

## Activation of Heat Shock Factor 1 DNA Binding Precedes Stress-induced Serine Phosphorylation

EVIDENCE FOR A MULTISTEP PATHWAY OF REGULATION\*

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**Exposure of mammalian cells in culture to the anti-inflammatory drugs sodium salicylate or indomethacin results in activation of heat shock factor 1 (HSF1) DNA binding activity. We have previously shown that the drug-induced HSF1 becomes associated with the heat shock elements of the hsp70 promoter, yet transcription of the hsp70 gene is not induced (Jurivich, D. A., Sistonen, L., Kroes, R. A., and Morimoto, R. I. (1992) *Science* 255, 1243–1245). In this study, we have examined the basis for uncoupling the heat shock transcriptional response. Comparison of heat shock and drug-induced forms of HSF1 has revealed that the transcriptionally inert drug-induced HSF1 is constitutively but not inducibly serine-phosphorylated, whereas heat shock-induced HSF1 is both constitutively and inducibly serine-phosphorylated. The transcriptionally inert intermediate represented by drug-induced HSF1 can be converted to the transcriptionally active state by a subsequent exposure to heat shock. The only detectable change in HSF1 is the acquisition of inducible serine phosphorylation. These data reveal that acquisition of the trimeric DNA binding state of HSF1 is independent of and precedes inducible phosphorylation and furthermore that inducible phosphorylation correlates with transcriptional activation.**

The genes that encode heat shock proteins are coordinately regulated in response to acute exposure of cells to a range of physiological and environmental trauma, including heat shock, amino acid analogues, heavy metals, oxidative stress, anti-inflammatory drugs, and arachidonic acid (2–5). The transcriptional induction of heat shock genes in eukaryotic cells is mediated by pre-existing heat shock transcription factors (HSF)<sup>1</sup> which, upon activation, bind as trimers to multiple arrays of

the heat shock element (HSE) which are located in the promoter region of genes encoding heat shock proteins and molecular chaperones (6–18). In vertebrate cells, the HSF gene family is comprised of four distinct HSF genes (HSF1–HSF4) which have been cloned in chicken (HSF1–HSF3), mouse (HSF1 and -2), and humans (HSF1, -2, and -4) (19–22).<sup>2</sup> The co-expression of multiple HSFs within the same cell type has led to the suggestion that these multiple factors could mediate the response to different forms of stress. Indeed, it has been shown that HSF1 corresponds to the stress-responsive factor, whereas HSF2 is not stress-responsive and is induced during early embryogenesis, spermatogenesis, and erythroid differentiation (23–28).

Overall, the HSFs exhibit a similar structure with a conserved amino-terminal localized DNA binding domain, multiple arrays of hydrophobic heptad repeats, and a carboxyl-terminal transcription activation domain (5, 13, 19–22, 29–33). HSF1 is maintained in the latent control state as a non-DNA binding monomer that is constitutively phosphorylated (16, 24, 34). Intramolecular negative regulation appears to be specified through interactions between leucine zippers 1–3 and 4, whereas zippers 1–3 have an additional function as an extended coiled-coil in forming stable trimers (17, 22, 29, 31). Upon heat shock, HSF1 rapidly translocates into the nucleus and exhibits the properties of a stable trimer which correlates with the acquisition of DNA binding activity; furthermore the transcriptionally active form of HSF1 becomes inducibly phosphorylated (11, 16, 24, 35).

Salicylate and indomethacin are non-steroidal anti-inflammatory drugs used to treat inflammation and other related chronic diseases (36). These drugs have been shown to influence the activity of transcription factors in yeast (37), plants (38), and mammalian cells (1, 39, 40). We have shown previously that salicylate or indomethacin treatment of human cells induces HSF1 DNA binding activity; however, the drug-induced form of HSF1 is transcriptionally inert (1, 40). In this study we have examined the basis of this uncoupling of HSF1 activities. Our results reveal that the heat shock transcriptional response is a multistep process in which trimerization and acquisition of DNA binding activity by HSF1 is necessary but insufficient for transcriptional activation. These data further demonstrate that acquisition of DNA binding activity by HSF1 is independent and precedes the event of inducible phosphorylation and finally that acquisition of transcriptional activity is linked to inducible serine phosphorylation.

### EXPERIMENTAL PROCEDURES

HeLa S3 cells were grown in Joklik's medium with 5% calf serum. Cell growth and heat shock conditions were as described (41). The concentrations and incubation periods for anti-inflammatory drug treatment were as described before (1, 40). The effect of cycloheximide on protein synthesis inhibition was verified by [<sup>35</sup>S]methionine labeling followed by trichloroacetic acid precipitation and quantitation of incorporation.

In experiments where the phosphorylated state of HSF1 was analyzed, cells were grown at 37 °C and carefully monitored in a temperature-controlled (±0.1 °C) incubator for 48 h prior to the addition of [<sup>32</sup>P]orthophosphate and heat shock. Six hours prior to treatment with anti-inflammatory drugs or heat shock, the cells were concentrated by centrifugation (600 × g for 5 min), resuspended at a density of 5 × 10<sup>5</sup>

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<sup>1</sup> The abbreviations used are: HSF, heat shock factor 1; HSE, heat shock element; PAGE, polyacrylamide gel electrophoresis.

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cells/ml in prewarmed (37 °C) phosphate-free Dulbecco's modified Eagle's medium for a period of 1 h, and labeled at steady state for 5 h with  $^{32}\text{P}$  (50  $\mu\text{Ci/ml}$  of medium). Unlabeled cells were treated in parallel. At selected times approximately  $5 \times 10^6$   $^{32}\text{P}$ -labeled cells were harvested, washed with ice-cold phosphate-buffered saline, and lysed in ice-cold radioimmune precipitation buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100 containing 50 mM NaF, 0.2 mM  $\text{NaVO}_4$ , 5 mM  $\text{PP}_i$ , 1 mM phenylmethylsulfonyl fluoride, 2  $\mu\text{g/ml}$  leupeptin A, 2  $\mu\text{g/ml}$  pepstatin A) for immunoprecipitation. The lysates were cleared by centrifugation and 3  $\mu\text{l}$  of rabbit anti-HSF1 polyclonal sera was added to the cell lysate supernatants. After 1 h at 4 °C, 30  $\mu\text{l}$  of a 1:1 slurry of phosphate-buffered saline/protein A-Sepharose was added and mixed for another hour at 4 °C. Beads were washed five times with ice-cold radioimmune precipitation buffer, 0.1% SDS buffer, resuspended in 25  $\mu\text{l}$  of Laemmli sample buffer, and boiled for 5 min. Protein was separated on an 8.5% SDS-PAGE gel and transferred to an Immobilon-P membrane (Millipore). Phosphoamino acid analysis was performed by partial acid hydrolysis (1 h at 110 °C in 6 N HCl) and thin layer electrophoresis as described (42).

Tryptic phosphopeptide analysis was performed on immunoprecipitated HSF1 eluted from gel slices, trichloroacetic acid-precipitated, oxidized with performic acid, and digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (Worthington) as described (42). The digested protein was desalted on a 10-ml Sephadex G-25 column. Phosphopeptide mapping was performed in two dimensions using TLC. The first dimension was electrophoresis using an HTLE-7000 apparatus (CBS Scientific) in pH 1.9 buffer (formic acid/glacial acetic acid/water (1:7.8:35.9)) at 1.0 kV for 35 min at 18 °C. The second dimension was chromatography in *n*-butyl alcohol/pyridine/glacial acetic acid/water (15:10:3:12). The plates were dried and exposed at -80 °C with an intensifying screen for 7 days. Quantitation of the individual peptides was performed using a Molecular Dynamics PhosphorImager.

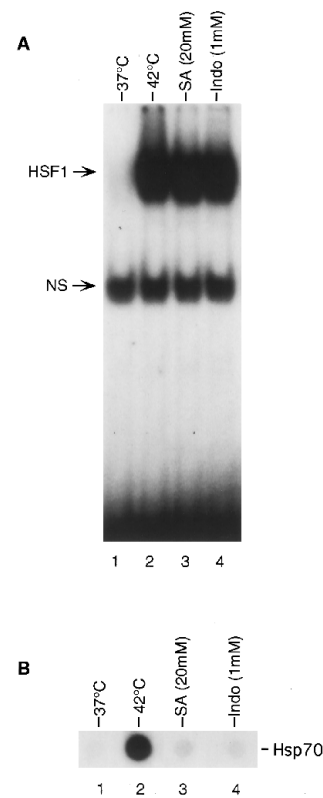
Transcription run-on assays were performed on nuclei from  $8 \times 10^6$  cells as described (10).  $^{32}\text{P}$ -Labeled RNA was annealed to plasmid DNA immobilized on nitrocellulose filters corresponding to the hsp70 gene (43), glyceraldehyde 3-phosphate dehydrogenase gene, and  $\beta$ -actin gene (44). The non-heat shock-inducible glyceraldehyde-3-phosphate dehydrogenase gene was used as a reference control, and the plasmid vector pBR322 was used as a nonspecific hybridization control. The intensity of the radiolabeled transcripts were quantified on a PhosphorImager 400A (Molecular Dynamics).

HSF DNA binding activity was analyzed using the gel mobility shift assay as described previously (10). Western blot analysis was performed using whole cell extracts (10  $\mu\text{g}$ ) and rabbit polyclonal anti-HSF1 antiserum (24). The immune complexes were analyzed using the ECL detection system (Amersham).

## RESULTS AND DISCUSSION

Exposure of human HeLa cells to anti-inflammatory drugs induces HSF1 DNA binding activity *in vivo* (1, 40). Moreover, the drug-induced form of HSF1 is bound to the promoter of the endogenous hsp70 gene, yet transcription of the hsp70 gene was not induced. These observations have suggested that activation of HSF1 is a multistep process. To further investigate the basis of this uncoupling between activation of DNA binding and transcription, we examined various properties of HSF1 induced in HeLa cells upon treatment with sodium salicylate or indomethacin. Aliquots of treated cells were analyzed for HSF1 DNA binding activity by gel mobility shift analysis. Similar levels of DNA binding activity are obtained upon exposure to either a 42 °C heat shock, indomethacin, or salicylate (Fig. 1A, lanes 2-4). However, as indicated by transcription run-on analysis of the hsp70 gene, transcription of the hsp70 gene is not induced in drug-treated cells (Fig. 1B, lanes 3 and 4) as compared with the elevated transcription of the hsp70 gene following heat shock (Fig. 1B, lane 2). Transcription of the reference control gene, glyceraldehyde-3-phosphate dehydrogenase was not altered by heat shock or treatment with any of the anti-inflammatory drugs (data not shown). These results reveal that activation of HSF1 DNA binding activity by either salicylate or indomethacin is insufficient for transcriptional activity and are consistent with previous observations (1, 40).

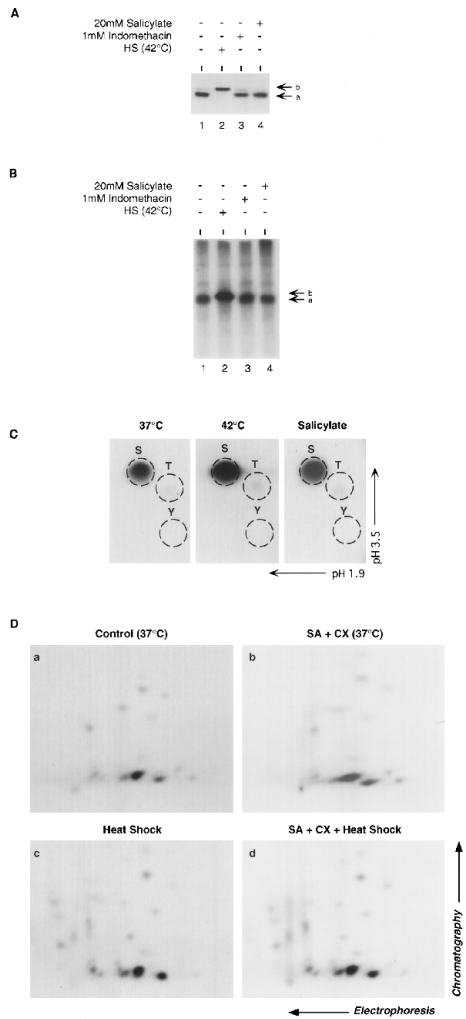
Previous studies on the biochemical properties of HSF1 from



**FIG. 1. Uncoupling of HSF1 DNA binding activity from transcriptional activation during treatment of HeLa cells with indomethacin and sodium salicylate.** A, gel mobility-shift analysis of whole cell extracts from control, heat-shocked, indomethacin- or salicylate-treated HeLa S3 cells. Cells were treated for 60 min with 42 °C heat shock as a positive control (lane 2), maintained at 37 °C as a negative control (lane 1), or treated with 1 mM indomethacin (lane 3) or 20 mM salicylate (lane 4) at 37 °C. B, transcription run-on analysis of the hsp70 gene from cells exposed to 37 °C (lane 1), 42 °C (lane 2), or treated with indomethacin (lane 3) or salicylate (lane 4).

control or heat-shocked cells have shown that the control form of HSF1 is constitutively phosphorylated, whereas the heat shock-activated form of HSF1 is inducibly phosphorylated (11, 17, 24, 35, 45). Therefore, we examined the phosphorylation state of indomethacin or salicylate-induced HSF1 using two complementary approaches. The inducibly phosphorylated form of HSF1 has a characteristic retarded mobility on SDS-PAGE (Fig. 2A, lanes 1 and 2) as compared with HSF1 from control cells. The drug-activated form of HSF1 exhibited an electrophoretic mobility which was indistinguishable from control HSF1 (Fig. 2A, lanes 1, 3, and 4), which suggests that the drug-induced form of HSF1 is not inducibly phosphorylated. This was corroborated by direct  $^{32}\text{P}$  steady state labeling experiments in which HSF1 was immunoprecipitated from control, heat-shocked, and drug-treated cells and analyzed by SDS-PAGE. HSF1 from heat-shocked cells contains a 2-3-fold increase in  $^{32}\text{P}$  incorporation over control levels (Fig. 2B, lanes 1 and 2), whereas the drug-induced HSF1 has a SDS-PAGE mobility and level of  $^{32}\text{P}$  incorporation comparable with that of HSF1 from control cells (Fig. 2B, lanes 1, 3, and 4). Taken together, these results reveal that the drug-induced form of HSF1 exhibits functional DNA binding properties while maintaining its constitutive phosphorylation state.

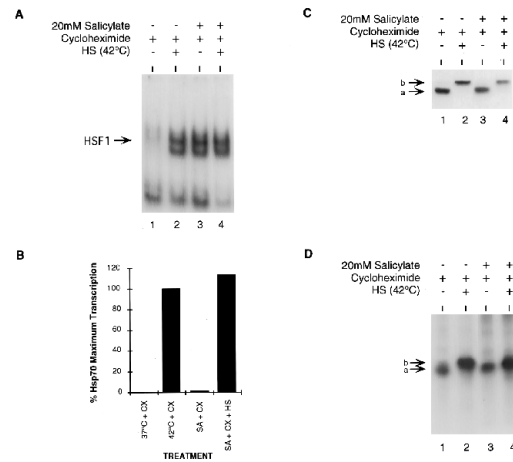
These results led us to further investigate the nature of HSF1 phosphorylation, specifically to compare the phosphoamino acid composition of control, stress-induced, and drug-induced HSF1. The results of phosphoamino acid mapping of steady state  $^{32}\text{P}$ -labeled HSF1 from control or heat-



**FIG. 2. The anti-inflammatory drug-induced form of HSF1 lacks inducible phosphorylation.** *A*, Western blot analysis of HSF1 from cells exposed to 37 °C (lane 1), 42 °C (lane 2), treated with indomethacin (lane 3) or salicylate (lane 4). The arrows correspond to the faster (a) and slower (b) migrating forms of HSF1. *B*, immunoprecipitation analysis of HSF1 in <sup>32</sup>P-labeled HeLa S3 cells corresponding to control (lane 1), heat shock (lane 2), indomethacin (lane 3), and salicylate (lane 4). *C*, phosphoamino acid analysis of HSF1 from control (37 °C) heat-shocked (42 °C) and salicylate-treated HeLa cells. The relative positions of phosphoserine, phosphothreonine, and phosphotyrosine are as indicated. The arrows indicate the direction and the pH at which the chromatography was performed. *D*, tryptic phosphopeptide acid analysis of HSF1 from control (a), salicylate-treated (b), heat shock (c), and salicylate and heat shock (d). The orientations of electrophoresis and chromatography are indicated.

shocked cells revealed phosphorylation at serine residues. Likewise, HSF1 from drug-treated cells is also serine-phosphorylated (Fig. 2C). Comparison of the tryptic phosphopeptide patterns revealed that HSF1 from control and drug treated cells was indistinguishable (Fig. 2D, panels a and b), whereas HSF1 from heat-shocked cells exhibited a characteristic phosphopeptide pattern corresponding to a mixture of constitutively phosphorylated peptides and stress-induced phosphopeptides (Fig. 2D, panel c).

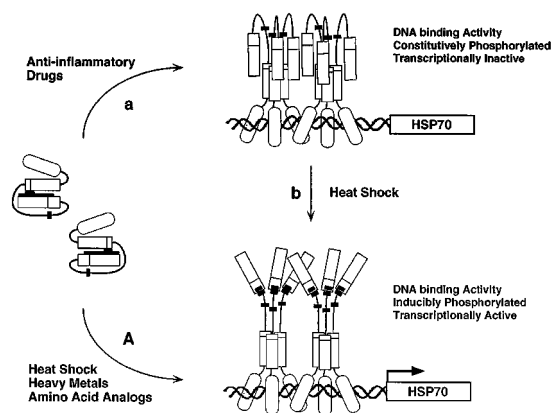
We next examined whether the drug-induced HSF1 corresponded to an inert state or whether this form of HSF1 was an intermediate that could be converted to a transcriptionally active state. To accomplish this, the salicylate-treated cells were treated with cycloheximide to prevent further synthesis of HSF1 and subjected to heat shock. Although heat shock did not affect the level of HSF1 DNA binding activity, there was a



**FIG. 3. The anti-inflammatory drug-induced form of HSF1 is a substrate for inducible phosphorylation, and heat shock can induce its transcriptional activity in the absence of protein synthesis.** *A*, HSF1 DNA binding was measured in HeLa S3 cells corresponding to cycloheximide + control (lanes 1), cycloheximide + heat shock (lane 2), cycloheximide + indomethacin + heat shock (lane 3), or cycloheximide + salicylate + heat shock (lane 4). *B*, levels of hsp70 gene transcription in HeLa cells exposed to sodium salicylate alone or the combination of salicylate and heat shock. HeLa cells were maintained at 37 °C (CX + 37 °C) or heat-shocked at 42 °C in the presence of cycloheximide (CX + 42 °C), exposed to cycloheximide + salicylate (CX + SA) or cycloheximide + salicylate + heat shock (CX + SA + 42 °C). *C*, Western blot analysis of whole cell extracts from samples isolated in *A*. The arrows correspond to the faster (a) and slower (b) migrating form of HSF1. *D*, immunoprecipitation analysis of HSF1 from steady state <sup>32</sup>P-labeled HeLa cells treated similar to *A*.

dramatic effect on hsp70 gene transcription (Fig. 3, A and B). We next examined the phosphorylation state of HSF1 and found that the mobility and phosphoprotein status of the salicylate HSF1 was now the same as from heat-shocked cells (Fig. 3, C and D, lanes 2–4). Furthermore, the HSF1 from cells initially treated with salicylate and subsequently by heat shock exhibited a tryptic phosphopeptide pattern which was identical to that of HSF1 from cells subjected to heat shock alone (Fig. 2D, panel d).

The data presented here provides evidence for the multistep process of HSF1 activation in which oligomerization from the negatively regulated monomer to the DNA binding trimer precedes inducible phosphorylation. This interpretation is predicated on the assumption that the mechanism of drug-induced activation of HSF1 is similar to the events which occur during heat shock. Despite the ability of the drug-induced HSF1 trimer to bind to the promoter of the endogenous hsp70 gene, this form of the factor is inert and transcription is not induced. We suggest that this state of HSF1 represents a stable intermediate, which may correspond to the constitutive DNA binding activity exhibited by *Saccharomyces cerevisiae* HSF (11, 46, 47). In part, this is based on the observation that the inert DNA binding trimer can be converted by heat shock to a transcriptionally active state which is accompanied by inducible phosphorylation. Likewise, despite the constitutive DNA binding activity of yeast HSF, transcriptional activity correlates with inducible phosphorylation. One interpretation of our data is that the absence of inducible serine phosphorylation in the drug-induced form of HSF1 is responsible for maintaining the factor in a transcriptionally inert state. This intermediate state of HSF1 is primed for activation by other stresses (1, 40, 48). A consequence of a multistep pathway for HSF1 activation is that certain inducers of the heat shock response, *i.e.* heat shock, heavy metals, and arachidonic acid, lead to trimerization, inducible phosphorylation, and transcriptional activity, whereas salicylate and indomethacin induce an intermediate state of



**FIG. 4. Multistep model of HSF1 activation.** Schematic representation of HSF1 multistep process of activation. In control cells, HSF1 is a constitutively serine-phosphorylated monomer. Exposure to anti-inflammatory drugs (a) induces trimerization and acquisition of HSF1-DNA binding activity; this form is not inducibly phosphorylated and is transcriptionally inert. Further exposure of the drug induced form of HSF1 to heat shock (b) leads to HSF1-inducible serine phosphorylation coincident with transcriptional activation.

HSF1 that is bound to DNA *in vivo* and is transcriptionally inert (Fig. 4).

What is the role of the stress-inducible serine phosphorylation of HSF1? Our demonstration that the events associated with HSF1 trimerization can occur in the absence of inducible phosphorylation reveals that inducible phosphorylation of HSF1 is not essential for conversion from the monomer to the trimer DNA binding state. Indeed, our data clearly establishes that DNA binding and oligomerization of HSF1 must precede the events of inducible phosphorylation. Is the inert trimer a substrate for stress-inducible phosphorylation? Our experiments reveal that the salicylate-induced trimers become inducibly phosphorylated; however, the studies presented here do not unequivocally establish whether the inert trimer is the substrate for the stress-induced kinase activity or whether the inert trimer is converted back to the monomer prior to inducible phosphorylation. The lack of inducible phosphorylation of the inert nuclear localized HSF1 trimer would seem to rule out the possibility that the kinase activity is constitutive and dependent upon the appearance of the HSF1 trimer for inducible phosphorylation to occur. These results suggest that the stress-induced HSF1 kinase activity is either activated by stress or requires stress for translocation to the nucleus. Finally, at issue is the role of inducible phosphorylation of HSF1 in regulating its activities. Previous studies on the *S. cerevisiae* and mammalian HSF have suggested that inducible phosphorylation is not essential for transcription (24, 49). Although the studies presented here offer the strongest evidence to correlate the acquisition of inducible phosphorylation of HSF1 with transcriptional activity, we cannot rule out the possibility that a stress-induced kinase or other consequences of heat shock have affected other components of the transcriptional machinery. Further investigation will be necessary to establish the role of stress-induced phosphorylation of HSF1 in regulating aspects of its activity during activation or attenuation of the heat shock transcriptional response.

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