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Analysis of the Phosphorylation of Human Heat Shock Transcription Factor-1 by MAP Kinase Family Members

Jynho Kim,[1,2] Arsenio Nueda,[1] Yong-Hong Meng,[1,2] William S. Dynan,[1] and Nahid F. Mivechi[1,2]*

[1]Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, Georgia 30912
[2]Department of Radiology, Medical College of Georgia, Augusta, Georgia 30912

Abstract The activation of heat shock transcription factor-1 (HSF-1) after treatment of mammalian cells with stresses such as heat shock, heavy metals, or ethanol induces the synthesis of heat shock proteins. HSF-1 is phosphorylated at normal growth temperature and is hyperphosphorylated upon stress. We recently presented evidence that HSF-1 can be phosphorylated by the mitogen activated protein kinase, ERK1, and that such phosphorylation appears to negatively regulate the activity of HSF-1. In this report, we have tested the ability of ERK1 to phosphorylate various HSF-1 deletion mutants. Our results show that ERK1 phosphorylation is dependent on a region of HSF-1 extending from amino acids 280 to 308. This region contains three serine residues that are potential ERK1 phosphorylation sites. The region falls within a previously defined regulatory domain of HSF-1. The possibility of protein kinases other than ERK1 phosphorylating HSF-1 was also examined using in-gel kinase assays. The results show that HSF-1 can be phosphorylated in a ras-dependent manner by other members of the MAP kinase family such as JNK and p38 protein kinases and possibly others. J. Cell. Biochem. 67:43–54, 1997.

Key words: HSF-1; heat shock; ERK1, phosphorylation; MAP kinases

Phosphorylation of transcription factors is a main mechanism regulating their nuclear translocation, DNA binding, or transcriptional activity. In many cases, multiple kinases can act on a single transcription factor [reviewed by Hunter and Karin, 1993]. The heat shock transcription factor-1 (HSF-1) is an example of a factor that is subject to complex regulation by phosphorylation. HSF-1 binds to conserved regulatory sequences known as heat shock elements and controls the expression of heat shock proteins in response to stress [Wu, 1984; Zimarino and Wu, 1987; Sorger et al., 1987; Larson et al., 1988; Abravaya et al., 1991; Mivechi et al., 1992].

Under normal growth conditions, mammalian HSF-1 exists in a latent, monomeric form [Baler et al., 1993; Sarge et al., 1993; Westwood and Wu, 1993]. This latent form is constitutively phosphorylated [Baler et al., 1993; Sarge et al., 1993; Mivechi and Giaccia, 1995; Cotto et al., 1996]. Upon heat shock, the latent form of HSF-1 is translocated into the nucleus, trimerizes, and is simultaneously hyperphosphorylated [Baler et al., 1993; Sarge et al., 1993; Rabindran et al., 1993]. The functional role of phosphorylation is not clearly understood. Because hyperphosphorylation accompanies transcriptional activation, it has been presumed to be important for this process [Baler et al., 1993; Sarge et al., 1993; Cotto et al., 1996]. In some cases, however, hyperphosphorylation of HSF-1 can be uncoupled from transcriptional activation [Sarge et al., 1993; Newton et al., 1996]. In addition, the phosphorylation of HSF-1 that accompanies activation of MAP kinases is associated with down-regulation of HSF-1 activity [Mivechi and Giaccia, 1995; Mivechi et al., 1994]. These data are suggestive of a complex pattern of regulation where phosphorylation of mammalian HSF-1 at different sites can be associated with either increases or decreases in activity.

Studies with yeast HSF reinforce the idea that a complex pattern of phosphorylation underlies changes in HSF activity. Yeast HSF is

Abbreviations: ERK, extracellular-signal regulated protein kinase; GST, glutathione-S-transferase; HSF-1, heat shock factor-1; HSP, heat shock protein; MBP, myelin basic protein.

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*Correspondence to: Dr. Nahid F. Mivechi, Medical College of Georgia, IMMAG, 1120 15th St., CB2803, Augusta, Georgia, 30912. E-mail: mivechi@immag.mcg.edu

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different than mammalian HSF-1 in that it binds constitutively to heat shock elements at normal growth temperature, but like mammalian HSF, it becomes hyperphosphorylated upon activation [Zimarino and Wu, 1987; Wiederrecht et al., 1988; Sorger, 1991]. Although it was originally proposed that this phosphorylation was important for activation, recent work has revealed that phosphorylation at some sites is actually correlated with the deactivation that occurs upon prolonged heat shock [HOJ and Jakobsen, 1994]. There are several other instances where mutations in the yeast MAP kinase or ras/cAMP signaling pathways alter the heat shock response, but it is not known if the effects are mediated through changes in the phosphorylation of HSF [Boorstein and Craig, 1990; Costigan and Snyder, 1994; Engelberg et al., 1994].

In the present study, we follow up on our previous observations that activation of mammalian MAP kinases inhibits the heat shock response, apparently by inducing changes in HSF-1 phosphorylation state [Mivechi and Giaccia, 1995; Mivechi et al., 1994]. The mammalian MAP kinase family includes a number of kinases that are activated by specific signals and that phosphorylate proteins at a pro-x-ser/thr-pro consensus sequence [Alvarez et al., 1993]. This family includes ERK1 and ERK2, which are activated principally by growth factors, the stress activated protein kinases (SAPKs in murine and JNKs in human), which are activated by ultraviolet light, pro-inflammatory cytokines, heat shock, or X-irradiation [Dam et al., 1995; Derijard et al., 1994; Sanchez et al., 1994; Adler et al., 1996], and the p38 kinase, which is activated by hyperosmolarity and heat shock [Han et al., 1994]. Most of the mammalian MAP kinases are known to exert their effects, at least in part, by phosphorylation of transcription factors. This phosphorylation can lead to either increases or decreases in transcriptional activity depending on the factor [Derijard et al., 1994; Sanchez et al., 1994; Whitmarsh et al., 1995; Janknecht et al., 1994; Hipskind et al., 1994; Gupta et al., 1995; Wang and Ron, 1996; Chen et al., 1993]. In this report, we have mapped the region of HSF-1 that is required for phosphorylation by ERK1. This region resides between amino acids 280 and 308 in the regulatory domain and contains 3 potential MAP kinase phosphorylation motifs at serine residues 292, 303, and 307. We have also used in-gel kinase assays to show that a number of other MAP kinase family members or proline-directed protein kinases phosphorylate HSF-1. Phosphorylation by these kinases is also dependent on specific sequences in the HSF-1 regulatory region.

MATERIALS AND METHODS

Cell Lines

NIH3T3 cells and NIH3T3 cells stably transfected with Ha-Ras (NIH3T3R) were a gift of Dr. Amato Giaccia (Stanford University, Stanford, CA). Cells were maintained in Dulbecco's modified Eagles medium supplemented with 10% FCS, and 400 μg/ml of G418 was routinely added to the cultures of NIH3T3R cells. Cells were incubated in the presence of 0.5% FCS 48 h prior to the onset of an experiment in order to reduce the activity of MAP kinases under normal growth conditions. NIH3T3R cells have been characterized in detail previously and have been shown to exhibit a transformed phenotype [Denko et al., 1994].

Generation of HSF-1 Deletion Mutants

The starting construct for mutagenesis was HSF-1 (1-450), which contains sequences encoding the first 450 amino acids of human HSF-1 inserted between NdeI and Hinc II sites of pET-23a (+) (Novagen, Madison, WI). The resulting protein, which is expressed under the control of the T7 promoter in *Escherichia coli*, contains a C-terminal histidine tag to facilitate purification. HSF-1 expressed in *E. coli* is constitutively active for DNA binding [Sarge et al., 1993; Rabindran et al., 1993]. Although missing 79 non-phylogenetically conserved amino acids at the C-terminus, the HSF-1 (1-450) protein is transcriptionally active, as measured in an in vitro transcription assay (S. Jesch and W.S. Dynan, unpublished results).

Specific restriction sites (SacII) were created along the HSF-1 sequence by site-directed mutagenesis using the method of Deng and Nickoloff [1992]. The selection oligonucleotide 5′TTCGCGGGATCGAATTCTCGGGCAGCGTTG-3′, was designed to change the Bgl II restriction site located at position 334 in the pET23 plasmid to an EcoRI site. The following mutation oligonucleotides were designed to introduce new SacII sites in the coding sequence of HSF-1; (0) 5′CTTTCTCTTCACCCGCGGGATCCGGTTTGAC-3′, (1) 5′ACTGCCGGCTATAC-

CGCGGCATGGAATGTGC-3′, (2) 5′CTCGTCT-ATGCTCCCCCGCGGGGAGGCCATGGG-3′, (3) 5′CTCCTCTACCCGCGGGCTCTGAGGC-3′, (4) 5′CTACGCTGAGGCA-CTTCCGCGG-GGTGGAGGTGGG-3′, (5) 5′CGGGCACGGT-CACCCGCGGGCT-GAACAGGTCC-3′, and (6) CCTGGGGGGCTCCCGCGGAGACAGGAG-CTC. Briefly, the selection oligonucleotide and different pairs of mutation oligonucleotides were hybridized to denatured HSF450pET23 plasmid. The annealed primers were extended using T4 DNA polymerase and ligated with T4 DNA ligase. The resulting mixture of hybrid and wild-type plasmid was digested with Bgl II and subsequently transformed into *E. coli* strain BMH71-18 mutS, defective in mismatch repair. Plasmid DNA was purified by alkaline lysis, extensively digested again with Bgl II and was used to transform *E. coli* DH5α cells. Clones containing the desired combination of Sac II sites were further digested with Sac II, relegated to create the final deletion mutants, and transformed into *E. coli*. All mutants were confirmed by DNA sequencing.

Purification of Histidine and GST-Tagged Fusion Proteins

The histidine-tagged HSF-1 deletion mutants were purified with Ni-NTA resin according to the manufacturer's instruction (Pharmacia, Gaithersburg, MD). GST-Jun (gift of Dr. Zon, Childrens Hospital, Boston) and GST proteins were purified using glutathione-agarose. Briefly, GST-fusion proteins were induced by growing the bacteria in 1 mM IPTG for 3 h. Bacteria were lysed in buffer containing Phosphate Buffer Saline (PBS), 100 mM EDTA, 1% Triton-X 100, 1% aprotinin, 1 mM PMSF, and 1 mM dithiotreitol (DTT). After centrifugation, glutathione-agarose beads (Sigma Chemical Co., St. Louis, MO) were added and rotated at 4°C for 30 min. After washing the beads with PBS plus 100 mM EDTA, the proteins were eluted with 20 mM glutathione in 100 mM Tris, pH 8.0, and the appropriate fractions were concentrated with UFV2BG filters (Millipore, Bedford, MA).

Immunoprecipitation and Immune Complex Kinase Assays

To assess MAP, JNK, or p38 protein kinase activity, cells were treated as described in Results and lysed in buffer containing sodium β-glycerophosphate (50 mM, pH 7.2), 10 mM $MgCl_2$, 5 mM EGTA, 1 mM EDTA, 10 mM KH_2PO_4, 1 mM Na_3VO_4, and 0.2 mM PMSF. Lysate was cleared by centrifugation, and the amount of protein was estimated by bicinchoninic acid (Pierce Chemical Co., Rockford, IL). Equal amounts of protein (300 µg) from each sample were immunoprecipitated with 5 µg of anti-ERK1, JNK, or p38 antibodies (Santa Cruz Biotech, Santa Cruz, CA) by incubating the mixture at 4°C for 1 h. The lysate/antibody mixture was incubated with Protein A-Sepharose for an additional 1 h. The antigen/antibody conjugated to protein A was washed 3 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 DTT, 1 mM Na_3VO_4, and 0.2 mM PMSF) [Mivechi and Giaccia, 1995; Dam et al., 1995; Kyriakis et al., 1994; Whitmarsh et al., 1995]. The complex was then incubated for 15 min at 30°C in 10 µl of kinase buffer with 50 µM of unlabeled ATP, 10 µCi of ^{32}P-γ-ATP, and 1 µg of purified wild-type HSF-1 (1-450) or HSF-1 deletion mutants as substrates. The reaction was terminated by the addition of 2 × SDS-sample buffer and samples were analyzed by SDS-PAGE.

In-Gel Kinase Assays

Following various treatments, cells were lysed in buffer containing 10 mM Tris, 50 mM β-glycerophosphate, 1.5 mM EGTA, 1 mM DTT, 0.1 mM Na_3VO_4, and 0.5% Triton-X 100 and cell lysates were cleared by centrifugation at 50,000*g* for 15 min. Protein concentration of the lysate was determined by bicinchoninic acid (Pierce Chemical Co.) and equal amounts of protein from each sample were added to 2× SDS Laemmli's sample buffer and heated to 95°C for 5 min [Mivechi and Giaccia, 1995]. The gel contained acrylamide/Piperazine diacrylamide (PDA) (29.2%:0.8%) (38%), 4× Tris pH 8.9 (28%), glycerol (3%), ammonium persulfate (0.1%), TEMED (0.1%), and 0.5 mg/ml of the Myelin Basic Protein (MBP) (UBI), Glutathione-S-Transferase (GST), GST-Jun or His-tagged HSF-1 fusion proteins. The stacking gel contained acrylamide/PDA (29.2%:0.8%) (16%), 4 × Tris pH 6.8 (24%), ammonium persulfate (0.15%), and TEMED (0.15%). Gels were loaded with 30–50 µg of protein from each sample and electrophoresed at 40 mA constant current. Gels were incubated twice in 100 ml of 50 mM Tris, pH 8.0, 20% 2-isopropanol for 30 min at 25°C; once in 250 ml of 50 mM Tris, pH 8.0, 0.2 mM

DTT for 1 h at 25°C; twice in 100 ml of 50 mM Tris, pH 8.0, 6 M guanidine HCl, 2 mM DTT for 30 min at 25°C; and in 1 liter of 50 mM Tris, pH 8.0, 0.04% tween-40, 2 mM DTT overnight at 4°C to renature the enzymes. Gels were then equilibrated with kinase buffer containing 40 mM HEPES, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EGTA, 2 mM DTT, 0.025 mM Mg^{2+} ATP, PKA, and CAM kinase/PKC inhibitors (1% final conc. of each inhibitor) (Sigma) for 30 min at 25°C. The kinase assay was performed with incubating the gel in kinase buffer supplemented with 10 μCi/ml of γ-^{32}P-ATP (6,000 Ci/mMol) for 1 h at 25°C. Gels were washed five times with 5% TCA, 0.1% sodium pyrophosphate for 3 h at 25°C, dried and exposed to X-ray film [Whitmarsh et al., 1995; Gupta et al., 1995] for 4–16 h at −70°C. For each in-gel kinase assay, a gel containing cell lysates but with no substrate embedded in the gel was also analyzed. These control gels provided detection of background autophosphorylation of protein kinases, which may occur and is independent of the addition of substrates into the gel.

RESULTS

Mutational Analysis of HSF-1 Phosphorylation by ERK1 Protein Kinase

Sequence analysis suggests that HSF-1 contains multiple potential ERK1 phosphorylation sites. To map the region of HSF-1 phosphorylation by ERK1, immune complex kinase assays were performed with purified wild-type histidine-tagged HSF-1 or a series of deletion mutants as substrates. ERK1 was immunoprecipitated from NIH3T3 cells that had not received any treatment (control), which were heated (43°C for 30 min), or which were pretreated with sodium vanadate (500 μM for 2 h at 37°C). The immunoprecipitated ERK1 protein kinase was then used to phosphorylate the purified wild-type HSF-1 protein (amino acids 1–450) or a mutant of HSF-1 protein that contained deletions between amino acids 203 and 445 (mutant Δ06) (Fig. 1). This deletion removes the C-terminal portion of HSF-1 that has regulatory and transcriptional activation functions [Shi et al., 1995; Green et al., 1995], but leaves the N-terminal DNA binding and trimerization domains intact (Fig. 1A). As shown in Figure 1B, wild-type HSF-1 was phosphorylated by immunoprecipitated ERK1. Consistent with previous results, the level of phosphorylation was increased when ERK1 was precipitated from cells that had been heat treated (wt, 43°C 30 min, 7-fold increase) or treated with sodium vanadate, which increases the level of ERK1 activity by inhibiting MAP kinase phosphatase (wt, SV 12-fold increase) [Mivechi and Giaccia, 1995].

When mutant Δ06 was used as a substrate, phosphorylation by ERK1 immunoprecipitated from control cells was reduced. Strikingly, the phosphorylation of mutant Δ06 was almost unaffected by heating or sodium vanadate treatment (1–2-fold). Coomassie blue staining of the gel performed as a control (Fig. 1C) shows that the Δ06 mutant protein was present at equal or greater levels as compared to wild-type HSF-1. These results suggest that the major region of phosphorylation of HSF-1 by ERK1 protein kinase resides between amino acids 203 and 450 and that there is little or no phosphorylation of DNA binding or trimerization domain located between amino acids 1 and 203.

We next performed immune complex kinase assays to analyze phosphorylation of HSF-1 deletion mutants containing mutational deletions located in the regions between amino acid residues 203 and 445. Mutant Δ34 had a deletion of sequences between amino acids 308 and 370, mutant Δ24 had a deletion of sequences between amino acids 280 and 370, mutant Δ36 had a deletion of sequences between amino acids 308 and 445, and mutant Δ56 had a deletion of sequences between amino acids 420 and 445 (Fig. 2A). As shown in Figure 2B, ERK1 immunoprecipitated from cells under control conditions, phosphorylates HSF-1 wild-type, mutants Δ34, Δ36, and Δ56 to the same extent. However, there was an approximate 7-fold reduction in phosphorylation of HSF-1 mutant Δ24. These results indicate that the phosphorylation of HSF-1 by ERK1 protein kinase is highly dependent on a sequence between amino acids 280 and 308. Similar results were obtained using ERK1 immunoprecipitated from heat-treated (43°C, 30 min) cells (Fig. 3C). The immune complex kinase assays performed with cells pretreated with sodium vanadate (500 μM) showed similar patterns of phosphorylation, that is, the HSF-1 mutant Δ24 showed a reduction in HSF-1 phosphorylation (data not shown). As shown in Figure 2A, the amino acids between 280 and 308 in HSF-1 protein contain 3 serine residues with potential ERK1 phosphorylation motifs. These serine residues are located at amino acid residues 292, 303, and 307. Figure 2D shows that all mutants

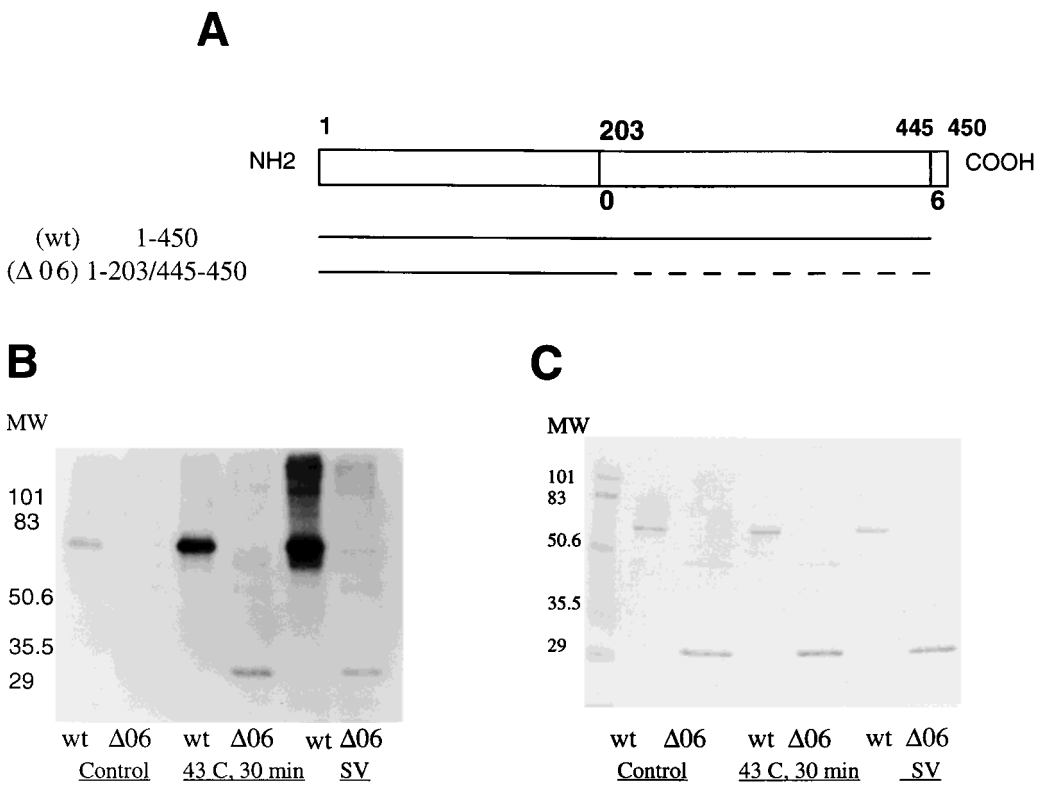

Fig. 1. Immune complex kinase assays using wild-type HSF-1 (1-450) and HSF-1 deletion mutant (Δ06) as substrates. **A:** Map of HSF-1 (1-450) (M_r 61,000) and mutant Δ06 (M_r 30,000). Dotted line indicates region absent in mutant (residues 203–445). **B:** Immune complex kinase assays of immunoprecipitated ERK1 protein kinase from control, heat shocked (43°C for 30 min) or sodium vanadate (SV) (500 μM for 2 h at 37°C) pretreated NIH3T3 cells. Reactions contained 1 μg of HSF-1 (1-450) or mutant Δ06 proteins as substrates. **C:** 1 μg of the purified wild-type HSF-1 (1-450) or mutant Δ06 were loaded in the gel, analyzed by SDS-PAGE, and stained with Coomassie blue. Molecular weight markers (MW) are shown in the left.

were present in approximately similar amounts, as measured by Coomasie blue staining.

Analysis of HSF-1 Phosphorylation by In-Gel Kinase Assays

We next performed in-gel kinase assays. In this method, equal amounts of protein are resolved in an SDS-PAGE that has had substrate added at the time of casting the gel. After cycles of denaturation and renaturation, incubation is carried out in situ with γ-^{32}P ATP, resulting in radiolabeled bands that indicate the position of the protein kinases able to phosphorylate the substrate present in the gel. Control, heat (43°C, 30 min) or sodium vanadate (500 μM) pretreated cells were lysed and equal amounts of each lysate were analyzed. Figure 3A shows results with myelin basic protein (MBP) as a substrate. As expected, the 44 kDa ERK1 and the 42 kDa ERK2 phosphorylate MBP under control conditions and MBP phosphorylation by these enzymes is dramatically increased under heat shock (43°C, 30 min) or sodium vanadate (500 μM) pretreatment conditions. This is consistent with previous reports that both ERK1 and ERK2 protein kinases are activated by heat shock as well as by sodium vanadate, an inhibitor of phosphatase, which regulates MAP kinases phosphorylation state [Mivechi and Giaccia, 1995; Mivechi et al., 1994; Klurlund, 1985; Tsao and Greene, 1991; Ward et al., 1994; Sun et al., 1993].

The phosphorylation of purified GST-Jun protein by JNK protein kinase in cell lysates obtained from control, heat-shocked (43°C, 30 min), or anisomycin (20 μg/ml for 20 min) pretreated cells is shown in Figure 3B. Both the 46 and 52 kDa JNK kinases are activated by heat and to a smaller extent by anisomycin, resulting in an increase in phosphorylation of GST-Jun transcription factor.

When purified HSF-1 was used as a substrate there was an increase in phosphorylation of HSF-1 under heat-shocked (43°C, 30 min)

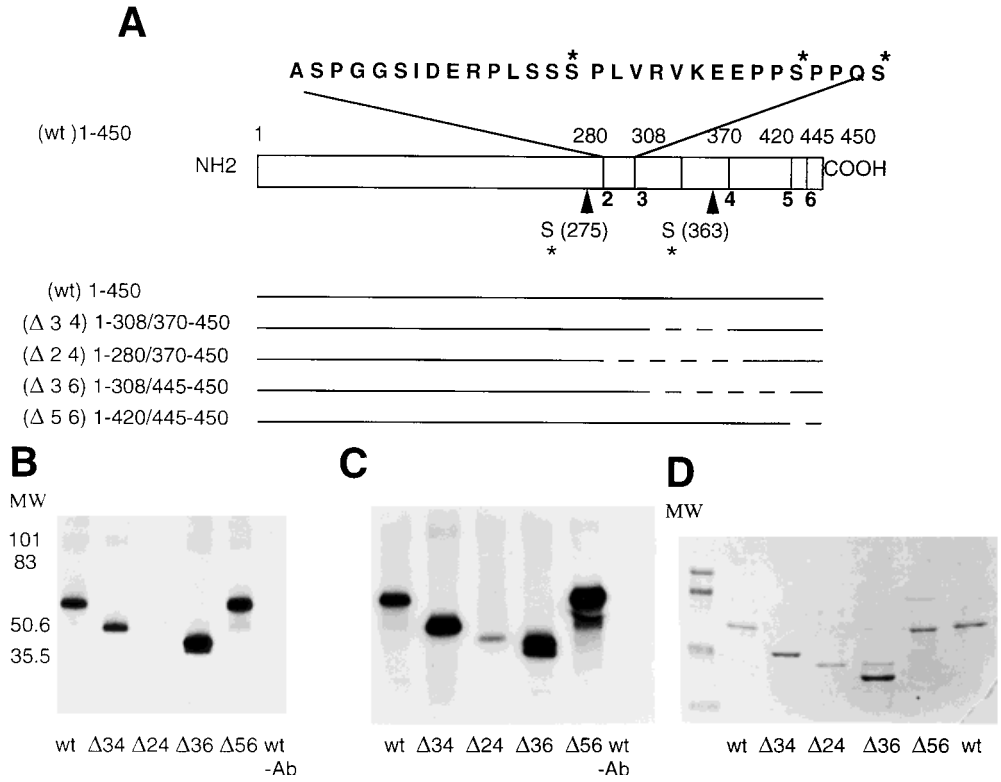

Fig. 2. Immune complex kinase assays using the wild-type HSF-1 (1-450) and individual HSF-1 deletion mutants as substrates. **A:** Map of HSF-1 (1-450) (M_r 61,000) and HSF-1 deletion mutants (Δ34, Δ24, Δ36, and Δ56 with M_r 54,000, 50,000, 43,000, 60,000, respectively). The dotted lines correspond to the sequences absent from each mutant. The serine residues (*) are potential MAP kinases phosphorylation sites. **B:** Immune complex kinase assays of immunoprecipitated ERK1 protein kinase from control NIH3T3 cells. The immunoprecipitated ERK1 was used to phosphorylate 1 µg of wild-type (wt) or the various deletion mutants indicated. The position of the molecular weight markers is shown on the left. **C:** Immune complex kinase assays of immunoprecipitated ERK1 protein kinase from heated (43°C for 30 min) NIH3T3 cells. Reaction contained 1 µg of wild-type (wt) or deletion mutants as substrates as indicated. The lane labelled as wt Ab in B and C indicate that no antibody was used during immunoprecipitation reaction. **D:** 1 µg of the purified wild-type HSF-1 (1-450) (wt) or deletion mutants analyzed by SDS-PAGE and stained with Coomassie blue. Molecular weight (MW) markers are shown on the left.

conditions by protein kinases with estimated molecular weights of 42–44 kDa, suggesting the activation of ERK1 or ERK2 protein kinases by heat shock (Fig. 3C). In sodium vanadate (500 µM) pre-treated cells (and to a lesser extent in the heated cells), protein kinases with higher molecular weights, possibly the 46 and 52 kDa JNKs, also phosphorylate HSF-1 (1-450).

Control in-gel kinase assays where either no substrate (Fig. 3D and F) was added to the gels, or when purified GST protein was used as a substrate (Fig. 3E) showed very little phosphorylation under these conditions, indicating that the assay is strongly substrate dependent.

Protein Kinases Phosphorylating HSF-1 Are Ras-Dependent

The MAP kinase family of protein kinases are regulated by ras protein [Khosravi-Far and Der, 1994; Crews et al., 1992]. To determine whether the protein kinases that phosphorylate HSF-1 show enhancement of their activities in the presence of activated Ha-ras, in-gel kinase assays were performed using cell lysates obtained from NIH3T3 or NIH3T3 R cells that contained activated ras. As shown in Figure 4, HSF-1 is phosphorylated intensely by kinases with molecular weights corresponding to ERK1 and ERK2 (44 and 42 kDa), JNK (52 and 46 kDa), as well as protein kinases with an estimated molecular weight of 38 kDa (p38) in lysates of NIH3T3R cells. There are also several protein kinases at high molecular weights (above 49 kDa molecular weight marker) that have not been identified. These data suggest that the protein kinases that phosphorylate HSF-1 are under the control of ras protein. This is in agreement with previous results that repressive effects of ERK1 potentiated in *ras* transformed cells [Mivechi and Giaccia, 1995].

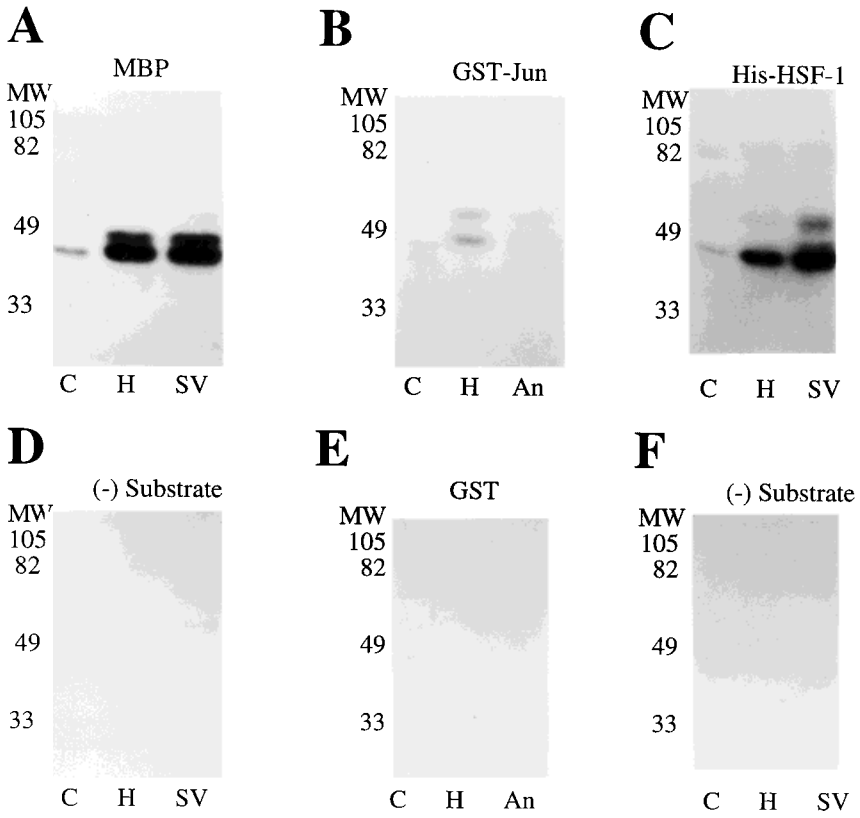

Fig. 3. Analysis of phosphorylation of HSF-1 (1-450) by using in-gel kinase assays. Equal amounts of protein from cell lysates of control (C), heated (43°C, for 30 min) (H), sodium vanadate (500 µM for 2 hours) (SV), or anisomycin (20 µg/ml for 20 min at 37°C) (An) pretreated cells were analyzed by in-gel kinase assays using (**A**) MBP as substrate. The protein kinases phosphorylating MBP represent ERK1 at 44 kDa and ERK2 at 42 kDa. **B:** GST-Jun as substrate. The protein kinases phosphorylating GST-Jun represent 46 and 52 kDa JNK. **C:** HSF-1 as substrates. In lanes indicated as C and H, the approximate molecular weights of the protein kinases phosphorylating HSF-1 are 44–46 kDa. In the lane indicated as SV, other protein kinases with approximate molecular weights of 46 and 52 kDa (most likely JNK kinases) also phosphorylate HSF-1. D–F are controls (no substrate or GST were added to the gels) for A–C, representing the level of autophosphorylation or phosphorylation of GST by protein kinases present in the cell lysates. **D:** Contained no substrate. **E:** Contained GST as a substrate. **F:** Contained no substrate. Molecular weight markers are shown at the left.

Further, the results strongly suggest that HSF-1 is the target of more than one member of the MAP kinase family of protein kinases.

To investigate whether the protein kinases that are activated following stable overexpression of Ha-ras in NIH3T3R cells regulate specifically the region between amino acids 280 and 370, the phosphorylation pattern of the wild type HSF-1 (amino acids 1–450) (Fig. 4) was compared with mutant Δ24 using in-gel kinase assays. Results with cell lysates from control, heated (43°C, 30 min) or sodium vanadate (500 µM) pretreated cells from NIH3T3 or NIH3T3R are shown in Figure 5. No phosphorylation of mutant Δ24 was detected by protein kinases present in the lysates obtained from NIH3T3 cells in agreement with results of immune complex kinase assays. However, mutant Δ24 can be phosphorylated by protein kinases with estimated molecular weights of 38 and 42 and/or 44 kDa (p38 and ERK 1 or 2) from lysates of NIH3T3R cells obtained from control or heat shock cells, and by a 52 kDa (JNK) after heat shock (minor level of phosphorylations) or sodium vanadate pretreated conditions. Although the specificity of this phosphorylation was not investigated in detail, a consensus MAP kinase phosphorylation site, which remains undeleted in mutant Δ24, corresponds to the serine residue 275 (Fig. 2). These data suggest that MAP kinases ERK1, ERK2, JNK, and p38 protein kinases have different affinities for the various sites present in HSF-1 protein and that specifically p38 protein kinase shows apparent expansion of specificity of MAP kinases phosphorylation in the presence of *ras*. It may be that very high levels of MAP or JNK kinases lead to utilization of lower affinity sites present in the

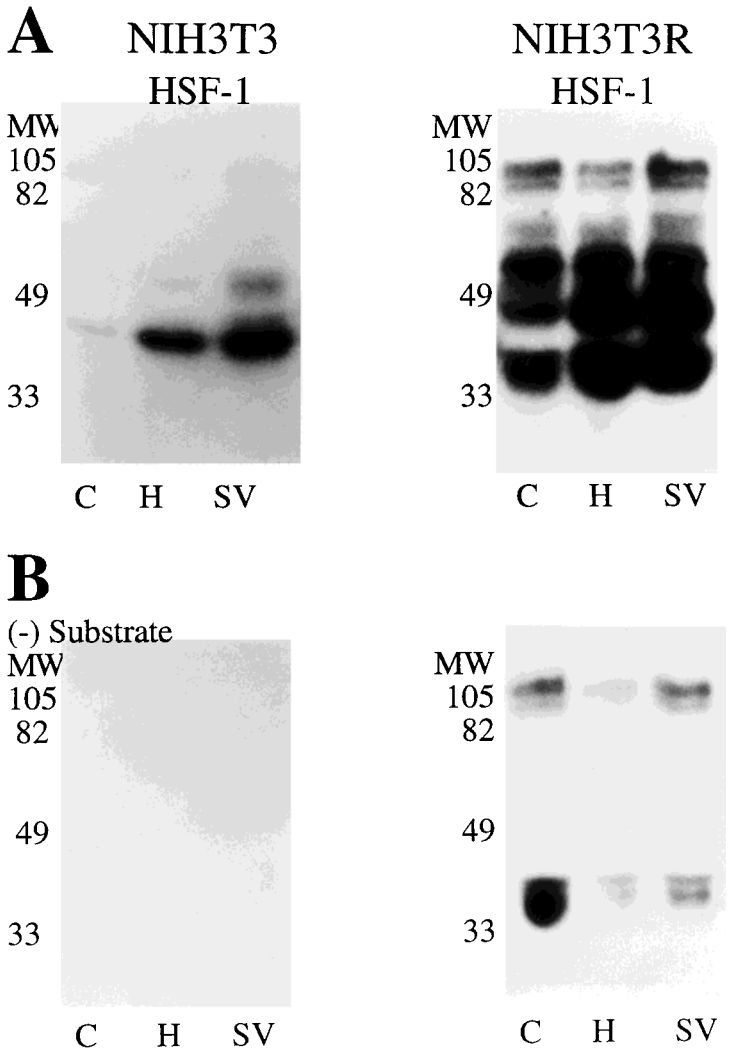

Fig. 4. Protein kinases phosphorylating HSF-1 are ras-dependent. **A:** Equal amounts of protein from lysates of control (C), heat shocked (43°C for 30 min) (H) or sodium vanadate (500 μM 2 h at 37°C) (SV) pretreated cells were analyzed by in-gel kinase assays using wild-type HSF-1 (1-450) as a substrate. The conditions shown in **B** are the same as in A, but no substrates were added to the gels to show the level of autophosphorylation of the protein kinases present in the cell lysates. Molecular weight markers are shown at the left.

HSF-1 protein. Figure 5B shows the background autophosphorylation (of the protein kinases present in the cell lysates) without the addition of any substrate. Differences in the background autophosphorylation observed in Figures 4B and 5B are due to the fact that two different sample preparations were used in the two sets of experiments and there were differences in the exposure time to X-ray film.

DISCUSSION

We have previously shown that activation of ERK1 negatively regulates heat shock protein expression. We proposed that this effect is mediated by ERK1 phosphorylation of HSF-1 [Mivechi and Giaccia, 1995]. These observations were extended here by determining the specific regions of HSF-1 that are required for phosphorylation by ERK1. Immune complex kinase assays show that this phosphorylation is highly specific both before and after heat shock. Deletion of amino acid residues in HSF-1 280 to 370 (mutant Δ24) virtually eliminated phosphorylation by ERK1. Other deletions from 308 to 370 (mutant Δ34) or 308 to 445 (mutant Δ36) were without effect. Thus, these results indicate that the regions absent from mutant Δ24 but present in mutants Δ34 and Δ36, that is, the region from 280 to 308, is critical for phosphorylation.

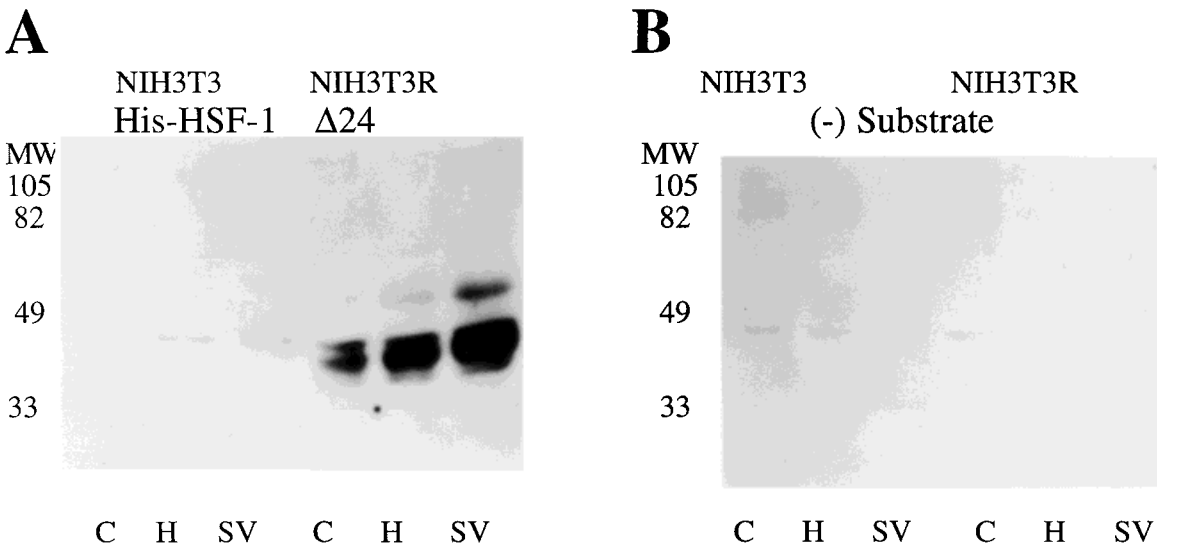

Fig. 5. HSF-1 mutant Δ24 is only phosphorylated in a ras-dependent manner. **A:** Equal amounts of protein from lysates of control (C), heat shocked (43°C for 30 min) (H) or sodium vanadate (500 μM 2 h at 37°C) (SV) pretreated NIH3T3 or NIH3T3R cells were analyzed by in-gel kinase assays using purified HSF-1 deletion mutant Δ24 as a substrate. The conditions shown in **B** are the same as in A, but no substrates were added to the gels to show the level of autophosphorylation of the protein kinases present in the cell lysates. Molecular weight markers are shown at the left.

The region from 280 to 308 contains MAP kinase consensus phosphorylation sites at positions 292, 303, and 307. A likely interpretation of the mutational data is that one or more of these positions are the actual sites of MAP kinase phosphorylation. However, we cannot exclude the possibility that the sequence between 280 and 308 constitutes a MAP kinase binding domain required for directing phosphorylation at other sites in HSF-1. It is unlikely that such sites could be located C-terminal to position 308, since phosphorylation was observed even when these sequences were deleted (for example, in mutant Δ36). However, such sites could be located N-terminal to position 280 (for example, the MAP kinase consensus site at position 275). Whether or not phosphorylation is actually within the region from 280 to 308, the results indicate that the interaction with ERK1 is highly specific, and that the increase in ERK1 activity after heat shock does not result in a random phosphorylation of the HSF-1 protein.

The results of in-gel kinase assays confirm that a protein with mobility corresponding to ERK1 (44 kDa) phosphorylates HSF-1. These assays also demonstrate the presence of a number of other kinases that are capable of phosphorylating HSF-1 under the conditions tested. Based on their apparent molecular weights, the finding that they are activated by heat and sodium vanadate, and the fact that their activity is strongly increased in *ras*-transformed cells, we believe these bands correspond to other members of the MAP kinase family. Because of their mobilities in SDS-PAGE, we have tentatively identified these as ERK2 (42 kDa) and as the stress-activated protein kinases JNK (52 kDa) and p38 (38 kDa). However, under conditions of maximal induction, in the presence of *ras*, the pattern of bands becomes quite complex, and it is possible that novel MAP kinase family members, specific to HSF-1, may be present. We are currently investigating this possibility through further biochemical fractionation of extracts from the induced cells.

Interestingly, phosphorylation of HSF-1 by the multiplicity of kinases identified in the in-gel kinase assays is dependent on the same sequences as are required for ERK1. That is, phosphorylation is much reduced by deletion of HSF-1 amino acids 280–370 (mutant Δ24). A similar dependence on these sequences was seen in immune complex kinase assays using antibodies against JNK or p38 (Fig. 6). The data raise the interesting possibility that HSF-1 phosphorylation in vivo may reflect the contributions of many different kinases that target the same region of HSF-1. This would be similar to the situation with ELK1 transcription factor [Whitmarsh et al., 1995; Janknecht et al., 1994], which is also phosphorylated by more

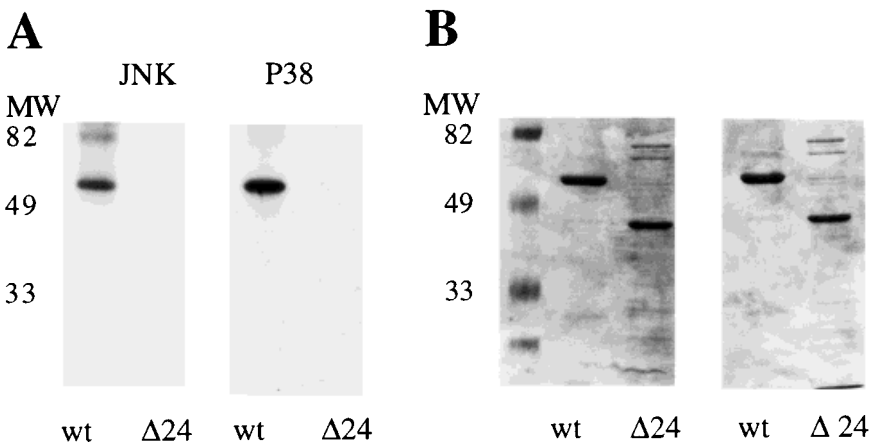

Fig. 6. Immune complex kinase assays of JNK and p38 protein kinases using the wild-type HSF-1 (1-450) and HSF-1 deletion mutant Δ24 as substrates. **A:** Immune complex kinase assays of immunoprecipitated JNK or p38 protein kinases from heat shocked (43°C for 30 min) NIH3T3 cells. Reactions contained approximately 1 μg of wild-type (wt) or deletion mutant (Δ24) as indicated. **B:** 1 μg of the purified wild-type HSF-1 (1-450) (wt) or deletion mutant Δ24 were analyzed by SDS-PAGE and stained with Coomassie blue. Molecular weight markers are shown on the left.

than one MAP kinase family member concurrently.

The region of HSF-1 that is targeted by MAP kinases is located within a phylogenetically conserved regulatory domain of HSF-1 that has been shown to repress the activity of chimeric GAL4-HSF-1 fusion proteins under unstressed growth conditions [Green et al., 1995]. Previous results suggest that ERK1 negatively regulates HSF-1 function. We suggest that phosphorylation of the regulatory domain by MAP kinases might stabilize the regulatory domain in an inactive conformation, reducing its activity.

Biologically, the induction of ERK1 and ERK2 by heat shock, and the subsequent phosphorylation of HSF-1 could represent a means for adaptation, that is, for a gradual down-regulation of the heat shock response after the initial burst of heat shock mRNA synthesis. This would parallel the situation in yeast, where HSF phosphorylation that occurs immediately after heat shock is associated with adaptation [Hoj and Jakobsen, 1994]. Adaptation is necessary so that normal growth and metabolic activity can be resumed after a period of stress. It is well established that mammalian ERK1 is involved in promoting transcription of growth-regulated genes. It is logical, therefore, that ERK1 might simultaneously function to turn off an alternative program of expression of stress-related genes. In contrast to ERK1, the activation of JNK or p38 protein kinases by heat shock may be associated with induction of an apoptotic response. The ability of JNK or p38 protein kinases to down-regulate HSF-1 may reflect the fact that there is no need to mount a heat shock response in cells destined to die.

Consistent with our studies, while this manuscript was under review other studies were published suggesting that serine 303 and serine 307 are the sites of potential phosphorylation by MAP kinases [Knauf et al., 1996; Chu et al., 1996; Kline et al., 1997]. Phosphorylation in this region exerts a negative regulatory effect on HSF-1. There are other potential sites of phosphorylation of HSF-1 protein; however, the protein kinases phosphorylating these sites or the significance of these phosphorylations in vivo is uncertain at the present time.

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