NP). The reaction mixture was analyzed by Western blotting (Panel B) for phosphorylation status of HSF1 and electrophoretic mobility shift assay (Panel C) for protein binding to ^{32}P-labeled HSE.

course of HSF1 and JNK phosphorylation is coincident (Fig. 2B, lanes 1–5). The mobility of HSF1 in SDS-PAGE began to shift upward and make a diffused band upon 15 min heat shock in comparison with control (compare lanes 1 and 2 of first panel) and at the same time frame, it began to induce JNK activation. The recovery for 90 min returned activated JNK into an almost basal level where HSF1 was dephosphorylated in terms of mobility shift. The presence of anisomycin led to consistent activation of JNK and HSF1 hyperphosphorylation up to 2 h post recovery (Fig. 2B, lanes 6–9). This seems to be driven by JNK activation because, without heat shock treatment, it induced sustained JNK activation and HSF1 hyperphosphorylation (lanes 10–13).

Anisomycin Induces the Hyperphosphorylation but not the Activation of HSF1

Although anisomycin led to HSF1 hyperphosphorylation, we found that the HSF1 was not converted into a transcriptionally active form. The hyperphosphorylated HSF1 was inactive both in DNA binding (up to 5 μg/ml of anisomycin, Fig. 3A) and transcriptional activa-

tion activities (data not shown). Therefore, HSF1 hyperphosphorylation seems to occur without coupling other activation process as we observed in HSF1 hyperphosphorylation by pervanadate [Park and Liu, 2000]. Protein synthesis inhibitors have been shown previously to inhibit the heat shock response by reducing the DNA binding activity of HSF1 [Baler et al., 1992]. In consistent with this finding, we observed that the HSF1 DNA binding activity induced by heat shock gradually declined with increasing concentrations of anisomycin (data not shown). To investigate if anisomycin-induced phosphorylation of HSF1 contributes to this inhibitory effect, the in vitro activation of HSF1 in S-100 cell extracts was used to compare intrinsic DNA binding activity between hyperphosphorylated HSF1 and a control. Figure 3B showed that the migration pattern of HSF1 was unchanged by in vitro heat activation of S-100 extracts prepared from control HeLa cells, suggesting that inducible phosphorylation of HSF1 does not occur in in vitro system. The basal DNA binding activity of S-100 extract prepared from control is comparable with one from anisomycin treated cells (Fig. 3C, lanes 1 and 3), and the maximum HSF1 DNA binding activity induced by either in vitro heat activation or NP-40 treatment was comparable regardless of its phosphorylation status (Fig. 3C, compare lane 2 with 4 and lane 5 with 6). Therefore, hyperphosphorylation seems to have little or no effect on the intrinsic DNA binding activity of activated HSF1, and the inhibitory effects of anisomycin to the DNA binding activity in vivo might be due to some inhibitors such as hsp70 which is known to increase upon the inhibition of nascent polypeptide synthesis and folding [Baler et al., 1992]. The in vitro heat treatment or NP40 might induce the dissociation of inhibitors from HSF1 prepared from anisomycin-treated HeLa cells and result in comparable DNA binding activity with control upon activation.

The Amount of Hyperphosphorylated HSF1 Upon Severe Heat Shock or Anisomycin Treatment Parallels With JNK Activity

SB203580, originally developed as a specific inhibitor of p38 MAP kinase at its low dosage, also reveals an inhibitory effect on certain JNK2 isoforms at higher concentration, suggesting that it could be used as an inhibitor of JNK [Cuenda et al., 1995; Whitmarsh et al., 1997]. To test if SB203580 could be used as an inhibitor of JNK, we analyzed its inhibitory effect on JNK and p38 MAP kinases at two different concentrations by an immune complex kinase assay (Fig. 4A). As reported previously, serum starvation led to the potent activation of p38 MAP kinase at 37°C, whereas severe heat shock inhibited its activation (Fig. 4A, bottom panel, compare lane 1 with 2) [Han et al., 1998]. Treatment of cells with 10 μM SB203580, the widely used working concentration to inhibit p38 MAP kinase, showed the maximum inhibitory effect for the serum starvation-induced activation of p38 MAP kinase in HeLa cells, and increasing the concentration to 100 μM had no additional effect. In contrast, only severe heat shock treatment and not serum starvation potently induced JNK activation (Fig. 4A, top panel). JNK activity was inhibited modestly by 10 μM SB203580 but reduced to the same extent as p38 MAP kinase at 100 μM. Therefore we decided to test the effect of SB203580 on HSF1 hyperphosphorylation.

The effect of SB203580 as a JNK inhibitor on the severe heat shock- or anisomycin-induced HSF1 hyperphosphorylation was assayed by a mobility shift of HSF1 on a SDS-polyacrylamide gel (Fig. 4B and C). Inhibition of JNK activity by treatment with SB203580 did not inhibit the mobility shift of HSF1 upon severe heat shock or anisomycin treatment, suggesting that partial inhibition of JNK is not enough to block the HSF1 mobility shift. However, we reproducibly observed that the amount of hyperphosphorylated HSF1 was reduced significantly by progressively increasing the concentration of SB203580. Treatment with 100 μM SB203580 reduced the hyperphosphorylated HSF1 into 67 and 45% upon heat shock and anisomycin treatment, respectively.

JNK Regulates hsp70 Promoter Activity

To assess the ultimate activity of the HSF1 pathway, we investigated heat shock promoter activity when heat shock is combined with a JNK inhibitor. Consistent with a positive role of JNK in hsp70 promoter activity, all inhibitors of the JNK signaling cascade tested here were suppressors of hsp70 promoter-driven reporter gene expression at 45°C (Fig. 5A). The inhibitory effects of SB203580 caused 77% reduction of CAT gene expression upon severe heat shock which might result from 76% reduction of JNK activity (Fig. 4A) and thereby a partial destabi-

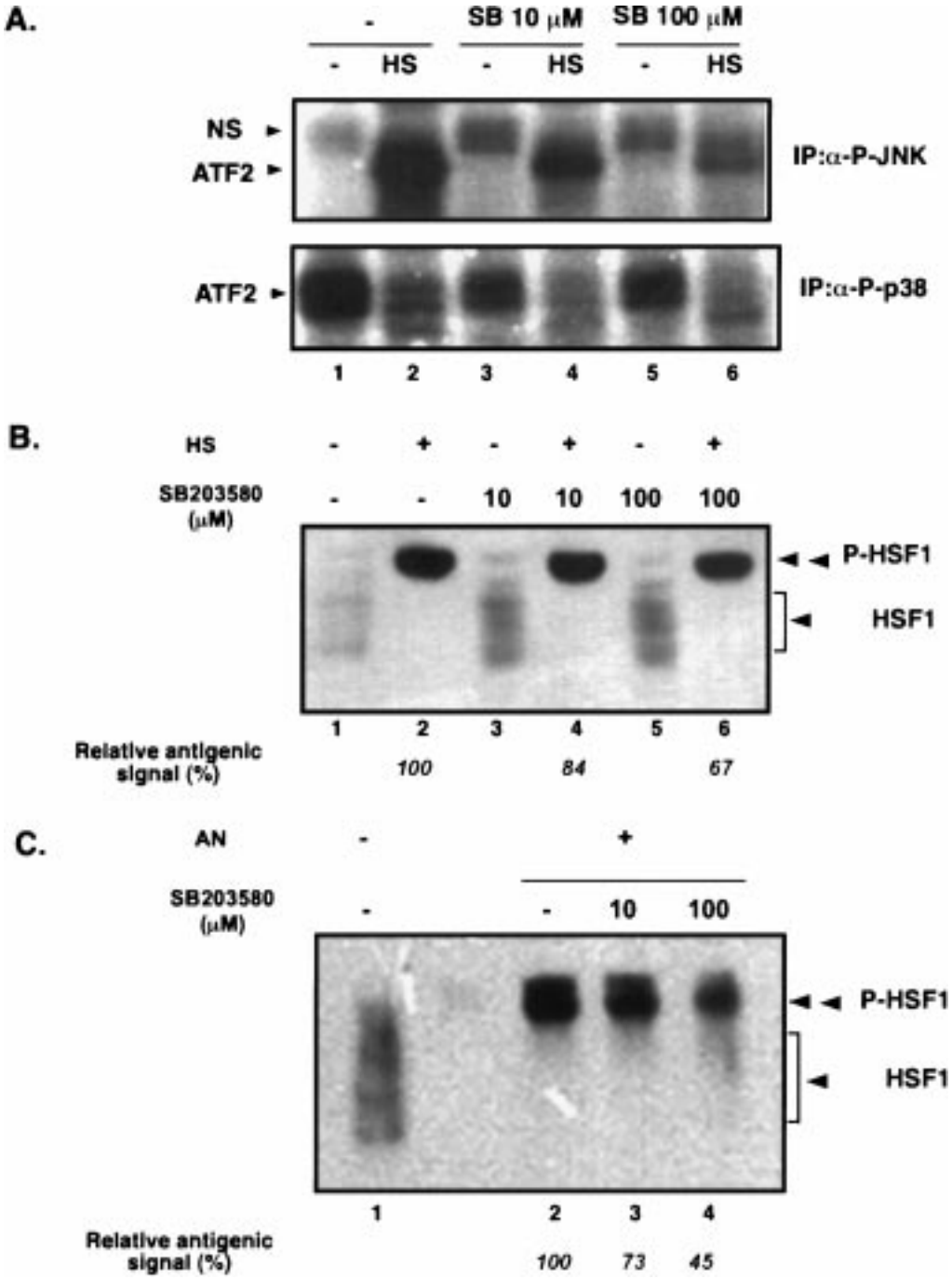

Fig. 4. The pharmacological inhibitor of JNK, SB203580, reduces hyperphosphorylated HSF1 upon heat shock or anisomycin treatment. **A:** Effects of SB203580 on JNK and p38 MAP kinases. HeLa cells were serum-starved for 12 h and then preincubated with SB203580 at the indicated concentration for 1 h. Cell plates were either kept at 37°C or heat shocked at 45°C for 30 min. Whole cell extract (500 μg) of each sample was immunoprecipitated with phospho-specific JNK1/2 and p38 kinase antibodies respectively, and analyzed for kinase activity by the immune complex kinase assay as described in Methods. NS; non-specific signal **(B)** SB203580 reduces the hyperphosphorylated HSF1 upon heat shock. HeLa cells were serum-starved for 12 h and then preincubated with SB203580 at the indicated concentration for 1 h. Plates were either kept at 37°C or heat shocked at 45°C for 30 min (− or +). Aliquots of cell extracts (30 μg) were analyzed by Western blotting for HSF1. Relative densitometric readings of hyperphosphorylated HSF1 are presented at the bottom of fluorogram. **C:** SB203580 reduces hyperphosphorylated HSF1 upon anisomycin treatment. HeLa cells were serum-starved for 12 h and then preincubated with SB203580 at the indicated concentration for 1 h. Cells were further incubated in the absence or presence of 100 ng/ml of anisomycin for 1 h. Aliquots of cell extracts (30 μg) were analyzed by Western blotting for HSF1.

lization of hyperphosphorylated HSF1 (Fig. 4B). In our cell line and assay condition, we could not detect JNK activation upon mild heat shock treatment at 42°C (data not shown). Consistent with this, all inhibitors of the JNK signaling cascade tested here have little or no effect on hsp70 promoter-driven reporter gene expression at 42°C, suggesting JNK plays a selective role on HSF1 at severe heat shock condition. The inhibitory effects of SB203580 or dominant-negative JNK seems to be specific to the heat shock promoter and not to the general transcriptional machinery because CMV promoter-driven CAT gene expression was not changed in control experiments (Fig. 5B).

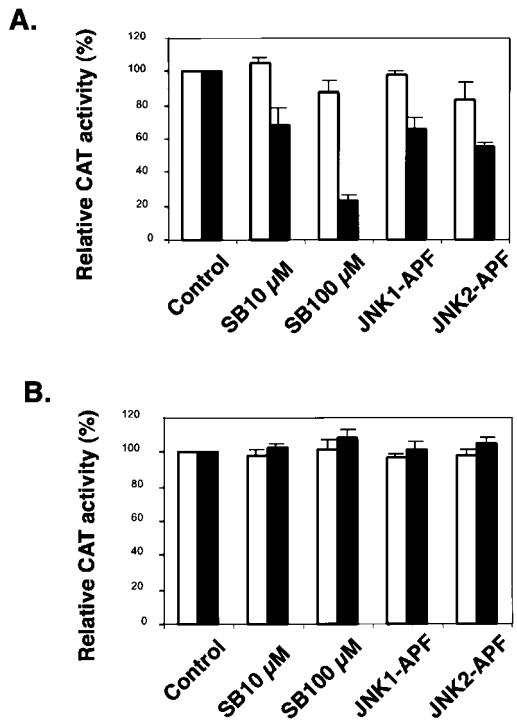

Fig. 5. JNK regulates heat shock promoter activity. Inhibitors of JNK selectively reduce hsp70 promoter activity upon severe heat shock. For SB203580 treatment, HeLa cells were co-transfected with pHB-CAT **(Panel A)** or pCMV-CAT **(Panel B)** as a reporter plasmid and pCMV-βGAL (0.25 μg) as an internal control. Cells were serum-starved for 12 h and then preincubated with SB203580 at the indicated concentration for 1 h. Cells were heat shocked at 42°C for 1 h or 45°C for 30 min and then incubated further for 6 h at 37°C. For the effect of dominant-negative JNK, HeLa cells were co-transfected with either JNK1-APF (0.5 μg) or JNK2-APF (0.5 μg), in addition to pHB-CAT (Panel A) or pCMV-CAT (Panel B) along with pCMV-βGAL (0.25 μg), and then processed as described above for the SB203580 treatment. Aliquots of whole cell extracts containing the same β-gal activities were used for a CAT assay. Error bars represent standard deviations of the mean for three independent experiments. White and black bars represent the relative CAT activity upon 42 and 45°C heat shock treatment, respectively.

DISCUSSION

In the present study, we report that the transcriptional activation domain of HSF1 is inducibly phosphorylated and targeted by the JNK2 isoform in vivo. The participation of JNK in the hyperphosphorylation of HSF1 upon anisomycin treatment or severe heat shock is further supported by the correlation between the activation profile of JNK and inducible HSF1 phosphorylation. Our results indicate that HSF1 hyperphosphorylation can occur prior to and independent of events involved in the activation process of HSF1. The in-vitro activation experiment also suggests that inducible phosphorylation does not contribute to the intrinsic DNA binding activity of activated HSF1. Functional investigation indicates that JNK-mediated HSF1 phosphorylation may enhance the stability of HSF1 and thereby increase the heat shock promoter activity as an initial cellular protection mechanism after the cessation of new protein synthesis and the activation of the proteasome degradation pathway by severe stress assault.

During the preparation of this manuscript, a contrasting result was reported in which JNK may phosphorylate the serine 363 motif to inactivate HSF1 [Dai et al., 2000]. JNK has been known to mediate both pro-apoptotic and anti-apoptotic signals probably depending on different JNK isoforms, the cell type or the duration of activation [Davis, 2000]. In this context, one difference is the use of JNK1 overexpression in the previous work compared to our results indicating the JNK2 isoform is most likely involved in HSF1 phosphorylation and its stabilization. Therefore, we speculate a differential role for JNK isoforms in the cellular protection against the stress where JNK1 serves as more pro-apoptotic signal to inhibit the transcriptional activity of HSF1 whereas JNK2 provides a more protective signal by enhancing HSF1 phosphorylation and its stabilization [Butterfield et al., 1997; Bost et al., 1999]. Even though we observed the inhibitory effect to the CAT gene expression when we overexpressed a dominant negative JNK1-APF which is apparently not compatible to binding and phosphorylation of HSF1 as shown in Figure 1, it could be explained by the fact that different isoforms of JNK have been known to

have at most ~2-fold difference in binding selectivity. Therefore, nevertheless JNK2 is the physiologically revelevant upstream kinase to HSF1, overexpressed JNK1-APF could compete away endogenous JNK2 to the binding and phosphorylation of HSF1 in our experimental condition.

The consensus phospho-acceptor sites for JNK deduced from c-Jun, ATF2, NFAT4, and Elk-1 is L(X)SP(X)D/E ~ R/K which is more stringent motif than the general MAP kinase phosphorylation site (PXS/TP). The transcriptional activation domain of human HSF1 contains only one potential phospho-acceptor site that exactly conforms to this consensus phosphorylation motif. The sequence surrounding S444 (LLSPQEPPR) in transcriptional activation domain 2 of human HSF1 is highly conserved in mammalian HSF1, but not found in the other HSF family members, HSF2, 3, and 4. Interestingly, chicken HSF1 does not have the conserved proline at the +1 position compared to the homologous human region, suggesting that it may not serve as a JNK substrate. This speculation is further supported by the fact that chicken HSF1 is not inducibly phosphorylated upon stress and is heat labile upon severe heat shock [Nakai et al., 1995]. Furthermore, chicken has the additional heat-stable HSF3 protein which has not been found yet in human and mouse. We speculate that hHSF1 has a JNK-mediated phosphorylation motif which plays a role in the stabilization of HSF1 upon severe heat stress.

JNK-mediated phosphorylation of its substrates requires a docking site (R/KXXXXLN/EL) located within 80 amino acids N-terminal to the phosphoacceptor site [Yang et al., 1998]. We found two consecutive putative JNK binding domains located at the proximal end of leucine zipper 4 of the suppressor domain (KCLSVACLDKNELSDHL) which loosely follow the consensus and which are highly conserved in the vertebrate HSF multi-gene family. This motif may explain why the truncated mutant of HSF1 and TAD can be efficiently phosphorylated by JNK in vitro and in vivo.

If JNK serves as a direct upstream kinase for HSF1, what would be the functional role of JNK-mediated HSF1 phosphorylation? Previously, inducible phosphorylation of HSF1 has been reported to have no effect on the regulation of DNA binding activity and subsequent activation [Sarge et al., 1993; Park and Liu, 2000]. Moreover, mild stress at 42°C is enough to turn on heat shock gene expression, under conditions in which there is no JNK activation in our HeLa cell line and assay conditions. Currently, we envision several possible roles of JNK-mediated HSF1 phosphorylation based on our results as well as the proposed role of phosphorylation for other JNK substrates. First of all, HSF1 phosphorylation by JNK would be critical to its stability. JNK can modulate the ubiquitin-dependent proteasome degradation pathway, where JNK can target most of its substrate to the proteasome by forming stable complexes [Fuchs et al., 1997, 1998]. JNK-dependent degradation relies on the phosphorylation status of its substrate because JNK-mediated phosphorylation protects the substrate from targeting and results in increased protein stability. Interestingly, Morimoto's group has reported that HSF2 is targeted by the proteasome degradation pathway [Mathew et al., 1998]. HSF2 contains a sequence similar to the putative JNK binding site in this study, but without an appropriate phospho-acceptor site. This may explain the shorter half-life of HSF2 compared to the long-lived, stable HSF1. It is also possible that JNK-mediated phosphorylation endows HSF1 with new properties, which might allow HSF1 to turn on certain sets of stress responsive genes distinct from heat shock protein. For example, certain stress responsive genes are highly expressed only with hyper-osmotic stress, but not with hypo-osmotic stress. Other genes are induced by 45°C and not by 42°C heat stress, or only induced by certain metabolic oxidative stress [Delpino et al., 1996; Okinaga et al., 1996; Lee and Corry, 1998; Richmon et al., 1998]

In general, the JNK signaling cascade appears to have a function and regulation that is opposite in function and regulation to that of ERK signaling cascade [Bokemeyer et al., 1996]. ERK signaling is activated by growth factors and it plays a major role in growth and proliferation, whereas JNK signaling is activated by environmental stress and it functions in growth arrest, apoptosis, and the stress response. In this context, previous findings that ERK induces constitutive phosphorylation of HSF1 in unstressed conditions to repress transcription activity seems to contrast with the role of JNK demonstrated in this study, where JNK enhances heat shock promoter activity by modulating HSF1 phosphorylation.

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