Transcriptional Activity of Heat Shock Factor 1 at 37 °C Is Repressed through Phosphorylation on Two Distinct Serine Residues by Glycogen Synthase Kinase 3α and Protein Kinases Ca and Cζ*

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Heat shock factor 1 (HSF1) is the key transcriptional regulator of the heat shock genes that protect cells from environmental stress. However, because heat shock gene expression is deleterious to growth and development, we have examined mechanisms for HSF1 repression at growth temperatures, focusing on the role of phosphorylation. Mitogen-activated protein kinases (MAPKs) of the ERK family phosphorylate HSF1 and repress transcriptional function. The mechanism of repression involves initial phosphorylation by MAP kinase on serine 307, which primes HSF1 for secondary phosphorylation by glycogen synthase kinase 3 on a key residue in repression (serine 303). In vivo expression of glycogen synthase kinase 3 (α or β) thus represses HSF1 through phosphorylation of serine 303. HSF1 is also phosphorylated by MAPK in vitro on a second residue (serine 363) adjacent to activation domain 1, and this residue is additionally phosphorylated by protein kinase C. In vivo, HSF1 is repressed through phosphorylation of this residue by protein kinase Ca or -ζ but not MAPK. Regulation at 37 °C, therefore, involves the action of three protein kinase cascades that repress HSF1 through phosphorylation of serine residues 303, 307, and 363 and may promote growth by suppressing the heat shock response.

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, HSP genes are regulated at the transcriptional level by heat shock factor 1 (HSF1), a sequence-specific transcription factor that interacts with heat shock elements (HSEs) in their promoters (3–5). Much evidence now suggests that although HSPs protect cells during hyperthermia, growth of cells under nonstress conditions is incompatible with the expression of the heat shock response (6). Heat shock arrests cells in G1 (7, 8), and HSF1 expression delays the progression of cells through G1, whereas HSP70 overexpression inhibits growth and development (6, 9).

In addition, HSF1 represses the ability of Ras protein to activate the promoters of immediate early genes such as c-fos (10). There evidently exists, however, a well-conserved mechanism to inhibit the activity of HSF1 at 37 °C and prevent these deleterious effects on cell growth. We have examined molecular mechanisms involved in the repression of HSF1 by signaling pathways involved in growth regulation.

Activation of HSF1 involves the conversion of a latent cytoplasmic monomer to a trimeric nuclear protein complex that controls the transcription of heat shock genes (3–5, 11). Trimerization is governed by arrays of amphipathic α-helical residues (“leucine zippers”) in the N-terminal domain and is negatively regulated by a fourth such domain in the C terminus (3, 12). However, although necessary, nuclear localization and DNA binding are not sufficient for the full transcriptional competence of HSF1, which can be activated to an intermediate state in which it binds to HSE sequences but does not stimulate transcription (13–16). Much evidence suggests a role for phosphorylation in the conversion of HSF1 from this intermediate state into a transcriptionally active form (13, 14, 16, 17). In addition, HSF from yeast and HSF1 from mammalian cells both undergo hyperphosphorylation during heat shock, and their hyperphosphorylation correlates well with transcriptional activation (17–19). Our previous studies showed that HSF1 is phosphorylated at multiple sites mostly on serine residues and that mitogen-activated protein kinases (MAPKs) of the ERK-1 family phosphorylate HSF1 on serine and repress the transcriptional activation of the heat shock protein 70B (HSP70B) promoter by HSF1 in vivo (20). These experiments and a number of other reports thus indicate that HSF1 is antagonized by Ras-MAPK signaling and that this may be a mechanism for HSF1 repression at 37 °C (20–24). The repressive effects of MAPK were transmitted through a specific serine residue (Ser-303) in a proline-rich sequence within the transcriptional regulatory domain of human HSF1 (20). However, despite the importance of Ser-303 in transmitting the signal from the MAPK cascade to HSP70 transcription, there

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2 The abbreviations used are: HSF, heat shock factor; HSE, heat shock element; GSK, glycogen synthase kinase; MAPK, mitogen-activated protein kinase; MAPKAP K2, MAPK-activated protein kinase 2; CAT, chloramphenicol acetyltransferase; PMA, phorbol myristate acetate; wt, wild-type; PKC, protein kinase C.

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was no evidence that Ser-303 could be phosphorylated by MAPK in vitro, although an adjacent residue (Ser-307) was avidly phosphorylated by MAPK (20). Preliminary studies suggested that Ser-303 is phosphorylated by glycogen synthase kinase 3 (GSK3) through a mechanism dependent on primary phosphorylation of Ser-307 by MAPK (20). In the present experiments, we have demonstrated the sequential phosphorylation by MAPK and GSK3 and its role in HSF1 repression.

**MATERIALS AND METHODS**

**Cell Culture**—NIH 3T3 cells were grown to confluence in Dulbecco’s modified Eagle’s medium containing 10% bovine calf serum and passaged at a 1:10 ratio.

**Site-directed Mutagenesis, Expression,** and **Purification of Recombinant HSF1**—Oligonucleotide-directed mutagenesis was performed using the pALTER-1 vector as described previously (20). Following mutagenesis, DNA sequences were checked by dideoxynucleotide sequencing and cloned into the pcDNA3.1 vector for mammalian expression and into the pET22B vector for expression in Escherichia coli. For purification, wild-type human HSF1 cDNA and point-mutated forms S303G, and S363G (25) were induced in E. coli by isopropyl-1-thio-

**N. G. Ahn** (University of Colorado, Boulder, CO) (32). For GSK3 plas-kinase 2 or calmodulin kinase II. To phosphorylate HSF1 with PKC and pp90rsk S6 kinase isoform RSK2 from rabbit muscle were obtained.

**aminoglycoside treatment, extracted, and purified to homogeneity as described (26).**

**In Vitro Phosphorylation of Recombinant** HSF1—Purified MAPK from P. ochraceus (p44,PKCα from rabbit brain and GSK3α, mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP K2), and p90RSK S6 kinase isofrom RSK2 from rabbit muscle were obtained from Upstate Biotechnology (Lake Placid, NY). In vitro phosphorylation of HSF1 by MAP kinase and GSK3 was carried out as described previ-ously (20). Purified enzymes (MAPK and GSK3α) were tested for contamination with other kinases that might phosphorylate HSF1 by assaying with peptides specific for MAPKAP K2, RSK2, and PKCα. PKCα RSK2 activity was assayed as described using substrate peptide RR LLSSRA, utilizing purified RSK2 enzyme as a positive control (27). Assays were carried out with or without MAPK addition, because MAPK is a potent activator of RSK2, MAPKAP K2 activity in the GSK3α preparation (with or without the addition of MAPK) was assayed as for RSK2 using MAPKAP K2 substrate peptide (KKLNRTLSSVA) (28). PKCα were purified to greater than 90% by high performance liquid chromatogra phy. Neither the MAPK nor GSK3α preparation was significantly contaminated with MAPKAP K2, RSK2, or PKCα. HSF1 can also be phos-phyphorylated in vitro with casein kinase 2 and calmodulin kinase II; however, the phosphopeptide maps obtained from HSF1 treated with either enzyme were completely different from the MAPK or GSK3α maps (data not shown), indicating that HSF1 phosphorylation by MAPK or GSK3α does not reflect contamination with either casein kinase 2 or calmodulin kinase II. To phosphorylate HSF1 with PKCα, 5 μg of HSF1 was incubated with enzyme (25 ng), phosphatidylinerine (100 μg), ATP (5 μM), and 32P-labeled ATP (1 μCi/μl) in buffer (20 mM HEPES, pH 7.4, 10 mM MgCl₂, and 0.2 mM EGTA) to a final volume of 50 μl. Incubations were for 12 min at 25 °C. Protein kinase C activity was assayed using the peptide QKRPSQRSKYL.

**Two-dimensional-Phosphopeptide Mapping and Peptide Sequenc ing**—Two-dimensional phosphopeptide mapping was carried out after digestion with trypsin as described previously (20, 28). For sequencing, peptides were eluted from the TLC medium and subjected to multiple cycles of Edman degradation, and amino acids were identified by high performance liquid chromatography elution time (29). Sequencing was performed by the Biopolymer Laboratory, Brigham & Women’s Hospi
tal, Boston, MA.

**Electrophoretic Mobility Shift Assay for HSF1-HSE Binding**—Bind ing of HSF1 or nuclear HSF1 to HSE was assayed by electrophoretic mobility shift assay as described previously (14, 30).

**Transfection**—HSF1 constructs were co-transfected with the chloro
amphenil-acyltransferase (CAT) construct p2500CAT, which con tains 2.5 kilobase pairs of 5'-noncoding sequence from the heat-inducible human HSP70 gene (31) (StressGen, Victoria, British Columbia, Canada). Cells were seeded at a density of 250,000 per 100-mm tissue culture dish 24 h prior to transfection carried out using calcium phos phate precipitation according to the manufacturer’s protocol (Promega). Cells were harvested 48 h after incubation with plasmids for assay of CAT protein expression (20). To control for transfection efficiency, cells were co-transfected with pCMV-β-galactosidase plasmid and assayed for β-galactosidase as described (10). In some experiments, HSF1 was co-transfected with MEK-1 expression vector (pCMV-MKK-1) from Dr. N. G. Ahn (University of Colorado, Boulder, CO) (32). For GSK3 plas-

**RESULTS**

In order to investigate the hypothesis that HSF1 is phosphorylated on the key regulatory serine residue 303 by GSK3 only after a priming phosphorylation by MAPK, we first examined the effect of mutating serines 303 and 307 on sequential phosphorylation by MAPK and GSK3 (Fig. 1). Wild-type recombina nant HSF1 (wtHSF1) was not phosphorylated by recombinant GSK3β (Fig. 1, lane 3) as shown previously with purified rabbit GSK3β (20) but was phosphorylated by purified GSK3α (lane 2). MAPK phosphorylated wtHSF1 (Fig. 1, lane 1) and led to a marked increase in phosphorylation by GSK3α and β (lanes 4 and 5). When similar experiments were carried out on HSF1 with a serine to glycine mutation (S303G), the levels of phosphorylation in incubations with MAPK, GSK3α, and GSK3β were similar to those in the wt control when used individually. (Fig. 1, lanes 6–8). However, we did not observe increased phosphorylation by GSK3α and β when combined with MAPK (lanes 9 and 10). Thus, inactivation of the putative GSK3 site (Ser-303) prevents MAPK stimulation of HSF1 phosphorylation by GSK3 (Fig. 1). The phosphorylation of HSF1 observed with GSK3α alone, without MAPK priming, is evidently at residues other than Ser-303 as similar levels of 32P uptake were observed in wtHSF1 and S303G when incubated with GSK3α (Fig. 1, lanes 2 and 7). Substitution of glycine for serine at Ser-307 (S307G) led to a HSF1 with similar properties to S303G, with phosphorylation by MAPK and GSK3α (Fig. 1, lanes 11 and 12), but no stimulation of GSK3-induced phosphorylation by MAPK (lanes 14 and 15). This indicates a requirement for intact Ser-307 and MAPK for GSK3 phosphorylation of HSF1 at Ser-303, consistent with a priming role for MAPK in HSF1 regulation by GSK3 (20). The experiments also show that despite loss of the phosphorylation site at serine 307, S307G is still phosphorylated by MAPK (lane 11). This, however, is consistent with previous studies indicating two major MAPK sites on HSF1 (Fig. 1; Ref. 20).

In order to confirm that the changes in levels of 32P incorpo-

**FIG. 1. Phosphorylation of HSF1 by MAPK and GSK3 in vitro.** Recombinant wtHSF1, S303G, or S307G was incubated with MAPK (p44,PKCα, or GSK3β) either singly or sequentially in the presence of [γ-32P]ATP. Incubations were terminated by boiling in SDS-polyacrylamide gel electrophoresis sample buffer, and phosphoproteins were analyzed by 10% SDS-polyacrylamide gel electrophoresis and autoradiography. For sequential treatment, HSF1 was preincubated with MAPK prior to incubation with GSK3α or GSK3β as described under “Materials and Methods.” wtHSF1 was incubated with MAPK alone (lane 1), GSK3α alone (lane 2), GSK3β alone (lane 3), MAPK + GSK3α (lane 4), or MAPK + GSK3β (lane 5). S303G was incubated with MAPK alone (lane 6), GSK3α alone (lane 7), GSK3β alone (lane 8), MAPK + GSK3α (lane 9), or MAPK + GSK3β (lane 10). S307G was incubated with MAPK alone (lane 11), GSK3α alone (lane 12), GSK3β alone (lane 13), MAPK + GSK3α (lane 14), or MAPK + GSK3β (lane 15). For controls, HSF1 (lane 16), S303G (lane 17), and S307G (lane 18) were incubated with [γ-32P]ATP in the absence of protein kinases. In lane 19, MAPK, GSK3α, and GSK3β were incubated together without added HSF1 in the presence of [γ-32P]ATP.
Hierarchical phosphorylation of HSF1 by MAPK and GSK3. A, two-dimensional phosphopeptide map of recombinant HSF1 after treatment with MAPK (p44<sup>mapk</sup>) in vitro. HSF1 was incubated with MAPK and [γ-<sup>32</sup>P]ATP, isolated, and subjected to tryptic two-dimensional mapping as described under “Materials and Methods.” B, HSF1 was incubated sequentially with p44<sup>mapk</sup> and GSK3<sub>a</sub> in the presence of [γ-<sup>32</sup>P]ATP and subjected to two-dimensional mapping as in A. C, HSF1 mutant protein S307G was incubated with p44<sup>mapk</sup> and GSK3<sub>a</sub> in the presence of [γ-<sup>32</sup>P]ATP and subjected to two-dimensional mapping as in B. D, HSF1 mutant protein S303G was incubated with p44<sup>mapk</sup> and GSK3<sub>a</sub> in the presence of [γ-<sup>32</sup>P]ATP and subjected to two-dimensional mapping as in C.

Repression of HSF-1 by GSK3 and PKC

We next examined the potential role of GSK3 in the regulation of HSF1 activity in vivo. We used a system described previously in which HSF1 expression from the pcDNA3.1.HSF1 (pHSF1) vector activates a heat shock promoter reporter construct in the absence of heat shock (20, 30, 31). HSF1 expression strongly induced HSP70B promoter activity in NIH3T3 cells, whereas co-expression of a GSK3<sub>a</sub> expression vector repressed the effects of HSF1 by 80%, and co-expression of pMEK-1, which increases cellular MEK-1 levels and specifically activates ERK1 and ERK2 (20), further reduced activity (Fig. 3). Similar results were obtained with a GSK3<sub>b</sub> expression vector (not shown). When similar experiments were carried out using HSF1 mutant S303G, we found that repression of HSP70B promoter activity by GSK3<sub>a</sub> was effectively abolished, identifying serine 303 as the target for HSF1 repression by GSK3<sub>a</sub> in vivo (Fig. 3). Neither mutation of HSF1 at Ser-303 or Ser-307 nor treatment with GSK3<sub>a</sub> or MAPK affected the binding of HSF1 to heat shock elements, indicating that the effects observed are exerted downstream of DNA binding, at the level of transcriptional transactivation.
Each culture was also co-transfected with control plasmid pCMV indexed to 2m with p2500CAT (12m NIH-3T3 cells were transfected of the HSP70B promoter by HSF1.

Phosphorylated by MAPK Ser-303, we next analyzed the second major site on HSF1 sive effects on HSF1 in addition to those mediated through effects of MAPK on HSF1 function (Fig. 3). Block the repression of HSF1 by MEK1, suggesting further (20) (see Fig. 7). However, Ser-303 mutation did not completely block the repression of HSF1 by MEK1, suggesting further effects of MAPK on HSF1 function (Fig. 3).

Because the data in Fig. 3 suggest that MAPK exerts repressive effects on HSF1 in addition to those mediated through Ser-303, we next analyzed the second major site on HSF1 phosphorylated by MAPK in vitro. We showed in Fig. 1A that two major phosphopeptides, a and b, are resolved in HSF1 after MAPK treatment in vitro. Phosphopeptide a contains serines 303 and 307 (Fig. 1). Analysis of phosphopeptide b isolated from the TLC plates after two-dimensional mapping indicated a sequence correspond to amino acids 362–372 in the human HSF1 sequence (25). To confirm this finding, we carried out site-directed mutagenesis on serine 363 contained within phosphopeptide b, which forms part of a sequence (PPSP) that conforms to a MAPK consensus motif (PX(S*/T*)P) (where the asterisk indicates the phosphorylation of a threonine or serine residue (34–36)). Mutation of Ser-363 to alanine, to create S363A, had a profound effect on the phosphopeptide map of HSF1 after treatment with MAPK (p44mpk). These experiments implicate Ser-363 as a critical residue (34–36) for the effect of MAPK on HSF1 function.

To investigate the functional importance of Ser-363, we next examined the ability of MEK-1 expression to repress transcriptional activation of the HSP70B promoter by the S363A mutant (Fig. 5). Contrary to expectations, although Ser-363 is avidly phosphorylated by MAPK in vitro (Figs. 1 and 4), the S363A mutant was equally sensitive to MEK-1 repression compared with the wt control (Fig. 5). Thus, either Ser-363 phosphorylation does not affect the transcriptional activity of HSF1 or this site is not phosphorylated by MAPK in vivo. This region in HSF1 (360–365; RPPSP) contains overlapping consensus motifs for both MAPK (PX(S*/T*)P) and PKC (RXXS*/T*) (36). We therefore examined the effect of expressing two isoforms of protein kinase C (PKCζ and PKCα) on the activities of co-transfected wtHSF1 and S363A (37). Recent studies have shown the existence of at least 11 PKC family members, which belong to three distinct classes based on structure and responsiveness to activators: PKC isoforms α, β, and γ are Ca2+- and phorbol ester-dependent; PKC isoforms δ, ε, η, and θ require phorbol esters but are Ca2+-independent; and the atypical isoforms ζ and λ require neither activator (38, 39). We chose PKCζ and PKCα as representative members of the family, ranging from the “classical” Ca2+- and phorbol ester-dependent PKCζ to the atypical PKCα, which is dependent on neither factor (37, 38). The transcriptional activity of wtHSF1 was inhibited by overexpression of MEK1, PKCζ, or PKCα (Fig. 5). One explanation for this finding could be that PKC acts indirectly through upstream acti-

**Fig. 3.** Effect of high-level expression of GSK3α on activation of the HSP70B promoter by HSF1. NIH-3T3 cells were transfected with p2500CAT (12 μg) alone or with expression plasmids pHSF1 (2 μg), pS303G (2 μg), pGSK3α (4 μg), or pMEK-1 (4 μg) as indicated. Each culture was also co-transfected with control plasmid pCMVβGAL (2 μg). Incubations and assays for CAT or β-galactosidase were carried out as described under “Materials and Methods.” CAT activity was indexed to β-galactosidase activity to control for transfection efficiency, and results were then expressed as a percentage of the activity in cells co-transfected with p2500CAT and pHSF1 (first column). Experiments were carried out twice, and representative results are shown. At the plasmid concentrations used in these experiments, wild-type HSF1 and S303G expression vectors were equally effective in activation of the HSP70B promoter.

**Fig. 4.** Effect of mutation of serine 363 on HSF1 phosphorylation by MAPK. Tryptic two-dimensional phosphopeptide map of recombinant S363A protein after treatment with MAPK (p44mpk) as described under “Materials and Methods.”

**Fig. 5.** Effects of HSF1 mutation at Ser-363 on repression by MEK-1, PKCα, and PKCζ expression. Cells were transfected with p2500CAT (12 μg), either without co-transfection or with pHSF1 (2 μg) or pS363A (2 μg). Effects of high level expression of protein kinase MEK-1, PKCα, or PKCζ were examined by co-expression with pMEK-1 (4 μg), pPKCα (4 μg), or PKCζ (4 μg), as indicated. Experimental conditions, assays, replication, and data analysis were as in Fig. 3. At the plasmid concentrations used here, wtHSF1 and S363A expression vectors were of similar effectiveness in activation of the HSP70B promoter, although at lower plasmid concentrations, S363A was slightly more effective.
viation of MAPK family members ERK1 and ERK2 (40). However, on examining the effects of S363A mutation on responses to PKC expression, we found that although it is not involved in inhibition by MEK1, S363A substitution prevented repression of HSF1 activity caused by high level expression of PKCa or PKCζ (Fig. 5). Mutation of HSF1 in the 303 position (S303G) did not, however, prevent HSF1 repression in cells overexpressing PKCa, indicating that the effects of PKC are exerted either directly or indirectly through phosphorylation of Ser-363 and do not involve Ser-303 (Fig. 6). To further probe the potential effects of PKC on HSF1 activity, we used calphostin C, which binds to the regulatory domain of PKC isoforms and specifically inhibits their activity (41). Incubation of cells co-transfected with pHSF1 and PKCa with calphostin C partially restored HSF1 activity, further implying a role for PKCa in HSF1 repression (Fig. 6). However, incubation with calphostin C did not markedly enhance HSF1 activity in the absence of PKCa co-transfection in these cells (Fig. 6). Thus, Ser-363, although avidly phosphorylated by purified MAPK in vitro, is not a major target for MAPK in vivo but instead mediates HSF1 regulation by members of the PKC family (Figs. 5 and 6).

We next determined whether HSF1 is directly phosphorylated by PKCa (Fig. 7). Recombinant HSF1 was phosphorylated by PKCa in vitro, whereas the phosphorylation of purified S363A under identical conditions was markedly reduced, suggesting that Ser-363 is phosphorylated by PKCa and directly mediates the effects of PKC expression on the transcriptional activity of HSF1 (Figs. 6 and 7).

Finally, in order to examine the effects of GSK3α and PKCa expression on the ability of HSF1 to form nuclear trimers capable of binding to heat shock elements, we carried out electrophoretic mobility shift assay analysis on nuclear extracts from control cells and transfectants (Fig. 8). HSF1 expression (Fig. 8, lane 5) led to the formation of a complex of similar electrophoretic mobility to HSF1-HSE complexes from heat shocked cells (Fig. 8, lanes 2 and 3). It is notable that although HSF1 expression increases activity of the HSP70B promoter by at least 100-fold, activation of HSF1-HSE binding increases only 2–3-fold in the transfectants (Figs. 3 and 7). This reflects the efficiency of transfection in the system used here (0.5–1.0%). Thus, increases in HSF1 binding in the transfectants are diluted by the 100-fold excess of untransfected cells. The trans-activation assay reports only on cells that have been co-transfected with HSF1 expression plasmid and the HSP70B promoter reporter construct and is unaffected by the presence of untransfected cells. Co-expression of GSK3α and PKCa did not inhibit the formation of the HSF1-HSE complex (Fig. 8, lanes 6 and 7). Thus, overexpression of these protein kinases represses HSF1 activity at step other than formation of DNA binding nuclear trimers.

**DISCUSSION**

These experiments demonstrate that HSF1 is phosphorylated by GSK3α on a residue (Ser-303) within the transcriptional regulatory domain (Fig. 1) (42, 43). In addition, HSF1 phosphorylation at Ser-303 by GSK3α was dependent on HSF1 phosphorylation by MAPK on an adjacent residue (Ser-307) (Figs. 1 and 2). HSF1 phosphorylation at Ser-303 thus involves hierarchical phosphorylation with primary phosphorylation by MAPK preceding secondary modification by GSK3. A similar indirect mechanism was demonstrated previously in the phosphorylation of protein substrates by GSK3 (44–46). Consistent with previous experiments showing that transfection of activated Ras protein and MEK-1 represses the heat shock response and that dominant negative constructs of ERK1 are activating, our data suggest that HSF1 is repressed under growth conditions through the Ras-MAPK pathway by primary phosphorylation by MAPK, leading to secondary phosphorylation by GSK3 (20–24). In addition, the finding that overexpression of GSK3α or GSK3β directly represses HSF1 suggests that
a subpopulation of HSF1 molecules may be constitutively phosphorylated at Ser-307 and that HSF1 may be directly regulated by the GSK3 pathway as well as indirectly through the Ras-MAPK pathway (Figs. 2 and 3). GSK3α or GSK3β is involved in transcriptionsal regulation and is regulated by a signaling pathway activated by cell surface receptors for the Wnt proteins and propagated through a kinase cascade involving phosphatidylinositol 3-kinase and protein kinase B (46–49). This process results in the inhibition of GSK3 activity, which is constitutive in noninduced conditions (48). Protein kinase B (also known as RAC-PK or Akt) is activated by heat shock and could potentially activate HSF1 through GSK3 inhibition (50). It is not clear whether one or both of the isoforms of GSK3 interact with HSF1 in vivo because both GSK3α and GSK3β can phosphorylate HSF1 in vitro and repress HSF1 when expressed at high levels in vivo (Figs. 1 and 2) (20, 33). It is also apparent that HSF1 repression by MEK-1 involves mechanisms in addition to priming HSF1 for GSK3 phosphorylation (Fig. 2). We therefore examined the role in HSF1 repression of a second residue phosphorylated by MAPK in vitro located at serine 363 within a MAPK consensus motif (Figs. 2 and 4). The analysis indicated, however, that Ser-363 is not involved in repression of HSF1 by the Ras-MAPK pathway, and in fact, Ser-363 mediates HSF1 repression by the PKC family (Figs. 5 and 6). PKCα and PKCβ both strongly repressed HSF1 function, and repression was relieved by mutation of Ser-363 but not by mutation of Ser-303 (Figs. 5 and 6). The findings that PKC phosphorylates HSF1 in vitro, that this effect is inhibited by loss of Ser-363, and that the repressive effects of PKCα expression are reversed by either S363A mutation or calphostin C exposure suggest that PKCα represses HSF1 largely through direct enzymatic modification (Figs. 5–7). Previous studies suggested a potential role for PKC in the heat shock response based on findings that phorbol esters enhance HSF1-HSE binding and HSP synthesis during heat shock (51). Similar observations were made in our unpublished studies and are seemingly at odds with a role for PKC in HSF1 repression. However, in the NIH 3T3 cell line used here, exposure to the active phorbol ester phorbol myristate acetate (PMA) leads to a progressive down-regulation of PMA binding activity, reaching levels only 20% of controls by 8 h exposure to $10^{-7}$ M PMA.4 In addition, such treatment leads to functional loss of PKC activity, as indicated by the finding that long term treatment with PMA eliminates subsequent PMA-induced MAPK induction in these cells (40). Stimulatory effects of PMA on HSF1 activity may thus reflect the down-regulation of PMA-binding PKC species. In addition, PMA has been shown to increase the cellular levels of HSF1 and HSF1 mRNA (52). Effects of PMA on HSF1 activity may therefore reflect alterations in HSF1 levels. However, our findings indicate that directly activating PKC by overexpression leads to HSF1 repression through phosphorylation on Ser-363 (Fig. 6). It is not clear, however, to what extent PKC is involved in HSF1 regulation under basal conditions. Treatment with calphostin C caused only a slight increase in HSF1 activity in NIH 3T3 cells (Fig. 6), although a larger (50%) increase was observed in HeLa cells (not shown). PKC may thus play a less prominent role, compared with MAPK in HSF1 repression during conditions of continuous growth (20). Repression through Ser-363 may be more significant in conditions leading to acute increases in PKC activity, such as the activation of serpentine receptors and binding of stimulatory ligands to growth factor receptors (38). It is apparent however, that the region in HSF1 containing Ser-363 is phosphorylated in cells in vivo, suggesting a potential role for Ser-363 in HSF1 regulation at 37 °C (20).

Repression by MAPK and GSK3 involves HSF1 phosphorylation at sites within the regulatory domain that control the activity of adjacent activation domains (42, 43). PKC repression is, however, exerted through a site (Ser-363) in a previously uncharacterized region of HSF1 between the regulatory domain (amino acids 220–310) and the C-terminal activation domains (amino acids 371–529) (42, 43). The close proximity of Ser-363 to activation domain 1 (amino acids 371–430) suggests a potential regulatory interaction (42, 43). Although we have not addressed here the role of phosphorylation in HSF1 activation by heat shock, these experiments suggest possible mechanisms. HSF1 activation by heat shock could involve antagonism of the mechanisms that repress HSF1 at 37 °C, an overriding regulatory change imposed by heat shock, or a combination of both mechanisms. That the reversal of negative regulation during heat shock is a potential mechanism for HSF1 activation is suggested by our findings that HSF1 overexpression in the absence of heat shock activates its function (Fig. 2) (10, 20, 30). These findings imply that the elevated HSF1 concentrations in transfectants titrate intracellular repressors and thus permit HSF1 activation at high concentrations. In addition, HSF1 mutants resistant to the inhibitory phosphorylations at Ser-303 and Ser-307 activate transcription at lower concentrations than those required for HSF70 promoter activation by wild-type HSF1, again suggesting the existence of titratable HSF1 inhibitors (20). However, our earlier studies suggested that HSF1 is phosphorylated in vivo on sites associated with HSF1 repression (Ser-303/307; peptide a) and Ser-363 (peptide b) before and after a heat shock (30 min at 42 °C) that activates HSP gene transcription (20). Thus, dephosphorylation of these residues may not be essential for HSF1 activation by heat shock. Therefore, although reversal of HSF1 repression may participate in activation, it seems likely that additional events unique to heat shock are involved in the full activation of HSF1 by heat, as discussed previously (17). A similar conclusion was reached in previous studies of the transcriptional regulatory domain of HSF1 showing that this domain has the property of dominantly repressing transcriptional activation domains at 37 °C and activating such domains during heat shock (43). The degree of transcriptional activation induced by heat shock exceeded the amount predicted to be caused by the reversal of repression (43).

In summary, therefore, HSF1 is a tightly regulated factor repressed at 37 °C by the action of protein kinase cascades terminating in the activation of MAPK, GSK3, and PKC, which lead to phosphorylation of inhibitory serine residues 307, 303, and 363. Coupling HSF1 repression to protein kinase activities associated with normal anabolic function may ensure suppression of HSF1 at 37 °C during growth and recovery from stress.

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