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c-Jun NH_2-terminal Kinase Targeting and Phosphorylation of Heat Shock Factor-1 Suppress Its Transcriptional Activity*

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The mammalian heat shock transcription factor HSF-1 regulates the expression of the heat shock proteins, molecular chaperones that are involved in cellular processes from higher order assembly to protein degradation. HSF-1 is a phosphorylated monomer under physiological growth conditions and is located mainly in the cytoplasm. Upon activation by a variety of environmental stresses, HSF-1 is translocated into the nucleus, forms trimers, acquires DNA binding activity, is hyperphosphorylated, appears as punctate granules, and increases transcriptional activity of target genes. As cells recover from stress, the punctate granules gradually disappear, and HSF-1 appears in a diffused staining pattern in the cytoplasm and nucleus. We have previously shown that the mitogen-activated protein kinase ERK phosphorylates and suppresses HSF-1-driven transcription. Here, we show that c-Jun NH_2-terminal kinase (JNK) also phosphorylates and inactivates HSF-1. Overexpression of JNK facilitates the rapid disappearance of HSF-1 punctate granules after heat shock. Similar to ERK, JNK binds to HSF-1 in the conserved mitogen-activated protein kinases binding motifs and phosphorylates HSF-1 in the regulatory domain. The overexpression of an HSF-1-green fluorescent protein fusion construct lacking JNK phosphorylation sites causes this HSF-1 mutant to form nuclear granules that remain longer in the nucleus after heat shock. Taken together, these findings indicate that JNK phosphorylates HSF-1 and suppresses its transcriptional activity by rapidly clearing HSF-1 from the sites of transcription.

In vertebrates, multiple heat shock transcription factors (HSFs)[1] bind to conserved regulatory DNA sequences known as heat shock elements (HSEs) (1–5). In response to chemical, environmental, and physiological stresses including heat shock, HSFs induce the expression of heat shock proteins (HSPs), which are molecular chaperones (6, 7). Accumulation of HSPs following heat shock or other stresses are crucial for cells to survive further injury as they confer protection to different cellular components; they also prevent apoptosis (8–10). So far, four HSFs have been identified, and the regulation of each is complex and not entirely understood (11–14). Among the various HSFs, the mammalian HSF-1 is regulated by phosphorylation (15–18). The nuclear translocation, DNA binding, and transcriptional activities of most mammalian transcription factors are regulated by phosphorylation. In many cases, multiple protein kinases can act on a single transcription factor (19). In the case of mammalian HSF-1, it appears to also be targeted by multiple protein kinases because it is phosphorylated under normal physiological growth conditions as well as after heat shock (15, 20–22). Following activation by heat shock, HSF-1 appears at the sites of transcription as several intensely staining punctate granules that become as large as 1.5–2.5 μm (23, 24). The functional role of phosphorylation in relation to HSF-1 granule formation and the signaling pathways controlling HSF-1 activity is not entirely clear. Phosphorylated forms of HSF-1 protein have been extensively studied by phosphopeptide mapping as well as mutational analysis. The data suggest that HSF-1 is phosphorylated on multiple serine residues and, perhaps, a threonine residue (18, 22, 25, 26). Constitutive phosphorylation of HSF-1 by survival-promoting signaling pathways, such as GSK-3 and ERK, on serine residues Ser^{303} and Ser^{307}, respectively, negatively regulates HSF-1 function, because mutations to alanine of either Ser^{303} alone or of both Ser^{303} and Ser^{307} cause constitutive transcriptional activation of HSF-1 (23, 25, 26). Transient overexpression of GSK-3, and to a lesser extent ERK, leads to a rapid diffusion of accumulated HSF-1 granules after heat shock, suggesting that sequential phosphorylation of HSF-1 by these two enzymes suppresses HSF-1 activity by perhaps forcing HSF-1 to disperse rapidly from the sites of transcription during recovery from heat shock (23). Phosphorylation of HSF-1 by these enzymes most likely holds HSF-1 in an inactive state under normal physiological growth conditions.

The MAPKs respond to diverse stimuli and consist of sequential protein kinase cascades. MAPKs are activated via phosphorylation of specific threonine and tyrosine residues by dual specificity MAPKs, which are known as MEK/MKKs. MEK/MKKs are phosphorylated and activated by MEK kinases (MEKKs/MKKK) (27, 28). There are three well characterized MAPK pathways: ERK1/ERK2, also known as p42/p44 MAPKs (29); the p38/RK/Mpk2/CSBP protein kinases (30, 31); and the c-Jun NH_2-terminal kinases (JNKs)/stress-activated protein kinases (32, 33). Activation of growth factor receptors, G protein-coupled receptors and some cytokine receptors activate ERKs (27). The p38 protein kinases are activated by proinflammatory cytokines and osmotic shock (31, 34, 35). JNKs, also

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[1] The abbreviations used are: HSF, heat shock transcription factor; HSE, heat shock element; HSP, heat shock protein; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; GFP, green fluorescent protein; CAT, chloramphenicol acetyltransferase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; HA, hemaglutinin; GST, glutathione *S*-transferase.

known as the stress-activated protein kinase, are activated by various cellular stresses such as UV, protein synthesis inhibitors, proinflammatory cytokines, G protein-coupled receptors, and growth factor receptors (33, 36, 37). JNK directs a wide range of cellular processes under physiological and pathological conditions, including the induction of apoptosis (38–40). JNKs preferentially phosphorylate sites containing the consensus sequence Pro-Xaa-(Ser/Thr)-Pro and sometimes requires the interaction with specific sequences that are prerequisite for efficient phosphorylation. A short region, known as the Δ domain, or D domain, that is conserved in a number of transcription factors in the TCF family, appears to be required for this interaction. However, different amino acid residues in the D domain are critical for ERK and JNK binding (36, 41, 42). Multiple transcription factors, including ATF2, SAP-1, TCFs/ElK1, MEF2C, CHOP, and c-Jun, have been shown to be phosphorylated, and their activity is regulated by various MAPKs (36, 43–47).

In this study, we show that similar to ERK, JNK also phosphorylates HSF-1 and suppress its transcriptional activity. Both ERK and JNK bind to a conserved residue on HSF-1 known as the D domain, and deletion of this domain reduces the ability of ERK and JNK to phosphorylate HSF-1 *in vitro*. Furthermore, the mutation of ERK or JNK phosphorylation sites on an HSF-1-GFP fusion protein causes HSF-1 to form granules, after heat shock, that remain in the nucleus for longer periods of time than the wild type HSF-1-GFP. These studies suggest that HSF-1 is the target of phosphorylation and suppression by two signaling pathways, ERK, which is involved in cell survival, and JNK, which is involved in cell death.

MATERIALS AND METHODS

Cell Culture—HeLa and H1299, human ovary and lung carcinoma cells, respectively, were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Plasmids—To construct HSF-1-GFP fusion proteins, full-length human HSF-1 cDNA was amplified using specific polymerase chain reaction primers. The COOH-terminal of the amplified fragment was fused to GFP in plasmid pEGFP-N1 (CLONTECH) at *Bgl*II and *Eco*RI restriction enzyme sites. Mutated polymerase chain reaction primers were used to create serine to alanine substitutions at Ser^{303} or Ser^{307} where nucleotides GCT were substituted by GGC, and for Ser^{363}, nucleotides TCC were substituted by GCC. All amplified HSF-1 cDNA fragments and the mutations were confirmed by sequencing. Other HSF-1 mutants have previously been described (48). The GAL4-HSF-1 fusion proteins were constructed by amplification of HSF-1 (amino acid residues 116–529) using specific primers, and the fragments were digested using appropriate restriction enzymes and subcloned into plasmid pSG424, which encodes GAL4 (1–147) DNA-binding domain.

Transient Transfection Analysis—Transient transfections were performed by calcium phosphate as well as GenePorter (GTS, San Diego, CA). Transfected DNA mixes included 2 μg of expression plasmid DNA and, when required, 1.5 μg of HSP-70-luciferase DNA and 0.1 μg of *Renilla* luciferase DNA with pBluescript carrier DNA were added to a total of 4 μg. The DNA mix was added to 3×10^5 cells. For luciferase assays, cells were plated in 35-mm culture dishes after transfection and left at 37 °C for 48 h before additional treatments. Luciferase assays were performed according to the manufacturer's instructions (Promega, Madison, WI). *Renilla* luciferase was used as an indicator of transfection frequency. For CAT assays following transfection of appropriate plasmids, cells were lysed, and CAT expression was determined using enzyme-linked immunosorbent assay (Roche Molecular Biochemicals). For CAT assays, firefly luciferase gene was used as an indicator of transfection frequency.

Indirect Immunofluorescence Analysis—Cells were transiently transfected with appropriate constructs using calcium phosphate and plated in 8-chamber tissue culture slides. After 48 h, cells were treated as described in the text, rinsed with PBS, and fixed with 4% paraformaldehyde for 30 min at 25 °C. Cells were permeabilized with a solution containing 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice and rinsed with PBS and were incubated in the blocking solution (5% goat serum and 5% bovine serum albumin in PBS) at 37 °C for 1 h. Cells were then incubated in the presence of the primary antibody for 1 h at 37 °C, rinsed with PBST (PBS + 0.1% Tween-20) and incubated in the presence of secondary antibody (conjugated with fluorescein isothiocyanate or Texas Red) for an additional 1 h at 37 °C. Cells were extensively rinsed with PBST and slides were mounted with Pro-Long Antifade (Molecular Probes, Eugene, OR) and examined by fluorescence microscopy. Cells transfected with plasmids containing GFP were fixed with 4% paraformaldehyde and analyzed.

Immunoprecipitation, Binding, and Immune Complex Kinase Assays—To assess protein kinase activity, cells were treated as described in the text and lysed in buffer containing 50 mM sodium β-glycerophosphate, pH 7.2, 5 mM $MgCl_2$, 6 mM EGTA, 10% glycerol, 1% Nonidet P-40, 1 mM 2-mercaptoethanol, 1 mM sodium vanadate, 0.2 mM PMSF. Lysates were microfuged, and equal amounts of protein (200–300 μg) from each sample were added to 1 μg of the appropriate antibody. After 1 h of incubation at 4 °C, 25 μl of a 50% solution of protein A-Sepharose beads was added, and the mixture was incubated at 4 °C for an additional 1 h. At this time, the mixture was washed four times with lysis buffer and once with kinase buffer (20 mM β-glycerophosphate, pH 7.3, 5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1 mM dithiothrietol, 1 mM sodium vanadate, and 0.2 mM PMSF). For phosphorylation experiments, 1 μg of each substrate was phosphorylated with [γ-^{32}P]ATP for 20 min at 30 °C with immunoprecipitated JNK1 (C17, Santa Cruz) or purified active JNK2 (UBI), ERK1 (United Biotechnology, Inc.), or ERK2 (New England BioLabs). Samples were analyzed by PAGE, and gels were exposed to x-ray film and quantitated with PhosphorImager. To follow the kinetics of ERK and JNK phosphorylation of wild type HSF-1 or HSF-1 deleted in the MAPK targeting domain, 1 μg of truncated wild type His-tagged HSF-1 or HSF-1 containing deletions were phosphorylated with various enzymes for 5 to 20 min at 30 °C in the presence of [γ-^{32}P]ATP. The phosphorylated products were analyzed by PAGE, exposed to x-ray film, and quantitated with PhosphorImager.

For *in vivo* binding assays, H1299, human lung carcinoma cells stably expressing HA-tagged JNK1 (HJ-16) or HA-tagged ERK1 (HE-10) were transiently transfected with pcDNA3-HSF-1 expression vector. After 48 h, cells were solubilized in Triton lysis buffer (20 mM Tris-Cl, pH 7.4, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium vanadate, 2 mM pyrophosphate, 1 mM PMSF, 0.5 mM dithiothrietol) (42), and JNK or ERK were immunoprecipitated using anti-JNK1 (C17, Santa Cruz) or anti-ERK1 (C14 and C16, Santa Cruz) antibodies. The immunoprecipitated materials were washed extensively with Triton lysis buffer and analyzed by PAGE followed by immunoblotting using anti-HSF-1 antibody. For *in vitro* binding of JNK1 or ERK1 to wild type HSF-1 or HSF-1 mutant Δ01 (deleted between amino acid residues 203 and 224), 1 μg of antibody to JNK1 or ERK1 was added to 350 μl of H1299 cell lysates (in Triton lysis buffer) that contains 300 μg of protein and were stably expressing HA-JNK1 or HA-ERK1 cDNAs. After 1 h of incubation at 4 °C, 25 μl of 50% solution of protein A-Sepharose beads were added, and the mixture was incubated at 4 °C for an additional 1 h. After this time, 3 μg of purified truncated wild type His-tagged HSF-1 (1–450) or truncated His-tagged HSF-1 mutant Δ01 proteins were added to the mixture and incubated at 4 °C for 2 h. The protein A-Sepharose beads were washed four times with Triton lysis buffer, and the immunoprecipitated materials were analyzed by SDS-PAGE followed by immunoblotting using antibody to HSF-1 (23). For control experiments, the same procedure was used, but no antibody to ERK or JNK was included during the immunoprecipitation.

Two-dimensional Phosphopeptide Mapping and Peptide Sequencing—Two-dimensional phosphopeptide mapping was performed following immunocomplex kinase assays and phosphorylation reactions using purified enzymes as described above using 10 μg of purified His-HSF-1 wild type or mutant protein as substrates. The phosphorylated HSF-1 protein was then analyzed by SDS-PAGE and extracted from the gel. The protein was then digested with trypsin and analyzed according to the standard methods (49, 50). The electrophoresis buffer contained *n*-butanol, pyridine, acetic acid, and water in ratios of 2:1:1:36, respectively. The phospho-chromatography buffer was *n*-butanol, pyridine, acetic acid, and water in ratios of 5:3:1:4.

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift analysis using whole cell extracts has been described in detail previously (2, 23). Briefly, after each treatment, cells were rinsed with PBS and lysed in 100 μl of extraction buffer (10 mM HEPES, pH 7.9, 0.4 mM NaCl, 0.1 mM EDTA, 0.5 mM dithiothrietol, 5% glycerol, 0.5 mM PMSF). The protein concentration of samples was estimated by the bicinchoninic acid method. Equal amounts of protein (10 μg) in extraction buffer (volume not exceeding 15 μl) were added to the reaction mixture, which contained 4 μl of binding buffer (37.5 mM NaCl, 15 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.5 mM dithiothrietol, 5% glycerol), 10 μg of yeast tRNA,

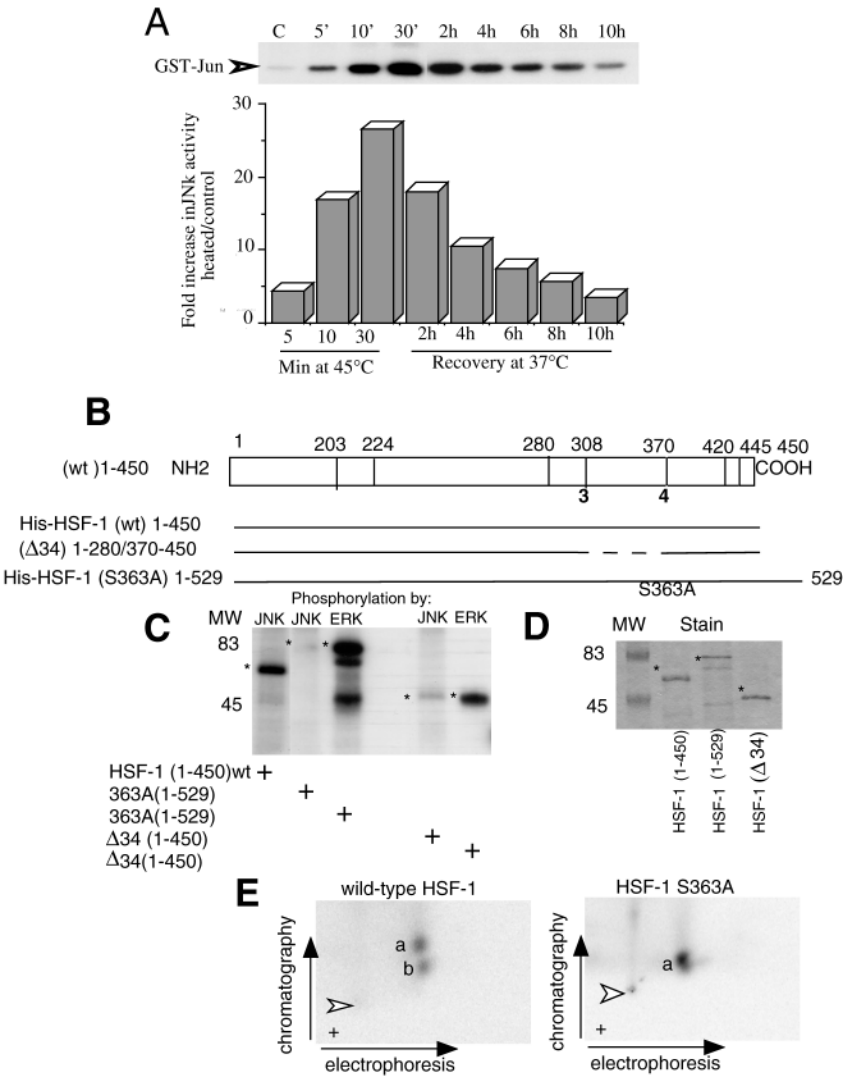

FIG. 1. **JNK is activated by heat shock and phosphorylates HSF-1 on serine 363.** *A*, immune complex kinase assays. HeLa cells were heated at 45 °C for 5, 10, or 30 min or were heated for 30 min and were incubated at 37 °C up to 10 h. JNK1 activity was determined by immunoprecipitation of JNK1 followed by a kinase reaction using GST-Jun as a substrate. *B–D*, immune complex kinase assays of HSF-1 wild type or mutants. *B*, diagram of wild type HSF-1 and mutant proteins. *Numbers above* indicate amino acid residues. *Numbers below* indicate start and end points of deleted region in mutant. Deletion is also indicated with *dashes* in the line diagrams of the mutant construct. *C*, JNK or ERK were immunoprecipitated from HeLa cells, and the immunoprecipitated material was used in immune complex kinase assays using HSF-1 wild type (1–450) or deletion mutants (Δ34) or His-HSF-1 S363A (1–529) as a substrate. Products were analyzed by SDS-PAGE and exposed to x-ray film. *D*, Coomassie Blue staining of HSF-1 wild type and mutant proteins used in *C*. *E*, peptide mapping of His-HSF-1 wild type and mutant. JNK1 was immunoprecipitated and HSF-1 wild type or mutant protein were phosphorylated in immune complex kinase assays. The phosphorylated proteins were analyzed by phosphopeptide mapping. Phosphopeptide b corresponds to amino acid residues 361–372 (56), which disappear upon S363A substitution.

1 μg of sheared *Escherichia coli* DNA, 10 μg of poly(dI-dC), and 1 ng of ^{32}P-labeled HSE oligonucleotide. The mixture was incubated for 15 min at 25 °C and resolved on a 4.5% nondenaturing polyacrylamide gel. After electrophoresis, gels were fixed in 7% (v/v) acetic acid for 5 min, rinsed once in distilled water, dried under vacuum, and exposed to x-ray film. The nucleotide sequence used for HSE was as follow: 5′-GTCGACGGATCCGAGCGCCTCGAATGTTCTAGAAAAGG-3′ (2). The double-stranded oligonucleotide was labeled using Klenow fragment of DNA polymerase I, deoxynucleotide triphosphates and [α-^{32}P]dCTP.

RESULTS

JNK Targeting and Phosphorylation of HSF-1—To investigate changes in JNK activity during and following a period of recovery after heat shock, immune complex kinase assays were performed. JNK was immunoprecipitated from lysates of heated HeLa cells and was used in kinase reactions using GST-Jun as a substrate. The results show that there is as much as a 20–30-fold increase in JNK1 activity during the first 10–30 min of heat shock (Fig. 1*A*). This heat-induced JNK activity is sustained 5–8-fold above that observed in untreated cells for as long as 8–10 h post-heat treatment.

Computer analysis of HSF-1 protein indicates at least five potential MAPKs phosphorylation motifs (Pro-Xaa-(Ser/Thr)-Pro). In human HSF-1 protein these sites are serine residues Ser^{275}, Ser^{292}, Ser^{303}, Ser^{307}, and Ser^{363}. Although ERK phosphorylates HSF-1 on Ser^{307} and perhaps other sites (25), phosphorylation of HSF-1 containing deletion and point mutations indicates that JNK mainly phosphorylates HSF-1 between amino acid residues 308 and 370 (Fig. 1, *B–D*). In this region, Ser^{363} is one potential MAPK phosphorylation motif. Substitution of this serine residue to alanine (S363A) or deletion of a fragment of HSF-1 containing Ser^{363} (Δ34) reduces HSF-1 phosphorylation by JNK, whereas these HSF-1 mutants can still be phosphorylated by ERK. Phosphopeptide mapping analysis with ^{32}P-labeled HSF-1 indicates that HSF-1 is phosphorylated on Ser^{363} by JNK, because the phosphopeptide corresponding to amino acid residues 361–372 containing Ser^{363} (25) is eliminated upon substitution of S363A (Fig. 1*E*). The remaining phosphorylated peptide corresponds to Ser^{307}, which has previously been shown to be the site of ERK phosphorylation as well (25). It is not clear, however, whether JNK can indeed phosphorylate this site as well *in vivo* when activated under some environmental stresses.

Similar to other transcription factors that are the target of MAPKs, HSF-1 also contains a recognition motif for ERK and JNK targeting that is located between amino acid residues 203–224. We therefore tested whether ERK or JNK binds HSF-1. Immunoprecipitation experiments were performed using extracts prepared from control or heated cells transfected with expression plasmids containing an epitope-tagged cDNA of ERK or JNK and cotransfected with plasmids containing wild type HSF-1. Immunoblot analysis using antibody specific to HSF-1 shows the presence of a phosphorylated form of HSF-1 (as apparent by its location in SDS-PAGE) in ERK

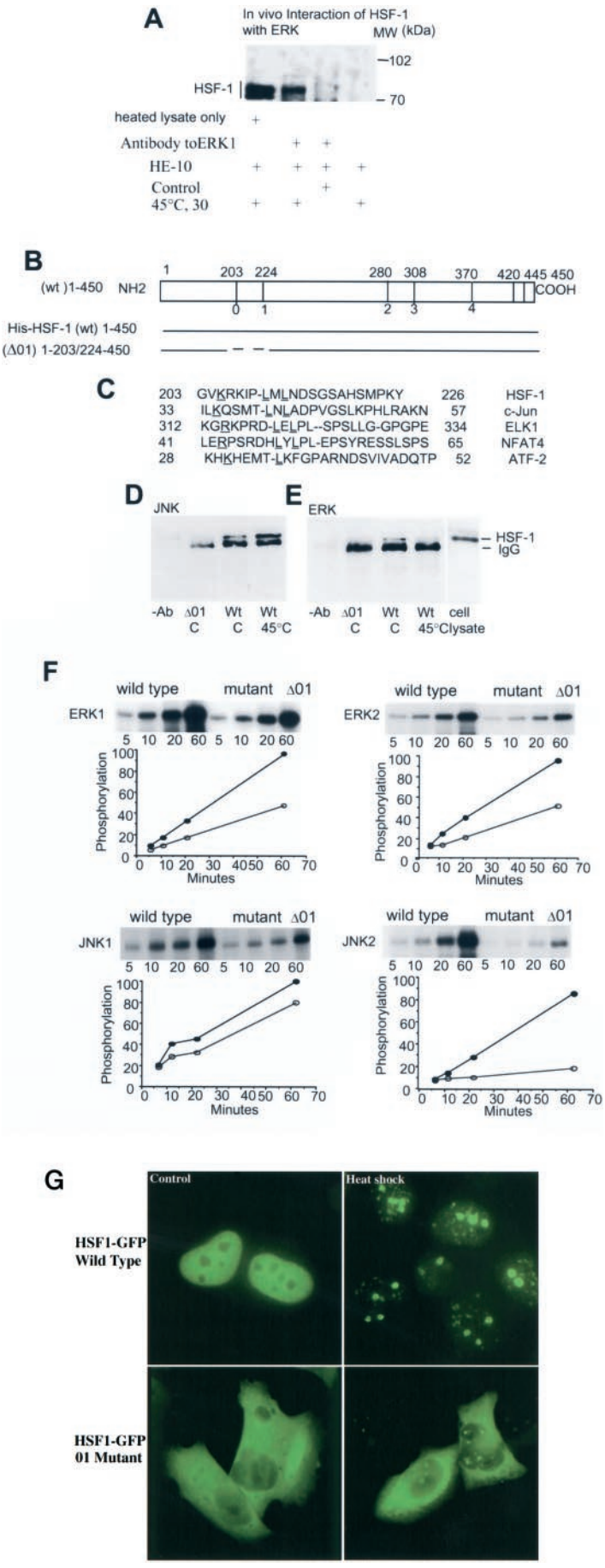

FIG. 2. **JNK targets HSF-1 to the D domain.** *A*, *in vivo* interaction of ERK1 with HSF-1. Human lung carcinoma cells H1299 stably expressing HA-tagged ERK1 (HE-10) were transiently transfected with full-length pcDNA3-HSF-1 expression vector. After 48 h, cells were lysed, and ERK1 was immunoprecipitated using anti-ERK1 antibodies. Immunoprecipitates were analyzed by PAGE and immunoblotted using anti-HSF-1 antibody (23). *B*, diagram of truncated wild type HSF-1 and deletion mutants. *Numbers above* indicate amino acid residues. *Numbers below* indicate start and end points of deleted region in mutant. *C*, the conserved sequence (*underlined*) of the D domain in HSF-1 and other transcription factors that are the target of MAPKs. *D* and *E*, binding of JNK1 and ERK1 to truncated wild type His-tagged-HSF-1 (1–450) and truncated His-tagged HSF-1 mutant Δ01 (deleted between amino acid residues 203 and 224). Anti-JNK1 or anti-ERK1 antibodies (*Ab*) were bound to protein A-Sepharose beads and incubated with unheated control (*C*) or heated (45 °C for 30 min) H1299 cell lysates stably expressing JNK1 (*D*) or ERK1 (*E*). Purified truncated wild type His-HSF-1 (1–450) or truncated His-HSF-1 mutant Δ01 were added to the protein A/JNK or ERK antibody mix. The immunoprecipitated materials were analyzed by PAGE, followed by immunoblotting using anti-HSF-1 antibody. The positions of the purified HSF-1 (1–450) and IgG bands are indicated. *F*, requirement of binding to HSF-1 for efficient JNK or ERK phosphorylation. Wild type HSF-1 or mutant Δ01 were phosphorylated for 5–60 min at 30 °C with immunoprecipitated activated JNK1 or purified active JNK2, ERK1, or ERK2. Samples were analyzed by PAGE, and gels were exposed to x-ray film. The gels were quantitated using PhosphorImager. All experiments were performed at least three times, and results were consistent. *G*, deletion of the amino acid residues 203–224 in HSF-1 protein prevents its nuclear localization. Representative immunofluorescence photographs (magnification, ×1000) of HeLa cells transfected with HSF-1-GFP (wild type), or HSF-1-GFP Δ01 (deleted between amino acid residues 203 and 224). HeLa cells were transiently transfected with the expression vectors. 48 h after transfection, cell were heated at 45 °C for 30 min and allowed to recover at 37 °C for 4 h, which is the best recovery time after heat shock to observe HSF-1 granules. GFP fluoresces green under blue light. *Wt*, wild type.

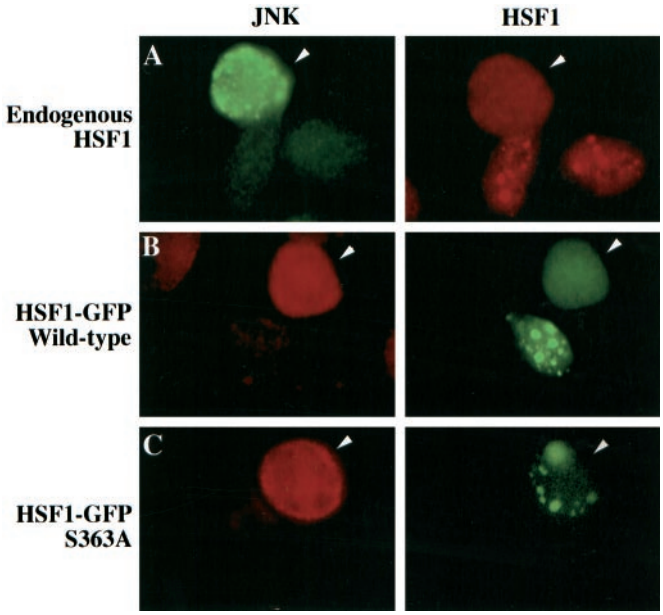

FIG. 3. **Overexpression of JNK leads to rapid HSF-1 inactivation.** *A*, representative immunofluorescence photographs (magnification, ×1000) of cells transfected with HA tagged-JNK1. HeLa cells were transiently transfected with the expression vectors. 48 h after transfection, cells were heated at 45 °C for 30 min and allowed to recover at 37 °C for 4 h (best recovery time after heat shock to observe granules). Overexpression of JNK1 was detected using antibody to HA and fluorescein isothiocyanate-conjugated secondary antibody (*A*, *left panel*). The endogenous HSF-1 was detected with antibody to HSF-1 and Texas Red-conjugated secondary antibody (*A*, *right panel*). *B* and *C*, representative immunofluorescence photographs (magnification, ×1000) of HeLa cells transfected with HA-tagged JNK1 and cotransfected with wild type HSF-1-GFP (*B*) or HSF-1-GFP (S363A) (*C*). 48 h after transfection, cells were heated at 45 °C for 30 min and incubated at 37 °C to recover for 4 h and were fixed and analyzed. Overexpressed JNK1 was detected as above except the secondary antibody was Texas Red-conjugated (*B* and *C*, *left panels*). GFP fluoresces green under blue light. *Arrowheads* in the *left panels* indicate cells with overexpressed JNK1 and in the *right panels* indicate HSF-1 in the same cells. The data are representative photographs of at least three separate experiments.

immunoprecipitates (Fig. 2*A*). *In vivo*, the hyperphosphorylated, activated form of HSF-1 is associated with retarded mobility during PAGE (15, 17, 18, 20, 26), and the monomeric, hypophosphorylated, and thereby inactive form of HSF-1 has a faster mobility (20). Our results show that the hyperphosphorylated forms of HSF-1 associated with ERK immediately upon cessation of heat shock. There was not any consistent interaction between HSF-1 and ERK under normal physiological growth conditions. In the case of JNK immunoprecipitates, we observed binding, although not consistently, of JNK to the hyperphosphorylated forms of HSF-1 under both physiological growth conditions and immediately upon heat shock. This hyperphosphorylated HSF-1 that was found to bind JNK had an apparent molecular mass 1–2 kDa higher than the HSF-1 species that was detected in ERK immunoprecipitates (data not shown).

We also tested whether ERK or JNK binds HSF-1 *in vitro* (Fig. 2, *B–E*). Immunoprecipitation experiments were performed using extracts prepared from control or heated cells transfected with expression plasmids containing an epitope-tagged cDNA of ERK or JNK and purified wild type His-tagged HSF-1 protein. Immunoblot analysis using antibody to HSF-1 shows the presence of HSF-1 in ERK or JNK immunoprecipitates (Fig. 2, *D* and *E*). To test whether the conserved MAPKs binding domain present in HSF-1 is required for JNK protein kinase targeting, we tested whether deletion of amino acid residues 203–224 abolishes binding of HSF-1 to JNK or ERK.

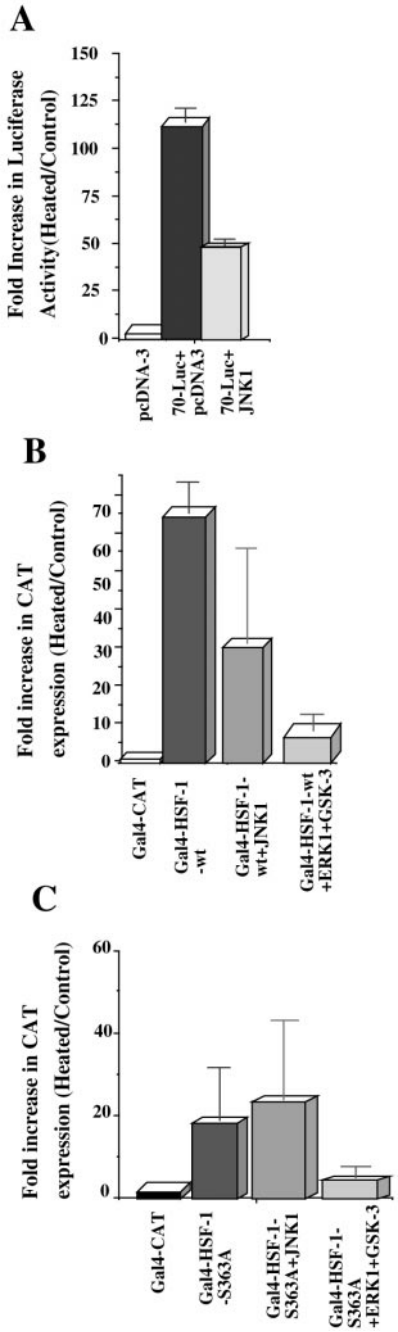

FIG. 4. **Overexpression of JNK suppresses wild type HSF-1 transcriptional activity but not HSF-1 lacking JNK phosphorylation site.** *A–C*, HeLa or H1299 cells were transiently transfected with plasmids containing HSP-70-luciferase (*A*) or GAL4-HSF-1 (residues 119–529) wild type (*B*) or S363A mutants (*C*) cotransfected with GAL4-CAT reporter gene and cotransfected with plasmids containing JNK1, ERK1, or GSK-3β as indicated. All transfections included plasmids containing *Renilla* luciferase for luciferase assays or firefly luciferase for CAT assays as an internal control. 48 h after transfection, cells were kept as unheated controls or were heated at 45 °C for 30 min and incubated at 37 °C for 6 h for the expression of reporter gene. Luciferase activity or CAT expression were determined in 20 or 50 μg, respectively, of cell lysates for each sample and adjusted for the expression of internal control. Data represented here are for H1299 cells and are shown as the fold change in luciferase activity or CAT expression in heated cells transfected without or with expression plasmids containing JNK1, ERK1, or GSK-3β. *Error bars* represent standard deviations of the mean of three independent experiments.

The results show that the deletion of these amino acid residues in mutant Δ01 abolishes the binding of HSF-1 to both JNK and ERK (Fig. 2, *D* and *E*).

Among the various known substrates of MAPKs, ELK1 transcription factor requires binding of JNK and ERK for their efficient phosphorylation, whereas this requirement is not as critical for the p38 protein kinases (42). To test whether the targeting of JNK or ERK to HSF-1 is required for the ability of these enzymes to efficiently phosphorylate HSF-1, phosphorylation experiments were performed using purified wild type HSF-1 or our HSF-1 deletion mutant that contains a deletion of amino acids 203–224 as substrates (48). The results indicate that efficiency of HSF-1 phosphorylation is reduced by different classes of MAPKs when the MAPKs targeting motif was deleted in mutant Δ01 as compared with wild type HSF-1 (Fig. 2*F*).

As it had been previously observed for ELK1 transcription factor, the sequences in the D domain encode the nuclear translocation signal. Interestingly, deletion of this region in HSF-1 protein prevents HSF-1 translocation into the nucleus. Immunofluorescent experiments using transient transfection of HSF-1-GFP Δ01 mutant into HeLa cells under both untreated or heated conditions show that unlike wild type HSF-1-GFP that translocates into the nucleus and forms granules after heat shock, this HSF-1 mutant is unable to translocate into the nucleus (Fig. 2*G*).

JNK Phosphorylation of HSF-1 Leads to Reduction in Its Transcriptional Activity—We then examined the role of JNK regulation and the consequence of substitution of Ser^{363} to alanine (S363A) in HSF-1 function *in vivo*. Because HSF-1 is translocated into the nucleus and accumulates as granules in the nucleus for several hours after heat shock (23), we analyzed the effect of JNK overexpression on the nuclear appearance of endogenous HSF-1. JNK was transiently overexpressed in HeLa cells, and control or heated cells were examined by indirect immunofluorescence analysis. Results indicate that cells overexpressing JNK show a rapid disappearance of HSF-1 granules (4 h to achieve 80% recovery as compared with 10–12 h in untransfected cells) that we have shown previously to be the sites of transcription (23), after heat shock (Fig. 3*A*). Furthermore, transient expression of plasmids containing JNK cotransfected with wild type HSF-1-GFP fusion construct also shows the same rapid recovery of HSF-1 granules after heat shock and 4 h of incubation at 37 °C (Fig. 3*B*). In contrast, cotransfection of plasmids containing JNK and HSF-1-GFP (S363A) does not result in the same pattern of HSF-1 recovery from punctate granules under the same conditions (Fig. 3*C*). This rapid diffusion of endogenous HSF-1 or wild type HSF-1-GFP granules from the sites of transcription is specific to cells overexpressing JNK and, as we have previously reported, GSK-3 and ERK (23). Overexpression of MNK1 (23) or p38 protein kinases (23) or GFP (data not shown) do not show this effect.

To investigate whether the rapid diffusion of endogenous HSF-1 granules or that of wild type HSF-1-GFP fusion protein

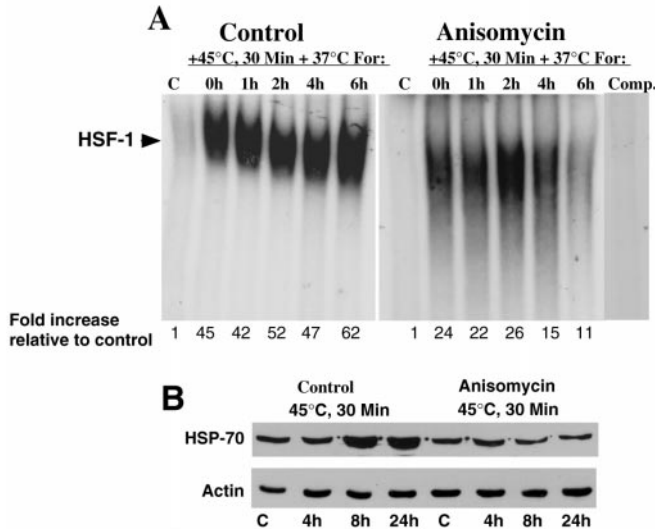

FIG. 5. **Treatment of cells with anisomycin accelerates the loss of HSF-1 DNA binding activity after heat shock.** *A*, gel mobility shift assays. HeLa cells were untreated (control) or treated with 20 μg/ml anisomycin for 20 min, rinsed with PBS, heated at 45 °C for 30 min, and allowed to recover at 37 °C for 0, 1, 2, 4, or 6 h. Samples in *lanes* labeled *C* were not heated. Whole cell extracts were used for gel mobility shift assays as described under "Methods and Materials." *Comp.* indicates the same lane as 6 h after heating but with 200-fold excess cold HSE added to the reaction. The amount of ^{32}P-HSE/HSF was quantitated for each lane with PhosphorImager and is presented relative to control. *B*, immunoblot analysis. Cells were untreated (control) or treated with 20 μg/ml of anisomycin for 20 min, rinsed with PBS, heated at 45 °C for 30 min, and allowed to recover at 37 °C for 4, 8, or 24 h. *Lanes* labeled *C* were not heated. 20 μg of TCA precipitated protein from each sample was immunoblotted and probed with antibody to inducible HSP-70 (using C92 antibody; Amersham Pharmacia Biotech) (*upper panel*) and reprobed with antibody against actin (*lower panel*) to show amount of protein loaded. Note that HeLa cells constitutively express an inducible form of HSP-70.

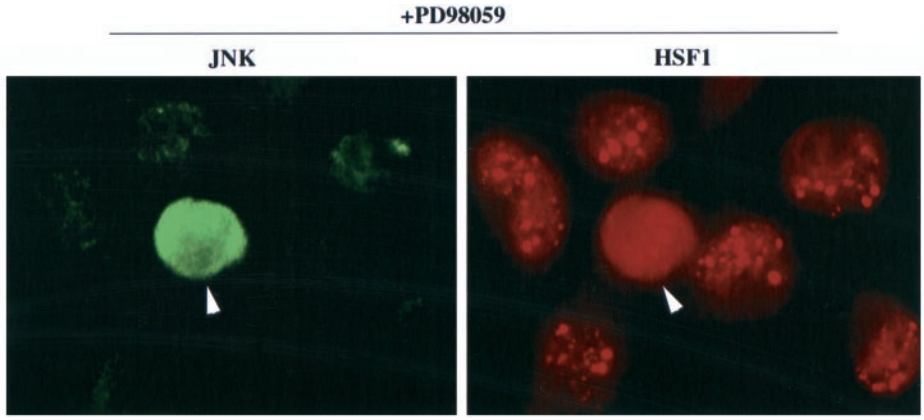

FIG. 6. **JNK effect on HSF-1 is independent of ERK cascade.** Representative immunoflurescence photographs (magnification, ×1000) of cells untreated or pretreated with PD98059 and transiently transfected with JNK1. HeLa cells were transiently transfected with 5 μg of HA-JNK1 DNA. 48 h after transfection, cells were untreated or were treated with 30 μM of PD98059 for 30 min, rinsed with PBS, heated at 45 °C for 30 min, and allowed to recover at 37 °C for 4 h. Cells were fixed and analyzed by indirect immunofluorescence. Overexpression of JNK1 was detected with mouse monoclonal primary antibody to HA and fluorescein isothiocyanate-conjugated secondary antibody. The endogenous HSF-1 was detected with rabbit polyclonal antibody to HSF-1 and Texas Red-conjugated secondary antibody. The *arrow* in the *left panel* indicates a cell with overexpressed JNK1, and the *arrow* in the *right panel* indicates the same cell stained for HSF-1.

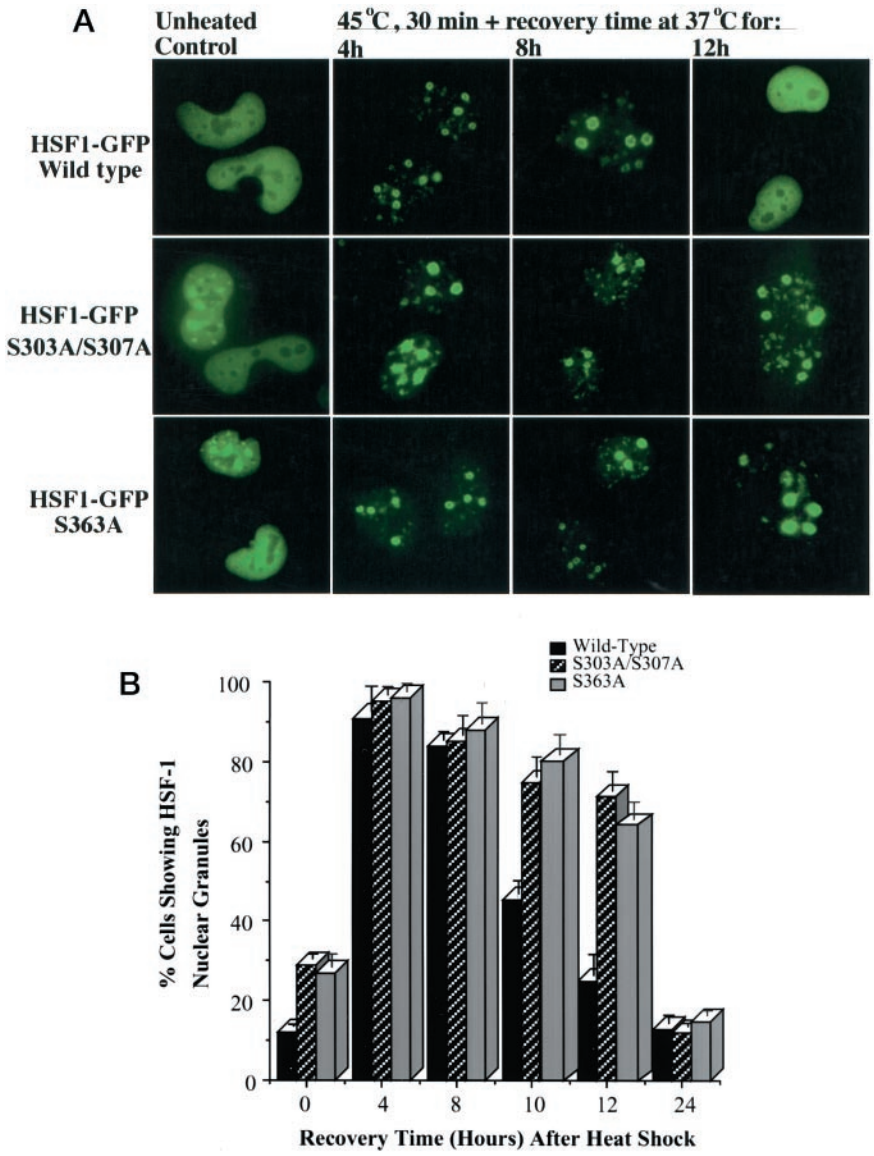

FIG. 7. **Kinetics of the disappearance of HSF-1 granules in HSF-1 mutants lacking JNK phosphorylation sites after heat shock.** *A*, representative immunofluorescence photographs (magnification, ×1000) of HeLa cells transfected with plasmids containing wild type HSF-1-GFP, HSF-1-GFP (S303A/S307A), or HSF-1-GFP (S363A). 48 h after transfection, cells were left unheated or were heated at 45 °C for 30 min and incubated at 37 °C for 4, 8, 10, 12, or 24 h for recovery. Cells were then fixed and analyzed. The data are representative photographs of at least three separate experiments. *B*, quantitation of the data shown in *A*. 0 (zero) on the *x*-axis represents untreated controls.

following JNK overexpression correlates with a reduction in HSF-1 transcriptional activity, cells were transiently transfected with either reporter constructs containing HSP-70 promoter fused to luciferase reporter (Fig. 4*A*) or GAL4-HSF-1 wild type (Fig. 4*B*) or a mutant of HSF-1 containing the S363A substitution (Fig. 4*C*) cotransfected with GAL4-CAT reporter gene. Results indicate that overexpression of JNK reduces the heat-induced HSP-70-luciferase expression over 50% (Fig. 4*A*) and that overexpression of JNK suppresses the heat-induced transcription of GAL4-CAT reporter after heat shock (Fig. 4*B*). The reduction in GAL4-HSF-1 transcriptional activity is also observed in cells cotransfected with expression plasmids containing ERK and GSK-3. This reduction in transcriptional activity is not observed in cells expressing HSF-1 S363A cotransfected with JNK expression plasmids, but it is observed in cells cotransfected with ERK and GSK-3β (Fig. 4*C*).

We also measured the transcriptional activity of HSF-1 when high levels of activated JNK are present in cells. For this, HeLa cells were pretreated with anisomycin and then heated, HSF-1 transcriptional activity was determined using gel mobility shift analysis as well as measuring the accumulation of HSP-70. As the data in Fig. 5*A* indicate, the HSF-1 DNA binding ability is reduced after heat shock, and this loss of HSF-1 DNA binding after heat shock is more rapid in cells pretreated with anisomycin. The heat-inducible HSP-70 accumulation was also reduced to almost undetectable levels (Fig. 5B). The concentration of anisomycin used here could conceivably reduce protein synthesis; however, our measurements of protein synthesis using [^{35}S]methionine incorporation into acid-insoluble fraction indicated that the rate of protein synthesis was 80% when compared with cells that were heated but were not pretreated with anisomycin at 6 h after heating. Therefore, the reduction in transcriptional activity of HSF-1 in cells containing high levels of JNK activity appears to be associated with a rapid loss of HSF-1 DNA binding activity and absence of HSP-70 production as well. The ability of JNK to suppress HSF-1 activity after heat shock is independent of the ability of ERK and GSK-3 repression of HSF-1 (23), because pretreatment of cells with PD98059, an inhibitor of MEK protein kinase (51) that completely abolishes GSK-3β dispersion of HSF-1 granules after heat shock (23) has no effect on the ability of JNK to disperse HSF-1 granules (Fig. 6).

Mutation of JNK Phosphorylation Site on HSF-1 Results in Delayed Recovery of HSF-1 Granules after Heat Shock—We then investigated the effect of the S363A substitution on the kinetics of HSF-1-GFP activity *in vivo*. HeLa cells were transiently transfected with plasmids containing wild type HSF-1-GFP or HSF-1-GFP (S303A/S307A) or HSF-1-GFP (S363A).

Cells were examined under unheated control conditions or after 4, 8, 10, 12, or 24 h of recovery time at 37 °C after heat shock (Fig. 7*A*). Similar to the overexpressed HSF-1 that appears diffuse throughout the nucleus (24), the wild type HSF-1-GFP, HSF-1-GFP (S303A/S307A), or HSF-1-GFP (S363A) are also found with a diffused staining pattern in the majority of cells under normal physiological growth conditions. As indicated in Fig. 7*B*, 12% of the cells transfected with the wild type HSF-1-GFP and 29% and 27% of the cells transfected with HSF-1-GFP (S303A/S307A) or HSF-1-GFP (S363A), respectively, show the overexpressed HSF-1 appearing with granular staining in the nuclei. After heat shock and 4 h of recovery time at 37 °C, over 90% of all transfected cells exhibit the presence of HSF-1 granules in their nuclei. At longer recovery times of 12 h, 25% of the cells transfected with wild type HSF-1-GFP show granules in their nuclei, whereas approximately 60–80% of cells with HSF-1-GFP (S303A/S307A) or HSF-1-GFP (S363A) show HSF-1 granules in their nuclei. These results indicate that phosphorylation of HSF-1 on Ser^{303}/Ser^{307} or Ser^{363} facilitates HSF-1 recovery after heat shock.

DISCUSSION

There is accumulating evidence that HSF-1 transcription is down-regulated by multiple mechanisms. These repression mechanisms include interaction with HSP-70, HSP-90, HSBP1, and perhaps other members of the HSF family (52–54). HSF-1 is also phosphorylated extensively, and therefore, it appears to be regulated by multiple protein kinases, whose identity have not all been established with certainty. The mitogen-activated protein kinases ERK and JNK recognize their substrates via a small domain known as the D domain. Deletion of this domain leads to severe reduction of the ability of these enzymes to phosphorylate their substrates (41, 42). In this study we have shown evidence that HSF-1 also contains sequences that are normally represented in the D domain where both ERK and JNK can bind. Furthermore, deletion of this domain reduces the ability of these enzymes to phosphorylate HSF-1 efficiently. Although both enzymes bind HSF-1 *in vitro*, we could specifically demonstrate ERK association with the hyperphosphorylated form of HSF-1 *in vivo* immediately after heat shock. However, ERK does not appear to be bound consistently to HSF-1 under physiological growth conditions. Interestingly, as the immunoblots in Fig. 2*A* suggest, ERK associates with the hyperphosphorylated form of HSF-1 after heat shock. Under similar experimental conditions, we were unable to consistently detect JNK association with HSF-1 *in vivo* (data not shown); however, in some immunoprecipitation experiments JNK was found to interact with what appears to be the high molecular weight, hyperphosphorylated form of HSF-1 in both untreated and heated cells. The inconsistency in detecting JNK association with HSF-1 could be their transient association with each other, or it could be that JNK may bind to HSF-1 for a short time after some recovery time after heat shock and not during the times that were tested here. The reason why ERK binds HSF-1 immediately after heat shock is unclear. However, ERK could bind but phosphorylate HSF-1 at later times when its phosphorylation site(s) becomes available, or because GSK-3β and ERK phosphorylation of HSF-1 have been postulated to occur sequentially, the limiting step may be GSK-3β phosphorylation of HSF-1, which could take place at a later time after heat shock. Our results also show that both JNK and ERK can bind HSF-1 *in vitro* and that deletion of the amino acid residues 203–224 in the HSF-1 protein reduces the ability of JNK1 and also JNK2, ERK1, and ERK2 to phosphorylate HSF-1. As we show in the data presented in Fig. 2*G*, the region of HSF-1 that is important for ERK and JNK binding also encodes the nuclear localization signal. This region, therefore, could be masked under normal physiological growth conditions by the interaction of the NH_2-terminal leucine zippers 1, 2, and 3 with the COOH-terminal leucine zipper 4 as had previously been suggested (55). Once HSF-1 protein is activated and becomes unfolded ERK and JNK could then bind to HSF-1.

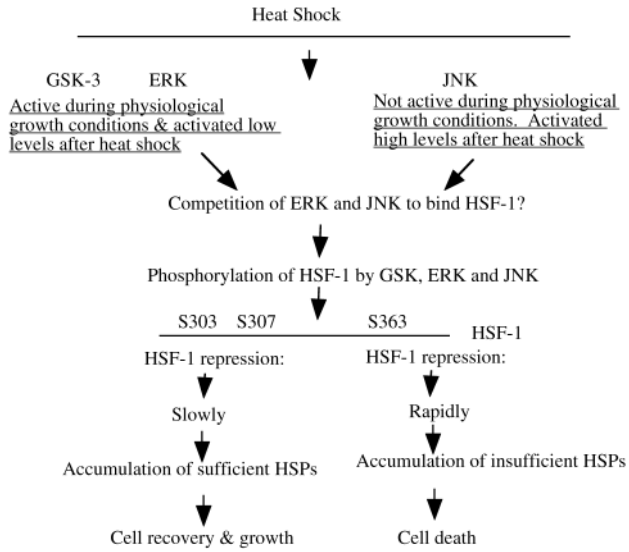

FIG. 8. **Schematic diagram showing HSF-1 regulation by ERK, GSK-3, and JNK signal transduction pathways.**

Previous experiments have shown that the ERK phosphorylation site on HSF-1 is Ser^{307}, and sequential phosphorylation by ERK and GSK-3 suppresses HSF-1 transcriptional activity (23, 25). JNK suppresses HSF-1 transcriptional activity by a similar mechanism as that observed for ERK and GSK-3, namely by causing HSF-1 to disperse from the sites of transcription more rapidly after heat shock. The paradox is how HSF-1 is suppressed by two opposing signaling pathways, namely, ERK and GSK-3, which are survival promoting signaling pathways, and JNK, a signaling pathway that is involved in apoptosis (Fig. 8). ERK, GSK, and JNK could cooperate to repress HSF-1 transcriptional activity for two reasons. First, because ERK and GSK are constitutively active under physiological growth conditions and their activities are only moderately enhanced by heat shock, they could repress HSF-1 constitutively, under physiological growth conditions, and gradually, during the recovery from heat shock for continuation of cell growth and survival. This is because retaining activated HSF-1 molecules could interfere with cell growth because of the partial or complete shut down of newly synthesized mRNAs and general protein synthesis that often accompanies the presence of large amounts of activated HSF-1, *e.g.* after heat shock. The gradual repression of HSF-1 in cells destined to survive after heat shock could result in accumulation of sufficient amounts of HSPs and protection of cells against further injury. Second, JNK is not normally active under physiological growth conditions, but it can be activated to high levels, and its activity is sustained for many hours after a severe heat shock. This could cause rapid repression of HSF-1 transcriptional activity and insufficient amounts of HSPs accumulation. Thus, damaged proteins remain unrepaired, and cells proceed into apoptosis.

In conclusion, we show evidence that JNK binds HSF-1 in its conserved domain and phosphorylates HSF-1 in its regulatory domain. Phosphorylation of HSF-1 by JNK leads to suppression of its transcriptional activity.

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c-Jun NH_2-terminal Kinase Targeting and Phosphorylation of Heat Shock Factor-1 Suppress Its Transcriptional Activity

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