Hyperphosphorylation of Heat Shock Transcription Factor 1 Is Correlated with Transcriptional Competence and Slow Dissociation of Active Factor Trimmers*

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In the course of its activation by heat and other stresses, the inactive monomer of human heat shock transcription factor 1 (HSF1) is converted to a DNA-binding homotrimer and is hyperphosphorylated. At least four Ser/Thr residues in HSF1 appeared to be inducibly phosphorylated during heat shock. Ser/Thr protein kinase inhibitors inhibited, and protein phosphatase inhibitor calyculin A and phosphor-ester enhanced, hsp70-CAT reporter gene expression but not heat shock element DNA binding activity in HeLa cells undergoing a moderate heat shock. Calyculin A (5–20 nM) caused hyperphosphorylation of HSF1, the extent of which was comparable to that produced by moderate to severe heat shock. Upon recovery from a 42 °C/30 min-heat shock, HSF1 trimers disassembled quantitatively within 2 h. Calyculin A interfered with the dissociation of HSF1 trimers. Thus, hyperphosphorylation increases the effective half-life of the HSF1 trimers, which may prolong factor activity subsequent to heat shock. Hyperphosphorylation also dramatically stimulated the transactivation function of HSF1: exposure to calyculin A of cells induced to form inactive HSF1 trimers resulted in the conversion of the inactive to active trimers. Given that deletion of certain sequences renders HSF1 constitutively active, these results suggested that the activation of HSF1 trimers by calyculin A was a consequence of hyperphosphorylation of HSF1 rather than of a downstream factor.

Many cellular regulators including transcription factors are regulated by phosphorylation and dephosphorylation. A phosphorylation/dephosphorylation event may activate DNA-binding ability, induce dimerization and specific DNA binding ability, prompt nuclear translocation, or cause association with a cofactor(s) or release from an inhibitor(s) (1). Heat shock transcription factor (HSF),1 the transcription factor responsible for the stress-regulated synthesis of heat shock proteins (hsp), has been shown to be hyperphosphorylated upon activation by heat and other stresses in several organisms including yeast (2–5). However, there is a scarcity of evidence linking this hyperphosphorylation to HSF function, except for a study with yeast suggesting that a heat-induced phosphorylation event is necessary for deactivation of the factor in this organism (6). Because stress profoundly alters cellular regulation, it seemed reasonable to expect that the cellular response to stress be integrated with normal signal transduction pathways. This study attempts to provide evidence for such integration through an examination of the effects of changes in the phosphorylation state of mammalian HSF1 on the activity of the transcription factor.

Heat shock and other stresses trigger a series of events that result in an increase in the expression of hsp genes that is proportional to the severity and duration of the stress (7). The mechanisms by which the cell determines the amounts of extra hsp needed to cope with a particular stress and ensures that these precise amounts are being synthesized are only incompletely understood. It is clear, however, that there are mechanisms operating at both the transcriptional and posttranscriptional levels (8). The transcriptional mechanism controls the activity of HSF1. In its inactive form, HSF1 appears to reside in the cytoplasm as a monomer or a heteromeric complex (7, 9). Stress activation of HSF1 involves the formation of homotrimers that are capable of specific DNA binding (to a heat shock element sequence, referred to herein as HSE) and of nuclear translocation. Homotrimerization, however, is not sufficient to enable the transactivation function of the factor. A second stress-induced event is required to render the factor transcriptionally competent. In the course of activation by heat and several other types of stress, or shortly thereafter, HSF1 also becomes hyperphosphorylated (4, 5). It is tempting to speculate that one or both of the above regulatory events may be modulated or controlled by phosphorylation.

Heat shock and other stresses are also known to cause rapid increases in the concentrations of inositol phosphates and diacylglycerol (Ref. 7, and references therein). Not unexpectedly, a recent report also documented increased nuclear protein kinase C activity. Heat shock may also activate the adenylate cyclase pathway. Furthermore, mitogen-activated protein kinases, such as Janus-activated kinase, stress-activated protein kinase, and RK/HOG1, are activated by heat shock and other stresses. One or more of these stress-activated kinases may be responsible for the hyperphosphorylation of HSF1.

Preliminary support for the notion that phosphorylation/dephosphorylation may regulate HSF1 activity has come from observations that Ser/Thr protein kinase inhibitors 1-[5-isooquinolinyl-sulfonyl]-2-methyl-piperazine (H7), staurosporine, 2-aminoquinine, and calphostin inhibit and that Ser/Thr phosphatase inhibitor okadaic acid potentiates hsp gene expression in cells exposed to a mild heat shock (10–12). These observa-

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1 The abbreviations used are: HSF, heat shock transcription factor; hsp, heat shock protein; HSE, heat shock element; CAT, chloramphenicol acetyltransferase; PMA, phorbol myristate acetate; PAGE, polyacrylamide gel electrophoresis; CMV, cytomegalovirus; H7, 1-[5-isooquinolinyl-sulfonyl]-2-methyl-piperazine; GF-X, 3-[1-(3-dimethylamino-propyl)-indol-3-yl]-maleimide.

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tions provided a point of departure for the present study. Using a potent protein phosphatase inhibitor, evidence was obtained that hyperphosphorylation of HSF1 trimers both increases their transcriptional activity and inhibits their dissociation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and [32P]Phosphate Labeling**—Human HeLa cells or HeLa-CAT cells (13) containing copies of an hsp70B promoter-driven chloramphenicol acetyltransferase (CAT) gene were cultured as described previously (9). In experiments with H7, 3-[1-(3-dimethylamino-propoyl)-indol-3-yl]-maleimide (GF-X), and phorbol ester (PMA), cells were preincubated with the chemicals for 3 h and in experiments with calyculin A for 30 min to 3 h. Calyculin A was obtained from Sigma. The chemical was found to rapidly lose activity in solution, even when stored in aliquots at −70 °C. All experiments were, therefore, carried out with solutions that were less than 1 week old. In experiments involving measurements of reporter activity, cells were washed after incubation with inhibitors for a defined time and were postincubated overnight at 37 °C. To label phosphoproteins, 70% confluent cells were washed with phosphate-free Dulbecco’s modified Eagle’s medium, starved for 40 min in the same phosphate-free medium, and then incubated with [32P]phosphate (100 μCi/ml) for 2 or 3 h. For experiments to assess calyculin A- or heat-induced phosphorylation, incubations were continued for 30 min either in the presence of calyculin A or at a heat shock temperature.

**Immunoprecipitation of HSF1 and Two-Dimensional Peptide Mapping**—Labeled cells were harvested and lysed in RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS with 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM sodium fluoride, and 5 μM sodium vanadate). The lysate was cleared by centrifugation, and HSF1 was immunoprecipitated from 200 μl with a rabbit anti-HSF1 polyclonal antibody and protein A-Sepharose (4). The immunoprecipitate was electrophoresed on 7.5% SDS-PAGE, and HSF1 was detected by autoradiography. For phosphopeptide analysis, HSF1 was eluted from the gel, precipitated with 10% trichloroacetic acid, oxidized with performic acid, and digested with N-tosyl-l-phenylalanyl chloromethyl ketone-trypsin or chymotrypsin. Two-dimensional separation of phosphopeptides was carried out as described previously (14). Plates were scanned using a PhosphorImager. Phosphoamino acid analysis was performed using a standard technique (14, 15).

**DNA Transfections**—Cells were transfected using Lipofectamine reagent (Life Technologies, Inc.). 3 × 106 cells in 35-mm dishes were transfected with 1 ml of a mixture containing 1 μg of CMV-HSF1 or a CMV-LexA-HSF1 construct, 0.5 μg of a CMV-β-galactosidase construct and, where applicable, 0.5 μg of a LexA-CAT reporter gene construct and 5 μl Lipofectamine reagent. DNA constructs used were described in Zuo et al. (16). After 5 h, cells were washed and incubated for 15 h in complete medium before exposure to heat shock or chemicals. Cells were typically collected immediately after treatment, except that reporter gene expression was measured after an additional 15-h incubation.

**Other Methods**—Native, limiting pore size gel electrophoresis and anti-HSF1 blots, Western blots, gel shift assays using a radiolabeled HSE probe, and CAT activity assays were carried out as described previously (16). CAT assay results were quantitated by PhosphoImager.

**RESULTS**

**Heat-induced Phosphorylation of HSF1**—HSF1 was isolated from cells preincubated for 2 h with [32P]phosphate at 37 °C and further incubated for 30 min at 37 °C or at 43 °C by immunoprecipitation using an anti-HSF1 antibody (4). The immunoprecipitated material was resolved on SDS-PAGE. Quantitation of the autoradiograph indicated that heat treatment increased the level of phosphorylation of HSF1 by up to 20-fold (Fig. 1A, left side; see also Fig. 3A). Anti-HSF1 Western blot analysis showed that extractable from heat-treated or control cells contained comparable amounts of HSF1 (Fig. 1A, right side). The blot also confirmed the previous observation (4, 5) that HSF1 from heat-treated cells has a lower mobility than HSF1 from control cells, presumably because of heat-induced phosphorylation of the factor. Similarly purified HSF1 from heat-treated and control cells was eluted from an SDS-PAGE gel and subjected to acid hydrolysis. Phosphoamino acids were resolved by two-dimensional electrophoresis. In contrast to

![Fig. 1. Inducible phosphorylation of HSF1.](image)

In A, HeLa-CAT cells were preincubated with [32P]phosphate at the control temperature and then further incubated for 30 min either at the control temperature in the presence or absence of 5 μM calyculin A or at 43 °C as described under “Experimental Procedures.” Cells were lysed in RIPA buffer. A first fraction of each lysate was used for immunoprecipitation of HSF1 by an anti-HSF1 antibody (left) and a second fraction for anti-HSF1 Western blot (right; arrow indicates HSF1). Immunoprecipitates were subjected to SDS-PAGE, which was followed by autoradiography of the dried gel (left). Relative incorporation of radiolabel into HSF1 bands was determined by using a PhosphorImager and is shown below the panel. B, two-dimensional analyses of tryptic (left) and chymotryptic (right) peptides of immunoprecipitated HSF1 labeled in vivo during heat shock. Arrows indicate the four tryptic peptides routinely resolved. C, phosphoamino acid analysis of immunoprecipitated HSF1 labeled in vivo during heat shock. The positions of phosphoamino acids are circled in the figure.

HSF1 from heat-treated cells (Fig. 1C), labeling of phosphoamino acids in HSF1 from control cells was virtually undetectable (data not shown). Results indicated that induced phosphorylation occurred mainly at Ser and Thr residues. Two-dimensional analysis of tryptic digests of HSF1 isolated from cells preincubated with [32P]phosphate and heat treated as before reproducibly separated four labeled peptides (Fig. 1B, left side). HSF1 peptides from control cells were only very weakly labeled (analysis not shown). Maps of chymotryptic peptides revealed three inducibly phosphorylated peptides (Fig. 1B, right side). Thus, during heat shock, HSF1 is phosphorylated at a minimum of four distinct Ser/Thr residues.

**Inhibitors of Protein Kinases and Phosphatases Modulate HSF1-dependent Gene Expression, without Affecting HSE DNA
Binding Activity—Two kinds of experiments were carried out to test the possibility that the activity of HSF1 is controlled by the ratio of protein kinase to phosphatase activities. Cultures of HeLa-CAT, a cell line derived from human HeLa cells containing integrated copies of a highly heat-inducible hsp70-CAT gene (13, 17), were exposed for 3 h in the presence (+) and absence (−) of kinase inhibitors and then either exposed to a 42 °C/30-min heat shock or kept at the control temperature. After several washes with complete medium, cells were incubated for another 15 h at the control temperature prior to preparation of extracts and CAT assays. In B, as in A, except that preincubation with calyculin A (Cal.) was for 30 min. Signals were quantified by PhosphorImager. Percentages of conversion of chloramphenicol to acetylated forms of chloramphenicol are given below the panels.

**FIG. 2.** Effects of protein kinase and phosphatase inhibitors on the expression of the hsp70-CAT reporter genes in HeLa-CAT cells. CAT assays are shown, with C indicating the position of [14C]chloramphenicol and AC that of the major acetylated product of [14C]chloramphenicol. In A, HeLa-CAT cells were preincubated for 3 h in the presence (+) and absence (−) of kinase inhibitors and then either exposed to a 42 °C/30-min heat shock or kept at the control temperature. After several washes with complete medium, cells were incubated for another 15 h at the control temperature prior to preparation of extracts and CAT assays. In B, as in A, except that preincubation with calyculin A (Cal.) was for 30 min. Signals were quantified by PhosphorImager. Percentages of conversion of chloramphenicol to acetylated forms of chloramphenicol are given below the panels.

**FIG. 3.** Effects of kinase and phosphatase inhibitors on HSF1 hyperphosphorylation and on HSE DNA binding activity. A, inhibition of HSF1 hyperphosphorylation. HeLa-CAT cells were placed in medium containing [32P]phosphate, exposed for 3 h in the presence or absence of kinase inhibitors, and then either heat-treated at 42 °C or incubated further at the control temperature for 30 min. HSF1 was immunoprecipitated from extracts and analyzed on SDS-PAGE. An autoradiograph is shown. Relative incorporation of radiolabel into HSF1 was quantified using a PhosphorImager and is shown below the panel. B and C, gel shift assays, using an end-labeled HSE oligonucleotide probe, of extracts from cells exposed to inhibitors and heat shock as described in Fig. 2. Positions of the HSE-HSF complex are indicated on the sides. Signals in the lower parts of the figures are due to binding of the Ku antigen to the probe (25).
学习贯彻HSE DNA binding activity and HSF1 hyperphosphorylation during heat shock and recovery from heat shock. A, gel shift assay using an HSE DNA probe. Parallel cultures of HeLa-CAT cells were preincubated for 3 h in the presence or absence of calyculin A, heat-shocked for 30 min, and then returned to the control temperature. At the times indicated, cultures were harvested, and extracts were prepared. Equal volumes of extracts were analyzed for DNA binding activity. Protein concentrations were measured to ensure that samples had similar concentrations. The position of HSE DNA-HSF1 complexes is indicated by an arrow. B, anti-HSF1 Western blot of the same samples.

hyperphosphorylation below) does not play a role in the regulation of HSF1 trimerization and DNA binding activity but may be involved in a subsequent step in the activation of the factor.

It was shown previously that okadaic acid, an inhibitor of protein phosphatases 1 and 2A, enhanced the heat-induced activity of a hsp promoter-driven reporter gene (11). This result was confirmed using calyculin A, a potent inhibitor of the same phosphatases (Fig. 2B). Note that in most experiments, calyculin A also caused a minor increase in CAT activity in the absence of stress (discussed below). Gel shift assays with extracts prepared immediately after heat shock failed to reveal increased HSE DNA binding activity in calyculin A-treated cells (Fig. 3C). Assuming that inhibition of Ser/Thr phosphatases results in HSF1 hyperphosphorylation, these findings are compatible with the above conclusion that hyperphosphorylation of HSF1 does not enhance its DNA binding activity but affects a later step in the activation of the factor.

Inhibition of Protein Phosphatases Results in HSF1 Hyperphosphorylation—As shown by increased incorporation of [32P]phosphate into HSF1 (Fig. 1A, left side) as well as by decreased mobility of the factor in SDS-PAGE (Fig. 1B, right side; Fig. 4B), exposure of cells to 5–20 nM calyculin A resulted in hyperphosphorylation of HSF1. The extent of this hyperphosphorylation was comparable to that produced by a moderate to severe heat shock. It is important to note that, in the absence of heat shock, even elevated concentrations (20 nM) of calyculin A failed to cause HSF1 homotrimerization (Fig. 5) or to induce substantial HSE DNA binding activity (Fig. 4A). Thus, the inhibitor is not by itself stressful to cells. Furthermore, hyperphosphorylation alone is insufficient to convert inactive HSF1 monomer/heteromer into active homotrimer.

Hyperphosphorylation Slows the Rate of Dissociation of HSF1 Homotrimers—hsp reporter gene expression may be stimulated by inhibition of Ser/Thr phosphatase activity because hyperphosphorylation of HSF1 causes the activation of its transactivation function or because phosphorylation activates a downstream factor. Alternatively, or in addition, hyperphosphorylation of HSF1 may result in a reduced rate of dissociation of active trimers subsequent to heat shock. Note that the experiments presented before did not address the latter question but only suggested that hyperphosphorylation was not required for HSF1 trimerization. To test the possibility that hyperphosphorylation affected the dissociation of HSF1 trimers, HeLa-CAT cultures preincubated with either 5 or 20 nM calyculin A and control cultures were subjected to a moderate heat shock (42 °C for 30 min) and then harvested either immediately after the stress or 30 min or 2 h after return to 37 °C. Extracts were prepared, and HSE DNA binding activity was analyzed by gel shift assay as before (Fig. 4A). As mentioned before, little HSE DNA binding activity was detected in unheated control cells and less in calyculin A-treated cells. High levels of activity were measured immediately following heat treatment in control as well as calyculin A-treated cells. By 2 h after heat shock, HSE DNA binding activity had been quantitatively inactivated in cells that had not received inhibitor. Mobility shift analysis (Fig. 4B) indicated that HSF1 was no longer hyperphosphorylated at this time. By contrast, DNA binding activity was still readily detected in calyculin A-treated cells 2 h after heat shock (Fig. 4A), suggesting that hyperphosphorylation of HSF1 reduced the rate of inactivation of HSE DNA binding activity (see Fig. 4B for evidence of HSF1 hyperphosphorylation in all samples that had received calyculin A).

The same extracts were analyzed by native, limiting pore size gel electrophoresis (native PAGE) and anti-HSF1 blot (Fig. 5). HSF1 from not-heated cells is known to appear as a monomer or heterodimer in this type of gel (4, 9). In extracts from heat-treated cells, HSF1 is found in large complexes representing HSF1 homotrimers (9). In cells pretreated with calyculin A, a fraction of monomeric HSF1 was apparently sequestered and not incorporated in trimers upon a 42 °C/30-min heat shock (Fig. 5). When calyculin A-exposed cells were subjected to a more severe heat shock, all monomers were converted to trimers (data not shown), suggesting that hyperphosphorylation did not block but only reduced the rate of trimerization. As discussed below, this phenomenon is not believed to be physiologically relevant. Two h after heat shock, HSF1 trimers had dissociated quantitatively in calyculin A-treated cells (Fig. 5). HSF1 from not-heated cells is known to appear as a monomer or heterodimer in this type of gel (4, 9). In extracts from heat-treated cells, HSF1 is found in large complexes representing HSF1 homotrimers (9). In cells pretreated with calyculin A, a fraction of monomeric HSF1 was apparently sequestered and not incorporated in trimers upon a 42 °C/30-min heat shock (Fig. 5). When calyculin A-exposed cells were subjected to a more severe heat shock, all monomers were converted to trimers (data not shown), suggesting that hyperphosphorylation did not block but only reduced the rate of trimerization. As discussed below, this phenomenon is not believed to be physiologically relevant. Two h after heat shock, HSF1 trimers had dissociated quantitatively in calyculin A-treated cells (Fig. 5).
Thus, hyperphosphorylation retarded dissociation of HSF1 trimers.

A reduced rate of HSF1 trimer dissociation should result in increased reporter activity, provided that trimers remain transcriptionally active. Trimerization was shown previously to be insufficient to render HSF1 transcriptionally active. In the following experiments, the possibility was investigated that HSF1 hyperphosphorylation can also mediate the activation of the transactivation function of the factor. If that were the case, hyperphosphorylation would increase HSF1 activity synergistically by increasing the transcriptional competence of the factor and by extending the half-life of the trimeric state during recovery from a stressful event. To obtain evidence for this possibility, the ability of calyculin A or PMA to activate the transactivation function of HSF1 was tested in situations in which the factor was present as a trimer having weak or undetectable transcriptional activity.

Activation by Hyperphosphorylation of Inactive HSF1 Trimers Resulting from HSF1 Overexpression—Cells transiently transfected with a CMV promoter-driven HSF1 DNA gene drastically overexpress HSF1. Overexpressed HSF1 appears to be almost quantitatively trimeric, nuclear-localized, and capable of binding HSE DNA in the absence of stress (16) (Fig. 6A). However, these HSF1 trimers are essentially incapable of activating an hsp promoter-driven reporter gene (16) (Fig. 6C). That overexpressed HSF1 from unstressed cells migrated more rapidly than factor from heat-shocked cells in SDS-PAGE gels (Fig. 6B) was compatible with the notion that the virtual inactivity of overexpressed factor was due to its incomplete phosphorylation (see Fig. 8B for a demonstration, by incorporation of $[^{32}P]$phosphate, that the related molecule LexA-HSF1 overexpressed from transfected genes is not hyperphosphorylated in the absence of heat shock).

Brief incubation of HeLa-CAT cells overexpressing HSF1 from transfected genes with 5 nM calyculin A caused a drastic increase in reporter expression in the absence of heat shock (Fig. 6C). A 3-fold lower increase in reporter gene activity in the experiment shown, or even lesser increases in experiments not shown, were observed when untransfected cells were similarly exposed to calyculin A. Together with the observation that calyculin A exposure caused hyperphosphorylation of overexpressed HSF1 (Fig. 6B; see also Fig. 8B), these results suggested that inactive HSF1 trimers can be activated by hyperphosphorylation. That calyculin A, although it does not enhance but rather inhibits trimerization, also weakly activated reporter gene expression in untransfected cells is compatible with this conclusion; unstressed cells contain variable (small) amounts of HSF1 trimers (see Fig. 5, third lane from the left) that should be activated by the same mechanism.

The transcriptional activity of HSF1 trimers could also be enhanced by stimulating protein kinase C activity. HeLa-CAT cells were transfected with an HSF1-expressing construct as before. Transfected cells were heat-treated for 30 min at 42 °C in the presence or absence of 320 nM PMA, an activator of protein kinase C. Although HSE DNA binding activity was not affected by the addition of PMA (Fig. 7B), exposed cells produced 5-fold higher levels of CAT activity than unexposed cells (Fig. 7A). Note that stimulation of reporter gene expression by PMA was only barely detectable in untransfected cells (data not shown) and could not be demonstrated at the control temperature (Fig. 7A). Apparently, PMA only weakly increased the activity of the protein kinase(s) involved in the activation of HSF1.

To more conclusively rule out an involvement of endogenous HSF1 in the above experiments, one such experiment was repeated, overexpressing LexA-HSF1 chimera 87/79 instead of

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**Fig. 6. Effects of calyculin A (Cal.) on overexpressed HSF1.** HeLa-CAT cultures were either transfected with CMV-HSF1 (lane T) as described under “Experimental Procedures” or left untransfected (lane N). Cultures either exposed to 5 nM calyculin A for 30 min or not exposed were subjected to heat shock or kept at the control temperature. For the analyses in A and B, cells were harvested immediately after treatments. For measurements of CAT activity, cells were washed after treatments and incubated for another 15 h at the control temperature prior to extract preparation and enzyme assay. A, gel shift assay using an HSE DNA probe. The region containing HSE DNA-HSF1 complexes is indicated on the left by an arrow. B, anti-HSF1 Western blot. The position of HSF1 signals is indicated by an arrow on the left. C, CAT activities. The positions of chloramphenicol and the major acetylated form of chloramphenicol are indicated by arrows. Signals were quantified by PhosphorImager. Percentages of conversion of chloramphenicol to acetylated forms of chloramphenicol are given below the figure.

HSF1. The chimera included all of the HSF1 sequence except for the 79 N-terminal amino acids containing the HSE DNA-binding domain, which were replaced with an 87-residue segment consisting of the DNA-binding domain of the *Escherichia coli* LexA protein. HeLa cells were cotransfected with CMV-87/79, a construct containing a CMV promoter-driven LexA-HSF1 chimeric gene, and LexA-CAT, a CAT reporter gene under the control of a basal promoter containing several binding sites for LexA. As was previously found, overexpressed LexA-HSF1 also forms inactive trimers capable of specifically binding LexA sites (16).2 Exposure of transfected cells to calyculin A rendered the chimeric protein transcriptionally competent (Fig. 8A). A $[^{32}P]$phosphate incorporation experiment (Fig. 8B, left side) demonstrated that calyculin A caused hyperphosphorylation of

2 W. Xia and R. Voellmy, unpublished observations.
the chimeric factor to a similar extent as a 42 °C/30-min heat shock. As expected, hyperphosphorylation of the chimera by calyculin A and heat shock also produced detectable mobility shifts (Fig. 8B, right side). Note that the transfection efficiency in these experiments was sufficiently high so that the signals detected in samples from transfected cells were predominantly due to chimeric factor and not to endogenous HSF1. These observations on the activation of chimeric HSF1 corroborate those made before on overexpressed wild-type HSF1. They are compatible with the notion that there are distinct steps in the pathway of activation of HSF1, a first step resulting in the trimerization of the factor, acquisition of DNA binding ability and nuclear translocation, and a second step that can be mediated by hyperphosphorylation, rendering the factor transcriptionally competent.

Certain deletions and substitutions in a region in the HSF1 sequence that includes the second hydrophobic repeat and sequences downstream from it cause HSF1 to become constitutively active (16). This region contains a regulatory domain that represses C-terminally located activation domains (16, 18, 19). Fig. 8A (last lane from the left) shows that a LexA-HSF1 chimera lacking this region constitutively activated the LexA-CAT reporter gene. Note that the level of reporter gene expression was similar to that produced by the unmutated LexA-HSF1 chimera in response to a 42 °C/30-min heat shock. Western blot suggested (Fig. 8C) that the deletion was still capable of being hyperphosphorylated during heat shock. Thus, hyperphosphorylation of other factors participating in transcription regulation by HSF1 is not required for HSF1 function. Furthermore, that hyperphosphorylation of the mutant factor is still heat-regulated but its transcriptional activity is not suggests that hyperphosphorylation of the transcription acti-

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**FIG. 7.** Effect of PMA on the transcriptional activity and DNA binding activity of overexpressed HSF1. Cultures of HeLa-CAT cells were transfected with CMV-HSF1. Transfected cultures were preincubated for 3 h in the presence or absence of 0.32 μM PMA and then subjected to a 42 °C/30-min heat shock. Cultures were then processed as in Fig. 6. A, CAT activity assay. B, gel shift assay using an HSE DNA probe. See Fig. 6 for other details.

![Image](https://via.placeholder.com/150)

**FIG. 8.** Transcriptional activity and phosphorylation status of LexA-HSF1 chimeras. HeLa cultures were cotransfected with CMV-LexA-HSF1 or CMV-LexA-HSF1 Δ202-315 constructs and with LexA-CAT reporter gene or were transfected with reporter gene alone (first two lanes from the left) as described under “Experimental Procedures.” Transfected cultures were preincubated for 30 min in the presence or absence of 5 nM calyculin A (Cal.) or, where appropriate, were heat-treated and then processed as in Fig. 6. A, CAT activity assay. Positions of chloramphenicol and the major acetylated form of chloramphenicol are indicated on the side, and values representing percentage of conversion of chloramphenicol to acetylated chloramphenicol are shown below the panel. In B, cultures, transfected with LexA-HSF1 or mock transfected, were exposed to [32P]phosphate as in Fig. 1 and were then incubated in the presence of the radiolabel with or without calyculin A for 30 min or were heat-treated. Cell lysate was then prepared and used for immunoprecipitation of anti-HSF1 reacting material, which was analyzed by SDS-PAGE and autoradiography (left) or was directly used for anti-HSF1 Western blot (right). See Fig. 1 for other details. C, anti-HSF1 Western blot using lysates from cells transfected with LexA-HSF1 Δ202-315 and treated for 30 min as indicated.
viation domains of HSF1 is not required for their function. What may be affected by hyperphosphorylation is the regulation of the transactivation function. This regulation was eliminated in the deletion.

**Activation of the Transactivation Ability of Endogenous, Inactive HSF1 Trimers**—Salicylate and the oxidative stressor menadione are known to induce trimerization of HSF1 in human cells, without causing increased hsp gene expression (20, 21). Based on the findings presented before, it was hypothesized that hsp synthesis was not induced by these chemicals because in their presence, HSF1 could not be phosphorylated to the extent required for activation of its transactivation function.

In agreement with the earlier observations, exposure of HeLa-CAT cells to salicylate or menadione failed to result in increased hsp70-CAT gene expression (Fig. 9, A and B). When HSE DNA binding activity was measured, it was found that both chemicals strongly induced DNA binding (Fig. 9C). If the above hypothesis was correct, co-exposure of cells to any of the two chemicals and calyculin A should result in HSF1 hyperphosphorylation and reporter gene activation. Results observed were as predicted. As is shown in Fig. 9D by incorporation into HSF1 of [32P]phosphate and in Fig. 9E by mobility shift assay, neither of the chemicals alone caused HSF1 hyperphosphorylation, the failure of which was corrected by the addition of calyculin A. Exposure of salicylate- or menadione-incubated cells to calyculin A resulted in levels of reporter gene activity exceeding greatly that produced by a 42 °C/30-min heat shock (Fig. 9, A and B). Only low levels of reporter gene activity were produced by calyculin A alone. These results suggested that hyperphosphorylation can play an important role in the activation of the transactivation function of endogenous HSF1.

**DISCUSSION**

Upon heat stress, HSF1 is hyperphosphorylated mainly on Ser and Thr residues. This hyperphosphorylation shows as an increase in the apparent monomer size of HSF1 in anti-HSF1 Western blots. Two-dimensional phosphopeptide analysis of tryptic and chymotryptic peptides of HSF1 indicates heat-induced phosphorylation of residues in at least four different peptides. Based on the results of the present study that are discussed in more detail below, phosphorylation of one or more of these residues appears to increase the activity of the transactivation function of HSF1 and to reduce the rate of HSF1 trimer dissociation.

Previous observations provided convincing evidence that activation of HSF1 involves at least two regulatory events that can be separated from one another. The first event converts inactive HSF1 monomers into DNA-binding and nuclear-localized trimers. The second event activates the transactivation function of these trimers. The first event is exemplified by findings that menadione, H2O2, or salicylate induce the forma-
tion of HSF1 trimers that are transcriptionally inert (20, 21). Furthermore, a mouse erythroleukemia cell line has been characterized in which HSF1(1) trimerizes in response to heat shock but remains unable to enhance hsp gene transcription (22). Moreover, PrPSc-infected mouse cells show normal HSF1 trimerization upon heat shock treatment but are essentially incapable of raising the rate of synthesis of hsp (23). Finally, overexpression of HSF1 in human cells transfected with a functional HSF1 CDNA gene results in the accumulation of DNA-binding and nuclear-localized trimers that are unable to activate hsp reporter genes (16). Similarly, expression in Xenopus laevis oocytes of mutant human HSF1 containing a single substitution in the third hydrophobic repeat (E293) results in inactive HSF1 trimers (16). Evidence for a second heat-regulated event was provided by the observation that a chimeric LexA-HSF1 protein overexpressed in human cells can be heat-activated to transcribe a LexA-CAT reporter gene. Like wild-type HSF1, overexpressed LexA-HSF1 is found in a DNA-binding form in human cells. Thus, heat shock triggered a second regulatory event, i.e., the conversion of inactive to active trimeric factor. Similar findings were made in the frog oocyte in which a LexA-HSF1 protein containing the E293 mutation was a transcriptionally inactive trimer that could be activated by heat shock. Finally, the transcriptional activity of a GAL4-HSF1 chimera lacking sequences required for trimerization could be enhanced by extreme heat shock (18, 19).

Evidence that hyperphosphorylation of HSF1 does not play a role in the first regulatory event is provided by several observations. As reported previously by others (10) and recapitulated herein for our cell line/reporter gene system, inhibitors of Ser/Thr protein kinases, here H7 and GF-X, inhibited the activation of hsp reporter genes in response to a moderate heat shock. The inhibitors did not reduce HSF1 DNA-binding activity measured in cells harvested at the end of the heat shock period. Conversely, protein phosphatase inhibitor calyculin A potentiated hsp reporter gene expression induced by a moderate heat shock. Similar observations were also made with phosphatase inhibitor okadaic acid (11). This effect of phosphatase inhibitors was not accompanied by a significant increase in HSE DNA-binding activity.

To find out whether the second regulatory event involved hyperphosphorylation of HSF1, the phosphorylation status of HSF1 in cells overexpressing HSF1 or exposed to menadione or salicylate was assessed by incorporation of \(^{32}\text{P}\)phosphate or by mobility shift assay. In each case, inactive trimers were found to be underphosphorylated when compared to HSF1 from heat-shocked cells. In each case, exposure to calyculin A resulted in hyperphosphorylation of HSF1 trimers that was similar in extent to that observed in moderately to severely heat-shocked cells. This hyperphosphorylation invariably accompanied activation of the transactivation function of the HSF1 trimers. Activation by calyculin A occurred in the absence of a significant increase in HSE DNA-binding activity. Thus, increased protein phosphorylation triggered the transcriptional activation of inactive HSF1 trimers. The most straightforward interpretation of these findings is that activation of the transactivation function of HSF1 depended on the phosphorylation of certain sites in the factor itself. The possibility that inductive phosphorylation of another factor(s), acting downstream from HSF1, was required for HSF1 to activate hsp genes could be excluded based on our previous identification of deletion mutants of HSF1 that are constitutively active in the absence of stress (16). However, it could not be ruled out that the second regulatory event involved a negative regulator of the HSF1 transactivation function, and that this regulator was inactivated by phosphorylation. Note that there is no evidence for the existence of such a regulator. Furthermore, that even extreme overexpression of HSF1 does not result in active factor appears to argue against a repressor-type mechanism of regulation. Nevertheless, the formal elimination of this possibility will require the identification of the residues inducibly phosphorylated in HSF1 and the demonstration that trimeric HSF1 mutated in these residues cannot be activated by calyculin A.

The present study uncovered an additional, unexpected role for HSF1 hyperphosphorylation. The critical experiment involved two sets of cultures. The first set was preexposed to calyculin A, and the inhibitor remained present for the duration of the experiment, whereas the second set did not receive the inhibitor. Cultures were subjected to a brief heat shock, and HSF1 was analyzed before and immediately after heat shock as well as at two different times after the return of the cells to the control temperature. HSE DNA binding assays revealed that DNA binding activity disappeared slower in the presence than in the absence of the inhibitor. Thus, hyperphosphorylation retarded the inactivation of HSF1 DNA binding ability. Native gel electrophoresis and HSF1 blot showed that, in the absence of calyculin A, most HSF1 monomers were converted to trimers during or shortly after the heat shock, and that the trimers were quantitatively disassembled within about 2 h following the return of cells to the control temperature. In the presence of calyculin A, however, i.e., in a situation in which HSF1 was hyperphosphorylated prior to heat shock, both HSF1 trimer formation in response to heat shock and their dissociation subsequent to heat shock was retarded. The severity of inhibition was dependent on the calyculin A dosage.

The observation that hyperphosphorylation of HSF1 causes inhibition of trimer formation upon heat shock may not be physiologically relevant. In our experiments, hyperphosphorylation was induced prior to the exposure of the cells to the stressful condition. Normally, trimerization of HSF1 in response to a stressful event may occur simultaneously with, or may even precede induced phosphorylation of the factor (24). By contrast, the inhibition of the dissociation of hyperphosphorylated HSF1 trimers may well be physiologically important. It would tend to potentiate the stimulatory effect of hyperphosphorylation on the transactivation function of the factor, further increasing the accumulation of hsp mRNA and hsp.

It was reported previously that exposure of cells to the amino acid analogue azetidine activates HSF1, without causing hyperphosphorylation of the factor as assessed by anti-HSF1 Western blot (5). This finding is in apparent conflict with the present observations and conclusions. However, there are several possible explanations for this anomaly. For example, mobility shift assays only provide information on overall changes in factor phosphorylation. They may not detect a change in phosphorylation of an individual residue that may be particularly important for factor regulation. In addition, mobility shift assays describe a property of the average HSF1 molecule and do not discriminate between active and inactive factor. Thus, the finding does not seriously detract from our observations but underscores the importance of further investigation aimed at defining the effects of individual phosphorylation events on the regulation of HSF1.

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