Repression of human heat shock factor 1 activity at control temperature by phosphorylation

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Human heat shock transcription factor 1 (HSF1) is responsible for stress-induced transcription of heat shock protein genes. The activity of the HSF1 transcriptional activation domains is modulated by a separate regulatory domain, which confers repression at control temperature and heat inducibility. We show here that two specific proline-directed serine motifs are important for function of the regulatory domain: Mutation of these serines to alanine derepresses HSF1 activity at control temperature, and mutation to glutamic acid, mimicking a phosphorylated serine, results in normal repression at control temperature and normal heat shock inducibility. Tryptic mapping shows that these serines are the major phosphorylation sites of HSF1 at control temperature in vivo. Stimulation of the Raf/ERK pathway in vivo results in an increased level of phosphorylation of these major sites and the regulatory domain is an excellent substrate in vitro for the mitogen-activated MAPK/ERK. We conclude that phosphorylation of the regulatory domain of HSF1 decreases the activity of HSF1 at control temperature, and propose a mechanism for modification of HSF1 activity by growth control signals.

[**Key Words:** heat shock factor; transcription factor; HSF1; Raf/ERK; phosphorylation]

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Transcription of mammalian heat shock protein genes is regulated by a family of heat shock transcription factors (for review, see Morimoto et al. 1992; Lis and Wu 1993). Heat shock factor 1 (HSF1) plays the primary role in regulating these genes in response to chemical and thermal stress (Sistonen et al. 1992). HSF1 is constitutively found in an inactive form in mammalian cells, and is activated by two processes. The ability of HSF1 to bind DNA is regulated by a structural transition from an inactive monomer to an active trimer (Sorger and Nelson 1989; Westwood and Wu 1993; Larson et al. 1995), and the ability of the transcriptional activation domains to stimulate transcription is regulated by a separate regulatory domain (Green et al. 1995; Shi et al. 1995; Zuo et al. 1995). This regulatory domain, located in the central portion of HSF1, can confer heat-induced transcriptional regulation on an activator that contains a heterologous DNA-binding domain and a heterologous transcriptional activation domain (Newton et al. 1996). This implies that the regulatory domain is sufficient to sense heat stress, and that this domain is a prime candidate to receive signals that modulate HSF1 activity.

Phosphorylation of HSF1 might be one mechanism by which HSF1 function is modulated. Heat shock factors

from yeast to humans have similar structures, and these factors are phosphorylated at serine and threonine residues at control temperature and are hyperphosphorylated following heat shock (Sorger 1990; Cotto et al. 1996). It does not appear that heat-induced hyperphosphorylation of the transcriptional activation domains is necessary for activation of human HSF1 following heat shock, as fusion proteins deleted for heat-induced phosphorylation sites remain heat-inducible (Newton et al. 1996). However, mutational analyses of HSF from the budding yeast *Kluyveromyces fragilis* suggest that hyperphosphorylation of serines might be involved in deactivation of HSF following heat shock (Hoj and Jakobsen 1994). The role of constitutive phosphorylation of HSFs has not yet been addressed.

Phosphorylation of transcription factors can occur through a number of different pathways in the cell, three of which are MAP (mitogen-activated protein) kinase pathways: the Raf/ERK (extracellular-signal-regulated kinase) pathway, the MEKK/SAPK(JNK) (stress activated protein kinase/Jun N-terminal kinase) pathway and the p38(RK)(HOG1) (reactivating kinase/high osmolarity glycerol response 1) pathway (for review, see Cano and Mahadevan 1995; Karin and Hunter 1995). These pathways result in the activation of kinases that phosphorylate Ser/Thr-Pro motifs and that are thus referred to as proline-directed kinases. The Raf/ERK pathway results

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in the stimulation of the most thoroughly studied MAP kinases, ERK1 and ERK2, through activation of receptor tyrosine kinases in response to growth factors. This pathway uses Ras/Raf signaling to activate MEK1/MEK2 (MAP kinase kinase), which phosphorylates ERK1 and ERK2, stimulating their activity. The SAPKs are a family of MAP kinases distinct from the ERKs that are activated by cellular stress as opposed to growth factors (Derijard et al. 1994; Kyriakis et al. 1994). The upstream regulation of this pathway is less well understood than the ERK pathway; however, SAPK is believed to be activated by a MEK equivalent called SEK1/JNKK1/MKK4 (Sanchez et al. 1994; Derijard et al. 1995; Lin et al. 1995), which appears itself to be regulated by MEKK (MAP kinase kinase kinase) (Lange-Carter et al. 1993; Yan et al. 1994). p38 is also a stress-activated kinase (Han et al. 1994; Rouse et al. 1994), which appears to be activated by other MEK family members, MKK3 and MKK6 (Derijard et al. 1995; Raingeaud et al. 1996) and MKK4. Both the ERK pathway and the SAPK pathway can be activated by a mechanism involving Ras, however Raf-1 contributes only to ERK activation but not SAPK activation (Minden et al. 1994).

In this study, we have examined phosphorylation of the regulatory domain of HSF1. Inspection of the sequence of this domain revealed six proline-directed serine motifs that are conserved between human and chicken HSF1. This observation raised the possibility that these serines might receive signals from MAP kinase-related pathways. We have found that the regulatory domain can be phosphorylated by ERK1/ERK2 *in vitro* and in a Raf-1-dependent manner *in vivo*. Mutational analysis of the proline-directed serine motifs in the regulatory domain indicates that phosphorylation of these serines causes decreased function of the transcriptional activation domains at control temperature, whereas the nonphosphorylated state increases function of the transcriptional activation domains. Thus, phosphorylation appears to down-regulate HSF1 via some of the same kinases that up-regulate transcription factors involved in cell growth decisions.

Results

Proline-directed serine motifs in the regulatory domain are necessary for full repression of activation at control temperature

Transcriptional activation by HSF1 is repressed at control temperature and heat shock inducible. Transcriptional activation is controlled by the regulatory domain, amino acids 221–310, that contains six conserved proline-directed serine motifs. To study a possible role for these serines in heat-induced regulation, we mutated a fusion protein containing the DNA-binding and dimerization domain of GAL4, the regulatory domain of HSF1 (amino acids 201–370) and the proximal activation domain of HSF1, AD1 (amino acids 401–420), as this construct was shown previously to be a potent, heat-inducible activator (Newton et al. 1996). Transcriptional acti-

vation was evaluated using S1 analysis following cotransfection of HeLa cells with a GAL4 expression plasmid and a CAT reporter containing five GAL4 sites. An internal reference plasmid was included that contains the basal *hsp70* promoter (lacking HSEs) with a deletion in the 5' untranslated region that results in a shorter product than the wild-type gene following S1 analysis.

Point mutation of the six proline-directed serines to alanine caused significant derepression of transcriptional activation at control temperature (Fig. 1A, lane 3). Basal activation was increased approximately fourfold. However, this construct was not completely derepressed, as heat shock induced activation a further three- to fourfold. We also mutated these serines into glutamic acid to mimic the negative charge of a phosphorylated serine and found that this mutation was fully repressed at control temperature and heat shock-inducible, similar to wild type (Fig. 1A, lanes 5,6). We then asked if all six serines were necessary for this effect and found that mutation of only serines 303 and 307 to alanine was sufficient to cause the derepressed phenotype (Fig. 1A, lanes 7,8), and once again mutation to glutamic acid appeared wild-type (lanes 9,10). These point mutations have no effect on either the stability or the binding of the GAL4 fusion proteins as shown by gel shift analysis using nuclear extracts made from transfected cells and a probe containing a single GAL4 binding site (Fig. 1B). The GAL4 specific bands are supershifted using a GAL4 specific antibody to distinguish GAL4 activity from the often obscuring nonspecific bands present in untransfected cells (Fig. 1B, lanes 21,22). Addition of the antibody also appears to stabilize the interaction between the DNA binding domain and the DNA, causing an increase in amount of complex in the supershift.

To determine whether the serine residues that are important for the function of the regulatory domain are phosphorylated *in vivo*, transfected 293T cells were pulse-labeled with $^{32}\text{P}_i$ for 1 hr followed by immunoprecipitation with a GAL4- or HSF1-specific antiserum. 293T cells, which gave similar transactivation results for the point mutants as HeLa cells (data not shown), were used for labeling experiments because of their high transfection efficiency. Endogenous HSF1 from mock-transfected cells is partly phosphorylated at 37°C and hyperphosphorylated following heat shock (Fig. 1C, lanes 9,10; Sarge et al. 1993). In contrast to this, the GAL4-HSF1 fusion containing the wild-type regulatory domain of HSF1 is phosphorylated at control temperature and shows no increase in phosphorylation after heat treatment (lanes 1,2). Substitution of serines 303 and 307 with alanine leads to decreased phosphorylation at both temperatures and an increased electrophoretic mobility of the GAL4 fusion protein, which is consistent with previously observed effects of dephosphorylation on HSF mobility (lanes 3,4). Mutation of all six proline-directed serines to alanine decreased phosphorylation and increased the mobility of the GAL4-fusion proteins (lanes 5,6), whereas mutation of all serines to glutamic acid also decreased phosphorylation, but decreased mobility

Figure 1. Proline-directed serine motifs 303 and 307 in the regulatory domain of HSF1 are necessary for transcriptional repression at control temperature. (A) S1 analysis of the effects of serine point mutations on activity of GAL4–HSF1 fusion proteins. Signals were quantified by a PhosphorImager, and transactivation was calculated by dividing the signal band by the internal reference (Int. Ref.) band. Heat shock (4-hr heat shock at 43°C) was performed as indicated. Fold heat induction is the level at 43°C divided by the level at 37°C. (WT) Wild-type; (AD1) activation domain 1. (B) Gel shift analysis comparing expression of mutant GAL4 fusion proteins with wild-type expression in cells transfected in parallel with those for A. The GAL4 DNA-binding domain-containing species (indicated by arrows) were supershifted with specific antisera. Lane 23 is probe alone. (C) In vivo phosphorylation of GAL4–HSF1 constructs. GAL4 fusion proteins were immunopurified from $^{32}\text{P}_i$ -labeled 293T cells transfected with GAL4–RD–AD1 constructs harboring the indicated point mutations within the regulatory domain (lanes 1–8). As a control phosphorylation of endogenous HSF1 from mock-transfected cells was analyzed (lanes 9,10). $^{32}\text{P}_i$ -labeling was performed for 1 hr at either 37°C (–) or 43°C (+). (D) Western blot analysis of parallel sets of transfected 293T cells as shown in C using a GAL4-specific antiserum.

(lanes 7,8). The expression levels of the different GAL4 constructs are equivalent, as shown by Western analysis (Fig. 1D). Both mutant constructs that contained serine to alanine mutations displayed decreased mobility following heat shock (Fig. 1D, lanes 3–6), implying mechanisms other than phosphorylation of these serines contribute to this characteristic mobility shift.

The experiments of Figure 1 suggest that the proline-directed serine motifs in the regulatory domain are phosphorylated at control temperatures, and that this phosphorylation is important for the ability of the regulatory domain to repress transcriptional activation domain activity at control temperatures. These mutations do not significantly affect induction at heat shock temperatures, suggesting that the phosphorylation state of the regulatory domain is not important following severe

stress and that dephosphorylation is not required for heat induction.

Mitogen-activated ERK is able to phosphorylate the regulatory domain of HSF1 in vitro

Because the regulatory domain of HSF1 is phosphorylated at proline-directed serine motifs, we tested the ability of three different MAP kinase family members (ERK, SAPK, and p38) to phosphorylate this domain in vitro. We first tested ERK kinase, as the in vivo labeling data indicated that the regulatory domain was phosphorylated during nonstress conditions. 293T cells expressing a HA-epitope tagged version of ERK1 were serum-deprived overnight and induced with PMA to stimulate the ERK/MAPK pathway. ERK1 was then immunoprecipi-

tated using 12CA5 mAb. Phosphorylation reactions were performed *in vitro* using recombinant GST-RD proteins containing either a wild-type or mutant regulatory domain. PMA treatment of the cells led to the activation of ERK, as demonstrated using the myelin basic protein (MBP) (Fig. 2A, lanes 1,2). The wild-type regulatory domain is an excellent substrate for ERK leading to phosphate incorporation of 7400 cpm/100 nmole compared with 2000 cpm/100 nmole when MBP is the substrate. Mutation of serine 303 and 307 partially decreased the level of phosphorylation by ERK to 4900 cpm/100 nmole (lanes 5,6). When all six serines are replaced by alanine, PMA-dependent phosphorylation is abolished (lanes 7,8).

We next tested the ability of the MAP kinase relatives SAPK and p38 to phosphorylate the regulatory domain. 293T cells were stimulated with anisomycin and endogenous SAPK or p38 were immunoprecipitated. Anisomy-

cin is a very potent inducer of SAPK (Kyriakis et al. 1994) and a 19-fold induction of SAPK activity was observed using GST-c Jun(1-135) as a control substrate (Fig. 2B, lane 1,2). The wild-type regulatory domain is weakly phosphorylated by induced, immunoprecipitated SAPK (lanes 3,4). This weak anisomycin-dependent phosphorylation is not affected by mutation of serine 303 and 307 or by mutation of all six serines and therefore probably reflects phosphorylation at other sites. Surprisingly, immunoprecipitated p38 phosphorylated the regulatory domain under these conditions to a similar level as ERK1 leading to phosphate incorporation of 45,000 cpm/100 nmole GST-RD (Fig. 2C, lanes 3,4) compared with 38,000 cpm/100 nmole of the S303/307A mutant (lanes 5,6) and 15000 cpm/100 nmole MBP as control substrate (lanes 1,2). When the Salla mutant of the regulatory domain is used as substrate, p38-dependent phosphoryla-

Figure 2. In vitro phosphorylation of the regulatory domain by different MAPK family members. (A) ERK immunocomplex kinase assay. 293T cells transfected with pcDNA HA-ERK1 (Price et al. 1992) were serum-deprived overnight and stimulated with 200 nM PMA for 20 min. HA-ERK1 was immunoprecipitated and *in vitro* kinase assays were conducted using 2 μ g of myelin basic protein (MBP, lanes 1,2), recombinant GST-RD proteins harboring the indicated point mutations (lanes 3-8), or GST (lanes 9,10) as substrates. Phosphorylation was quantified by PhosphorImager analysis. (B) SAPK and (C) p38 immunocomplex kinase assay. Endogenous SAPK or p38 was immunopurified from 293T cells stimulated with 50 μ g/ml anisomycin for 40 min. *In vitro* kinase assays were performed as indicated in A using a GST fusion protein containing the amino-terminal 135 amino acids of c-Jun as a control substrate for SAPK (B, lanes 1,2), and MBP as a control substrate for p38 (C, lanes 1,2). (D) Phosphorylation by crude extracts from control temperature and heat shocked cells. Hela cells were heat-shocked at 43°C for the times indicated. Kinase assays were performed as in A, except using GAL4-HSF1 fusion proteins both with (amino acids 201-529) and without (amino acids 371-529) the regulatory domain and GAL4-AH (amphipathic helix) as a control. One microgram of each protein was used and phosphate incorporation was 800 cpm in lanes 1-3, 6000 cpm in lanes 4-6, and 1500 cpm in lanes 7-9. The background of phosphorylated cellular proteins is attributable to the use of crude extracts.

tion is abolished (lanes 7,8). We conclude that the regulatory domain is not a good substrate for SAPK, but is a good substrate for ERK and p38.

The result that p38 could phosphorylate the regulatory domain was not expected as there is no increase in phosphorylation level in vivo of the regulatory domain upon heat shock (Fig. 1C), which would induce the activity of p38. Therefore, we tested whether extracts from heat-shocked cells are capable of increased phosphorylation of the regulatory domain in vitro. There is no increase in the phosphorylation level of the regulatory domain in kinase reactions using extracts from HeLa cells heat-shocked for 10 or 30 min in comparison to extracts from control temperature cells (Fig. 2D). Similar results were obtained using extracts from 293T cells (data not shown). These are conditions that strongly induce the activity of p38 (Rouse et al. 1994), and thus the lack of induced phosphorylation indicates that p38 is likely not the predominant regulatory domain kinase in HeLa or 293T cells. Taken together, these results prompted us to further investigate the phosphorylation by ERK1/ERK2, which remained a candidate to be the predominant regulatory domain kinase.

To confirm that specifically serines 303 and 307 were phosphorylated by ERK, we performed tryptic mapping of the regulatory domain phosphorylated in vitro with purified ERK1/ERK2. Tryptic digest of amino acids 201–370 of HSF1 should produce four fragments that contain proline-directed serine motifs; Ser-303 and Ser-307 are on the same tryptic fragment (Fig. 3A). Analysis of a GST fusion containing the wild-type regulatory domain pro-

duced a pattern with seven spots (Fig. 3B, panel 1), two of which are missing when Ser-303 and Ser-307 are mutated to alanine (Fig. 3B, panel 2; marked with bracket and dotted circle). These two spots are either a result of the monophosphorylated peptide and the diphosphorylated peptide, or a result of incomplete digestion at lysine 298 attributable to inhibition by glutamate 299 (Boyle et al. 1991). We conclude that either Ser-303 or Ser-307, or both are phosphorylated by ERK1/ERK2 in vitro.

Stimulation of the Raf/ERK pathway increases phosphorylation of Ser303/307 in vivo

Phosphorylation of the regulatory domain in the above experiments was measured using fusion proteins. In intact HSF1, the factor undergoes a transition from monomer to trimer following heat shock, and this apparently requires a conformational change in the protein (Westwood and Wu 1993; Larson et al. 1995) that might affect accessibility of the regulatory domain at control or heat shock temperatures. To test whether the regulatory domain of intact HSF1 is phosphorylated by the Raf/ERK signal transduction pathway in vivo, serine mutations were introduced into an epitope-tagged full-length HSF1 vector and wild-type or mutant forms of this vector were cotransfected with an expression vector encoding a constitutively active Raf. Cells were labeled metabolically and HSF was immunoprecipitated using an anti-FLAG antibody. Expression of Raf increased HSF phosphorylation 4.8-fold under control (Fig. 4A; lanes 1,2) or 5.1-fold under heat shock conditions (lanes 3,4). Mutation of the six proline-directed serines to alanine led to a significant decrease in Raf-dependent phosphorylation to 2.4-fold and 1.6-fold at control and heat shock temperature, respectively (lanes 5–8). Both HSF1 constructs show higher phosphorylation following heat shock compared with control temperature; this is consistent with the observation of heat-inducible phosphorylation of endogenous HSF1, which is believed to occur at positions outside the regulatory domain. Expression levels of the FLAG epitope-tagged HSF1 constructs were constant, as shown by Western blot analysis (Fig. 4B).

To determine whether serines 303 and 307 are phosphorylated in vivo, tryptic digests were performed on endogenous HSF that had been immunoprecipitated following metabolic labeling of HeLa cells. The tryptic map produces one major spot at control temperature (Fig. 4C, indicated by bracket). Mixing experiments with wild-type and mutant regulatory domain phosphorylated in vitro (samples from Fig. 3) showed that this major spot comigrated with the fragment containing Ser303/307 (data not shown). In addition, cotransfection of tagged HSF1 and constitutive Raf led to an increased phosphorylation of this spot (Fig. 4D, cf. panels 1 and 2). This spot disappears when Ser303 and Ser307 are mutated to alanine (Fig. 4D, panels 3,4). We conclude that the regulatory domain of intact HSF1 is phosphorylated at proline-directed serine motifs 303/307, and that this phosphorylation can be modulated by Raf coexpression.

Figure 3. Phosphopeptide map of HSF1 regulatory domain phosphorylated in vitro by ERK1/ERK2. (A) Schematic of GST-RD fusion protein indicating position of trypsin cleavage sites relative to serine 303 and serine 307. (GST) Glutathione S-transferase; (RD) regulatory domain. (B) Tryptic phosphopeptide analysis of in vitro phosphorylated GST-RD. Five micrograms of recombinant GST-RD containing the wild-type regulatory domain (1) or the S303,307A double point mutation (2) were phosphorylated in vitro using purified ERK1/2 and 2-D phosphopeptide analysis was performed. The two phosphopeptides absent in the double point mutant are indicated by a bracket and a dotted circle.

Figure 4. Proline-directed serine motifs 303 and 307 are targets for Raf-dependent phosphorylation in HSF1 in vivo. (A) In vivo phosphorylation of transfected FLAG epitope tagged HSF1 constructs. 293T cells were cotransfected with the indicated HSF1 construct with or without RSV-Raf-BXB [Bruder et al. 1992]. Cells were serum-deprived overnight, $^{32}\text{P}_i$ -labeled for 1 hr at 37°C (–HS) or 43°C (+HS), and cell extracts were immunoprecipitated using anti-FLAG M2 agarose. Proteins were quantified by PhosphorImager analysis. (B) Expression levels of FLAG epitope-tagged HSF1 constructs in parallel sets of transfected and serum-deprived 293T cells as shown in Fig. 3A obtained by Western blot analysis using M2 antibody. (C) 2-D tryptic phosphopeptide map of endogenous HSF1 immunoprecipitated from HeLa cells labeled with $^{32}\text{P}_i$ for 1 hr. The major spot, indicated with a bracket, comigrates with the bracketed spot in Fig. 3. (D) 2-D tryptic phosphopeptide map of FLAG epitope-tagged HSF1 wild-type and S303,307A mutant with or without Raf BXB coexpression. Immunoprecipitated HSF1 constructs from serum-deprived, $^{32}\text{P}_i$ -labeled 293T cells were resolved by SDS-PAGE (autoradiography is shown at top) and subjected to phosphopeptide analysis. Phosphopeptide maps of HSF1 wild-type (1,2) and HSF1 S303,307A (3,4) are shown without (1,3) or with (2,4) coexpression of constitutively active Raf. The major phosphopeptide in wild-type HSF1 that is absent in the S303,307A mutant is indicated with a bracket.

Two specific charged residues in the vicinity of serine 303 and 307 are critical for regulatory domain function

The evidence presented above implies that phosphorylation of serines 303 and 307 plays an important role in defining the activity of the regulatory domain of HSF1. To determine whether this region of HSF1 is particularly important for function, we mutated surrounding amino acids and examined their phenotype in GAL4 fusions by cotransfection into HeLa cells. Alanine scanning of charged residues between amino acids 296 and 311 revealed that lysine 298 [Newton et al. 1996] and glutamic

acid 300 are critical for repression of transcriptional activation at control temperature and heat inducibility (Fig. 5A, lanes 7,8,11,12). Mutation of four other charged residues in the region to alanine had no effect on either repression or heat induction. Mutation of valine 297 to alanine gave partial derepression at control temperature. Gel shift analysis showed that the mutations had little effect on the expression level or DNA-binding ability of the fusion proteins (Fig. 5B). Taken together, these specific charged residues, along with the phosphorylated serine residues define a region that appears to be critical for regulatory domain function.

It is possible that the derepressed phenotype caused by

Figure 5. Two specific charged residues in the vicinity of serines 303 and 307 are critical for regulatory domain function. (A) Effects of point mutations of charged residues within the regulatory domain on transcriptional activity of GAL4-HSF1 fusion proteins as measured by S1 analysis. Heat shock and quantification are as in Fig. 1A. (B) Gel shift analysis of the expression of mutant GAL4 fusion proteins relative to wild-type expression. Extracts were prepared from cells transfected in parallel with those for A. The GAL4 species (indicated by arrows) were supershifted in the indicated lanes with GAL4 antisera.

mutating lysine 298 to alanine and glutamic acid 300 to alanine is attributable to an effect of these mutations on phosphorylation of serine 303 and 307. To test this hypothesis we combined an R296A, K298A mutation, which gave a derepressed phenotype, with the all serine to glutamic acid mutation, which gave a wild-type phenotype. The R296A, K298A mutation was dominant over the serine to glutamic acid mutation, as the combined mutant gave a completely derepressed phenotype, implying that this mutation functions independently of the serines (data not shown). In addition, the regulatory domain with the K298A mutation had the same level of phosphate incorporation as the wild-type regulatory domain as determined by *in vivo* $^{32}\text{P}_i$ labeling experiments (data not shown).

Function of the regulatory domain in HSF1 at control temperature

The above data demonstrate that amino acids K298, E300, and proline directed serines are important for the activity of the regulatory domain in GAL4 fusion proteins. To verify that these mutations also affect activity in more natural settings, we introduced these mutations into either wild-type HSF1 or into a variant of HSF1 that contains the LexA DNA-binding domain. We took advantage of the observation that a portion of transfected HSF1 is activated to bind to DNA at control temperature (Rabindran et al. 1993) to analyze the effects of these mutations on transcriptional activation (note that these mutations appear to have no phenotype at 43°C, Figs. 1 and 5). The LexA fusion proteins contain amino acids 84–529 of HSF1, which includes the HSF1 trimerization domain, and have been shown previously to be regulated in a manner similar to intact HSF1 at the levels of both DNA binding and transcriptional activation (Zuo et al. 1994; data not shown). HeLa cells were co-transfected with LexA-HSF1 expression plasmids containing the different mutations in the regulatory domain and a LexA-driven CAT reporter. A growth hormone expression vector was used as internal control for transfection efficiency. CAT assays were performed to monitor the LexA-HSF activity within the cells at 37°C. The K298A mutation in this context led to a 18.5-fold derepression compared with wild type, whereas the mutation of proline-directed serine to alanine led to a 6.3-fold derepression (Fig. 6A). As expected, mutation of these serines to glutamic acid had very little effect.

To determine whether mutations in these residues affect function of intact HSF1, we introduced wild-type and mutant HSF1 plasmids along with an HSE-driven CAT reporter into HeLa cells. Transfection of wild-type HSF1 causes only a marginal increase in CAT activity compared with mock-transfected cells (Fig. 6B). Expression of HSF1 containing the K298A mutation led to 11-fold higher CAT activity, and mutation of proline-directed serine to alanine showed a 3.6-fold stimulation. These proteins were expressed at similar levels, as shown by Western analysis (Fig. 6C). We conclude that mutations in the regulatory domain have similar effects in full-length HSF1, LexA fusion proteins that contain all amino acids carboxy-terminal of 84, and in GAL4 fusion proteins that contain smaller regions of HSF1.

Discussion

The results presented here suggest a mechanism for repression of HSF1 activity at control temperatures by phosphorylation of proline-directed serine motifs. Although it is not yet clear which of several kinases with this specificity might phosphorylate HSF1, our results establish the Raf/ERK pathway as a candidate to phosphorylate this domain *in vivo*. The mutational analyses indicate that the consequence of this phosphorylation is an inhibition of the ability of HSF1 to activate transcription. These results provide a mechanism by which HSF1

Figure 6. The regulatory domain represses the transactivation potential of HSF1 at control temperature. (A) Transcriptional activity of LEXA-HSF1 constructs. HeLa cells were transfected with an LEXA-CAT reporter, the internal reference plasmid pXGH5 and pCMV5 (lane 1) or pCMV LEXA(1-87) HSF1(84-529) constructs harboring the wild-type regulatory domain (lane 2) or the indicated point mutations (lanes 3-5). Relative CAT activities (average from three different experiments with standard deviation) determined 36 hr post-transfection and corrected for transfection efficiencies are shown. (B) Transcriptional activity of full-length HSF1 constructs. HeLa cells were transfected with pCMV5 (lane 1), pCMV-HSF1 flag (lane 2), or the appropriate HSF1 constructs harboring the indicated point mutations within the regulatory domain (lanes 3-5) along with an HSE-CAT reporter and pXGH5 as internal reference. Relative CAT activities were determined as A. (C) Western blot showing expression level of constructs from B, using anti-FLAG M2 antibody.

activity might be modulated by signaling pathways that respond to growth conditions.

Positive and negative regulation by MAP kinase related pathways

Phosphorylation by MAP kinase-related pathways has been shown to modulate transcriptional activator func-

tion, and can affect stability, nuclear localization, DNA-binding, and transactivation (for review, see Hunter and Karin 1992; Hill and Treisman 1995). In HSF1, phosphorylation occurs in the regulatory domain, and is necessary for full repression of the transcriptional activation domains. In contrast, stimulation of the Raf/ERK pathway results in up-regulation of activator function in most of the systems studied. In mammals, phosphorylation of the ternary complex factors Elk-1 and SAP-1 increases the transcriptional activation potential of these proteins (Marais et al. 1993; Zinck et al. 1993), as does phosphorylation of Ets-1 and Ets-2 (Yang et al. 1996). In *Drosophila* eye development two Ets-related proteins, yan and pointed^{P2}, are both downstream targets of ERKA, and phosphorylation of pointed increases its transactivation potential, whereas phosphorylation of the repressor yan inhibits its ability to repress (O'Neill et al. 1994). Increased repression following phosphorylation by ERK kinases has been proposed for c-Fos in mammals and Bicoid in *Drosophila*, although the mechanism by which phosphorylation functions in these cases has not been established (Ofir et al. 1990; Chen et al. 1993; Ronchi et al. 1993; Okazaki and Sagata 1995). Thus, two separate mechanisms have evolved that allow similar or identical kinase pathways to regulate activators by either increasing function (e.g., Elk-1) or decreasing function (e.g., HSF1).

There are several proline-directed serine/threonine kinases in the cell (Cano and Mahadevan 1995). In addition to ERK, p38 is capable of phosphorylating the regulatory domain (Fig. 2), and there are numerous other kinases, including CDKs, that might also have this activity. Even more kinases with similar substrate specificity are being discovered. This raises the possibility that several different kinases might modulate HSF1 activity via the mechanisms that we propose here. Further characterization of these different kinase pathways and their effects on HSF1 phosphorylation will be required to establish how broad this mechanism of regulation of HSF1 activity is.

Implications for regulation of HSF1 activity in vivo

HSF1 is normally found in a non-DNA-binding monomeric state that is converted to a DNA-binding trimer in response to a wide variety of cellular stresses, pharmacological treatments, and physiological conditions (Morimoto et al. 1992). Under mild stress conditions, inhibition of the transactivation potential of HSF1 by phosphorylation might provide an additional level of regulation to prevent a high level of heat shock gene induction when it is not needed or is detrimental for cell growth. HSPs fulfill protective functions under cellular stress conditions mainly because of their ability to act as molecular chaperones, but under normal conditions artificial overexpression can lead to an inhibition of cell proliferation (Feder et al. 1992; Knauf et al. 1992). Consistent with the experiments reported here, two recent studies have shown an inhibitory effect of the Raf/ERK pathway on heat shock gene transcription. Overexpression of a dominant inhibitory mutant of ERK1 increases

hsp70 reporter gene activity in NIH-3T3 cells after a short heat treatment (Mivechi and Giaccia 1995), whereas in Ha-ras-transformed rat fibroblasts the induction of hsp68 transcription is decreased (Engelberg et al. 1994). The ability of HSF1 to integrate signaling pathways via phosphorylation of the regulatory domain might be one mechanism to modulate the cellular stress response in response to different growth conditions. A direct test of this hypothesis will require the development of systems that allow characterization of HSF1 function under more physiological induction conditions, and that allow a direct determination of which kinase phosphorylates the regulatory domain under those conditions.

Phosphorylation does not appear to affect regulatory domain function under the severe stress of heating to 43°C. Mutation of the proline-directed serine motifs in the regulatory domain has no phenotype under heat shock conditions; both wild-type and mutant proteins are maximally induced (Fig. 1). Dephosphorylation of the regulatory domain is not necessary for heat shock-induced transcription, as the phosphorylation level of the GAL4-HSF1 constructs is not affected by heat treatment and mutants in which the serines have been changed to glutamic acid are still fully heat shock-inducible (Fig. 1). This is consistent with a role for the regulatory domain in receiving growth signals: Under conditions of severe stress, HSF might be expected to be maximally activated regardless of cellular growth conditions because proper induction of heat shock gene expression is necessary to ensure cell survival. Thus, modulation of the regulatory domain of HSF1 by phosphorylation might be an important mechanism that provides the appropriate level of HSF1 activity under various normal growth conditions, but is overridden upon severe stress.

A model for regulatory domain function

The regulatory domain of HSF1 can function in the absence of other portions of HSF1. When this domain is placed in a protein with the GAL4 DNA-binding domain and a portion of the VP16 activation domain, it represses activation domain function at normal growth temperatures and confers heat inducibility (Newton et al. 1996). We have mapped a critical region of this domain: mutation of charged amino acids at 298 or 300 eliminates function of the regulatory domain; mutation of serines 303 and 307 to glutamic acid, which mimics a phosphorylated state, results in full function of the regulatory domain, whereas mutation of serines 303 and 307 to alanine inhibits the ability of the regulatory domain to repress transcription. Thus, phosphorylation of serines 303 and 307 would add two more charged side chains to an important region of the regulatory domain that contains other key charged amino acids. This region might form a charged interface with a separate protein that either shields the transcriptional activation domains or represses transcription directly. Alternatively, the regulatory domain might shield transcriptional activation domain function directly via specific contacts. Although

we cannot rule out this latter possibility, several lines of evidence suggest the contrary. None of the amino acids in activation domain 1, when mutated, had an effect on heat regulation (Newton et al. 1996). The regulatory domain can also repress a heterologous activation domain (Newton et al. 1996). In addition, no interaction was detected between the regulatory domain and the activation domains when tested in the two-hybrid system (M. Green, unpubl.). Therefore, to understand the mechanism by which this region of HSF1 functions, it will be important to identify any proteins that interact directly with this charged region of the regulatory domain.

Materials and methods

Plasmid constructions

Point mutagenesis Point mutations were made by Kunkel mutagenesis as described previously (Newton et al. 1996), confirmed by sequencing and then subcloned back into the GAL4-RD-AD1 expression vector (Newton et al. 1996).

Expression vectors Mutations were introduced into the full-length HSF1 coding region by subcloning the *EcoRI* fragment of HSF1 cDNA (Rabindran et al. 1991) into pUC18 and replacement of the *NgoMI* fragment with the appropriate fragments of GAL4-RD-AD1 variants. The *EcoRI* fragments coding for wild-type and mutant HSFs were subcloned into the mammalian expression vector pCMV5. The carboxy-terminal FLAG epitope was added using the polymerase chain reaction (PCR) with a forward primer containing the *PstI* site of HSF1 and a reverse primer incorporating the appropriate FLAG sequence, a stop codon, and a *BglII* site. PCR products were subcloned into the different pCMV HSF1 plasmids.

The LexA[1–87]HSF1[84–529] vector was constructed by subcloning a blunt-ended *TaqI*-*EcoRI* fragment of HSF1 into *EcoRI*-digested, blunt-ended pSH-2-1 (Hanes and Brent 1989). The *HindIII*-*BglII* fragment of this plasmid was subcloned into pCMV5 and the RD mutations were introduced using the *SphI*-*BglII* fragments of the different pCMV HSF1 FLAG constructs as described above. The GST expression vectors were constructed by amplifying (PCR) the 170 amino acid wild-type RD sequence with primers containing *BamHI* (forward) and *EcoRI* (reverse) sites and subcloning into *BamHI*/*EcoRI*-digested pGEX-2TK (Pharmacia). The RD mutations were introduced subsequently by subcloning of the appropriate *NgoMI* fragments of the different GAL4-RD-AD1 constructs.

Reporter plasmids The CAT reporter constructs used here were G540CAT (Taylor 1991), HSECAT (Greene et al. 1987), and X₄G₂CAT (Bunker and Kingston 1994), which contain five GAL4 DNA-binding sites, two synthetic HSE sites, or four LEXA plus two GAL4-binding sites, respectively, at –40 of the hsp70 basal promoter driving the expression of the CAT gene. The internal reference plasmid pIR17-84 contains hsp70 promoter sequences to –84 (thus deleting the HSEs) with a deletion in the 5' untranslated region driving the expression of the CAT gene (Kingston et al. 1986). This produces an S1 reference signal that is 130 nucleotides compared with 230 nucleotides for the reporter.

Transient transfections and CAT assays

HeLa and 293T cells were grown in DMEM + 10% calf serum at 37°C in 5% CO₂. Plates at ~90% confluency were split 1:10 the day before transfection into 10-cm dishes. Transfections were done by the CaPO₄ method (Ausubel et al. 1989) and contained 15 µg of total DNA. For HeLa cells, 5 µg expression plasmid, 5 µg G540CAT reporter plasmid, and 5 µg pIR17-84 (reference for S1 assay) or 1 µg pXGH5 (reference plasmid for CAT assay) and pSK+ as carrier DNA were used. Chloroquine was added to the media at a final concentration of 100 µM immediately prior to addition of precipitate. Cells were washed with PBS and placed into fresh medium 4 hr later, and heat shock was induced ~24 hr posttransfection (43°C for 4 hr). Transfectants were harvested either immediately for S1 analysis or 12 hr later for CAT assay. Cell extracts and phase-extraction CAT assays were performed as described previously (Ausubel et al. 1989). Transfection efficiency was measured using a human growth hormone-based radioimmunoassay (Nichols Institute).

293T cells were transfected for 3 hr without chloroquine-treatment using various expression plasmids (0.5 µg if not otherwise indicated) and pSK+ as carrier DNA. If indicated, cells were serum-deprived (0.1% calf serum) 24 hr posttransfection for ~16 hr, stimulated with phorbol-12-myristate-13-acetate (PMA, ICN Biochemicals) or anisomycin, and harvested immediately.

RNA harvest and S1 analysis

Total cellular RNA was isolated from transfected cells using RNeasy prep columns from Qiagen according to manufacturer's instructions. Single-stranded 5' end-labeled S1 probe was prepared as described previously (Kingston et al. 1986) and contains sequences from +229 to -133 of the hsp70-CAT fusion reporter construct. Total cellular RNA (20 µg) was mixed with S1 probe (5 × 10⁴ dpm), and hybridization, S1 nuclease digestion, and analysis on 8% denaturing polyacrylamide gels were done by standard procedures (Ausubel et al. 1989). Gels were quantitated using a Molecular Dynamics PhosphorImager.

Electrophoretic mobility shift assay

Transfected HeLa cells were washed with ice-cold PBS, duplicate plates were pooled and nuclear extracts were prepared as described previously (Lee et al. 1988). Total protein concentrations were determined by Bradford assay (Biorad). Protein (2 µg) was used in a total volume of 10 µL buffer D (20 mM HEPES at pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT) and 11 µL of a salt mix was added (20 mM HEPES at pH 7.5, 16.67 mM KCl, 8.83 mM MgCl₂, 16.67 µM ZnCl₂, and 6.63 mM spermidine). GAL4 antibody (RK5C1, Santa Cruz Biotechnology Inc.) (0.2 µg) was added and the mixture was incubated on ice for 30 min. Poly[d(I-C)] (Pharmacia) (5 µg) and 2.5 ng double-stranded radiolabeled probe containing a single GAL4 site (5'-CGGAGTACTGTCCTCCG-3') was added, the mixture was incubated at 30°C for 15 min, and resolved on a native 4.5% acrylamide gel in 0.5× TBE.

Protein purification and kinase assays

GST fusion protein expression vectors were transformed into BL21 DE3 strain of *Escherichia coli*. Protein induction and purification were as described (Smith and Johnson 1988). The amount of protein was estimated by the Bio-Rad protein assay.

For immunopurification of ERK1, SAPK or p38, cells were washed with ice-cold phosphate-buffered saline and lysed in

lysis buffer [20 mM HEPES at pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 2 µM leupeptin, 400 µM phenylmethylsulfonyl fluoride (PMSF), and 10 kallekrein-inhibiting units Trasylol per ml]. Extracts were cleared by centrifugation and preincubation with protein G-Sepharose (Pharmacia). Kinases were precipitated using the anti-HA mAb 12CA5 (Boehringer Mannheim) or a rabbit antiserum against SAPK (Kyriakis et al. 1994) or p38 (Rouse et al. 1994) bound to protein G-Sepharose. The immunoprecipitates were washed twice with LiCl buffer (500 mM LiCl, 100 mM Tris at pH 7.6, 1 mM DTT, and 0.1% Triton X-100) and three times with kinase assay buffer (20 mM MOPS at pH 7.2, 2 mM EGTA, 10 mM MgCl₂, 1 mM DTT, and 0.1% Triton X-100). Kinase reactions were initiated by addition of 2 µg of substrate proteins [GST-RD, GST-c Jun(1-135), myelin basic protein (MBP) (Sigma)] and 100 µM [γ-³²P]ATP (5000 cpm/pmol) in a final volume of 60 µL of kinase buffer. The reactions were stopped after 20 min at 30°C by addition of SDS sample buffer and analyzed by SDS-PAGE and autoradiography. Quantitation was performed using a Molecular Dynamics PhosphorImager.

Western blotting

Similar amounts of whole-cell extracts were resolved by SDS-PAGE on 10% gels, blotted onto nitrocellulose (Schleicher & Schuell), and subjected to Western blotting analysis using M2 anti-Flag mAb (IBI Kodak) or rabbit antiserum against GAL4 (kindly provided by M. Ptashne, Harvard University, Cambridge, MA). The antibody-antigen complexes were visualized by the enhanced chemiluminescence detection system (Amersham).

In vivo labeling and immunoprecipitation

Transfected 293T cells were washed in prewarmed (37°C) phosphate-free minimal essential medium (Gibco BRL) and labeled in 1 ml of medium supplemented with 2% dialyzed fetal bovine serum (omitted in serum-deprived samples) and 0.5 mCi of ³²P_i (DuPont/New England Nuclear) for 60 min at 37 or 43°C. Cells were washed with ice-cold phosphate-buffered saline, and lysed in radioimmune precipitation buffer [RIPA: 10 mM Tris at pH 8.0, 0.1 M NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] supplemented with 50 mM β-glycerophosphate, 1 mM Na₃VO₄, 40 mM NaF, 2 µM leupeptin, 400 µM PMSF, and 10 kallekrein-inhibiting units of Trasylol per ml. The lysates were cleared by centrifugation and equal counts from all samples were incubated with a rabbit antiserum against HSF1 (kindly provided by R. Morimoto, Northeastern University, Boston, MA) or GAL4 (kindly provided by M. Ptashne). Two hours later protein A-Sepharose (Pharmacia) was added and incubation was continued for 1 hr. Flag-tagged proteins were immunoprecipitated using M2 affinity gel (IBI Kodak) and modified RIPA buffer (10 mM Tris at pH 7.4, 0.15 M NaCl, 1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, and 1% Triton X-100). Immune complexes were washed 4× with the appropriate RIPA buffer, which was supplemented with 1 M NaCl in the first washing step. Pellets were boiled in SDS sample buffer and analyzed by SDS-PAGE (10% acrylamide) and autoradiography.

Tryptic phosphopeptide analysis

In vitro phosphorylation of GST-RD fusion proteins was performed using a purified ERK1/2 preparation (Mukhopadhyay et al. 1992). GST proteins (5 µg) were phosphorylated in kinase

assay buffer at 30°C with 100 μ M [γ - 32 P]ATP (20,000 cpm/pmole). Reactions were stopped 60 min later by adding SDS sample buffer.

For phosphopeptide analysis of in vivo labeled HSF1, Hela cells were 32 P_i-labeled using 2 mCi/ml and HSF1 was immunoprecipitated. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose. The appropriate bands were cut and proteins were digested with TPCK-treated trypsin (Worthington) as described (Boyle et al. 1991). Labeled peptides were lyophilized, spotted onto microcellulose glass-backed TLC plates (Merk) and analyzed by 2-D peptide mapping. Electrophoresis was carried out on LKB Multiphor II (Pharmacia) at 1.0 kV for 25 min using pH 1.9 buffer [25 ml formic acid (88%), 78 ml glacial acetic acid, and 897 ml H₂O]. Ascending chromatography was performed in *n*-butyl alcohol/pyridine/glacial acetic acid/water (15:10:3:12) for 7–9 hr. Plates were exposed at –80°C with an intensifying screen for 2–3 days.

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