Redox regulation of mammalian heat shock factor 1 is essential for Hsp gene activation and protection from stress

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The activation of eukaryotic heat shock protein (Hsp) gene expression occurs in response to a wide variety of cellular stresses including heat shock, hydrogen peroxide, uncoupled oxidative phosphorylation, infection, and inflammation. Biochemical and genetic studies have clearly demonstrated critical roles for mammalian heat shock factor 1 (HSF1) in stress-inducible Hsp gene expression, resistance to stress-induced programmed cell death, extra-embryonic development, and other biological functions. Activation of mammalian Hsp gene expression involves the stress-inducible conversion of HSF1 from the inactive monomer to the DNA-binding competent homotrimer. Although Hsp activation is a central conserved process in biology, the precise mechanisms for stress sensing and signaling to activate HSF1, and the mechanisms by which many distinct stresses activate HSF1, are poorly understood. In this report we demonstrate that recombinant mammalian HSF1 directly senses both heat and hydrogen peroxide to assemble into a homotrimer in a reversible and redox-regulated manner. The sensing of both stresses requires two cysteine residues within the HSF1 DNA-binding domain that are engaged in redox-sensitive disulfide bonds. HSF1 derivatives in which either or both cysteines were mutated are defective in stress-inducible trimerization and DNA binding, stress-inducible nuclear translocation and Hsp gene *trans*-activation, and in the protection of mouse cells from stress-induced apoptosis. This redox-dependent activation of HSF1 by heat and hydrogen peroxide establishes a common mechanism in the stress activation of Hsp gene expression by mammalian HSF1.

[*Keywords*: Heat shock factor; oxidative; heat; stress; transcription; hydrogen peroxide]

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The heat shock response was originally identified as the occurrence of *Drosophila* salivary gland chromosomal puffs, indicative of increases in transcriptional activity, that occurred in response to elevated temperatures, salicylate, or chemicals that result in uncoupling of oxidative phosphorylation (Ritossa 1962; for review, see Morimoto et al. 1996; Pirkkala et al. 2001). Over the past forty years an explosion of interest in the heat shock response has revealed the presence of a family of heat shock proteins (Hsps) that function in protein folding, trafficking, maturation, degradation, signal transduction, and cell stress protection that are conserved from bacteria to humans (Welch 1992; Morimoto et al. 1994; Feder and Hofmann 1999; Christians et al. 2002). Whereas many Hsps exhibit basal levels of expression consistent with their roles in protein maintenance, cell growth and division, and other crucial cellular functions, a common feature of Hsp genes is their dramatic transcriptional induction in response to a wide variety of environmental, chemical, and pathophysiological stressors (Morimoto et al. 1996; Morimoto 1998; Pirkkala et al. 2001).

In eukaryotic cells the expression of Hsp genes is activated in response to stress by *cis*-acting promoter elements composed of variations of an inverted repeat (nGAAn), called heat shock elements (HSE), and a homotrimeric DNA-binding transcription factor, heat shock factor (HSF). Both HSF and HSEs are conserved in their fundamental structures from yeast to humans (Wu 1995; Morano and Thiele 1999; Pirkkala et al. 2001). In general, HSF molecules contain an N-terminal DNA-binding domain of the helix-turn-winged helix type, one or more coiled-coil trimerization domains, nuclear localization domains, and a C-terminal *trans*-activation domain. Additional HSF functional domains have been identified that modulate DNA-binding site selectivity, homotrimerization, and regulation of *trans*-activation (for review, see Pirkkala et al. 2001).

Although mammals harbor at least three genes encoding HSF proteins, the HSF1 isoform and the corresponding single HSF of *Drosophila* are known to respond to stress to activate Hsp gene transcription (Goodson and Sarge 1995; Mercier et al. 1999; Mathew et al. 2001; Pirkkala et al. 2001). Many studies have demonstrated that the activation of metazoan stress-responsive HSF pro-

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teins is a multistep process that involves conversion from the inactive monomer to the homotrimer, nuclear accumulation, DNA binding, and target gene activation (Sarge et al. 1993; Zuo et al. 1995; Orosz et al. 1996; Zhong et al. 1998). Although the activation of both *Drosophila* HSF and mammalian HSF1 from the monomer to the homotrimer is known to be modulated via direct interactions with Hsps and other factors, as well as putative intramolecular interactions (Abravaya et al. 1992; Rabindran et al. 1993; Shi et al. 1998; Zou et al. 1998; Bharadwaj et al. 1999), the precise manner in which stress-responsive HSF isoforms sense stress is not well understood. Both kinetic analysis of HSF activation in response to stress in vivo and the observation that this activation occurs in the absence of new protein synthesis suggest that the stress signal is transmitted rapidly to HSF and not via a multistep elaborate signal transduction cascade (Zimarino et al. 1990; Cotto et al. 1996). Furthermore, purified recombinant human HSF1, and HSF purified from *Drosophila* SL2 cells, are able to undergo conversion from the inactive monomer to the homotrimer that is competent for high-affinity binding to HSEs, upon exposure to stress in vitro (Goodson and Sarge 1995; Larson et al. 1995; Zhong et al. 1998; Mercier et al. 1999; Ahn et al. 2001). Although these observations demonstrate that *Drosophila* and mammalian HSF have intrinsic stress-sensing capacity, the mechanisms by which stress is sensed and by which this signal triggers the formation of an HSF homotrimer are not well understood. Furthermore, it is unclear how the multitude of apparently distinct stresses including heat shock, hydrogen peroxide, infection and inflammation, nonsteroidal antiinflammatory drugs and others all function to activate HSF homomultimerization. The critical roles of mammalian HSF1 in stress-responsive activation of Hsps, fertility, and extra-embryonic development (McMillan et al. 1998; Xiao et al. 1999; Christians et al. 2000; Zhang et al. 2002) underscore the importance of understanding the mechanisms by which HSF1 is activated in response to stress.

In this report we demonstrate that purified recombinant mammalian HSF1 directly senses both heat and oxidative stress in vitro and that HSF1, activated by either means, can be reversibly inactivated by reductant. Two cysteine residues (C35 and C105), localized within or nearby the HSF1 DNA-binding domain, are required for disulfide bond formation in response to stress, which leads to the formation of HSF1 homotrimers, DNA binding, nuclear accumulation, target gene activation, and protection from stress-induced apoptosis. These observations establish commonalities in the mechanism for how diverse stresses activate HSF1 function and, consequently, the expression of heat shock proteins.

Results

Direct and reversible activation of mammalian HSF1 in vitro

Although the heat shock response is ubiquitous in eukaryotic cells, little is known about the precise mechanisms by which the stress signal is transmitted to and interpreted by the stress-responsive HSF proteins to activate the transition from an inactive monomer to the homotrimeric DNA-binding competent form. We purified mouse HSF1 and HSF2 to homogeneity from *Escherichia coli* under conditions where they are maintained as inactive monomers (Ahn et al. 2001), and we ascertained whether they can be activated by heat or hydrogen peroxide in vitro. As shown in Figure 1A, in the absence of stress, purified mouse HSF1 migrated as a monomer in a protein cross-linking SDS-PAGE assay. However, after incubation at 42°C or in the presence of 200 μM H_2O_2 for 5 min, HSF1 formed electrophoretic species consistent with dimers, trimers, and higher-order homomultimers. Incubation of lower concentrations of purified HSF1 under these stress conditions resulted in essentially all of the monomer being converted into higher-order multimers (data not shown). Furthermore, HSF1 homomultimerization was activated at concentrations of H_2O_2 as low as 100 nM, and pretreatment of H_2O_2 with catalase prior to addition to HSF1 abolished HSF1 multimerization (data not shown). As shown in Figure 1B, a purified HSF1 truncation derivative containing only amino acids 1–290, encompassing the N-terminal DNA-binding domain through the coiled-coil motif, was also activated by both heat and H_2O_2 to form homomultimers. These conditions did not elicit changes in the multimerization state of recombinant mouse HSF2, an isoform whose multimerization in vivo is not altered in response to these stress conditions.

Because HSF1 homotrimerization is essential for high-affinity binding to its cognate HSE element, electrophoretic mobility shift analysis (EMSA) experiments were used to independently assess the specificity of direct stress activation of HSF1 multimerization. As shown in Figure 1C, using an Hsp70 HSE DNA element, both heat shock and H_2O_2 treatment activated recombinant HSF1(1–290) DNA binding, whereas the low level of HSF2(1–280) binding was not stimulated in response to these stress conditions.

Previous experiments suggested that activation of the heat shock response in vivo is inhibited under conditions of hypoxia, redox-active metal limitation, or in the presence of powerful reductants such as dithiothreitol (DTT; Huang et al. 1994; Davidson et al. 1996). Using the HSF1 multimerization assay, we tested whether DTT was capable of inactivating purified recombinant HSF1 that had been activated in vitro by heat shock or H_2O_2. As shown in Figure 1D, heat shock and H_2O_2 activation of HSF1 multimerization was readily reversed by subsequent incubation with DTT, as was binding to the Hsp70 HSE DNA sequence (data not shown). To ascertain whether this inactivation of HSF1 multimerization by DTT was reversible, DTT was removed by dialysis and HSF1 was either untreated or subjected to another round of heat shock or H_2O_2 exposure. As shown in Figure 1D, HSF1 that had been inactivated with DTT was capable of being reactivated by heat shock or H_2O_2 treatment upon removal of DTT by dialysis. Taken together, these data demonstrate that mammalian HSF1, through the N-ter-

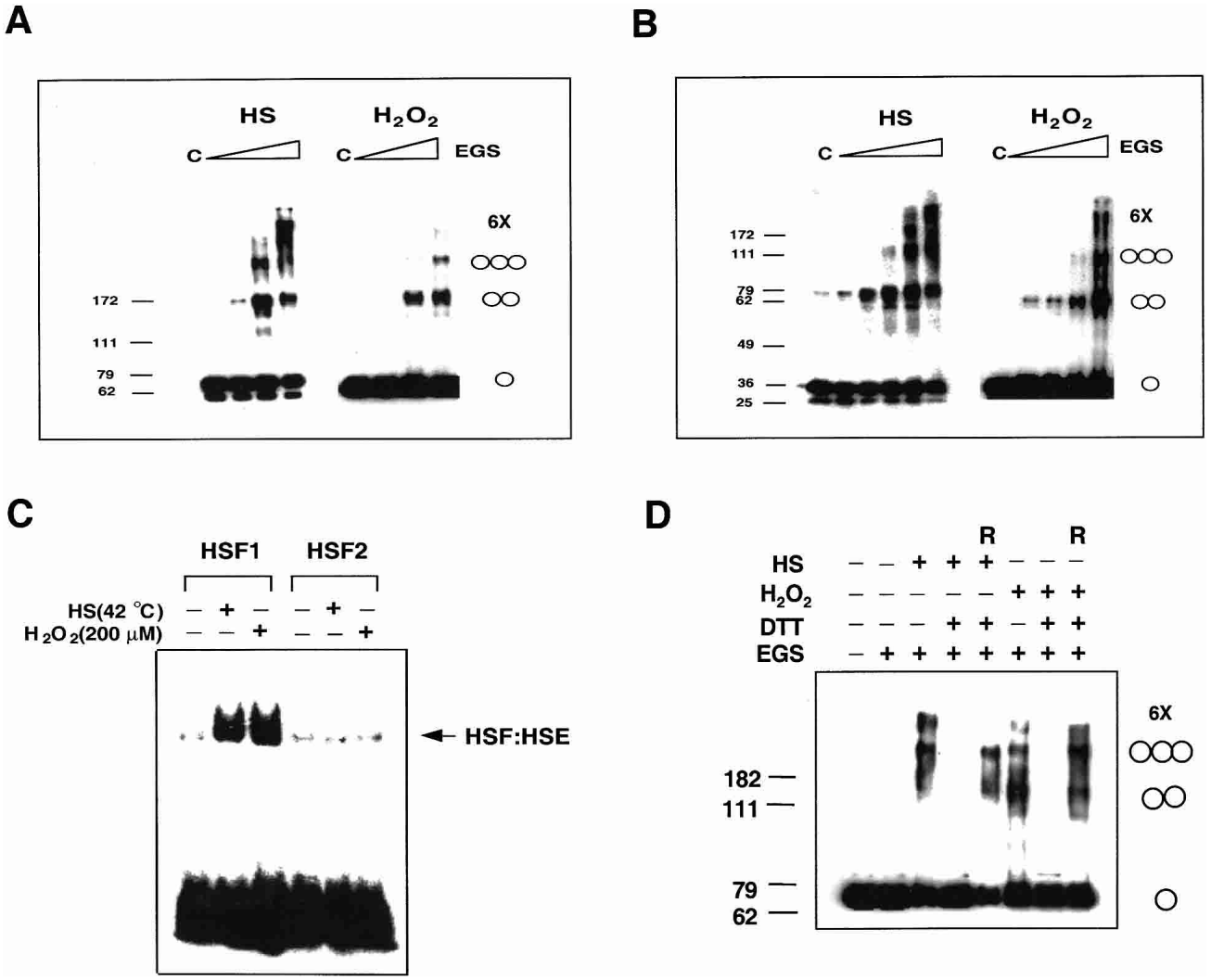

Figure 1. HSF1 is reversibly activated in vitro in a redox-regulated manner. (*A*) Recombinant mouse HSF1 was purified as a monomer and treated by either heat shock (HS) at 42°C for 5 min or with H_2O_2 (200 μM at 15°C for 5 min), cross-linked with increasing concentrations of EGS (wedge) at room temperature for 20 min, resolved by SDS-PAGE, and detected by immunoblotting. The positions of protein mass standards are shown on the *left*, and the positions of HSF1 monomers, dimers, trimers and higher-order multimers are shown on the *right*. (*B*) Activation of HSF1 multimerization is independent of the C-terminal region. HSF1(1–290) was treated as for the full-length protein in *A*. (*C*) In vitro activation by heat shock and H_2O_2 is HSF1 isoform-specific. Recombinant HSF1(1–290) and HSF2(1–280), purified as described for full-length HSF1 were treated (+) with heat shock or H_2O_2 as described in *A*, followed by incubation with a ^{32}P-labeled HSE DNA fragment and analysis by EMSAs. The arrow indicates the position of the HSF–HSE protein DNA complex. (*D*) HSF1 activated by heat shock (HS) or H_2O_2 is reversibly inactivated by reductant. Following HSF1 activation by heat shock or H_2O_2 as in *A*, DTT was added to a final concentration of 5 mM for 30 min. DTT was removed by dialysis and samples restressed (R) as in *A*, followed by EGS cross-linking and resolution by SDS-PAGE and immunoblotting.

minal 290 amino acid residues, is specifically and directly activated in vitro by heat and H_2O_2 in a reversible redox-regulated manner.

HSF1 activation by heat or H_2O_2 initiates disulfide bond formation

The direct activation of HSF1 in vitro by both heat and H_2O_2, and its reversibility by DTT, strongly suggest that HSF1 directly senses these stresses via changes in redox state. This notion is further supported by previous observations in vivo showing that the presence of DTT, or hypoxic conditions, inhibits heat shock activation of Hsp gene expression (Huang et al. 1994). Because cysteine residues are responsive to cellular redox states, we inspected the mouse HSF1 sequence for the presence and location of cysteine, and we detected five cysteine residues throughout the length of HSF1. Three cysteines, located within HSF1 amino acid residues 1–290, map to positions 35, 105, and 152 with cysteine 35 localized within the β1 strand of the highly conserved HSF DNA-binding domain (Harrison et al. 1994). Cys105 is found in

the linker domain immediately after β4 of the DNA-binding domain, and Cys152 is located within the HSF1 trimerization domain. Because our mutational analysis demonstrated no significant contribution of Cys152 to stress-inducible activation of HSF1, we focused on Cys35 and Cys105, conserved in all mammalian species for which the HSF1 sequence is available, represented on the three-dimensional structural model of the HSF DNA-binding domain shown in Figure 2A.

To address whether either or both of the Cys35 or Cys105 residues are important for the redox-dependent stress-responsive activation of HSF1 in vitro, we independently altered Cys35 and Cys105 to serine by site-directed mutagenesis, purified the recombinant proteins, and assayed for stress-inducible trimerization by SDS-PAGE under reducing and nonreducing conditions. Spectral analysis of the HSF1 wild-type and point mutants, before and after the heat or H_2O_2 stress treatments, indicated no overall defects in protein structure or aggregation (data not shown). As shown in Figure 2B, wild-type HSF1 is detected as stress-inducibly multimerized species on nonreducing SDS-PAGE, in response to both heat shock and H_2O_2, in a redox-dependent manner. This stress-induced multimerization pattern is completely inhibited by inclusion of β-mercaptoethanol in the SDS-PAGE. In contrast, mutagenesis of either Cys35 (Fig. 2C) or Cys105 (Fig. 2D) to serine abolished stress-inducible HSF1 homomultimerization and DNA binding (data not shown), and the HSF1 C35S,C105S double mutant had an indistinguishable behavior from that of ei-

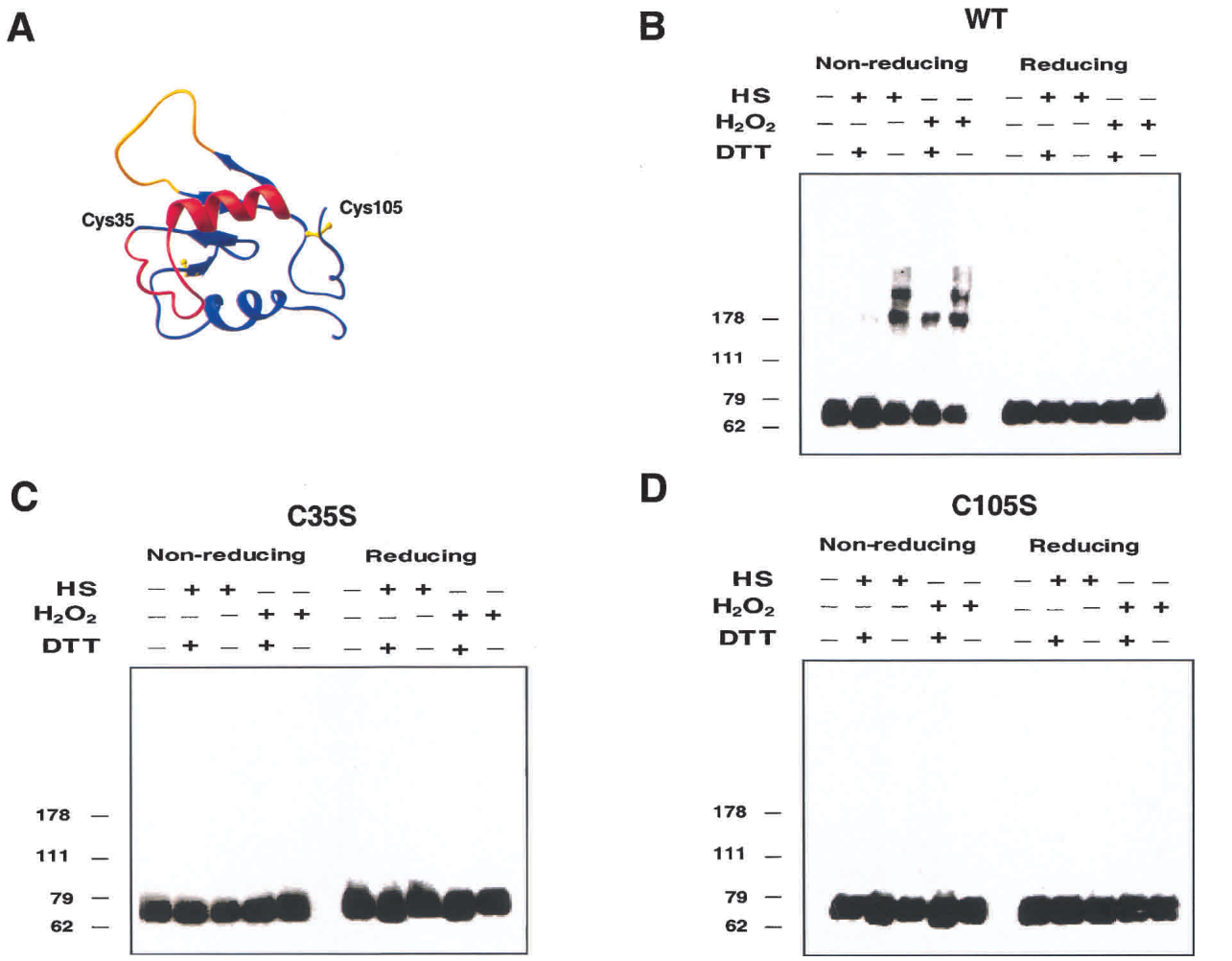

Figure 2. HSF1 cysteine residues are required for the activation and stability of homomultimers. (*A*) A structural model of the mammalian HSF1 DNA-binding domain, and the positions of Cys35 and Cys105 modeled into the structural coordinates for the *Kluyveromyces lactis* HSF DNA-binding domain structure (Harrison et al. 1994). The HSF1 helix-turn-helix DNA-binding domain is shown in red, the loop domain is shown in yellow, and cysteines at positions 35 and 105 are shown in ball-and-stick models in yellow. This model predicts that C35 and C105 are separated by ~20Å. (*B–D*) Stress-induced HSF1 multimerization correlates with disulfide bond formation. The purified HSF1 wild-type, HSF1 C35S, or HSF1 C105S proteins were incubated in the absence of stress (–) or in the presence of heat shock (HS) or H_2O_2 (+) as in Figure 1D. DTT was added to a final concentration of 5 mM for 30 min at room temperature; samples were mixed with equal volumes of 2× SDS sample buffer with or without β-ME, followed by SDS-PAGE and immunoblotting with anti-HIS antiserum.

ther single mutant (data not shown). The specificity of these results is supported by the observations that a C152S or a V56A mutation had no discernible effect on HSF1 activation in vitro or in vivo (data not shown; see below).

We observed that heat shock or H_2O_2 activation of mammalian HSF1 homomultimerization is dependent on two cysteine residues (C35, C105) and is reversed by reducing agents. These data suggest the possibility that a redox-dependent disulfide bond, involving C35 and C105, may be important for the stress-dependent conversion of recombinant HSF1 from inactive monomers to DNA-binding competent homotrimers. To further test this hypothesis, the reactivity of HSF1 cysteine residues towards modification with the thiol specific alkylating agent ^{14}C-iodoacetic acid was probed before and after stress-induced activation. HSF1(1–290) was heat shocked at 42°C or treated with 200 μM H_2O_2 for 10 min, carboxymethylated with ^{14}C-iodoacetic acid, followed by resolution on SDS-PAGE and PhosphorImaging. As shown in Figure 3, wild-type HSF1(1–290) was highly carboxymethylated when treated under control conditions, and this modification was significantly reduced after the protein was either heat shocked or treated with H_2O_2. A small but significant level of residual modification occurred with HSF1(1–290) that had been incubated under activating conditions. In contrast to the wild-type HSF1(1–290) protein, there was no significant change in carboxymethylation before or after treatment with heat shock or H_2O_2 for both the HSF1(1–290) C35S and HSF1(1–290) C105S mutants, although the overall carboxymethylation levels were decreased compared to the wild-type HSF1(1–290), due to the absence of one cysteine in these mutants that would otherwise be carboxymethylated. Consistent with a normal HSF1 activation phenotype for the HSF1(1–290) C152S mutant, and the V56A mutant, carboxymethylation of these HSF1 derivatives was reduced in a manner similar to that of wild-type HSF1(1–290) in response to either heat shock or H_2O_2 treatment. Coomassie blue staining of all of the samples used in these experiments demonstrated that equivalent amounts of wild-type and mutant forms of HSF1 protein were used in all reactions. Taken together, the reduction in HSF1 reactivity with ^{14}C-iodoacetate upon activation, and the loss of this change in reactivity with the C35S or C105S mutants, support the hypothesis that disulfide bond formation is an integral part of the HSF1 intrinsic stress activation mechanism.

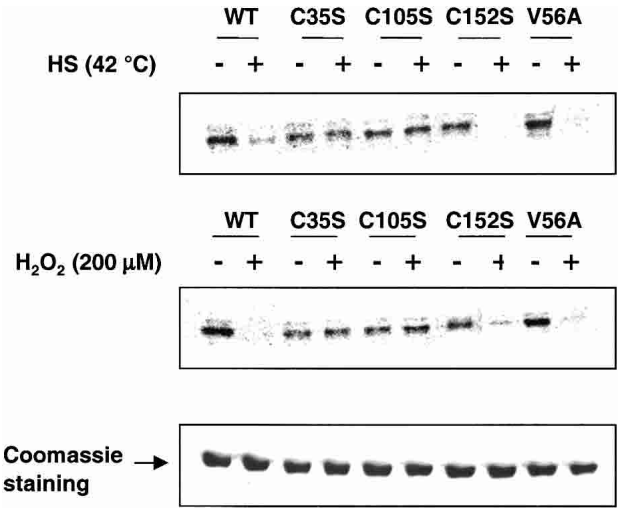

Figure 3. Stress protects HSF1 cysteine residues from modification. Wild-type HSF1 and the HSF1 C35S, HSF1 C105S, HSF1 C152S, or HSF1 V56A mutants were incubated under control (–), heat shock (HS) conditions, or in the presence of H_2O_2 (+) and carboxymethylated with ^{14}C-iodoacetate, followed by SDS-PAGE and image analysis. The *bottom* panel shows Coomassie blue staining of the purified HSF1 used in these experiments.

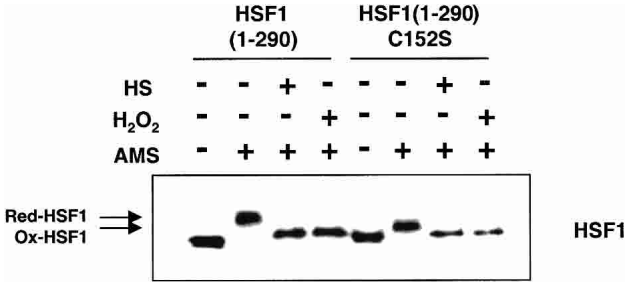

Figure 4. Changes in HSF1 redox state in vivo as a function of heat shock and H_2O_2 stress. MEF *hsf1*$^{-/-}$ cells were transfected with plasmids expressing either HSF1(1–290) or HSF1(1–290) C152S, and either untreated (–) or treated with heat shock at 42°C or 500 μM H_2O_2 for 1 h (+). Cell lysates were incubated in the presence (+) or absence (–) of AMS, followed by SDS-PAGE and immunoblotting with anit-HSF1 antiserum. Arrows indicate the positions of oxidized (Ox) and reduced (Red) HSF1 as inferred from AMS reactivity.

The results described above demonstrate that both heat shock and H_2O_2 treatment in vitro directly activate mammalian HSF1 from the inactive monomer to a homotrimeric form capable of high-affinity sequence-specific DNA binding. In parallel with this activation, the reactivity of Cys35 and Cys105 to alkylating agents is dramatically reduced. To ascertain whether these same stresses elicit changes in HSF1 redox state in vivo, we used a method previously shown to monitor the oxidation state of the bacterial H_2O_2 sensing transcription factor OxyR in vivo, via the cysteine alkylating agent 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS; Åslund et al. 1999). The alkylation of a cysteine residue by AMS adds ~500 Da to target proteins, and can be observed by changes in electrophoretic mobility by SDS-PAGE and immunoblotting. Mouse embryonic fibroblasts (MEF) hsf1$^{-/-}$ cells were transfected with vectors expressing either HSF1(1–290) or the HSF1(1–290) C152S mutant, treated with or without stress, followed by AMS modification of total cellular reduced cysteine residues and immunoblotting with anti-HSF1 antiserum. As shown in Figure 4, HSF1(1–290) from cells that were treated with AMS under control culture conditions migrated more slowly through SDS-PAGE than unmodified HSF1(1–290), consistent with alkylation at HSF1(1–290) cysteine residues. However, consistent with C35

and C105 engaging in disulfide bonds, AMS treatment after either heat shock or H_2O_2 stress resulted in only a slight decrease in electrophoretic mobility of HSF1(1–290). Consistent with this residual HSF1(1–290) modification occurring at cysteine 152 after stress, the HSF1(1–290) C152S mutant displayed little if any electrophoretic mobility change when comparing cell extracts not treated with AMS to extracts treated with AMS following either heat shock or H_2O_2 stress (Fig. 4). Taken together, these data suggest that the redox activation of HSF1 homotrimerization observed in vitro is paralleled by stress-dependent changes in HSF1 redox state in vivo.

Activation of HSF1 in vivo requires Cys35 and Cys105

The activation of HSF1 in vivo is a multistep process involving the monomer to trimer transition, nuclear accumulation, DNA binding, and Hsp gene *trans*-activation. To begin to understand the physiological importance of HSF1 activation by the redox-dependent disulfide bond formation observed in vitro, we analyzed the ability of the wild-type HSF1 protein and the C35S and C105S mutant proteins to participate in multiple steps in this activation pathway. Because HSF1 multimerization is required for sequence-specific, high-affinity binding to HSEs, we examined the ability of the wild-type HSF1 protein, and the C35S and C105S mutants that are defective in multimerization and HSE binding in vitro, to be stress-activated for DNA binding in vivo. As tools for these experiments, a series of isogenic stable cell lines derived from *hsf1*$^{-/-}$ MEFs were generated in which the expression vector pCDNA3.1/hgy, the same vector expressing wild-type HSF1, or the indicated HSF1 mutant alleles was introduced. As shown in Figure 5A, cell lines were identified by immunoblotting that express indistinguishable levels of either wild type or the indicated mutant derivatives of HSF1. As shown in Figure 5B and C by EMSA analysis using nuclear extracts, treatment of cells expressing wild-type HSF1 (WT) with either heat shock (42°C for 1 h) or 500 μM H_2O_2 at 37°C for 1 h gave

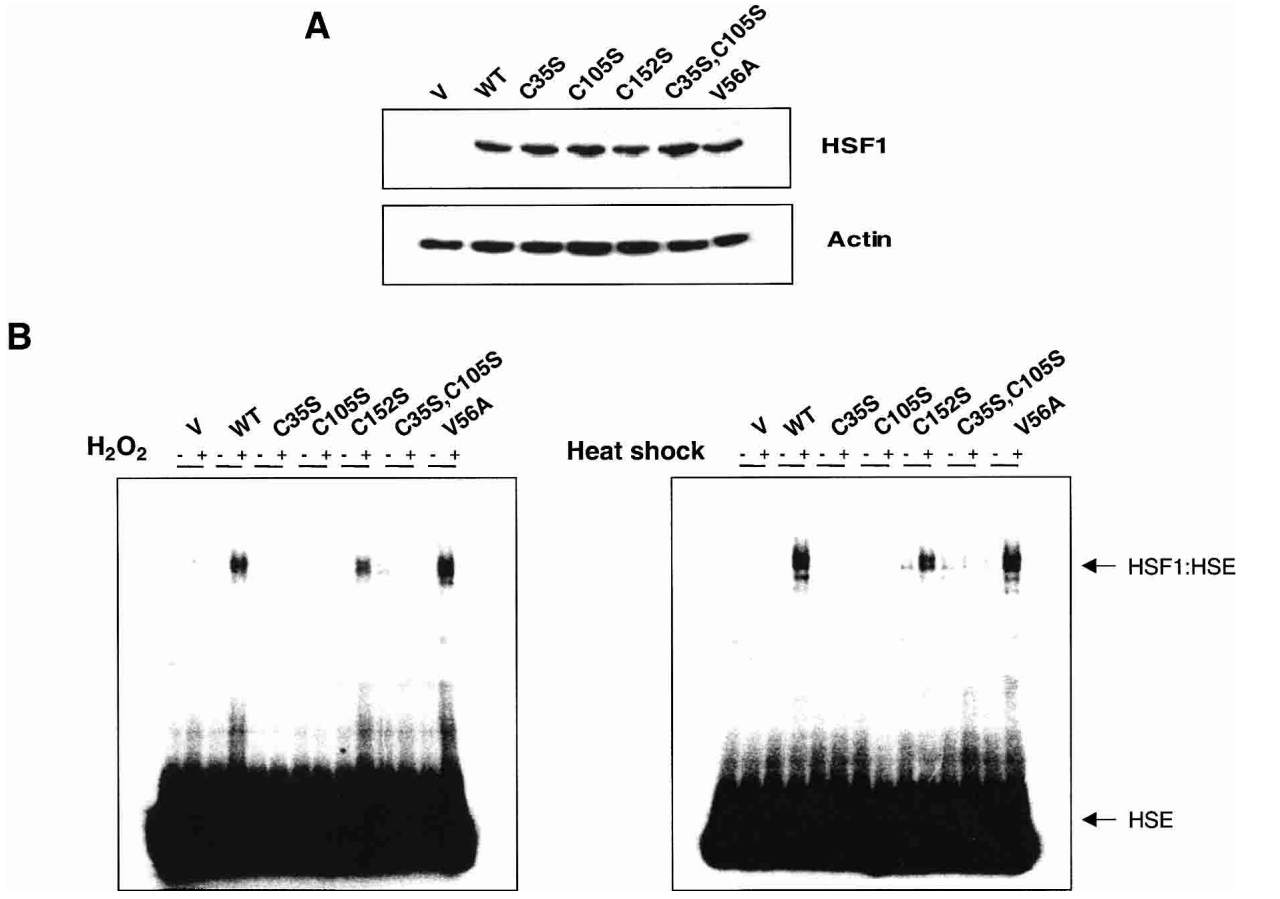

Figure 5. Redox regulation is essential for HSF1 activation in vivo. (*A*) *hsf1*$^{-/-}$ MEFs were used to generate stable cell lines harboring the empty vector (V) or expressing wild-type (WT) or the indicated mutants of HSF1. Immunoblotting, using actin levels as a loading control, demonstrates that the cell lines selected express equivalent levels of HSF1 protein. (*B*,*C*) The stress-inducible DNA-binding activity from cell lines expressing wild-type and the indicated mutant forms of HSF1 was assayed by EMSA using a ^{32}P-labeled HSE DNA fragment from the Hsp70 promoter. Cell cultures were treated at 37°C for control conditions (–) or with stress conditions (+) via 500 μM H_2O_2 at 37°C (*B*) or heat shock at 42°C for 1 h (*C*). The arrows indicate the positions of the free HSE DNA fragment and the HSF1–HSE protein DNA complex.

rise to activated HSF1 that robustly bound the Hsp70 HSE element. Furthermore, little HSE binding activity was observed in nuclear extracts from either unstressed cells or *hsf1*$^{-/-}$ cells that had stably integrated the vector alone. In contrast, neither the HSF1 C35S, nor the C105S or the double mutant (HSF1 C35,105S) were activated by heat stress or H_2O_2 treatment, whereas the C152S and V56A mutants, fully functional in our in vitro assays, were stress-activated in a manner essentially indistinguishable from the wild-type HSF1 protein. We also observed HSF1 EGS-cross-linking results which paralleled the DNA-binding data, using whole cell extracts from control, heat shock-, and H_2O_2-treated cells (data not shown). Taken together, these data demonstrate that the redox activation of HSF1 that is dependent on C35 and C105 in vitro is essential for stress activation of HSF1 DNA-binding activity in nuclear extracts.

Previous studies established that, in many cell types, stress-activated HSF isoforms are cytosolic in the absence of stress and are translocated to the nucleus as one key step in response to stress (Sarge et al. 1993; Zuo et al. 1995; Zandi et al. 1997). Because the HSF1 C35S, HSF1 C105S, and HSF1 C35S,C105S mutants are defective in stress-induced multimerization and DNA binding in vitro, and nuclear extracts from cells expressing the C35S or C105S mutants exhibit no stress-inducible HSE DNA-binding activity, we analyzed these mutant derivatives for their subcellular location under control conditions and in response to heat shock or H_2O_2. Mouse *hsf1*$^{-/-}$ cells were transiently transfected with plasmids expressing wild-type HSF1-Flag, or the Flag epitope-tagged HSF1 C35S, HSF1 C105S, HSF1 C35,105S or HSF1 V56A mutants. Indirect immunofluorescence microscopy demonstrated that under normal culture conditions, each of the HSF1 derivatives were diffusely localized in cells under steady-state conditions (Fig. 6A). In response to either heat shock or H_2O_2 treatment, HSF1-Flag and the Flag-epitope-tagged functional HSF1 V56A mutant concentrated in cell nuclei. However, neither the HSF1 C35S, HSF1 C105S nor the HSF1 C35,105S double mutant concentrated in the nucleus in response to either stress (Fig. 6A). To independently assess HSF1 subcellular localization in response to stress, the Flag-tagged HSF1 wild-type and C35,105S mutant derivative were expressed in *hsf1*$^{-/-}$ cells, and their distribution was assessed by biochemical fractionation and immunoblotting experiments before and after exposure to heat shock or H_2O_2. As shown in Figure 6B (top panel), whereas both wild-type HSF1 and the HSF1 C35S,105S mutant were found predominantly in the cytosolic fraction before stress, wild-type HSF1 accumulated essentially quantitatively in the nucleus in response to either heat shock or H_2O_2 exposure. In contrast, the HSF1 C35,105S derivative accumulated predominantly in the cytosol in response to both stress conditions, with little stress-induced accumulation in the nuclear fraction evident. Immunoblotting using these same extracts revealed that the *c-fos* protein was nuclear in all cases (Fig. 6B, middle panel) and did not change its distribution in response to stress, whereas actin remained largely cytosolic under all conditions (Fig. 6B, lower panel). Analysis of the HSF1 C35S or C105S mutants gave subcellular distributions indistinguishable from that of the HSF1 C35,105S double mutant (data not shown). Taken together, these experiments demonstrate that the redox-regulated formation of disulfide bonds in response to stress that is essential for HSF1 activation in vitro is also essential for HSF1 stress activation and nuclear accumulation in vivo. Furthermore, because the HSF1 C35,105S mutant is defective for stress-activated multimerization and DNA binding in vitro and in vivo, these experiments suggest that HSF1 multimerization is a prerequisite for stress-induced nuclear translocation.

Function of HSF1 in gene activation and stress protection in vivo requires redox regulation

Biochemical and genetic studies have clearly demonstrated critical roles for mammalian HSF1 in stress-inducible Hsp gene expression, resistance to stress-induced programmed cell death, extra-embryonic development, and other biological functions (McMillan et al. 1998; Xiao et al. 1999; Christians et al. 2000; Zhang et al. 2002). To ascertain whether the redox regulation of HSF1 plays an important role in Hsp target gene activation, the *hsf1*$^{-/-}$ MEF stable cell lines expressing wild-type or mutant HSF1 derivatives were treated under control conditions or with heat shock or H_2O_2, and the expression of known HSF1-dependent target genes (McMillan et al. 1998) was analyzed by immunoblotting. As shown in Figure 7A and B, *hsf1*$^{-/-}$ cells that ectopically express wild-type HSF1 restore inducible expression of Hsp70 and Hsp27 in response to both heat shock and H_2O_2 treatment. In contrast, neither the HSF1 C35S, C105S nor the C35,105S double mutant restored normal induction of these two target genes in response to either stress, although Hsp70 exhibited slightly less dependency on wild-type HSF1 compared to Hsp27. This residual activation of Hsp70 expression could be a consequence of the previously observed stress-induced posttranscriptional stabilization of Hsp70 mRNA (Kaarniranta et al. 1998).

It is well established that the presence of the HSF1 gene, and the expression of Hsps including Hsp70 and Hsp27, are important to protect cells from stress-induced apoptosis (McMillan et al. 1998; Xiao et al. 1999). We ascertained whether the redox activation of HSF1, via C35 and C105, is important for the protection of mouse embryonic fibroblasts from stress-induced apoptosis by quantitative ELISA assays for apoptosis-specific antigens using the stable cell lines expressing wild-type HSF1 and mutant derivatives. As shown in Figure 8, and as previously demonstrated for heat shock (McMillan et al. 1998), MEF cells lacking a functional *hsf1* gene are highly sensitive to both heat shock- and H_2O_2-induced apoptosis. However, these cells are largely protected from both heat shock and H_2O_2-induced apoptosis in the stable cell line in which wild-type HSF1 expression has been restored. In contrast, expression of the HSF1 C35S, HSF1 C105S or HSF1 C35,105S double mutant in *hsf1*$^{-/-}$

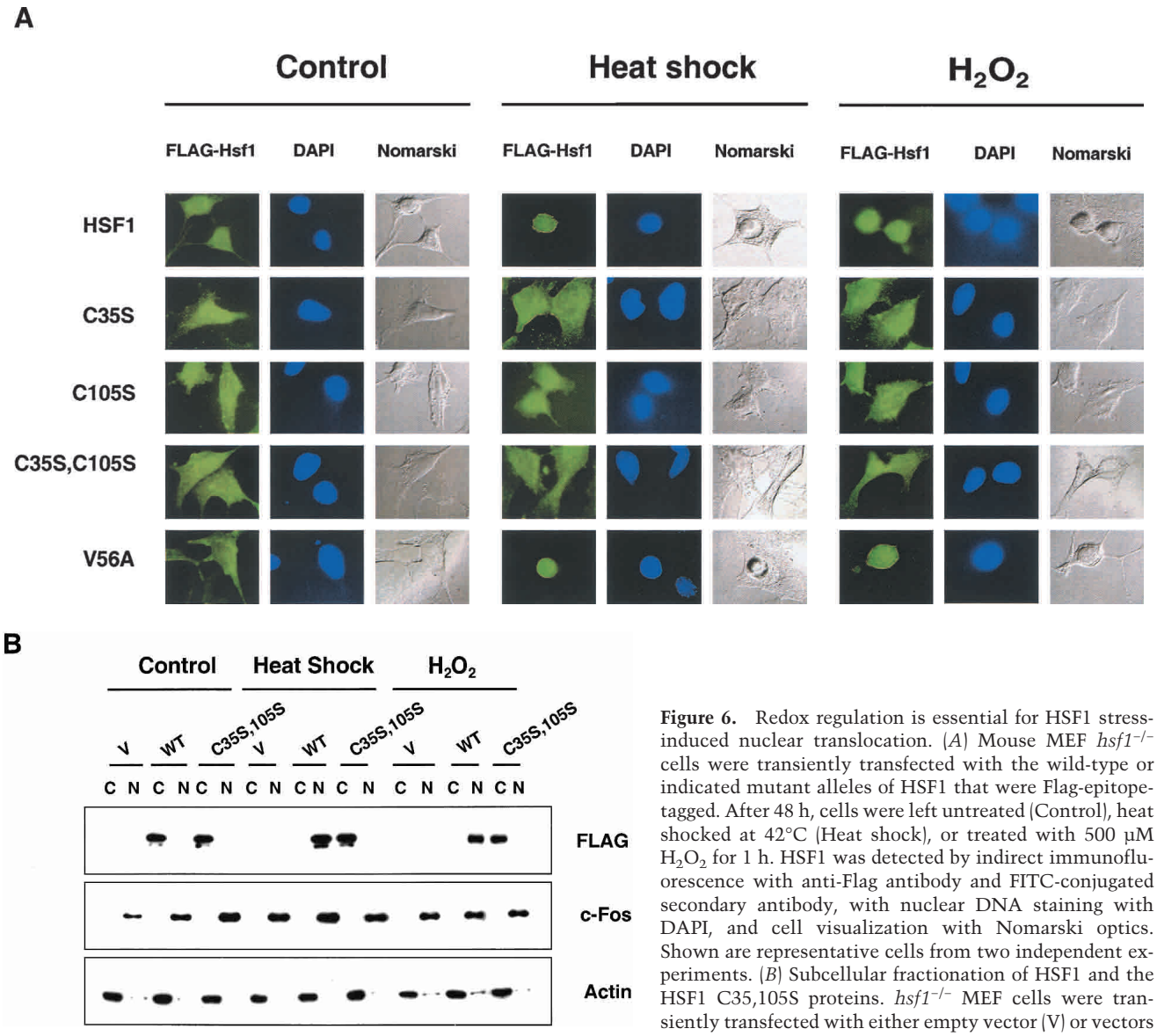

Figure 6. Redox regulation is essential for HSF1 stress-induced nuclear translocation. (*A*) Mouse MEF *hsf1*$^{-/-}$ cells were transiently transfected with the wild-type or indicated mutant alleles of HSF1 that were Flag-epitope-tagged. After 48 h, cells were left untreated (Control), heat shocked at 42°C (Heat shock), or treated with 500 μM H_2O_2 for 1 h. HSF1 was detected by indirect immunofluorescence with anti-Flag antibody and FITC-conjugated secondary antibody, with nuclear DNA staining with DAPI, and cell visualization with Nomarski optics. Shown are representative cells from two independent experiments. (*B*) Subcellular fractionation of HSF1 and the HSF1 C35,105S proteins. *hsf1*$^{-/-}$ MEF cells were transiently transfected with either empty vector (V) or vectors expressing wild-type (WT) or the C35,105S HSF1 mutant. Cells were treated under control (–), heat shock, or H_2O_2 (+) conditions, fractionated into nuclear and cytosolic components, and resolved by SDS-PAGE and HSF1-Flag; actin and c-fos were detected by immunoblotting with ant-Flag, anti-actin, and anti-c-fos antibody as described in Materials and Methods.

cells only slightly protected MEF cells from heat- or H_2O_2-induced apoptosis. Taken together, the redox-activation of HSF1 via C35 and C105 is essential for the expression of stress-inducible anti-apoptotic Hsp genes and for the protection of mouse embryonic fibroblasts from stress-induced apoptosis.

Discussion

The eukaryotic heat shock response can be induced by a variety of environmental, chemical, and pathophysiological conditions (for review, see Morimoto et al. 1996; Morano and Thiele 1999; Santoro 2000; Pirkkala et al. 2001). Although these stressors are distinct in origin, they have the commonality that they activate the multimerization and DNA-binding activity of the stress-responsive mammalian HSF1 isoform. Nonetheless, the precise mechanisms by which this multitude of diverse signals is transmitted to and interpreted by HSF1 are not yet clear. While the strength and duration of the heat shock response are modulated by HSF intramolecular interactions, a number of protein kinases, Hsps, and other cellular factors, HSF1 from mammals and *Drosophila* HSF possess intrinsic stress-sensing capability (Goodson and Sarge 1995; Morimoto 1998; Zhong et al. 1999; Ahn et al. 2001). In this work we demonstrate that homotrimerization of mammalian HSF1 is directly activated in vitro by both heat stress and H_2O_2, in a redox-regulated,

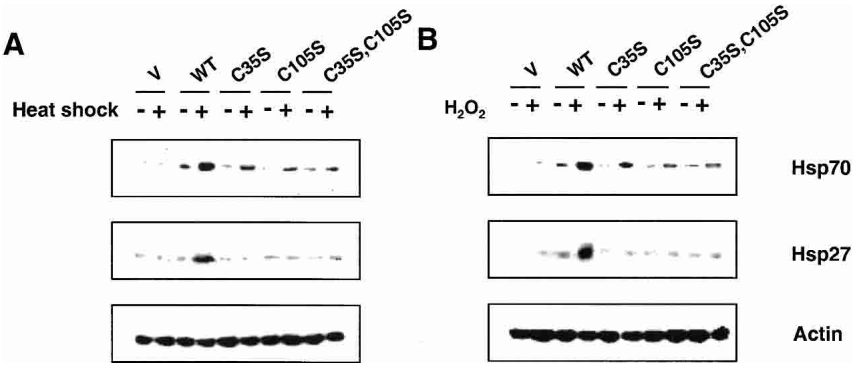

Figure 7. HSF1 redox activation is required for Hsp induction in response to heat shock and H_2O_2 stress. *hsf*$^{-/-}$ MEF stable cells lines with either vector or expressing the wild-type and indicated HSF1 alleles were treated under control (37°C), heat shock (42°C for 1 h; *left* panel), or H_2O_2 (500 µM for 24 h; *right* panel) conditions. After a 24-h recovery, total cell extracts were prepared, then fractionated by SDS-PAGE, and Hsp70, Hsp27, and actin were detected by immunoblotting with the respective protein-specific antibody.

reversible fashion that requires cysteine residues at two positions within or adjacent to the DNA-binding domain. Cysteine residues required for HSF1 redox regulation are essential for stress-activation of HSF1 multimerization, DNA binding, nuclear accumulation, Hsp target gene activation, and the protection of mammalian cells from stress-induced apoptosis.

Our data demonstrating redox-regulated HSF1 activation involving C35 and C105 strongly suggest that these two cysteine residues are engaged in disulfide bonds, the formation of which is essential for HSF1 homomultimerization in vitro and in vivo. Although the precise nature and function of the disulfide bonds that are formed are currently unknown, our circular dichroism results (S.-G. Ahn and D.J. Thiele, unpubl.) demonstrate that in concert with stress-induced disulfide bond formation, HSF1 undergoes significant conformational changes. These structural changes may either facilitate homomultimerization or be a consequence of multimerization. One possibility is that C35 and C105 could form intramolecular disulfide bonds, which may poise or stabilize HSF1 in a conformation that allows intermolecular homotrimerization via other protein–protein interaction domains such as the conserved coiled-coil region. Alternatively, intermolecular interactions could form such that C35 from one monomer is engaged in disulfide bonds with C105 from another member of the homotrimer. In either case, the formation of HSF1 multimers could be facilitated or stabilized by contributions from the coiled-coil domains, the loop within the DNA-binding domain, the linker, or other regions known to contribute to HSF1 multimerization and DNA-binding cooperativity (Rabindran et al. 1993; Liu and Thiele 1999; Ahn et al. 2001). However, our studies demonstrate that the maintenance of this homotrimeric structure is redox-regulated in vitro, and both heat shock and H_2O_2 stress induce concomitant changes in HSF1 redox state in vivo.

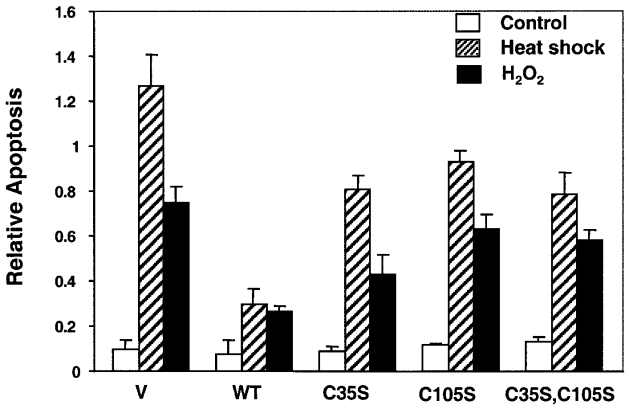

Figure 8. HSF1 redox activation protects cells from stress-induced apoptosis. Hsf1$^{-/-}$ stable cell lines harboring the empty vector (V), wild-type HSF1 (WT), or the indicated mutant derivatives were incubated under control conditions (37°C), exposed to heat shock (44°C for 1 h followed by 6 h at 37°C), or 500 µM H_2O_2 at 37°C for 6 h. Relative apoptosis of these cultures was determined as described in Materials and Methods and plotted on the *y* axis as the Relative Apoptosis index.

It is interesting to note that whereas C35 is conserved in essentially every stress-responsive metazoan HSF isoform, C105 is positionally conserved in HSF1 from mouse, rat, and humans, organisms that encode a family of heat shock transcription factors with specialized and distinct functions (for review, see Wu 1995; Pirkkala et al. 2001). *Drosophila* HSF, previously demonstrated to undergo direct heat and H_2O_2 sensing in vitro (Zhong et al. 1998), lacks a C105 equivalent. Although it is currently unknown whether C35 is important for *Drosophila* stress sensing, organisms with a single HSF gene may utilize mechanisms that are similar, though not identical to that of the dedicated and specialized stress-sensing HSF1 isoform of mammals. It is also interesting to note that the single *Saccharomyces cerevisiae* HSF completely lacks cysteine residues. Although yeast HSF appears to have both constitutive and stress-enhanced DNA-binding activity, the biochemical mechanisms by which stress signals are transmitted to this protein are not currently understood.

Our analysis of HSF1 and mutant derivatives by indirect immunofluorescence microscopy and subcellular fractionation experiments demonstrates that the HSF1 C35S, HSF1 C105S and the HSF1 C35,105S mutants, defective in stress-induced multimerization in vivo and in vitro, are also defective for stress-induced nuclear accumulation. This observation suggests that HSF1 multimerization may be a prerequisite for nuclear accumu-

lation, perhaps by exposing an otherwise quiescent nuclear localization signal in the monomer. Alternatively, the HSF1 homotrimer could mask a nuclear export signal or abrogate interactions with a nuclear export protein as a consequence of homotrimerization. Such stress-dependent changes in nuclear export have been demonstrated for the yeast Yap1 protein, which engages in redox-regulated interactions with a nuclear export protein, Crm1 (Kuge et al. 1998; Delaunay et al. 2000). Consistent with a requirement for HSF1 multimerization as a prerequisite for nuclear accumulation, previous studies have shown a tight temporal association between HSF1 trimerization and nuclear localization, and that HSF1, when overexpressed in HeLa cells, accumulated as the monomeric form in the cytoplasm and the multimeric form in the nucleus (Sarge et al. 1993; Zuo et al. 1995). Although additional studies must be conducted to test this hypothesis, it is clear that redox-regulation of HSF1 multimerization, and nuclear accumulation are early and tightly linked steps in HSF1 activation.

In this report we demonstrate that both heat stress and H_2O_2 activate HSF1, in vitro and in vivo, via redox regulation of disulfide bonds involving C35 and C105. Hydrogen peroxide-induced disulfide bond formation is well established as a regulatory mechanism in proteins such as the OxyR transcription factor and the Hsp33 stress-activated protein chaperone (Zheng et al. 1998; Åslund and Beckwith 1999; Jakob et al. 1999; Choi et al. 2001; Kim et al. 2002). However, it is currently unclear how heat shock would stimulate the formation of disulfide bonds, or whether the precise biochemical mechanisms that initiate HSF1 disulfide bond formation are identical with respect to heat and H_2O_2 stress. Metals such as copper or iron, present in the in vitro reactions and in vivo, could engage in well characterized redox chemistry with oxygen that would generate reactive oxygen species capable of initiating disulfide bond formation (Stadtman 1990; Demple et al. 1999). Although a direct analysis of how heat and H_2O_2 activate HSF1 trimerization must be carried out, a number of previous observations support a mechanistic relationship between changes in cellular redox state and conditions that cause activation of the heat shock response. Studies in yeast have demonstrated that oxidative stress protection pathways play an important role in protection from heat-induced cell death and that the cytotoxic effects of heat shock require oxygen (Davidson et al. 1996; Jamieson 1998; Davidson and Schiestl 2001). Secondly, studies in mammalian cells have demonstrated that the activation of HSF1, and the transcriptional induction in response to heat shock, are dramatically inhibited under conditions of hypoxia or in the presence of reducing agents such as DTT (Huang et al. 1994; Jacquier-Sarlin and Polla 1996). Furthermore, the observations that specific genes are induced in bacterial cells by both heat shock and H_2O_2 strongly support the possibility that these two stresses could generate similar intracellular signals or consequences (Morgan et al. 1986; Zheng et al. 2001).

Studies have demonstrated that, in addition to heat shock, treatment of eukaryotic cells with uncouplers of oxidative phosphorylation, cadmium, H_2O_2, alcohol, pro-oxidants, nonsteroidal antiinflammatory drugs and other chemicals, as well as infection and inflammation and other pathophysiological states will activate the trimerization and DNA binding of HSF1 (Mosser et al. 1990; Jurivich et al. 1992; Jacquier-Sarlin and Polla 1996; Morimoto et al. 1996; Santoro 2000; Pirkkala et al. 2001). Although these disparate conditions all activate HSF1 multimerization in vivo, previous work from *Drosophila* (Zhong et al. 1998) and our work on mammalian HSF1 (S.-G. Ahn and D.J. Thiele, unpubl.) indicate that only a subset of these conditions activate HSF in vitro. One potential common feature of these HSF-activating conditions is the generation, either directly or through metabolic transformations in vivo, of reactive oxygen species that could initiate the formation of disulfide bonds to trigger HSF1 homomultimerization. HSF1 is known to be associated with Hsps and other proteins both before and after activation, and these interactions could serve to set thresholds for HSF1 activation in response to conditions that could generate changes in cellular redox status demanding transcriptional regulatory responses. Perhaps different levels of oxidative stress protection and distinct levels of cellular reductant such as glutathione, in different cells, tissues, or organisms, may play a role in marking the distinct set-point temperature at which HSF is known to be activated.

Materials and methods

Generation and growth of cell lines

Mouse embryonic fibroblast (MEF) cells derived from congenic wild-type mice and *hsf1*$^{-/-}$ mice were a generous gift from Dr. Ivor Benjamin (University of Texas Southwestern Medical Center, Dallas, TX). The MEF cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. DMEM medium supplemented with 10% fetal bovine serum was used as a growth medium for all cell lines. To establish stable cell lines, HSF1 was amplified from pBK-*hsf1* containing the mouse *hsf1* cDNA by PCR and subcloned into the expression vector pCDNA3.1/hgy. The wild-type *hsf1* (pCDNA3.1/hgy-*hsf1*) or HSF1 cysteine mutants (pCDNA3.1/hgy-C35S, pCDNA3.1/hgy-C105S) were transfected into the MEF *hsf1*$^{-/-}$ cells by electroporation using conditions described previously (Ahn et al. 2001). After 48 h, transfected cells were selected in media containing 200 μg/mL hygromycin, and purified clones were further cultured in medium containing 200 μg/mL hygromycin. For heat shock treatment, culture plates were wrapped with parafilm and immersed in a water bath for the times and temperatures specified in the figure legends. Hydrogen peroxide at specified concentrations was added directly to culture dishes, and cultures were incubated at 37°C for the indicated times. Whole-cell lysates were prepared as described (Ahn et al. 2001) and resolved by denaturing SDS-PAGE followed by immunoblotting. Specific proteins were detected using the following antibodies: inducible Hsp70 with anti-Hsp70 (sc-24, Santa Cruz Biotechnology), anti-Hsp27 (SPA-815, StressGen), and anti-actin (C-11, Santa Cruz Biotechnology) and HSF1 and HSF2 with either specific polyclonal antiserum or anti-His antibody (QIAGEN).

Electrophoretic mobility shift analysis

Nuclear extracts were prepared from heat shock- or H_2O_2-treated MEF cell lines as described (Ahn et al. 2001). Five mi-

crograms of extract was incubated with ^{32}P-labeled HSE (heat shock element) consensus sequence oligonucleotide for 15 min at room temperature in binding buffer [20 mM HEPES at pH 7.6, 5 mM EDTA, 1 mM DTT, 150 mM KCl, 50 mM $(NH_4)_2SO_4$, 1% Tween-20 (v/v)]. Following electrophoresis on native 5% polyacrylamide gels, HSF1–HSE DNA complexes were visualized by autoradiography.

Purification of recombinant HSF1 and HSF2

The mouse GST-tagged HSF1 and HSF2 open reading frame was subcloned into the bacterial expression vector pGEX6p-1 and used as a template for single or double cysteine mutant constructs. The cysteine mutants of *hsf1* were constructed by site-directed mutagenesis, and all mutations were confirmed by DNA sequencing prior to protein expression in bacteria. *E.coli* BL21 (DE3) Codon Plus cells (Stratagene) were transformed with the respective expression plasmids. Cells were grown in 2× YT medium containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol to an OD_{600} of 0.7, and then the expression of GST-HSF1 fusion protein was induced by addition of 0.4 mM IPTG for 12 h at 20°C. For in vitro trimerization studies, HSF1 was purified from cells grown at 20°C, a condition under which HSF1 exists in the monomeric form (Ahn et al. 2001). Cells were harvested by centrifugation and resuspended in ice-cold lysis buffer containing 20 mM Tris-HCl at pH 8.0, 2 mM EDTA, 2 mM DTT, 300 mM NaCl, 1 mM benzamidine, 1 mM PMSF, 1 µg/mL aprotinine, leupeptin, and pepstatin, respectively. Resuspended cells were disrupted by sonication, and then Triton X-100 was added to 0.5% (v/v). The crude lysate was centrifuged at 12,500 rpm for 20 min at 4°C to remove the insoluble debris. The soluble extract was then incubated with glutathione Sepharose 4B affinity resin for 2 h at 4°C to bind the GST-tagged fusion protein, and the resin was washed three times for 5 min each with lysis buffer containing 0.5% Triton X-100, followed by three times for 5 min each wash with lysis buffer without detergent. Then the resin was washed two times for 10 min with a buffer containing ATP (20 mM Tris-HCl at pH 8.0, 20 mM MgCl, 5 mM ATP, 300 mM NaCl, 2 mM DTT) to remove any associated *E. coli* Dna-K protein. Prescission protease (Amersham Pharmacia Biotech) was added, and cleavage was carried out at 4°C for 10–12 h. For the purification of the resulting C-terminal His-tagged HSF1 fusion protein, Ni^{2+}-agarose affinity resin was used as described (Goodson and Sarge 1995). The purity of the HSF proteins was determined by Coomassie blue staining on SDS-polyacrylamide gels, and the absence of associated DnaK was verified by immunoblotting with anti-DnaK polyclonal antiserum. The lack of protein aggregation for either the wild-type HSF1 protein or all mutant derivatives was demonstrated by UV-VIS spectrophotometry before and after either heat shock or H_2O_2 treatment. Before or after stress treatment, samples were centrifuged at 4°C at 16,000*g* for 30 min, and the supernatants were removed and spectrophotometrically analyzed.

In vitro HSF cross-linking

For cross-linking experiments, purified HSF proteins were diluted into phosphate-buffered saline (PBS) to a final concentration of 4 µM and heat shocked (42°C) or H_2O_2-treated (200 µM, 15°C) for 5 min. EGS [ethylene glycol bis(succinimidyl succinate)] was added to final concentrations specified in figure legends, followed by incubation at 22°C for 15 min. After quenching of the cross-linking reactions with excess glycine, samples were resolved by electrophoresis through a 6% SDS-polyacrylamide gel and analyzed by immunoblotting with anti-His (QIAGEN) or anti-HSF isoform-specific antibody.

[^{14}C] carboxymethylation experiments

Wild-type HSF1 and HSF1 cysteine mutant derivatives were treated with H_2O_2 for 10 min at 15°C and then incubated with [^{14}C] iodoacetic acid (final concentration of 2.4 mM, 7.9 mCi/mmol, ICN) for 30 min. The carboxymethylation reaction was quenched by a 5-min incubation in the presence of 100 mM DTT, and the samples were resolved by electrophoresis on 12% SDS-PAGE, electrotransfer to nitrocellulose membrane, and PhosphorImaging (Denu and Tanner 1998).

Analysis of HSF1 redox state in vivo

Assays monitoring the oxidation state of HSF1 in vivo via alkylation of cysteine residues with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) were performed as described (Åslund et al. 1999). MEF *hsf*$^{-/-}$ cells were transfected with plasmids expressing either HSF1(1–290) or HSF1(1–290) C152S. After 48 h, cells were incubated under control or stress conditions as specified in the legend to Figure 4, and total cell extracts were prepared and incubated on ice for 1 h with trichloroacetic acid at a final concentration of 10% to protonate all thiols and precipitate total cell protein. The precipitates were centrifuged at 16,000 *g* for 15 min at 4°C, and the pellets were dissolved in 0.1% SDS/0.67 M Tris-HCl, at pH 8/15 mM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS; Molecular Probes). The samples were incubated at 37°C for 2 h, fractionated on 15% SDS-PAGE minigels (Bio-Rad), transferred to a nitrocellulose membrane, and probed with anti-HSF1 polyclonal antibodies.

Subcellular fractionation and indirect immunofluorescence microscopy

MEF *hsf1*$^{-/-}$ cells were transiently transfected with plasmids expressing wild-type or the indicated mutants of Flag-tagged *hsf1* by electroporation, and cells were plated on two-chamber tissue culture slides in complete medium. After 48 h, cells were rinsed twice with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, washed twice with PBS, and then permeabilized for 2 min with a solution containing methanol/acetone (1/1). The cells were incubated with anti-Flag monoclonal antibody (M2, Sigma-Aldrich) diluted 1:500 in 2% bovine serum albumin (BSA) and then with FITC-conjugated anti-mouse immunoglobulin G (IgG) antibody (1:1000 dilution, Sigma-Aldrich). Immunolabeled cells were visualized by fluorescence microscopy and photographed using a Hamamatsu ORCA-2 cooled CCD camera.

For subcellular fractionation experiments, mouse *hsf1*$^{-/-}$ cells were transfected with the Flag-tagged wild-type HSF1 or C35,105S mutant. After 48 h, cells were washed in PBS, resuspended in 400 µL of buffer A containing 10 mM HEPES at pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 5 mg/mL leupeptin, 0.5 mM PMSF and incubated on ice for 5 min. NP40 was added to a final concentration of 0.6%, and the mixture was centrifuged at 12,000 rpm at 4°C for 1 min. The supernatant (cytosolic fraction) was removed and the pellet (nuclear fraction) was resuspended in buffer C containing 20 mM HEPES at pH 7.9, 0.4 M NaCl, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.01% Triton X-100, and 0.5 mM PMSF. The samples were mixed, incubated on ice for 15 min, and centrifuged at 4°C, 12,000 rpm for 15 min, and the supernatant was then used as a source of nuclear extract. Nuclear and

cytosolic extracts were separated by SDS-PAGE, and nuclear and cytosolic proteins were detected by immunoblotting. Relative levels of HSF1-Flag in the cytosol and nucleus were estimated by densitometric scanning using the GeneGenius bioimaging system (Syngene).

Determination of HSF1 oxidation states by nonreducing SDS-PAGE

Recombinant HSF1 was treated with heat shock (for 5 min at 42°C) or H_2O_2 (for 5 min at 15°C). Immediately after stress treatments, samples were mixed with an equal volume of 2× SDS sample buffer containing βME (reducing conditions) or without βME (nonreducing conditions), incubated at 100°C for 5 min, followed by resolution on 6% SDS-PAGE and immunoblotting using anti-His antibody.

Apoptosis measurements

$Hsf1^{-/-}$ stable cell lines harboring the empty vector (V), wild-type HSF1 (WT), or the indicated mutant derivatives were incubated under control conditions (37°C), exposed to heat shock (44°C for 1 h followed by 6 h at 37°C), or to 500 μM H_2O_2 at 37°C for 6 h. Apoptosis was measured using a cell death detection ELISA kit (Boehringer Mannheim). Relative apoptosis correlates with absorption at 405 nm with a reference wavelength of 490 nm per the manufacturer's instructions.

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