Mapping of tryptic phosphopeptides of heat shock factor 1 (HSF1) from non-stressed or moderately heat-stressed HeLa cells, labeled in vivo by $^{32}$P-orthophosphate, revealed four major phosphopeptides A to D. Heat stress drastically increased phosphopeptide signals. To identify target peptides and amino acids and to correlate phosphorylation and transactivation function, phosphopeptide maps were produced of LexA-human HSF1 chimeras and mutant derivatives thereof, and transactivation activities of original and mutant chimeras were compared. LexA-HSF1 chimeras were previously shown to be regulated identically to HSF1, except that they transactivate promoters with LexA-binding sites instead of hsp promoters. The patterns of phosphopeptides of LexA-HSF1 and endogenous HSF1 were similar. Analysis of single residue substitutions suggested that phosphopeptide C is peptide VKEEPPSP-PQSPR (297–309) phosphorylated on Ser-307 but not Ser-303. Substitution of Ser-307 but not Ser-303 caused decrease of factor activity. Mapping several constitutively active chimeras associated unphosphorylated peptide C with the transcriptionally active HSF1 conformation, suggesting that dephosphorylation of this peptide (at Ser-307) may either be an integral step in the activation process or serve to maintain the active conformation of HSF1. Exploiting this correlation, indirect evidence was obtained that activation domains of HSF1 interact with the distantly located regulatory domain to maintain the factor in an inactive state.

Stress-induced expression of heat shock protein (hsp) genes is controlled by heat shock factor (HSF) (HSF1 in vertebrate species; Refs. 1–5). Activation of HSF1 is generally thought to be triggered, and in others, e.g. overexpression from a transfected HSF1 gene (11), the first step is bypassed. Mutagenesis experiments identified elements in the 529-residue-long human HSF1 sequence, located between residues 186 and about 315 and collectively referred to herein as regulatory domain, that are involved in keeping trimeric factor in an inactive state.

Although their physiological relevance remains uncertain, these observations demonstrate that, under conditions of maximal activation of the MAP kinase pathway, HSF1 is phosphorylated by MAP kinase, and its activity is inhibited.

Inhibitors of Ser/Thr kinases partially inhibit heat-induced hyperphosphorylation of human HSF1 and prevent increased Hsp expression but do not affect HSF1 trimerization and HSE DNA-binding activity (17), suggesting that induced phosphorylation is required for the second but not the first step in the activation of HSF1. In agreement with these observations, HSF1 hyperphosphorylation is induced more slowly than trimethylation on HSF1 histones in vivo (10, 12, 13), e.g. exposure to sodium salicylate (10) or menadione (12), only the first step can be triggered, and in others, e.g. overexpression from a transfected HSF1 gene (11), the first step is bypassed. Mutagenesis experiments identified elements in the 529-residue-long human HSF1 sequence, located between residues 186 and about 315 and collectively referred to herein as regulatory domain, that are involved in keeping trimeric factor in an inactive state in the absence of stress (11, 14, 15).

HSF1 is phosphorylated at serine and threonine residues in unstressed cells (16, 17). Following heat shock or when cells are exposed to one of several chemical stressors, the transcription factor becomes hyperphosphorylated (1, 4, 18). During recovery of cells from such stress, HSF1 is dephosphorylated to its pre-stress state.

Regarding the possible role of constitutive phosphorylation, Mivechi and Giaccia (19) found that overexpression of a dominant inhibitory mutant of ERK1 increases the expression of an hsp70 promoter-driven reporter gene in NIH-3T3 cells. Knauf et al. (20) have shown that expression of activated Raf in human 293T cells results in increased phosphorylation of a flag epitope-tagged HSF1 expressed from a transfected gene. Chu et al. (21) performed experiments involving co-transfection of NIH-3T3 cells with an hsp70-CAT reporter gene and with expression vectors for human HSF1 and MEK-1 and noted that overexpression of MEK-1 reduces reporter gene expression. Although their physiological relevance remains uncertain, these observations demonstrate that, under conditions of maximal activation of the MAP kinase pathway, HSF1 is phosphorylated by MAP kinase, and its activity is inhibited.

Inhibitors of Ser/Thr kinases partially inhibit heat-induced hyperphosphorylation of human HSF1 and prevent increased Hsp expression but do not affect HSF1 trimerization and HSE DNA-binding activity (17), suggesting that induced phosphorylation is required for the second but not the first step in the activation of HSF1. In agreement with these observations, HSF1 hyperphosphorylation is induced more slowly than trimethylation and correlates better with the acquisition of transcriptional competence (16). A role of induced phosphorylation in activating the transcription function of the factor is also implied by experiments involving overexpression of HSF1 in human cells from a transfected gene. The exogenous HSF1 that is trimeric but transcriptionally inert can be activated by calyculin A, an inhibitor of Ser/Thr protein phosphatases (17). Although suggestive, these data are correlative in nature, and would be expected to be confirmed with other techniques.
there is no direct proof for any functional role of HSF1 hyperphosphorylation.

While most HSF1 is in a non-trimeric form in unstressed human cells, a low level of transcriptionally inert HSF1 trimers could be detected (17). If this finding reflects the continuous assembly and disassembly of HSF1 trimers, the substrate of constitutive phosphorylation may be either the non-trimeric or the trimeric factor, or both. For the purposes of this study, the question of whether non-trimeric or trimeric factor is the target of phosphorylation did not need to be resolved since similar phosphopeptide patterns were obtained from non-trimeric and trimeric endogenous HSF1. The substrate for stress-induced phosphorylation most likely is the inactive HSF1 trimer since trimerization (measured as DNA-binding activity) precedes induced phosphorylation of the factor (16). Further support is provided by findings that exposure of cells to certain chemicals or transfection with an HSF1 gene results in trimeric, underphosphorylated and transcriptionally inert HSF1 that is hyperphosphorylated and activated by heat shock or by addition of an inhibitor of protein phosphatases (9, 17). Thus, the preferred substrate for in vivo mapping of phosphoamino acids is trimeric HSF1. In the present study, to allow for parallel assays of phosphorylation and transcription factor function, a trimeric (because expressed from transfected genes) chimeric HSF1 was used in which the HSE DNA-binding domain was substituted by the DNA-binding domain (only) of bacterial repressor LexA. This chimeric factor is known to be regulated in a fashion that is indistinguishable from wild-type HSF1, except that it binds a LexA recognition site rather than an HSE sequence (11, 22). Initial in vivo mapping experiments confirmed that the chimeric factor is also phosphorylated similarly to endogenous HSF1.

Previous studies, mapping phosphopeptides of GAL4-human HSF1 chimeras or of recombinant HSF1 phosphorylated in vitro, concluded that HSF1 is constitutively phosphorylated on residues 303 and 307, and transactivation assays suggested that phosphorylation at residue 303 plays an important role in repressing the transcriptional ability of the factor (16, 20, 21). The present study is relying on phosphopeptide mapping of a fully functional, trimeric HSF1, i.e. LexA-HSF1 chimera, and of mutant derivatives thereof labeled in vivo and on transactivation assays with the same molecules to both revisit and extend previous observations. Our experiments revealed four major, 32P-labeled, tryptic peptides of HSF1 (phosphopeptides A to D). Mapping data obtained from mutant LexA-HSF1 chimeras are consistent with the view that the phosphopeptide C corresponds to peptide VKEEPPSPPQSPR (residues 297–309) phosphorylated at Ser-307 but not Ser-303. Transactivation assays matched the mapping results closely: substitution of Ser-307 but not of Ser-303 results in deregulation of the transcription function of HSF1. Apparently, peptide C needs to be phosphorylated in transcriptionally inactive HSF1. In various constitutively active mutants of HSF1 (LexA-HSF1), peptide C is not phosphorylated effectively, suggesting that dephosphorylation of the peptide is a hallmark of activated HSF1. These findings also imply that rephosphorylation of peptide C cannot trigger the return of the factor to an inactive state during recovery from heat stress, which kind of mechanism, i.e. dephosphorylation of HSF of the stress, has been suggested to play a role in the inactivation of yeast HSF (23). Extension of the analysis of the phosphorylation status of peptide C to additional deletion mutants suggested that most of or, perhaps, the entire region between residue 186 and the end of the HSF1 sequence plays a role in keeping HSF1 in a transcriptionally inert state, i.e. that much of the HSF1 sequence participates in the regulation of the transcription function, and that activation of HSF1 involves global changes in the structure of the molecule.

MATERIALS AND METHODS

Cell Culture and 32P/Orthophosphate Labeling of Proteins—Hela or Hela-CAT cells containing stably transformed chloramphenicol acetyltransferase (CAT) reporter genes controlled by the human hsp70B promoter (24) were grown in 100- or 60-mm dishes in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum at 37 °C and 5% CO2. For experiments involving [32P]orthophosphate labeling of proteins, transfected (about 2 × 106 cells) or untransfected Hela-CAT cells (about 105 cells) were washed with phosphate-free DMEM, preincubated for 40 min in phosphate-free DMEM supplemented with 5% dialyzed calf serum, and then incubated for 2 h with [32P]orthophosphate (200 μCi/ml) at 37 °C prior to a 30-min heat treatment at 42–43 °C. Control cultures were labeled at 37 °C for 2.5 h.

Plasmids—All LexA-HSF1 genes were under the control of the CMV promoter present in pcDNA vectors (Invitrogen). Construction of the LexA-HSF1 chimera and of all HSF1 deletions used herein was described previously (11, 22). HSF1 deletions were introduced into the chimera by standard subcloning procedures, making use of conveniently located restriction sites. Single or multiple amino acid substitutions were made by replacing short restriction fragments containing the sites to be mutated by corresponding fragments resulting from restriction digestion of polymerase chain reaction fragments into which the desired mutations had been introduced by means of appropriately designed primers. The presence of substitutions was verified by DNA sequence analysis.

Transfections—Transfections were performed using the LipofectAMINE™ reagent (Life Technologies, Inc.). For experiments involving [32P]orthophosphate labeling of proteins, 70% confluent Hela-CAT cells in 100-mm dishes were incubated for 5 h with 3 ml of a mixture containing 1 μg of a construct containing a CMV promoter-driven β-galactosidase reporter gene (22), 10 μg of a construct containing a LexA-HSF1 gene, and 15 μl of LipofectAMINE™ reagent in serum-free medium. Subsequent to this incubation, cells were washed and incubated for about 40 h in complete medium. For transactivation experiments, 70% confluent HeLa cells in 60-mm dishes were used. Transfections were carried out as before, except that cultures were incubated with a 1-ml mixture including 1 μg of a LexA-HSF1 construct, 0.5 μg of a construct containing a CAT reporter gene functionally linked to a minimal promoter supplemented with LexA binding sites (LexA-CAT; Ref. 11), and 0.5 μg of the CMV-β-galactosidase reporter gene construct. Following transfection, cultures were incubated in complete medium for 15 h prior to heat treatment.

For experiments involving phosphopeptide mapping, cells were harvested immediately after heat treatment. For transactivation experiments, cells were harvested after an additional 24-h incubation at 37 °C subsequent to heat (or control) treatment. Heat treatment was by incubation of cultures in a 42–43 °C waterbath for 30 min.

Enzyme Activity Assays—CAT assays were performed as described by Gorman et al. (25). β-Galactosidase assays employed a standard colorimetric procedure (6).

Immunoprecipitation of HSF1 or LexA-HSF1, and Phosphopeptide Mapping—Cells (about 2 × 107; about 106 cells in mapping experiments with endogenous HSF1), [32P]orthophosphate-labeled as described before, were collected and washed with phosphate-buffered saline. They were lysed in 0.4 ml of radioimmuno precipitation buffer (50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1% sodium deoxycholate, 1% Triton X-100, 5 mM sodium vanadate, 0.5 mM sodium fluoride, 0.5 mM phenylmethylsulfonyl fluoride), and the lysate was clarified by centrifugation. HSF1 or LexA-HSF1 was immunoprecipitated in the cold from 400 μl of cleared lysate using a previously described (1) rabbit anti-HSF1 polyclonal antibody bound to protein A-Sepharose CL-4B. The immunoprecipitate was dissolved in sample buffer and fractionated by 7.5% SDS-polyacrylamide gel electrophoresis. The gel was dried and exposed to autoradiography. Regions containing HSF1 or LexA-HSF1 (or mutant derivatives) were excised, and the gel slices were dispersed in 1 ml of 50 mM ammonium bicarbonate, pH 7.4. After addition of 50 μl of β-mercaptoethanol and 1 μl of 100% SDS, the gel was incubated for 5 min and then incubated at room temperature for 3 h. Following removal of gel pieces and addition of 20 μg of bovine serum albumin, HSF1/LexA-HSF1 was precipitated with 20% trichloroacetic acid. The pellet was washed with cold ethanol and taken up in ice-cold performic acid, and the solution was incubated on ice for 1 h to oxidize protein. After two cycles of lyophilization, oxidized protein was dissolved in 50 μl of 50 mM ammonium bicarbonate, pH 7.4, and 2 μg of TPCK-trypsin

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were added. Proteinase digestion was for 15 h at 37 °C. TPK-C-trypsin (2 μg) was added twice again over the course of the reaction. After two cycles of lyophilization, peptides were resuspended in electrophoresis buffer (1:3.5:4.0, formic acid/glacial acetic acid/water (pH 1.9)) and, after addition of markers (Tramine, Xylene cyanol FF, and d-DNP-lysine), were applied to a cellulose TLC plate (EM Laboratories). Phosphopeptides were separated in the first dimension by electrophoresis (1030 volts for 35 min at 15 °C) using an HYTEL 7000 apparatus (C.B.S. Scientific) and in the second dimension by chromatography in n-butanol, pyridine, glacial acetic acid, and water (5:3:3:1:4) (26). Radiolabeled phosphopeptides were detected by PhosphorImager (Molecular Dynamics).

**Phosphorylation of Peptides in Vitro**—For assays involving protein kinase C, synthetic peptide (20 μg) corresponding to residues 295–311 of human HSF1 (VRKVEEPPSPPQSPRVE) was added to reaction mixtures (50 μl) containing 20 μM MOPS, pH 7.2, 25 μM β-glycerophosphate, 15 μM MgCl₂, 1 μM CaCl₂, 1 μM sodium orthovanadate, 1 μM dithiothreitol, 90 μM ATP, 0.1 mg/ml diglycerides, 0.1 mg/ml phosphatidyserine, 10 μCi [³²P]ATP and 0.1 μg of protein kinase C (mixture of α, β, and γ isomers from rat brain). For MAP kinase assays, reaction mixtures (50 μl) contained 20 μM MOPS, pH 7.2, 25 μM β-glycerophosphate, 15 μM MgCl₂, 5 μM EGTA, 1 μM sodium orthovanadate, 1 μM dithiothreitol, 90 μM ATP, 20 μg of peptide, 10 μCi [³²P]ATP, and 0.2 μg of recombinant, active p42 Erk1 or 0.1 μg of recombinant, active p42 Erk2. All protein kinases were from Upstate Biotechnology. Following incubation at 30 °C for 1 h, peptides were precipitated with 20% trichloroacetic acid in the presence of 20 μg of bovine serum albumin. Precipitates were washed, dissolved, digested with trypsin, and thin layer-electrophoresed and chromatographed as described in the previous section.

## RESULTS

**LexA-Human HSF1 Expressed from Transfected Genes Is Similarly Hyperphosphorylated as Endogenous Human HSF1**—To radiolabel phosphorylatable residues in HSF1, HeLa-CAT cells were incubated for 2 h at 37 °C and then for an additional 30 min either at 42–43 °C (heat shock) or at 37 °C (control) in the presence of [³²P]orthophosphate. Cells were collected and lysed in the presence of phosphatase and protease inhibitors, and HSF1 was immunoprecipitated with an HSF1-specific antibody, subjected to SDS-polyacrylamide gel electrophoresis, eluted from the gel, carefully oxidized, and digested to completion with trypsin. Peptides were resolved by thin layer electrophoresis and chromatography, and radiolabeled phosphopeptides were detected by PhosphorImager (C.B.S. Scientific) and in the second dimension by chromatography in n-butanol, pyridine, glacial acetic acid, and water (5:3:3:1:4) (26). Phosphopeptides were first separated by chromatography and second by electrophoresis. Results from two independent experiments with LexA-HSF1 are shown in panels C and D. A schematic map of LexA-HSF1 is shown at the top of the figure.

![Fig. 1. Two-dimensional tryptic phosphopeptide maps of endogenous HSF1 (A and B) and of LexA-HSF1 expressed from transfected genes (C and D). Transcription factors were labeled in vivo with [³²P]orthophosphate at 37 °C (Co) or at 42–43 °C (panels labeled "HS", or not labeled). Note that about five times more cells were used in mapping experiments involving endogenous HSF1 than in experiments involving LexA-HSF1 or mutant derivatives of LexA-HSF1 (See subsequent Figs.). Phosphopeptides were prepared as detailed under "Materials and Methods" and applied to a TLC plate (arrow). Phosphopeptides were first separated by chromatography and second by electrophoresis. Results from two independent experiments with LexA-HSF1 are shown in panels C and D. A schematic map of LexA-HSF1 is shown at the top of the figure.](image)
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Fig. 2. Identification of Ser-307 as a target of phosphorylation. Tryptic phosphopeptide maps of singly or multiply alanine-substituted LexA-HSF1 chimeras are shown in panels A through D. Positions of residues substituted are indicated on top of panels. See Fig. 1 for other experimental details.

with different mutations were tested in HeLa cells (using the transactivation assay described below), it was observed that, unlike the unmutated chimera, chimeras including an alanine substitution of Ser-307 (S307A) were active at non-heat shock temperature (not shown). Based on these results, it was tentatively concluded that the absence of a phosphorylated residue at position 307 resulted in deregulation of the chimeric HSF1, and that phosphopeptide C corresponded to peptide VKEEPPSPQR (297–309) phosphorylated at Ser-307. Confirmation was obtained from a mapping experiment with a LexA-HSF1 chimera with a single alanine substitution at Ser-307 (S307A; Fig. 2B). As expected, phosphopeptide C was virtually absent from the map. Surprisingly, phosphopeptide A was also absent, and new phosphopeptide A’ appeared. Since phosphopeptides A and A’ have similar chromatographic properties, and A’ migrates twice as far as A in the electrophoretic dimension, it is probable that phosphopeptides A and A’ are identical except that phosphopeptide A is phosphorylated on two residues and A’ on only one. Phosphorylation of one of the two phosphorylatable residues in peptide A may be dependent on a conformational change brought about by phosphorylation of Ser-307. The above result also indicates that phosphorylation of Thr-120, Thr-124, Ser-174, and Ser-199 does not contribute to the phosphopeptides appearing in the map of LexA-HSF1.

The sequence around Ser-307 not only corresponds to a protein kinase C recognition sequence but also includes a MAP kinase consensus sequence (PX(S/T)P). Thus, Ser-307 could have been phosphorylated either by a MAP kinase or by a protein kinase C. Peptide C also contains a second MAP kinase consensus sequence centered around Ser-303. The above mapping experiment with the S307A chimeras suggested that Ser-303 was not phosphorylated; if phosphopeptide C was doubly phosphorylated, the S307A mutant should have produced a singly phosphorylated peptide with the same chromatographic characteristics as phosphopeptide C but with twice its electrophoretic mobility. The appearance of a peptide with the predicted coordinates was not observed in the map of the S307A mutant factor. To confirm that Ser-303 was not phosphorylated in vivo, a mutant LexA-HSF1 chimera with an S303A substitution was prepared and subjected to phosphopeptide mapping (Fig. 2C). As predicted, the mobility of phosphopeptide C was unaffected by the mutation. Again, like in the case of the S307A mutant factor, new phosphopeptide A’ appeared at the expense of phosphopeptide A. The map of a chimera with alanine substitutions of both Ser-303 and Ser-307 (S303A/S307A) is shown in Fig. 2D. In agreement with the previous results, phosphopeptide C was absent. Interestingly, unlike with the single substitutions, the map of the S303A/S307A factor contained phosphopeptide A but not A’, providing further evidence for the notion that the conformation of the peptide C region influences phosphorylation of peptide A. Note that although the differences between phosphopeptide patterns of wild-type and mutant chimeras were quite obvious from visual inspection, results were quantified by measuring mobilities in both the chromatographic (suffix c) and the electrophoretic (suffix e) dimensions of phosphopeptide signals and the DNP-lysine standard and calculating relative \( R_f \) values. \( R_f \) values for signals A, A’, B, C, and D were 0.063, 0.067 ± 0.005, 0.043 ± 0.005, 0.121 ± 0.009, and 0.483 ± 0.005, respectively. \( R_f \) values were 0.84, 1.50 ± 0.05, 1.26 ± 0.08, 1.46 ± 0.04, and 1.05 ± 0.05, respectively. The consistency of \( R_f \) values calculated from different experiments provides further support for our interpretation of results.

Peptide vRVEELSSPPQSPRve (small letters refer to HSF1 amino acids outside of tryptic peptide C) was prepared synthetically and was reacted with protein kinase C or MAP kinase in the presence of [\(^{32}P\)ATP]. Thin layer electrophoresis and chromatography (after trypsin digestion) revealed that reaction with protein kinase C resulted in a phosphopeptide with similar electrophoretic mobility as that of phosphopeptide C (not shown), suggesting that Ser-307 can be phosphorylated by protein kinase C. MAP kinase (either p44 Erk1 or p42 Erk2), used under conditions under which it effectively phosphorylates the model substrate myelin basic protein, failed to phosphorylate the peptide.

To test the functional consequences of substitutions of Ser-303 and Ser-307, parallel cultures of HeLa cells were co-transfected with an unmutated or mutated LexA-HSF1 gene construct, a β-galactosidase gene construct and a CAT reporter gene under the control of a minimal promoter supplemented with LexA binding elements (LexA-CAT). One day after transfection, one of each set of duplicate cultures was heat-treated at 42–43 °C for 30 min, and all cultures were harvested the following day. β-Galactosidase assays were carried out to verify that parallel cultures had been transfected with comparable efficiencies (not shown). Reporter gene expression was assessed by the standard chromatographic CAT assay (25), and typical results are shown in Fig. 3. Chromatograms were scanned by PhosphorImager to estimate the regulatability of the transcription function of the different LexA-HSF1 chimeras (Table I). The LexA-HSF1 and the S303A chimeras were similarly heat-activatable. In contrast, both the S307A and S303A/S307A chimeras were deregulated, i.e. they showed significant activities in the absence of heat shock. Thus, dephosphorylation of Ser-
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Transactivation ability of LexA-HSF1 chimeras. HeLa cells were co-transfected with a LexA-HSF1 construct (indicated at the top of panels), a β-galactosidase construct, and reporter gene LexA-CAT, heat-treated for 30 min at 42 °C (+) or not heat-treated (−), and processed 1 day after heat treatment (10). Chromatographic CAT assays are shown. The arrow points to the major acetylated product of chloramphenicol.

Table I

Transactivation ability and phosphorylation of peptide C

| Construct          | Fold heat induction of LexA-CAT reporter activity | Fraction of radioactivity incorporated in peptide C ([(A+B+C+D)/I]) |
|--------------------|--------------------------------------------------|---------------------------------------------------------------|
| LexA-HSF1          | 7.8                                              | 0.25                                                          |
| LexA-HSF1 S303A    | 12                                               | 0.46                                                          |
| LexA-HSF1 S307A    | 1.2                                              | <0.05                                                         |
| LexA-HSF1 S303A/S307A | 2.0                                              | <0.05                                                         |
| LexA-HSF1 S275A    | 2.0                                              | <0.05                                                         |
| LexA-HSF1d186–202  | Deregulated*                                     | <0.05                                                         |
| LexA-HSF1d202–316  | Deregulated*                                     | <0.05                                                         |
| LexA-HSF1d453–523  | No transactivation                               | 0.08                                                          |
| LexA-HSF1d409–465  | No transactivation                               | <0.05                                                         |

*Transactivation ability of these mutants (either in the HSF1 or the LexA-HSF1 background) was determined previously (Refs. 11 and 30).
served, but the abundance of the most slowly migrating bands is increased drastically relative to the faster migrating forms, indicating a heat-induced shift to more highly phosphorylated HSF1 species. The fact that this shift in distribution can be observed by Western blot implies that heat-induced phosphorylation events occur in most HSF1 molecules. Since inducible phosphorylation involves addition of a radiolabeled phosphate group to a previously unphosphorylated residue during heat shock, the specific radioactivity of a phosphopeptide including a newly phosphorylated residue should be at least equal to or higher than that of a phosphopeptide containing a residue 32P-labeled by turnover or during recovery. Thus, a truly inducibly phosphorylated peptides should be represented by one of the major signals present in our maps.

Two previous studies highlighted the importance of phosphorylation at Ser-303 (16, 21), and a third did not discriminate between phosphorylation of Ser-303 and Ser-307 (20). The study by Chu et al. (21) relied mostly on in vitro phosphorylation experiments. It showed that incubation of recombinant HSF1 with MAP kinase results in molecules phosphorylated on Ser-307 and that the latter molecules can be further phosphorylated on Ser-303 by glycogen synthase kinase 3. The significance of these observations remains unknown. The study by Kline and Morimoto (16) detected in excess of ten tryptic phosphopeptides originating from HSF1 32P-labeled in vivo. The specific radioactivity of about half of these phosphopeptides was increased modestly (the “constitutive phosphopeptides”) and of the other half increased more drastically (the “inducible phosphopeptides”) during heat treatment. A similar phosphopeptide pattern was observed for a GAL4-mouse HSF1d1–123 chimera expressed in COS monkey cells. The same experimental system was then used to produce maps of similar chimeras containing additional deletions or substitutions in the HSF1 sequence. Substitution in the original chimera of Ser-303 and Ser-307 led to the disappearance of four of the five major constitutive phosphopeptides. Substitution of either Ser-303 or Ser-307 deleted three of the same four phosphopeptides. In functional assays, S303A and S303A/S307A substitutions were found to be constitutively active. A single substitution of Ser-307 was not tested. Ser-303 and Ser-307 lie within the same tryptic peptide (referred to herein as peptide C). Thus, even if phosphorylation of these residues was incomplete, thin layer electrophoresis and chromatography could only distinguish singly and doubly phosphorylated peptide C, i.e. no more than two signals should be attributable to the peptide. As we pointed out, the appearance of four different signals originating from peptide C is indicative of some technical problem with the preparation of phosphopeptides. Presumably because oxidation and proteolytic cleavage reactions ran to completion in our experiments, the resulting maps revealed a much simpler pattern, consisting of only four major signals. S303A/S307A and S307A substitutions led to the disappearance of phosphopeptide C. These findings support the conclusion that peptide C is identical with peptide VKEEPPSPPQSPR (297–309), containing Ser-303 and Ser-307. In contrast, phosphopeptide C still was present as a major signal in the map of an S303A substitution, whereas an S303A substitution was normally heat-regulated. We conclude from these results that Ser-307 but not Ser-303 is phosphorylated and suggest that phosphorylation of Ser-307 plays a regulatory role. Although the observation (discussed more fully below) that active HSF1 is underphosphorylated further supports a regulatory role of Ser-307 phosphorylation, it is noted that the alternative interpretation of our transactivation assays with the S307A substitution mutant, which is that the substitution itself resulted in an activating conformational change, cannot be categorically ruled out.

As discussed before (in the Introduction), the substrate for stress-inducible phosphorylation most likely is the HSF1 trimer. Constitutive phosphorylation may occur in either or both, non-trimeric and trimeric, inactive HSF1. While the question which of these forms of HSF1 is the true substrate for consti-
tutive phosphorylation may not be easily resolved, it may not be critical from a practical point of view. Similar phosphopeptide patterns were obtained with (endogenous) HSF1 from un-stressed (HSF1 largely monomeric) and stressed (HSF1 largely trimeric prior to phosphorylation) cells. The present study employed LexA-HSF1 chimeras that are trimeric under the experimental conditions used and are regulated identically with wild-type HSF1 (11, 22). In contrast, the Kline and Morimoto study (16) was performed with GAL4-HSF1 molecules that include the dominant dimerization domain of GAL4 in addition to or instead of the HSF1 trimerization domain. Presumably, the GAL4-HSF1 chimeras are dimeric, and the HSF1 sequences contained in the chimeras fold improperly because of the presence of HSF1 trimerization domains that are unable to assemble into a trimeric structure or because of the lack of these domains, as the case may be. In the resulting, atypically folded chimera, but not in native HSF1, Ser-303 may become a target of phosphorylation, and phosphorylation of this residue that lies in the regulatory region of the molecule may affect the functional properties of the chimeras.

Phosphorylation of HSF1 is influenced by conformational changes in the molecule. Whereas endogenous HSF1 and the wild-type and S303A chimeras produce an intense phosphopeptide C signal, this signal is either weak or absent in constitutively active proteins (d186–202, d202–277, d202–316, S275A and S279A/S283A). This observation suggests that peptide C, i.e. Ser-307, is only phosphorylated when HSF1 is in an inactive conformation but not when it is in the active conformation. Presumably, phosphoserine 307 is dephosphorylated in the course of HSF1 activation. Since phosphorylation of Ser-307 apparently cannot occur in the active conformation, this event is unlikely to play a pivotal role in the inactivation of the factor subsequent to a stressful event. Rather, Ser-307 may be dephosphorylated after active HSF1 has been converted to an inactive form by another mechanism. In contrast, as substitution of Ser-307 by a non-phosphorylatable amino acid suffices to activate HSF1, it is conceivable that dephosphorylation of phosphoserine 307 is an intermediate step in the activation of the factor. Alternatively, dephosphorylation of the residue may serve to stabilize the activated conformation of HSF1. Note that because phosphoserine 307 is likely to be dephosphorylated during heat shock, heat-inducible incorporation of [32P]orthophosphate at Ser-307 may be best explained by the third mechanism discussed before (involving dephosphorylation of the residue in the presence of [32P]P4 subsequent to dephosphorylation).

Finally, advantage was taken of the observation of a close correlation between activation of HSF1 and dephosphorylation of phosphoserine 307 to ask whether mutations in HSF1 sequences distal to the regulatory domain (located between residues 186 and 315) also cause the factor to acquire an active conformation. The region near the carboxyl terminus of the 529-residue-long HSF1 sequence contains at least two activation domains (15). Somehow the conformational state of the regulatory domain determines whether or not the distant activation domains function. One possible mechanism by which this regulation at a considerable distance (100–300 residues) could be achieved would involve an interaction (direct or via an intermediary protein) between the regulatory domain and the activation domains, an interaction which is disrupted in the course of activation. A related mechanism may involve interactions between the activation domains and sequences preceding the regulatory domain. If one of these mechanisms were regulating the activation domains, one would predict that certain mutations in either the regulatory domain or the activation domains could cause the factor to assume an activated conformation. While this is clearly the case for the regulatory domain as shown by the ability of HSF1 molecules mutated in the regulatory domain to transactivate a reporter gene in the absence of stress, it has not been possible to similarly analyze the activation domains for obvious reasons. Our results suggest that dephosphorylation of phosphoserine 307 can serve as an indicator of the active conformation that is independent of the factor’s transcriptional activity, providing us with a tool for examining the regulatory influence of carboxyl terminal located HSF1 sequences. We found that a deletion mutant lacking the carboxyl-terminal end of HSF1 (d453–523) and an internal deletion (d409–465) produced maps with drastically reduced Ser-307 (phosphopeptide C) signals. These observations support a model of regulation in which the regulatory region and carboxyl-terminal located sequences including the activation domains are involved in establishing an inactive conformational fold that includes over half of the sequence of HSF1. The phosphopeptide mapping approach may be used to further delineate elements located in the carboxyl half of the HSF1 sequence that participate in the regulatory mechanism.

Note that the assignment of Ser-307 as a phosphorylation target, although fully supported by the available evidence, cannot be made with absolute certainty. One of the observations made in the present study is that activated HSF1 mutants or carboxyl-terminal deletions produce a much reduced phosphopeptide C signal. This finding raises the possibility that a single amino acid substitution may alter HSF1 conformation to such an extent that dephosphorylation of phosphopeptide C is sufficiently accelerated to reduce the level of [32P]-labeled phosphopeptide C to below the limit of detection.

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