Heat Shock Factor 1 Represses Ras-induced Transcriptional Activation of the c-fos Gene*

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Heat shock factor 1, the critical molecular regulator of the stress response is conserved throughout eukaryotic organisms and activates the transcription of heat shock genes. We now show that heat shock factor 1 inhibits the expression of c-fos, an immediate early gene that controls responses to extracellular stimuli for growth and differentiation. Heat shock factor 1 inhibits the transcription of the c-fos gene and antagonizes the activating effects of the signal transducing protein Ras on the c-fos promoter and on the promoter of another Ras responsive gene uPA. This property was specific for heat shock factor 1; c-fos repression was not seen with the structurally related protein heat shock factor 2. Repression involved different molecular mechanisms compared with those involved in transcriptional activation by heat shock factor 1 and specifically did not require binding to the c-fos promoter. Thus, in addition to its known role as a transcriptional activator of the cellular heat shock response, heat shock factor 1 also antagonizes the expression of Fos, a key component of the ubiquitous AP-1 transcription factor complex and as such could influence multiple aspects of cell regulation.

Heat shock results in the activation of a pre-existing transcription factor, heat shock factor 1 (HSF1) through a process that includes trimerization and nuclear translocation (1–3). Activated HSF1 binds to heat shock elements (HSE) in heat shock protein (hsp) gene promoters and induces expression of hspS involved in the assembly, folding, and transport of other proteins and conferring thermotolerance on expressing cells (4–7). Our recent studies indicate that in addition to being a transcriptional activator, HSF1 functions as a repressor of a number of genes including IL1B (8). This suggests that at elevated temperatures HSF1 possesses both activation and suppression functions, depending on the nature of the target promoter (1, 2, 8). The function of the heat shock response in mammals is somewhat mysterious considering the tight regulation of body temperature in homeotherms. However, the response may have become adapted to function during fever, the neuroendocrine response to disease that modulates elevation in body temperature (9). Because one function of interleukin 1β, the product of the IL1B gene and a target for HSF1 repression is the mediation of fever, HSF1 could function as a feedback inhibitor of IL1B during fever (8). We are interested to know whether HSF1 affects other genes involved in cell proliferation, differentiation, and viral infection that might be involved in the heat shock response and the mediation of fever. One such gene is c-fos, an early response gene involved in regulating normal cellular proliferation and differentiation as well as being expressed in many types of tumors (10, 11). c-fos encodes the nuclear Fos protein, which associates with Jun proteins to form a heterodimeric transcription factor with AP-1 site selectivity that is a nuclear target of the Ras signaling pathway and is a participant in the replication of some viruses (12–14). Ras, which serves as a signal transducer in a manner similar to other signal GTP-binding proteins, transmits signals from the plasma membrane to nuclear targets such as the transcription factors mentioned above (18). We have therefore examined the effect of HSF1 on the expression of the c-fos gene and the activity of the c-fos promoter in mitogen and Ras-stimulated Chinese hamster ovary (CHO K1) fibroblasts.

EXPERIMENTAL PROCEDURES

Human HSP70B promoter construct p25000CAT was purchased from StressGen (Victoria, BC, Canada), and the human HSP27 promoter was a gift from Drs. Lee Weber and Eileen Hickey (Department of Biology, University of Nevada). Ha-Ras expression plasmid pB67T1, control plasmid Homer 6, and mouse urokinase-type plasminogen activator (uPA) gene promoter-luciferase reporter plasmid (uPA-Luc) were generous gifts from Dr. M. C. Ostrowski (Ohio State University), the CHO K1 cell line was from the American Type Tissue Culture Collection, and culture media and supplements were from Life Technologies, Inc.

pGL reporter Constructs—pGLhs70B was constructed by inserting the HSP70B gene promoter fragment from BglII site to HindIII site (1.44 kilobases) of p25000-CAT into pGBl. Basic 3 at the corresponding sites. pGL.hsp27 was constructed by inserting the HSP27 gene promoter fragment (19) from BamHI site to HindIII site (730 base pairs) into pGL.Basic 3 at the BglII and HindIII sites. pfos 2000 was constructed by inserting the human c-fos promoter from −2000 to +42 relative to the transcription start site into pGL.Basic 2. For pfos 396, a fragment of the c-fos promoter was deleted from −2000 to −396. Pfos 396D219-81 was derived from pfos 396 by deletion from −219 to −81, which increases promoter activity (18).

Expression Constructs—Human HSF1 cDNA (20) was inserted into the pDNA3.1(−) expression vector at the XhoI and EcoRI sites, termed as pHSF1 (+ or −) indicate the orientation of the polycloning site in pDNA3.1). Human HSP2A cDNA was inserted into the pDNA3.1(+) EcoRI and XhoI sites to produce HSP2A. Then the fragment from pHSF1 to VspI sites of pSV-β-Galactosidase plasmid, containing SV40 early promoter and enhancer segments, lacZ coding region and SV40 poly(A) signals, was inserted into the pHSF plasmids at NsiI and SapiI sites to replace the Neomycin resistance gene, and these constructs were termed p-B-HSF1 and p-B-HSF2, respectively. pHSF1mut was derived

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1 The abbreviations used are: HSF1, heat shock factor 1; HSF2, heat shock factor 2; hsp, heat shock protein; uPA, urokinase-type plasminogen activator; CHO, Chinese hamster ovary; MAPK, mitogen-activated protein kinase; TCF, ternary complex factor; SRF, serum response element; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; SRF, serum response factor.
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**Northern Analysis**

Total RNA was isolated, fractionated on 1% agarose/formaldehyde gels, and transferred to membranes as described. Specific mRNAs were detected by hybridization with a rat c-fos cDNA probe (2.2-kilobase cDNA fragment) and a murine HSP 70.1 cDNA probe (22) radio-labeled with α-[^32]PdCTP using a Rediprime kit (Amersham). Double stranded DNA templates were prepared from minipreps as described.

**Transient Transfection, Luciferase, and β-Galactosidase Assay**

CHO K1 cells were dispensed into 6-well plates at 2.2 × 10^6 cells/well and left to grow for 20–24 h prior to liposome-mediated transfection with reporter plasmids, expression plasmids, and control [pHSV-β-Gal expression plasmids] as described. Cell extracts were prepared as described, and luciferase and β-galactosidase assays were performed according to the manufacturer’s protocol (Promega). β-Galactosidase and luciferase activity was measured as described. Luciferase activity was normalized to β-galactosidase activity, which was used as an internal transfection efficiency control. Results are expressed as fold activity of control. Analysis of variance with Tukey’s multiple range test was employed for statistical analysis of luciferase values.

**Stable Transfection**

After transfection, cells were maintained in Ham’s F-12 medium containing 10% FCS for 24 h. Neomycin (G418) was added to 300 ng/ml until surviving cells reached confluence. Surviving clones were then selected and amplified.

** EMSA**

Nuclear extracts were prepared according to Schreiber et al. (23), incubated with a double stranded, ^32^P-labeled HSE-containing oligonucleotide probe, and analyzed by EMSA as described.

**RESULTS**

**Heat Shock Inhibits Expression of the c-fos Gene**—We first determined whether the serum induction of c-fos mRNA was inhibited by heat shock. Exposure of quiescent CHO K1 cells to serum strongly induced c-fos expression but did not affect hsp70 expression. In contrast, heat shock (42 °C) carried out simultaneously with serum exposure inhibited c-fos expression while inducing hsp70 expression. Cells cultured continuously in 10% serum showed a low level expression of c-fos that was reduced by heat shock (Fig. 1). There was no change in the intensity of the 28S rRNA band in cells exposed to any of the treatments, indicating even recovery and loading of RNA (Fig. 1). These results differ from previous reports showing that c-fos mRNA increased in some cell types following 2–6 h of heat shock at 44 °C and recovery at 37 °C for 15 min to 24 h (24, 25).

c-fos is, however, an early response gene induced rapidly after serum exposure and expression seen several hours after stimulation is likely due to secondary effects on message stability or transcription factor expression (24, 25).

**The Effects of Heat Shock Factor Expression on c-fos Promoter Activity**—Because the expression of hsp70 and c-fos mRNAs was reciprocally affected by heat shock and because HSF1 is the activator of hsp70 promoters, we examined the possibility that heat shock represses the c-fos promoter through activation of HSF1 (Fig. 2). We investigated the effects of HSF1 expression on the activity of the c-fos promoter. Expression of HSF1 inhibited promoter activity by approximately 70% using either the entire c-fos promoter (plasmid pfos 2000) or reporter plasmid pfos396D219-81 (Fig. 2A). In subsequent transfection experiments, therefore, the more active (pfos396D219-81) plasmid was used. To determine whether repression involved direct effects of HSF1 or was due to a downstream product of HSF1 transcription, we constructed an HSF1 expression plasmid with an inactivating mutation in the DNA binding domain (HSF1mut) that prevented binding to HSP promoters (shown subsequently). Expression of HSF1mut repressed c-fos transcription, indicating a direct mechanism for repression independent of secondary products of HSF1-activated transcription (Fig. 2A). Expression of HSF2, a transcription factor structurally related to HSF1, had no effect on either of the c-fos promoters, indicating that repression is specific for HSF1 (Fig. 2A). To confirm these findings, we next established cell lines stably expressing HSF1 and HSF1mut, which were then co-transfected with c-fos reporter plasmids and pSV-β-Gal control plasmids. c-fos promoter activity in the cell lines stably expressing HSF1 or HSF1mut was only 7% of the activity in wild type cells not expressing HSF1, further indicating repression of the c-fos gene by HSF1 (Fig. 2B).

To control for functional activity of our HSF expression system, we analyzed the effects of HSF1 and HSF2 expression on the promoters of two hsp genes (HSP 70B and HSP 27) (19, 27). HSF1 strongly induced the HSP 70B promoter causing a 13.6 ± 0.8-fold induction while producing only a minor effect (2.4 ± 0.12-fold) on the HSP 27 promoter (Fig. 2C). HSF2 also showed a minor induction of both the HSP 70B and HSP 27 promoters (Fig. 2C). These experiments indicated that HSF1 is specific for the HSP 70B promoter and that HSF2, although ineffective in repressing the c-fos gene, activates the hsp promoters. By contrast, HSF1mut did not activate the HSP70B promoter (Fig. 2C). Similar experiments were carried out in cells stably expressing wild type HSF1 and HSF1mut (Fig. 2D). Cells were transfected with pGL-hsp70B or pfos 396D219-81 along with pSV-β-Gal plasmids, respectively. Wild type HSF1 and HSF1mut both repressed the c-fos promoter, but only wild type HSF1 activated the HSP70B promoter, further indicating that although HSF1 activation of the HSP70B promoter requires DNA binding, c-fos repression is independent both of DNA binding and transcription of hsp genes (Fig. 2D).

We next examined the effects of HSF1 expression on Ras-activated c-fos activity using a system involving co-transfection of activated Ha-Ras expression plasmid (28) with the c-fos reporter plasmid (Fig. 2E). Activated Ras protein specifically stimulated the c-fos promoter (by at least 5-fold) while not activating the hsp promoters (HSP 70B and HSP 27) (not shown). When activated Ha-Ras was co-transfected with HSF1 or HSF2 expression plasmids, HSF1 completely blocked activation of the c-fos promoter by Ras, whereas HSF2 expression was ineffective (Fig. 2E). To determine whether HSF1 has a general repressive effect on Ras-activated gene expression, we also examined the Ras-responsive mouse uPA gene (29). Gene reporter plasmid pUPA-Luc was strongly activated by Ras ex-
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**FIG. 2. Effects of HSF expression at 37°C on c-fos and HSP promoter activity.** A, effects of HSF1 and HSF2 expression on c-fos promoter activity. HSF expression plasmids (1 μg/well) were co-transfected with 2 μg/well of c-fos-luciferase reporter plasmids into CHO K1 cells. After incubation for 20–24 h with 0.5% FCS, cells were harvested for luciferase assay. Each experimental condition was repeated in triplicate. Luciferase activity is expressed as fold activity over the empty plasmid control. Data points are means ± S.E. of three or four independent transfection experiments. B, repression of the c-fos promoter in cells stably expressing HSF1. c-fos-luciferase reporter plasmids (2 μg/well) and 1 μg/well of β-galactosidase reporter (control) plasmids were co-transfected in triplicate into cells stably expressing HSF1 or HSF1mut and wild type CHO K1 cells. After incubation for 20–24 h with 0.5% FCS, cells were harvested for luciferase assay. Luciferase activity is expressed as fold activity and normalized to a value of 1-fold for the stable HSF1 expressors. C, activation of heat shock promoters by HSF1 or HSF2 expression. HSF expression plasmids (1 μg/well) were co-transfected with hsp promoter-luciferase reporter plasmids (2 μg/well) in triplicate into CHO K1 cells. Incubations, assay conditions and data analysis are as in A. D, activity of the c-fos and HSP70B promoters in cells stably expressing HSF1 and HSF1mut. Cells were transfected with, respectively, pG5.hsp70B (2 μg/well) or pfos396D219-81 plasmids (2 μg/well) along with βSV-β-galactosidase plasmid (1 μg/well) in triplicate. Incubation and assay conditions and data analysis are as in A. E, repression of Ras-activated transcription by HSF1. HSF expression plasmids or empty control (pcDNA3.1) plasmid (1 μg/well) and Ras expression plasmid HoT61 or control (empty) Ras expression plasmid Homer 6 (1 μg/well) were co-transfected in triplicate with ras-luciferase reporter plasmids (2 μg/well) into CHO K1 cells and incubated for 20–24 h with 0.5% FCS. In addition, to analyze the effects of HSF1 on the uPA gene promoter, HSF1 expression plasmids or empty control (1 μg/well) and Ras expression plasmid HoT61 or Ras expression control plasmid Homer 6 (1 μg/well) were co-transfected in triplicate with uPA-luciferase reporter plasmid (2 μg/well) and incubated as for the c-fos experiments. Luciferase activity is expressed as fold activity over the empty control plasmid. Data points are means ± S.E. of three to six independent experiments.

To confirm that the stable transfectants contained activated HSF1, extracts from wild type and HSF1 expressing CHO cells were analyzed by EMSA (Fig. 3). Cell lines transfected with HSF1 contained a novel, high M₁ HSE binding activity (marked HSF1) in addition to constitutive complexes (Fig. 3). The unique band was supershifted with anti-HSF1 antibody, consistent with it being a HSF1-HSE complex (Fig. 3). This was confirmed in the extract from heat shocked cells, which contained a similar HSE binding activity also supershifted with anti-HSF1 antibody (Fig. 3). Untransfected cells, cells transfected with empty plasmid, and cells transfected with HSF1mut did not contain activated HSF1 (Fig. 3). Thus the addition of one leucine residue at position 22 within the DNA binding domain inhibits HSF1 activation to a DNA binding form in vivo, confirming that binding of HSF1 to the c-fos promoter is not required for c-fos repression. This was further confirmed by EMSA analysis of HSF1 binding to pfos396D219-81, the construct repressed by HSF1 (Fig. 2A). We examined the binding of HSF1 in nuclear extracts from heat shocked cells to oligonucleotides spanning the +12 to −81 and −219 to −396 regions, the sequences from the c-fos promoter. The sequences from the c-fos promoter that are contained in pfos396D219-81. Although HSF1 from the extracts bound avidly to a consensus HSE and these HSF1-HSE complexes were supershifted by anti-HSF1 antibody, binding of HSF1 to sequences from the c-fos promoter was not observed (not shown).

**DISCUSSION**

These studies indicate that the mitogenic stimulation of c-fos is blocked by heat shock at least partially through transcriptional repression of the c-fos promoter (Figs. 1 and 2). However c-fos repression did not require the binding of HSF1 to the c-fos promoter because a HSF1 construct deficient in HSE binding...
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activity and transcriptional activating function still repressed the c-fos promoter and no evidence was obtained for HSF1 binding to the promoter (Fig. 2). The mechanisms of c-fos repression by HSF1 thus contrast with those underlying transcriptional activation, which are dependent on HSF1 binding to HSE sequences within hsp promoters (1, 2). Transcriptional repression may involve a number of contrasting mechanisms (30–32). The present experiments argue against the displacement of activating factors, because binding to HSE-like elements is unnecessary for HSF1 repression (Fig. 2). In addition, the fact that HSF1 activates hsp promoters while repressing c-fos suggests that HSF1 does not globally repress the activity of basal transcription factors (Fig. 2). HSF1 may thus repress the c-fos promoter either by interaction with an upstream signal transduction component or a specific co-activator. Upstream regulation of the c-fos promoter is complex, involving serum activation of the Ras pathway and induction of serum response factor (SRF) to bind to the SRE and complex with TCF in sequence in the C-terminal 300 amino acids containing repressing and "leucine zipper" trimerization domains while diverging in sequence in the N-terminal DNA binding domains involved in repression (Fig. 2). The present experiments argue against the displacement of activating factors, because binding to HSE-like elements is unnecessary for HSF1 repression (Fig. 2). In addition, the fact that HSF1 activates hsp promoters while repressing c-fos suggests that HSF1 does not globally repress the activity of basal transcription factors (Fig. 2). HSF1 may thus repress the c-fos promoter either by interaction with an upstream signal transduction component or a specific co-activator. Upstream regulation of the c-fos promoter is complex, involving serum activation of the Ras pathway and induction of serum response factor (SRF) to bind to the SRE and complex with TCF in sequence in the C-terminal 300 amino acids containing repressing and "leucine zipper" trimerization domains while diverging in sequence in the N-terminal DNA binding domains involved in repression (Fig. 2). HSF1 and HSF2 sequences are highly homologous in the N-terminal DNA binding and "leucine zipper" trimerization domains while diverging in sequence in the C-terminal 300 amino acids containing regulatory and activation domains (1, 2, 20, 37, 38). Regions mediating repression might thus be located within the C terminus.

In conclusion, therefore, HSF1 functions as a Ras antagonist by repressing Ras-mediated activation of the c-fos promoter. Transcriptional repression may constitute a significant component of HSF1 function in the cellular heat shock response as well as in systemic responses, such as fever involving elevated temperatures.

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