Mammalian heat shock genes are regulated at the transcriptional level by heat shock factor-1 (HSF-1), a sequence-specific transcription factor. We have examined the role of serine phosphorylation of HSF-1 in the regulation of heat shock gene transcription. Our experiments show that mitogen-activated protein kinases (MAPKs) of the ERK-1 family phosphorylate HSF-1 on serine residues and repress the transcriptional activation of the heat shock protein 70B (HSP70B) promoter by HSF-1 in vivo. These effects of MAPK are transmitted through a specific serine residue (Ser-303) located in a proline-rich sequence within the transcriptional regulatory domain of human HSF-1. However, despite the importance of Ser-303 in transmitting the signal from the MAPK cascade to HSF70 transcription, there was no evidence that Ser-303 could be phosphorylated by MAPK in vitro, although an adjacent residue (Ser-307) was avidly phosphorylated by MAPK. Further studies revealed that Ser-303 is phosphorylated by glycogen synthase kinase 3 (GSK3) through a mechanism dependent on primary phosphorylation of Ser-307 by MAPK. Secondary phosphorylation of Ser-303 by GSK3 may thus repress the activity of HSF-1, and its requirement for priming by MAPK phosphorylation of Ser-307 provides a potential link between the MAPK cascade and HSF-1. Our experiments thus indicate that MAPK is a potent inhibitor of HSF-1 function and may be involved in repressing the heat shock response during normal growth and development and deactivating the heat shock response during recovery from stress.

Sequential Phosphorylation by Mitogen-activated Protein Kinase and Glycogen Synthase Kinase 3 Represses Transcriptional Activation by Heat Shock Factor-1

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Exposure of cells to elevated temperatures leads to the expression of the heat shock response in which the induction of a cohort of heat shock proteins (HSPs) is accompanied by the expression of heat resistance (1, 2). In mammalian cells, heat shock genes are regulated at the transcriptional level by heat shock factor-1 (HSF-1), a sequence-specific transcription factor that binds to heat shock elements (HSE) in their promoters (3–5). Although the mechanisms involved in HSF-1 activation are not fully understood, they operate at the posttranslational level and involve the conversion of HSF-1 from a latent cytoplasmic form to a nuclear protein that controls the transcription of heat shock genes (3–5). HSF family members are unique in binding to DNA in a homotrimeric complex (3, 6). Trimerization is governed by arrays of amphipathic α-helical residues (“leucine zippers”) in the amino-terminal domain and is negatively regulated by a fourth “leucine zipper” domain in the carboxyl terminus (3, 7). However, although heat-inducible nuclear localization and DNA binding are necessary steps in HSF-1 activation, these events are not sufficient for full transcriptional competence; HSF-1 can be activated to an intermediate state in which it binds to HSE sequences but does not stimulate transcription (8–11). Much preliminary evidence suggests a role for phosphorylation in the conversion of HSF-1 from this intermediate state into a transcriptionally active form (8, 9, 11). In addition, HSF from yeast and HSF-1 from mammalian cells both undergo hyperphosphorylation during heat shock (12, 13). The regulation of transcriptional activity in trimeric HSF-1 involves a number of functional domains which could mediate stepwise activation of HSF-1 and may be targets for phosphorylation (14–18). The transcriptional activation domains in the carboxyl terminus of the protein are suppressed under non-stress conditions by interaction with leucine zipper 2 in the amino-terminal core domain containing the DNA binding and oligomerization regions (16, 17). In addition, a centrally located regulatory region mediates the heat inducibility of the COOH-terminal activation domains independently of the core domain (14, 15, 18). The regulatory domain contains a number of serine residues within a proline-rich region essential for heat shock activation of HSF-1, which are potentially phosphorylated during heat shock (amino acids 220–310). As a number of these sequences conform to consensus phosphorylation domains for the mitogen-activated protein kinase (MAPK), we have examined the role of MAPK phosphorylation in regulating the transcriptional activating functions of HSF-1.

MAPK activity in mammalian cells was first ascribed to two closely related proteins, extracellular signal regulated kinase (ERK 1 and 2), that are rapidly activated in response to stimuli such as signals for proliferation and differentiation and stresses such as oxidative stress and ionizing radiation (19–22). The central importance of ERK 1 and 2 in cell proliferation is indicated by the findings that they are essential components in the signal transduction pathways leading from growth factor receptor tyrosine kinases to the initiation of mitogenesis (23). The ERK family was recently joined by two related gene families, the Jun/SAP kinases and the p38/Hog-1 family (24, 25).
These three MAPK families share a number of properties, including a downstream position in a three kinase cascade composed of: MAP kinase kinase kinase, its substrate MAP kinase kinase (MKK), and finally the MAPK itself with the unique property of being activated by phosphorylation on adjacent threonine and tyrosine residues by MKK (20, 21, 24, 25). The ERK, JunK, and p38 families have distinct targets as well as overlapping substrates, and each family is activated by heat shock (24–28). As the MAP kinases regulate other transcription factors, including TCF-62, c-Jun, and ATF-2, we have therefore examined the potential role of MAPK in HSF-1 regulation (23). The present study focuses on the role of phosphorylation in HSF-1 function. Our studies show that HSF-1 is phosphorylated on serine residues by ERK-1. Increasing cellular ERK activity by overexpressing a specific MKK (MEK-1) inhibited the transcriptional activating potential of HSF-1. Point mutation studies identified a specific residue, Ser-303, as essential for transmitting the inhibitory influence of MAPK to HSF-1. Paradoxically, two-dimensional tryptic phosphopeptide mapping of the wild type and mutant forms of HSF-1 after incubation with MAPK in vitro indicated that Ser-303 is not phosphorylated by MAPK. Instead Ser-303 is phosphorylated by glycogen synthase kinase 3 (GSK3). Repression of HSF-1 by MAPK appears to involve the phosphorylation of HSF-1 on an adjacent residue, Ser-307, which primes HSF-1 for subsequent phosphorylation on Ser-303 by GSK3. Thus serial phosphorylation of HSF-1 by MAPK and GSK3, although not interfering with the ability of HSF-1 to bind to HSE represses its function at a later step in transcriptional initiation or elongation.

**MATERIALS AND METHODS**

**Cell Culture—**THP-1 cells (ATCC TIB 202) from the American Type Culture Collection were cultured in endotoxin-free RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 μg/ ml β-mercaptoethanol, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in 5% CO2, 95% air atmosphere and routinely subcultured by dilution at a 1:3 ratio. NIH 3T3 cells were grown to confluence in Dulbecco’s modified Eagle’s medium containing 10% bovine calf serum. Confluent cultures were serum-starved in medium containing 0.1% bovine calf serum 24 h prior to treatment.

**32p Labeling of Cells and Immunoprecipitation of HSF-1—**THP-1 cells (70 x 10^6 to 100 x 10^6 cells) were incubated in 5 ml of phosphate-free RPMI 1640 containing 10% complete medium and 5 μCi of [32P]orthophosphate (8500–9120 Ci (314–337 TBq)/mmol, DuPont NEN) for 4 h at 37°C in a 5% CO2, 95% air humidified incubator. The cells were collected by centrifugation at 400 g for 10 min, resuspended in 1 ml of phosphate-buffered saline, and centrifuged at 10,000 x g for 2 min. The cell pellet was resuspended in 450 μl of 20 mM HEPES, pH 7.9, 0.4 mM NaCl, 25 mM glycerol, 1 mM EDTA, 2.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, 5 mM NaF, 0.5 mM sodium vanadate, 1 μg/ml okadaic acid, left on ice for 20 min, and frozen at –80°C. The cell extracts were thawed on ice and centrifuged at 10,000 x g for 15 min. The supernatant was collected and 1 ml of 50 mM Tris-HCl, pH 7.4, 0.15 μM NaCl, 1% Nonidet P-40, 0.25% deoxycholate, 2 mM EGTA containing inhibitors as above was added. Protein-A-Sepharose beads (100 μl) were added and the samples incubated for 2 h at 4°C on a rocking apparatus. The beads were pelleted by centrifugation, the 100 μl of Ab68–3 anti-HSF-1 antibody was added, and the samples incubated for 2 h at 4°C as above. Protein-A-Sepharose beads were added as before, the mixture incubated for 30 min at 4°C, and the beads collected and washed three times in 500 μl of incubation buffer and repeated centrifugation. The final pellet was resuspended in reducing electrophoresis sample buffer, boiled 3 min, and processed for two-dimensional mapping and/or phosphorarnine acid analysis.

**Expression and Purification of Recombinant HSF-1—**The human HSF-1 cDNA was excised from its parent vector (pBHSF1) by digestion with XhoI, the recessed strands filled with Klenow fragment, and the resulting fragment cloned into the NdeI and EcoRI sites of the pET22b vector (Novagen, Madison, WI) in order to produce the native protein, containing no additional amino acid. The plasmid provided ampicillin resistance to transform BL21(DE3) cells, which express large amounts of recombinant protein under isopropyl-1-thio-β-D-galactopyranoside induction. HSF-1 was isolated and purified by heparin-Sepharose chromatography, Mono-Q ion-exchange chromatography, and Mono-Q ion-exchange chromatography. The resulting material is pure by SDS-PAGE and C8 reverse phase chromatography binds the specific heat-shock element.

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and assayed as above (9).

**Mutagenesis—**Oligonucleotide-directed mutagenesis was performed using pALTER-1 vector as described in the Altered Sites II In Vitro Mutagenesis system manual (Promega, Madison, WI). The full-length EcoRI cDNA insert of HSF-1 was cloned into the EcoRI site of pALTER-1 vector. This construct in the desired orientation was used for the oligonucleotide-directed mutagenesis. For this purpose, three oligonucleotides (GTCCCTGGCCGCCCCCTGCGCT, AGCCCCGGCCGCCGCCTC, and CGCCCTAGGGCCGCCGCTG) were employed in mutating serine residues at positions 275 (S275F), 303 (S303G), and 307 (S307G) to glycine. In addition, oligonucleotides AGGCCCCCGACCCCGCTC and CGCCCTAGGGCCGCCGCTG were used to mutate Ser-303 and Ser-307 to aspartate. The point mutations were confirmed by deoxynucleotide sequencing.

**Transfection—**Wild type and mutated HSF-1 were force-cloned into XhoI/EcoRI sites of the pcDNA3.1 vector, which contains the cytomegalovirus promoter (Invitrogen, San Diego, CA). The HSF-1 cDNA contains an XhoI site at a position 5 base pairs upstream of the ATG start codon. The transfection was carried out using calcium phosphate precipitation as described in the Profection Mammalian Transfection System technical manual (Promega). NIH 3T3 cells were seeded at a density of 250,000/100-mm tissue culture dish 24 h prior to transfection. The HSF-1 constructs were co-transfected with the HSF107B promoter chloramphenicol acetyltransferase (CAT) construct p2500CAT that contains 2.5 kilobase pairs of 5′ non-coding sequence from the heat-inducible human HSF107B gene (32) including at least two heat shock elements, the TATA box region, and most of the leader sequence (Stressgen, Victoria, BC, Canada). The amounts of plasmids used in each transfection are indicated in the figure legends. Cells were harvested 48 h after incubation with plasmids for expression of CAT protein. CAT expression was then determined using an enzyme linked immunoassay for CAT protein according to the manufacturer's recommendations (Boehringer Mannheim). In some experiments, HSF-1 was co-transfected with a pCMV-MEK-1 plasmid obtained from Dr. N. G. Ahn, Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO (33).

**RESULTS**

**Serine Phosphorylation of HSF-1 in Vivo and in Vitro—**In order to determine whether human HSF-1 is phosphorylated in vivo, THP-1 human monocytes were labeled with [32P]orthophosphate and whole cell extracts were prepared either without or with heat shock prior to HSF-1 isolation by immunoprecipitation. Analysis of the immunoprecipitated HSF-1 by trypsin digestion and two-dimensional phosphopeptide mapping indicated multiple phosphorylated species both in control and heat-shocked cells (Fig. 1, A and B). The phosphoamino acid analysis of the isolated proteins showed that the immunoprecipitated HSF-1 incorporated phosphate almost exclusively on serine residues whether analyzed before or after heat shock (Fig. 1C). In order to investigate the nature of protein kinases involved in the phosphorylation of HSF-1, we next analyzed the efficiency of a wide range of kinases, including protein kinase A, calmodulin-dependent protein kinases II and IV, casein kinase II, and the MAP kinases, to phosphorylate HSF-1 in vitro. We focused on MAP kinase because MARK1 activity is potentially involved in HSF-1 phosphorylation (34). Transefction of pCMV-MEK-1 repressed the ability of HSF-1 to activate the HSF107B promoter by 70–80% (Fig. 2A). We next attempted to determine whether the effects of MAPK were exerted at the first step in HSF-1 activation (trimerization, nuclear localization/HSE binding) or at a later stage. To investigate potential effects of MAPK phosphorylation on the HSE binding ability of HSF-1, we incubated purified rHSF-1 with MAPK in vitro as described in Fig. 1D (except that unlabeled ATP was used) and measured HSE binding by EMSA (Fig. 2B). rHSF-1 differed from HSF-1 extracted from human cells in being competent to bind HSE in the absence of heat shock. rHSF-1 (with or without MAPK treatment) was diluted with MKIV buffer over a 50-fold concentration range prior to the gel shift assay (Fig. 2B). Incubation with MAPK did not alter the magnitude of rHSF-1 to HSE binding and the concentration of labeled HSF-1-HSE complexes was similar at each dilution with or without MAPK treatment (Fig. 2B).

**Role of MAPK Phosphorylation in HSF-1 Function—**We next examined the potential role of MAPK kinase in the control of HSF-1 activity. Cellular MAPK activity was stimulated by overexpressing MEK-1, the proximal activator of MAPK (33). We then determined the effect of elevated MEK-1 on the ability of HSF-1 expressed from the pCMV-HSF-1 vector to activate the HSF107B promoter in NIH 3T3 cells under non-heat shock conditions (Fig. 2A). MEK-1 was chosen for its specificity in activating the ERK kinases and because our control experiments indicated that NIH 3T3 cells stably expressing the pCMV-MEK-1 vector exhibited MAPK activity 4–8-fold greater than controls (not shown). NIH 3T3 cells were used here instead of the THP-1 cells used earlier as, in contrast to NIH 3T3 cells, HSF-1 expression does not appreciably activate the HSF107B promoter in THP-1 cells in the absence of heat (34). Transcription from the HSF107B promoter was strongly activated by HSF-1 expression in NIH 3T3 cells, while in controls transfected with the HSF-1 cDNA in inverse orientation, there was no evidence of transcriptional activation (Fig. 2A). Co-transfection of pCMV-MEK-1 repressed the ability of HSF-1 to activate the HSF107B promoter by 70–80% (Fig. 2A). We next attempted to determine whether the effects of MAPK were exerted at the first step in HSF-1 activation (trimerization, nuclear localization/HSE binding) or at a later stage. To investigate potential effects of MAPK phosphorylation on the HSE binding ability of HSF-1, we incubated purified rHSF-1 with MAPK in vitro as described in Fig. 1D (except that unlabeled ATP was used) and measured HSE binding by EMSA (Fig. 2B). rHSF-1 differed from HSF-1 extracted from human cells in being competent to bind HSE in the absence of heat shock. rHSF-1 (with or without MAPK treatment) was diluted with MKIV buffer over a 50-fold concentration range prior to the gel shift assay (Fig. 2B). Incubation with MAPK did not alter the magnitude of rHSF-1 to HSE binding and the concentration of labeled HSF-1-HSE complexes was similar at each dilution with or without MAPK treatment (Fig. 2B). This was a consistent finding in replicate experiments using incubation periods ranging from 30 min to 6 h and employing multiple applications of MAPK to in order to approach stoichiometric labeling of HSF-1 (data not shown). These in vitro studies therefore indicate that phosphorylation of HSF-1 with MAPK does not inhibit its binding to HSE (Fig. 2B). Similar observations were made in additional control gel shift experiments in vivo, which indicated that co-transfection of the pCMV-MEK-1 vector did not inhibit the ability of HSF-1 expressed in the same cells to

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3 J. Landry, F. Soncin, and S. K. Calderwood, unpublished results.
Fig. 1. **Serine phosphorylation of HSF-1 in vivo and in vitro.**

*A*, two-dimensional tryptic phosphopeptide map of HSF-1 from control THP-1 cells labeled with $^{32}$P in vivo. Cells were prelabeled with $[^{32}P]$orthophosphate prior to lysis, HSF-1 immunoprecipitation, gel purification, and tryptic phosphopeptide mapping. *B*, two-dimensional tryptic phosphopeptide map of HSF-1 from heat-shocked cells labeled with $^{32}$P in vivo and recovered by immunoprecipitation as in *A*. *C*, phosphoamino acid analysis of HSF-1 recovered from control or heat-shocked THP-1 cells by immunoprecipitation with anti-HSF-1 antibody (as in *A*). The relative migration of phosphoserine, phosphothreonine, and phosphotyrosine was determined by co-migration with standards. *D*, phosphorylation of purified recombinant human HSF-1 and MBP by MAP kinase (p44/42). Either HSF-1 or MBP was incubated with or without MAP kinase and [$^{32}$P]ATP as described under “Materials and Methods.” HSF-1 was separated from free ATP by 10% SDS-PAGE and MBP by 15% PAGE. The electrophoretic mobility of protein standards of known molecular weight ($\times 10^{-3}$) is indicated under column *Mr*. *E*, phosphoamino acid analysis of rHSF-1 after treatment *in vitro* with MAPK as in *D*. *F*, two-dimensional tryptic phosphopeptide map of rHSF-1 after treatment with MAPK *in vitro* as in *D*, trypsin cleavage, and two-dimensional thin layer electrophoresis as described under “Materials and Methods.”
trimerize, localize to nuclei, and become competent for HSE binding (data not shown). Thus activation of the MAPK cascade inhibits the ability of HSF-1 to activate heat shock promoters at a step distal to the inducible DNA binding step.

**Investigation of Serine Residues Involved in Mediating the Effects of MAPK on HSF-1 Function**—In order to localize potential target serines for MAPK within HSF-1, we scanned the HSF-1 sequence for MAPK consensus phosphorylation sites within regions of HSF-1 shown to mediate transcriptional regulation. A number of such sites were found in the transcriptional regulatory domain, a region of HSF-1 shown to repress HSF-1 activity at normal temperatures and to mediate the stimulation of the COOH-terminal transcriptional activation domains during heat shock (14, 15). The regulatory domain includes amino acids 221–310 and contains three potential MAP kinase sites centering on serines 275, 303, and 307 (15). The MAP kinases phosphorylate substrates containing the consensus sequence FX(S/T)P (where asterisk indicates phosphorylation of serine or threonine residue) (20, 21, 29). To test these residues as authentic targets for MAPK phosphorylation, we first prepared synthetic model peptides corresponding to the regions encompassing the MAPK sites and measured their activity as MAPK substrates. These peptides included H275, which contains Ser-275; H300, containing Ser-303 and Ser-307; and H350, a sequence bearing the MAPK consensus that occurs just outside the regulatory domain (30). Each of the model peptides was efficiently phosphorylated by MAPK, with the H300 peptide (column 4) proving the best substrate (Fig. 3A). H275 (column 3) and H350 (column 5) were phosphorylated at approximately 20–30% the rate of H300 (Fig. 3A). The negative control pp90^ras^S^6^ kinase peptide (column 2) was not phosphorylated, while MBP, a well characterized substrate for MAPK exhibited activity of a similar order to H275 and H350, approximately 25% the activity of H300 (Fig. 3A, column 6). Ser-275, Ser-303, and Ser-307 are thus potential targets for MAPK, and, to test the importance of these residues in HSF-1 function, we therefore investigated the effects of mutating each serine residue to glycine (Fig. 3B). Mutations were introduced at Ser-275, Ser-303, and Ser-307 (to produce S275G, S303G, and S307G), mutants were cloned into the CMV expression vector, and co-transfection experiments with the *HSP70B* promoter-reporter construct carried out as above (Fig. 2A). In addition, a triple mutant containing glycine substitutions at all three sites (S275G/S303G/S307G, or Triple G) was constructed and cloned into the expression vector. The wild-type and mutant HSF-1 expression vectors were co-transfected at a range of concentrations into NIH 3T3 cells with the *HSP70B* promoter reporter construct (Fig. 3B). The S303G mutant showed a marked gain in activity over wild-type, with significant promoter activation at a plasmid concentration of 100 ng, reaching a plateau level at 500 ng/culture (Fig. 3B). In contrast, the activity of wtHSF-1 was lower and reached maximal activation at 4000 ng/culture, a marked deficit compared with the S303G mutation (Fig. 3B). CAT activity did not increase if the concentration of wt plasmid was increased to 8000 ng, indicating maximal activation of wtHSF-1 at 4000 ng (not shown). Although not as effective as S303G, mutagenesis of Ser-307 did lead to an intermediate increase in activity over the wtHSF-1 vector evident at 500 and 1000 ng (Fig. 3B). The activity of S275G also appeared greater than wtHSF-1, although the differences were not significant at most concentrations (Fig. 3B). The S275G/S303G/S307G triple mutant exhibited an enhancement of transcriptional activity over wtHSF-1, which was comparable with the gain in function of S303G (Fig. 3B). In order to confirm that the effects of the point mutations were not due to altered DNA binding or stability properties of HSF-1, we carried out control gel shift experiments with HSF-1 extracted either from *Escherichia coli* expressing the HSF-1 constructs from the pET 22 vector or from the nuclei of NIH 3T3 cells transfected with the mutants.

![Fig. 2. Effect of MAP kinase on the transcriptional activation of the HSP70B promoter by HSF-1. A, effect of co-transfection of a MEK-1 expression vector on the ability of pCMV-HSF-1 to activate the human HSP70B promoter in NIH 3T3 cells. Cells were transfected with the HSP70B promoter-CAT construct p2500CAT (10 μg) either with 4 μg of pCMV-HSF-1 (column 3), without expression plasmid (column 1), with HSF-1 cloned into the expression vector in reverse orientation (column 2) or with a combination of pCMV-HSF-1 and pCMV-MEK-1 (column 4). All cultures were then incubated at 37 °C for 24 h to allow expression of CAT. Results are expressed as an average percentage of the CAT activity observed in extracts from cells co-transfected with p2500CAT and pCMV-HSF-1 (column 3). The results are a representative sample from three replicate assays. B, effect of HSF-1 phosphorylation with MAPK on HSE binding avidity. HSF-1 was cold-phosphorylated with p44^MPK^ and unlabelled ATP using the conditions described in Fig. 1D and then incubated at a range of dilutions with ^32P^-labeled HSE as described. Control samples were sham-phosphorylated with p44^MPK^ in the absence of ATP and then incubated with labeled HSE as for the MAPK-treated samples. All samples were then analyzed by EMSA as described under “Materials and Methods.”](image-url)
FIG. 3. Investigation of serine residues in HSF-1 involved in mediating the effects of MAPK on the HSP70B promoter. A, phosphorylation by purified MAPK of model peptides derived from the HSF-1 sequence containing putative MAPK consensus phosphorylation sites.
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in the CMV-based expression vector (Fig. 3C). Each of the HSF-1 species (including wt, S307G, S303G, S275G, and Triple G) bound to the HSE oligonucleotide with similar avidity and migrated at a similar rate (Fig. 3C). Thus none of the mutations markedly altered the DNA binding properties of HSF-1 (Fig. 3C). In order to control for potential confounding effects of the mutations on the behavior of HSF-1 in mammalian cells, such as altered stability or nuclear localization, we prepared nuclear extracts from control and transfected NIH 3T3 cells (Fig. 3C). HSE binding activity was minimal in untransfected cells, while in the HSF-1 transfectants, specific HSE binding activity was detected (Fig. 3C). When the radioactivity in the specific HSF-1 bands was quantitated, the relative amounts of HSE binding activity was found to be similar in extracts from each transfectant, indicating that the concentration of trimeric HSF-1 competent to bind DNA was similar in the nuclei of wt and mutant HSF-1 transfectants (Fig. 3C). The control experiments in Fig. 3C therefore suggest that the activating effects of the S303G and S307G mutations are not due to gross alterations in HSF-1 structure and exert their effects on mechanisms other than trimerization, nuclear localization, or HSE binding (Fig. 3C). It is notable that, although wtHSF-1 was a less effective transcriptional activator than the S303G or S275G/S303G/S307G Triple G mutants at lower concentrations of plasmid, at the higher concentrations (4000 ng) it was as effective as the mutants (Fig. 3B). Mutation at Ser-303 thus decreases the threshold concentration for HSF-1 activation but not the amplitude of transcriptional activation (Fig. 3B). The existence of a threshold concentration for HSF-1 activation suggests that at higher plasmid concentrations, increased intracellular expression of trimeric HSF-1 may titrate an endogenous cellular inhibitor, which antagonizes the activity of lower concentrations of HSF-1 in the presence of an intact serine residue at amino acid 303. This endogenous inhibitor of HSF-1 function could be cellular MAPK activity repressing the transcriptional activity of HSF-1 through Ser-303. To further explore this possibility, the HSF-1 mutants were co-transfected into NIH 3T3 cells with p2500CAT in the presence of pCMV-MEK-1 in order to increase the activity of MAPK, the putative HSF-1 antagonist (Fig. 3D). As mentioned above, and consistent with the threshold model, co-transfection with MEK-1 suppressed the ability of wtHSF-1 to induce the HSP70B promoter by approximately 80% (Fig. 3D). In contrast, the 303G and S275G/S303G/S307G Triple G mutants were less sensitive to MEK-1 co-transfection, with only 40–50% inhibition of HSP70B activation by MEK-1 expression compared with wtHSF-1 (Fig. 3C). The S307G mutant behaved similarly to S303G, exhibiting 40–50% inhibition by MEK-1 expression (Fig. 3C). These experiments further suggest that activated MAPK represses HSF-1 function through serines 303 and 307. To determine whether the repressive effects of Ser-303 phosphorylation could be due to the resultant increase in negative charge, a further mutation was prepared in which serine 303 was replaced with aspartate (S303D) (Fig. 3E). These experiments were carried out at the higher plasmid concentrations (4000 ng/culture) at which wtHSF-1 was fully active (Fig. 3B). Compared with wtHSF-1, the activity of the S303D mutant was decreased by approximately 50%, indicating that the introduction of negative charge in the 303 position inhibits the transcription activating potential of HSF-1, consistent with a role for phosphorylation in negative control at this site (Fig. 3D).

We next investigated Ser-275, Ser-303, and Ser-307 as direct substrates for MAPK phosphotransferase activity using two-dimensional phosphopeptide mapping of the wt and mutant HSF-1 constructs after expression in E. coli, purification, phosphorylation with MAPK and trypsin digestion as described in Fig. 1D. Examination of the two-dimensional map from the S275G/S303G/S307G Triple G mutant indicated loss of the major spot a and minor spot c compared with wtHSF-1 (Fig. 4, A and B). Similar results were obtained with a double serine-aspartate mutation of residues 303 and 307, a finding that appears to exclude Ser-275 as a major site for MAPK phosphorylation (Fig. 4C). This was confirmed by examination of the two-dimensional map from the S275G mutant, which was similar to the pattern from wtHSF-1 (Figs. 4, A and D). Significantly, the two-dimensional map from S303G also showed loss of the majority of peptide a, although a slight trace of labeling remained in contrast to the complete loss of the spot in the triple or double mutants (Fig. 4E). Phosphopeptide a is thus likely to be composed of amino acids 299–309 (EDEPSSEPQPSR), as this is the only peptide containing Ser-307 predicted to be produced by trypsin cleavage. Surprisingly, examination of the two-dimensional map from S303G showed a similar pattern to wtHSF-1, indicating that although Ser-303 is essential for the regulation of HSF-1 by MAPK, it is not a direct substrate for the kinase (Fig. 4F). These findings were confirmed using model peptides H300S303D and H300S307G with glycine for serine substitutions at, respectively, positions 303 and 307 within the H300 peptide as described under “Materials and Methods.” While H300S307G was a good substrate for phosphorylation by MEK-1, a negative control peptide shown previously to be a substrate for pp90ras (2), with peptide H275 (3), with peptide H300 (4), with peptide H350 (5), and with a positive control substrate MBP (6). Assays were carried out in triplicate as described under “Materials and Methods,” and results are presented as a percentage of the rate of phosphate incorporation into the most active substrate (H300) ± 1 standard deviation. B, effect on transcriptional activation of the HSF70 promoter of serine to glycine mutation of MAP kinase consensus sequences within of HSF-1. Wild type and mutant HSF-1 cDNAs were cloned into the pCDNA3.1 eukaryotic expression vector to produce (wt, S275G, S303G, S307G, S275G/S303G/S307G Triple G), and the constructs were co-transfected into NIH 3T3 cells with p2500CAT as described under “Materials and Methods.” Cells were transfected with the expression plasmids at a range of concentrations (100, 250, 500, 1000, and 4000 ng/ml, each) and accumulation of CAT protein measured at 48 h. Values are expressed as a percentage of the concentration of CAT protein expressed in cells transfected with 1.0 µg of wt HSF-1 expression plasmid. C, binding of wt and mutant HSF-1 species to the heat shock element after expression in E. coli (lanes 1–7) or transfection into NIH 3T3 cells. Recombinant HSF-1 was isolated from E. coli as described under “Materials and Methods” and binding to HSE assessed as described in Fig. 2B. Extracts were made from untransformed E. coli (C, lane 1), from wtHSF-1 transformants (lane 2) and from cells transformed with S307G (lane 3), S303G (lane 4), S275G (lane 5), S275G/S303G/S307G Triple G (lane 6), and S303D/S307D (lane 7). Control experiments indicated that the retarded species in each case was authentic HSF-1 that could be competed for with a 10-fold molar excess of cold HSE and supershifted by anti-HSF-1 antibody (data not shown). Nuclear HSF-1 was extracted from untransformed NIH 3T3 cells (lanes 1, U) or cells transfected with 4 µg of, respectively, wt HSF-1 (set, lane 2), S307G (lane 3), S303G (lane 4), S275G (lane 5), S275G/S303G/S307G Triple G (lane 6) prior to incubation with [32P]HSE and analysis of binding by EMSA. As described above, control competition and antibody supershift experiments indicated that the species indicated was authentic HSF-1 (data not shown). D, effect of serine to glycine mutation on the sensitivity of HSF-1 to transcriptional inhibition by co-transfection with pCMV-MEK-1. NIH 3T3 cells were co-transfected with p2500CAT (10 µg) and either wt pCMV-HSF-1, S303G, or S303D (4 µg of plasmid each). Experimental details and replication were as in B.
Hierarchical Phosphorylation of HSF-1 by MAPK and GSK3β—In order to reconcile these findings, we next examined the hypothesis that Ser-303 is phosphorylated by a kinase other than MAPK whose activity is nevertheless modified by MAPK. One potential candidate is GSK-3, which phosphorylates serine or threonine residues in acidic, proline-rich domains of proteins (35). The sequence adjacent to Ser-303 conforms to this consensus (30). In addition, phosphorylation sites in some GSK-3 substrates are phospho-unless activated (primed) by upstream phosphorylation with another (primary) kinase to create the consensus sequence S**XXXS** (35, 36). Phosphorylation of Ser-307 with MAPK would create S**XXXS** at Ser-303 in HSF-1 (S**PPPQS**). We therefore examined the ability of MAPK to prime HSF-1 for phosphorylation by GSK-3. A number of GSK3 cDNAs have been sequenced, and the functions of the two most extensively studied of these (GSK3 α and β) appear to be similar (35). We used GSK3β due to ease of availability. The first experiments were carried out using the H300 model peptides described above. Peptides were pretreated with MAPK and unlabeled ATP, subsequently isolated from the MAPK by ultrafiltration, and then incubated with GSK3β in the presence of [32P]ATP (Fig. 5A). As predicted from the known properties of GSK3β, although incubation of non-pretreated H300 with GSK3β led to a relatively small amount of phosphorylation, MAPK pretreatment led to a greater than 100-fold increase in H300 phosphorylation by GSK3β (Fig. 5A). Substitution of Ser-303 to create model peptide H300S303G leads to loss of GSK3β phosphorylation, even though this peptide is a good substrate for MAPK, indicating that HSF-1 is phosphorylated primarily on Ser-307 by MAPK and secondarily on Ser-303 by GSK3β (Figs. 4G and 5A). Finally, glycine for serine substitution at Ser-307 creates a peptide (H300S307G) that is neither a good primary substrate for MAPK nor a good secondary substrate for GSK3β, again supporting the hypothesis outlined above (Figs. 4G and 5A). In order to determine if this mechanism operates at the level of the whole protein, we examined the ability of GSK3β to phosphorylate purified rHSF-1 (Fig. 5B). While unmodified HSF-1 was only weakly phosphorylated by GSK3β (lane G), rHSF-1 that was pretreated with MAPK underwent a doubling in phosphate incorporation after GSK3β treatment (Fig. 5A). The relative ratios of incorporation were 1 (GSK3β), 60 (MAPK), and 125 (MAPK + GSK3β). Although [32P] incorporation into rHSF-1 was low in the HSF-1 treated with GSK3β alone, this labeling was not at background levels and indeed reflected true phosphorylation at multiple sites in HSF-1. This was indicated in two-dimensional mapping experiments, which showed considerable overlap in the phosphopeptide patterns after treatment by GSK3β alone or MAPK + GSK3β; the differences were largely in the amount of [32P] incorporation which was greatly increased by combination with MAPK (Fig. 5C).

The two-dimensional map from rHSF-1 treated with GSK3β alone contained four main phosphopeptides (w, x, y, z), as well as two minor species that migrate with rF values similar to phosphopeptides A and B from the MAPK map (compare Fig. 5C with Fig. 1F). Combining MAPK phosphorylation with GSK3β led to a complex pattern that contained peptides w, x, y, and z from the GSK3β alone map as well as intensely labeled MAPK peptides A and B (Fig. 1D). In order to compare the phosphopeptide patterns in Fig. 5 (C and D), the plate treated with GSK3β alone was exposed to x-ray film for a much longer period, and thus the relative incorporation of [32P] into the peptides are not reflected by the intensity of the spots. Quantitative analysis of the spots by PhosphorImager indicates that [32P] incorporation into the specific phosphopeptides w, x, y, and
z was 40–50-fold greater in the MAPK treated samples, indicating that MAPK phosphorylation is a powerful activator of HSF-1 phosphorylation by GSK3β. Phosphate incorporation by peptides A and B was, respectively, 355-fold and 325-fold stronger with MAPK, and these phosphopeptides probably result primarily from direct MAPK phosphorylation (Fig. 5, C and D). In addition, the two-dimensional map from rHSF-1 after combined MAPK/GSK3β treatment contains number of novel phosphopeptides that are not evident with either kinase alone (Fig. 5, C and D). These include species with retarded electrophoretic mobility but similar chromatographic behavior to peptide A that could include peptide A labeled on Ser-303 and Ser-307 (Fig. 5D). The abundant phosphopeptide species evident after double phosphorylation with MAPK and GSK3β suggests that this combination could cause hyperphosphorylation (Fig. 5D). A further preliminary observation is that combining MAPK and GSK3β phosphorylation leads to a phosphorylation pattern that resembles the pattern derived from HSF-1 phosphorylated in vivo (Figs. 1A and 5D). Although further study is clearly required to confirm this observation, these experiments suggest that MAPK and GSK3β play an important part in determining the phosphorylation state of HSF-1 and may play important regulatory roles in the protein.

**DISCUSSION**

These experiments indicate that MAPK phosphorylates HSF-1 on serine residues in vitro and that HSF-1 is phosphorylated on similar serine residues in vivo, suggesting a role for MAPK in HSF-1 function (Fig. 1). A physiological role for MAPK in HSF-1 regulation is also indicated by the findings that stimulation of cellular MAPK activity represses HSF-1 (Fig. 2A). As the transcriptional activity of HSF-1 in mammalian cells is regulated by at least two independent mechanisms including: 1) heat-inducible trimerization, nuclear translocation and DNA binding of HSF-1 and 2) heat stimulation of constitutively repressed transactivation domains, it is likely that MAPK acts on one or both of these processes (3–5, 14–18). The balance of evidence in this study indicates that the effects of the MAPK cascade are not transmitted through the first mechanism and favor an effect distal to DNA binding on step 2 (Figs. 2B and 3C). Such a mechanism is also favored by the location of the MAPK consensus phosphorylation sites within the HSF-1 sequence relatively far from the NH₂-terminal core.
domains and within the regulatory domain, a region of HSF-1 shown to control the COOH-terminal activation domains (3, 4, 14, 15, 18). Functional analysis of the MAPK consensus sites by mutagenesis and co-transfection with an HSP70B promoter reporter construct showed that Ser-303 is the key residue that confers MAPK-mediated repression on HSF-1, as shown by the finding that Ser-303 mutation produces an HSF-1 molecule with enhanced transcriptional activating properties resistant to the inhibitory effects of MEK-1 (Fig. 3, B and D). Mutation of the adjacent Ser-307 produces a similar although weaker activating effect on HSF-1 (Fig. 3, B and D). Interestingly, mutations of both Ser-303 and Ser-307 (S275G/S303G/S307G) caused a similar activation of HSF-1 function to the single S303G mutation, suggesting that signals transmitted through both serines are redundant and may work through a common mechanism (Fig. 3, B and D). The finding that Ser-303 is phosphorylated by GSKβ rather than MAPK and becomes primed for GSKβ phosphorylation by MAPK phosphorylation at Ser-307 is consistent with the mutagenesis studies indicating that S303G produces a stronger effect on HSF-1 function than the S307G mutation and that mutations at the two sites are redundant (Fig. 5, A–D and Fig. 3B). The S307G mutation working indirectly through its influence on Ser-303 would be expected to have a weaker phenotype than S303G. These in vitro studies therefore strongly implicate GSKβ as the kinase that phosphorylates Ser-303 in HSF-1, although further in vivo studies will be required to confirm the physiological significance of the findings (Fig. 6).

Our experiments therefore indicate that serial phosphorylation of HSF-1 on Ser-307 and Ser-303 by MAPK and GSKβ represses the activity of the transcription factor at a step distal to the DNA binding stage. Thus in cells with active MAPK, HSF-1 is constitutively repressed (Figs. 2 and 3). Similar conclusions were made in an earlier study, which showed activation of heat shock genes by expression of dominant interfering mutants of ERK-1 (37). Therefore, in growing cells there appear to be at least two barriers to HSF-1 activation, one of which operates on cytoplasmic HSF-1 to maintain the monomeric, extra-nuclear, non-DNA binding form and a second inhibitor that acts on nuclear, trimeric HSF-1 through the mechanism outlined above to inactivate HSF-1 that escapes barrier 1 (3–5, 7). The findings that HSF-1 repression at each stage is overcome in a dose-dependent manner by HSF-1 expression suggest the existence of intracellular inhibitors that can be titrated by increasing levels of HSF-1 (3–5) (Figs. 2 and 3). Heat shock may thus activate HSF-1 by antagonizing the inhibition of steps 1 and 2. Although the nature of the repressor of cytoplasmic HSF-1 is not certain, much evidence implicates HSP70 as a feedback inhibitor in this process (3–5). ERK-1 could be the second inhibitor, antagonizing the function of trimeric HSF-1 through serial phosphorylation of serines 307 and 303 (Figs. 2–5). This model is consistent with studies showing that HSF-1 activation is a multistep process (9, 10). It may be significant that Ser-307 and Ser-303 fall within a region of HSF-1 (amino acids 290–310) required for the heat induction of the COOH-terminal activation domains by the regulatory domain (14, 15). Phosphorylation of HSF-1 on serine 303 by the mechanism outlined above could thus mediate the negative control exerted on the activation domains by the regulatory domain (Fig. 6). Introduction of negative charge at position 303 by GSKβ phosphorylation (or the substitution of an aspartate residue, Fig. 3E) could alter a charge-dependent interaction of this domain with other regions within HSF-1 or affect its binding to other proteins involved in transcriptional initiation or elongation. The regulation of HSF in eukaryotic cells appears to be highly conserved at the functional level as indicated by comparison of these mechanisms to the regulation of HSF from the yeast Kluyveromyces lactis. In this organism, phosphorylation of the transcriptional regulatory domain (CE2) modulates the repression of HSF through the COOH-terminal activation domains, a mechanism reminiscent of the one described above for human HSF-1 (38). In addition, the key serine residue in phosphorylation control of the CE2 domain, Ser-460 is also a potential GSKβ phosphorylation site with the sequence MS**SSSS, although the remainder of the CE2 domain diverges from the sequence of the regulatory domain in human HSF-1 (38, 39).

The exact place of the molecular mechanisms described above in the physiological regulation of the heat shock response is still uncertain. However, one might suggest a mechanism involving the relief of the transcriptional repression exerted by Ser-303 phosphorylation through the activation of a phosphatase or the inhibition of a kinase. Serine 303 could then be re-phosphorylated during the recovery stage when heat shock transcription is deactivated and ERK1 and 2 are activated (21, 23, 26, 28). The repression of HSF-1 through phosphorylation of Ser-303 may also have other consequences for cells in addition to terminating transcription during recovery from stress. This mechanism could also repress the heat shock response during cell proliferation, when MAP kinases are activated (20, 23). In support of this hypothesis, it has been shown in yeast that the mechanisms regulating growth control and the stress response are mutually antagonistic and in mammalian cells that cellular ras expression reduces HSP70 mRNA synthesis without affecting the ability of HSF-1 to bind to DNA (21, 37, 40).

In conclusion, therefore, HSF-1 is a substrate for phosphorylation by MAP kinases of the ERK1 family in vitro and consequently to the purified enzyme leads to the phosphorylation of HSF-1 on at least two independent sites. Activation of MAPK in vitro leads to functional inhibition of HSF-1, as determined by reduced activation of the HSP70B promoter. These effects of MAP kinases appear to be transmitted through serine 303 situated in a proline-rich region within the transcriptional regulatory domain of human HSF-1. Repression of HSF-1 by MAPK appears however to be indirect and is mediated through
the phosphorylation of HSF-1 on Ser-307, which permits the secondary phosphorylation of Ser-303 by GSK3. As both Ser-303 and Ser-307 fall within an essential region of the transcriptional regulatory domain of HSF-1, the data suggest a role for serial MAPK, GSK3 phosphorylation in regulating this domain. Repression of HSF-1 by MAPK may thus function to deactivate the heat shock response during recovery from stress and the resumption of normal growth and development (Fig. 6).

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