Two Distinct Disulfide Bonds Formed in Human Heat Shock Transcription Factor 1 Act in Opposition To Regulate Its DNA Binding Activity[†]

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ABSTRACT: Under circumstances of heat stress, heat shock transcription factor 1 (HSF1) plays important roles in heat shock protein expression. In this study, an increasing concentration of dithiothreitol (DTT) was found to either enhance or inhibit the heat-induced trimerization of HSF1, suggesting the involvement of dual redox-dependent HSF1 activation mechanisms. Our in vitro experiments show that the heat-induced bonding between the cysteine C36 and C103 residues of HSF1 forms an intermolecular disulfide covalent bond (SS-I bond) and that it directly causes HSF1 to trimerize and bond to DNA. Gel filtration assays show that HSF1 can form intermolecular hydrophobic interaction-mediated (iHI-m) noncovalent oligomers. However, the lack of a trimerization domain prevents HSF1 activation, which suggests that iHI-m noncovalent trimerization is a precondition of SS-I bond formation. On the other hand, intramolecular SS-II bond (in which the C153, C373, and C378 residues of HSF1 participate) formation inhibits this iHI-m trimerization, thereby preventing SS-I bond formation and DNA binding. Thus, HSF1 activation is regulated positively by intermolecular SS-I bond formation and negatively by intramolecular SS-II bond formation. Importantly, these two SS bonds confer different DTT sensitivities (the SS-II bond is more sensitive). Therefore, a low concentration of DTT cleaves the SS-II bond but not the SS-I bond and thus improves DNA binding of HSF1, whereas a high concentration DTT cuts both SS bonds and inhibits HSF1 activation. We propose that these interesting effects further explain cellular HSF1 trimerization, DNA binding, and transcription when cells are under stress.

Heat shock transcription factor 1 (HSF1)[1] is a central regulator of the synthesis of inducible heat shock proteins (HSPs) in mammals. In the normal state, HSF1 exists in the cytoplasm in an inert monomeric state. However, when cells are stressed, HSF1 forms a homotrimeric complex and is translocated to the nucleus, where it specifically binds heat shock element (HSE), a conserved regulatory DNA sequence comprised of at least three contiguous inverted repeats of 5′-nGAAn-3′ upstream of heat shock genes (*1*–*6*).

Voellmy (*7*) offered the opinion that HSF1 trimer formation is repressed by its association with HSPs and other cochaperons, as in unstressed cells, HSF1 monomers form heterocomplexes with HSPs and other cochaperones. Thus, these cochaperones, which include HSP90, P23, and immunophilin, play negative roles in HSF1 trimerization (*8*–*14*). However, when cells are exposed to stress, HSF1 could be released from heterocomplexes, since the accumulation of non-native proteins is capable of competing strongly for molecular chaperones like HSP70 and HSP90 (*14, 15*). Recently, circular dichroism (CD) spectroscopy was used to show that the unfolded structure of the carboxyl-terminal domain of HSF1 under physiological conditions changes to a more compact structured state under stress, which suggests that HSF1 and its cochaperones separate under stress (*16*), which allows HSF1 monomers to self-assemble and form HSF1 homotrimers, and thus HSF1 gains its DNA binding activity. A series of experiments, including internal mutagenesis experiments (*17, 18*), indicated that these two steps (trimerization and DNA binding) are inseparable. In addition, recent observations (*19, 20*) provided a new viewpoint, namely, that an RNA (HSR1) and a translation factor (eEF1A) are involved in mammalian HSF1 activation. This result indicates that some intracellular factors repress HSF1 trimerization, while other factors (co-activators) are essential for HSF1 activation. In fact, many co-activators of HSF1 trimerization, such as ASC-2 and HSC70 (*21, 22*), have been proven to bind activated hHSF1, but their effects on HSF1 activation differed. Moreover, after stress, some chaperones, such as HSBP1 (*23*), promoted breakdown of the HSF1 trimer to the inactive monomeric state.

Previous studies have indicated that mammalian HSF1 trimer formation depends on intermolecular hydrophobic

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[1] Abbreviations: HSF1, heat shock transcription factor 1; HSE, heat shock element; iHI-m, intermolecular hydrophobic interaction-mediated; DTT, dithiothreitol.

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noncovalent interactions in its trimerization domain (*17, 24, 25*). EGS [ethylene glycol bis(succinimidyl succinate)], a unique sulfhydryl (SH) group cross-linker, was added during the majority of HSF1 trimerization experiments, which strongly suggests that intermolecular disulfide bonds [SS bond(s)] may participate in HSF1 trimerization. In fact, many transcription factors (like p53, AP-1, and NF-κB) are redox-regulated via highly conserved cysteine residues (*26, 27*). In previous study, Manalo et al. (*28*) showed that redox changes can influence human HSF1 trimerization and DNA binding in vivo and in vitro, implying that cysteine residues play a role in HSF1 activation. In human and mouse HSF1, there are five cysteine residues (*3*) (C1−C5 at amino acid positions 36, 103, 153, 373, and 378 of human HSF1, respectively) (Figure 1A). Manalo et al. (*29*) showed that under oxidative conditions, the formation of an intramolecular SS bond between C3 and C4/C5 residues prevents HSF1 trimerization and thus inhibits HSF1 DNA binding activity, and they also showed that C1 and C2 residues have no effect on the redox sensitivity of human HSF1 (*29*). On the other hand, Ahn and Thiele (*30*) observed that another SS bond between C1 and C2 residues is essential for activity in the mouse homologue.

Thus, two different SS bonds were found in mouse and human HSF1 (*29, 30*). However, it is not known how these two types of SS bonds concurrently regulate HSF1 heat-induced activation. To investigate the roles of SS bonds in hHSF1, we prepared hHSF1 and a series of cysteine mutants of hHSF1 and quantified their trimerization and DNA binding activities in various redox environments. On the basis of previous papers (*29, 30*) and our results obtained, a new redox-dependent model was built, to further our understanding of hHSF1 activation.

MATERIALS AND METHODS

Plasmids and PCR-Mediated Mutagenesis. Expression vector pET21b-human HSF1 (hHSF1) was constructed by inserting full-length hHSF1 cDNA (*31*) into the pET21b vector (Novagen). The deletion mutants pQE30-hHSF1 were constructed by inserting amino acid residues 1−290 or 1−120 of hHSF1 into the pQE30 (Qiagen) expression vector. Cysteine residues of hHSF1 (C1−C5, corresponding to cysteine residues at amino acid positions 36, 103, 153, 373, and 378, respectively) were replaced with serine residues by PCR-mediated site-directed mutagenesis (*32*). All plasmids were isolated from *Escherichia coli* JM109 and verified by DNA sequencing.

Cell Culture and Protein Purification. E. coli BL21(DE3) was transformed with full-length hHSF1 (amino acid residues 1−529) or its cysteine mutants (C1S−C5S single and C4/5S double mutants of hHSF1). *E. coli* SG13009 (pREP4) was transformed with deletion mutants hHSF1-(1−290) or hHSF1-(1−120). *E. coli* were cultured in LB medium to an OD_{600} of 0.6, and the targeted proteins were induced by adding 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) and culturing for 12 h at 20 °C (*33*). Cells were collected by centrifugation and then resuspended in binding buffer containing 20 mM Tris-HCl (pH 8.0), 30 mM imidazole, 200 mM sodium chloride, 1 mM benzamidine, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF). After sonication, crude lysate was centrifuged at 13000 rpm for 40 min at 4 °C. Supernatant was loaded onto a HisTrap HP column by FPLC (GE Healthcare), and hHSF1 was eluted using an elution buffer containing 150 mM imidazole. hHSF1 was further purified using a mono Q HR 5/5 column (GE Healthcare), as described previously (*34*). The fractions that were at least 90% pure as determined by Coomassie staining were collected and stored at −80 °C.

In Vitro hHSF1 Trimerization (intermolecular SS-I bond formation) Assays. For SS-I bond assays, samples (6 μg) were dialyzed in TGE buffer [20 mM Tris-HCl (pH 7.9), 25% glycerol, 0.5 mM EDTA, 100 mM sodium chloride, and protease inhibitor] at 4 °C and then treated with various amounts of redox chemicals, i.e., diamide (0.1, 1, or 10 mM), dithiothreitol (DTT) (0.1, 1, or 10 mM), and hydrogen peroxide (H_2O_2) (0.1, 1, or 10 mM) for 10 min at 4 °C. Treated samples were then heat-activated for 30 min at 42 °C, then mixed with 5× nonreducing sample buffer [60 mM Tris-HCl (pH 6.8), 25% (v/v) glycerol, 2% (w/v) SDS, and 0.1% (w/v) bromophenol blue, without β-mercaptoethanol] in the presence of 5 mM *N*-ethylmaleimide (to alkylate the free sulfhydryl groups of hHSF1), and then incubated for 10 min. Samples were then boiled for 10 min at 95 °C to completely denature proteins and then loaded onto a SDS−PAGE gel to determine the relative amounts of hHSF1 monomers, dimers, and trimers.

Electrophoretic Mobility Shift Assays (EMSAs). Two complementary single-stranded HSE oligonucleotides were annealed and used in an EMSA, as described previously (*35*). HSE was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (*36*). For EMSAs, samples (100 ng) were pretreated as described in above SS-I bond assays and then heat-activated (30 min at 42 °C). They were then incubated with ^{32}P-labeled HSE [∼30000 cpm (<1 ng)] for 20 min at room temperature (25 °C), and finally, the hHSF1−HSE complexes were separated from free HSE on a 5% Tris-borate-EDTA native gel and visualized by autoradiography.

Gel Filtration Chromatography Assays. FPLC ÄKTA explorer (GE Healthcare) was used for the gel filtration chromatography assay. hHSF1 solutions were dialyzed in GF buffer [50 mM phosphate buffer and 150 mM sodium chloride (pH 7.2)] and then analyzed on a Superdex 200 10/300 GL gel filtration column (GE Healthcare). All the experiments were performed at 20 °C, and the gel filtration column flow rate was set at 0.4 mL/min. Fractions were mixed with 5× sample buffers containing 3.5 mM β-mercaptoethanol, analyzed by SDS−PAGE, and then Western blotted. A rat monoclonal antibody HSF1 Ab-1 (Neomarkers) was used as a primary antibody. The Western blotting procedures were followed those from ref *29*.

RESULTS

Intermolecular SS-I Bond Formation Participates in Heat-Induced Human HSF1 DNA Binding. As reported previously by others in vivo (*37*) and in vitro (*33, 38, 39*), HSF1 forms a trimer that interacts with HSE in response to heat shock. In this study, the DNA binding activity of hHSF1 was examined by an electrophoretic mobility shift assay (EMSA) (Figure 1B), and trimer formation was detected by denaturing gel electrophoresis (SDS−PAGE) (Figure 1C). Moreover, it should be noted that in these SDS−PAGE experiments,

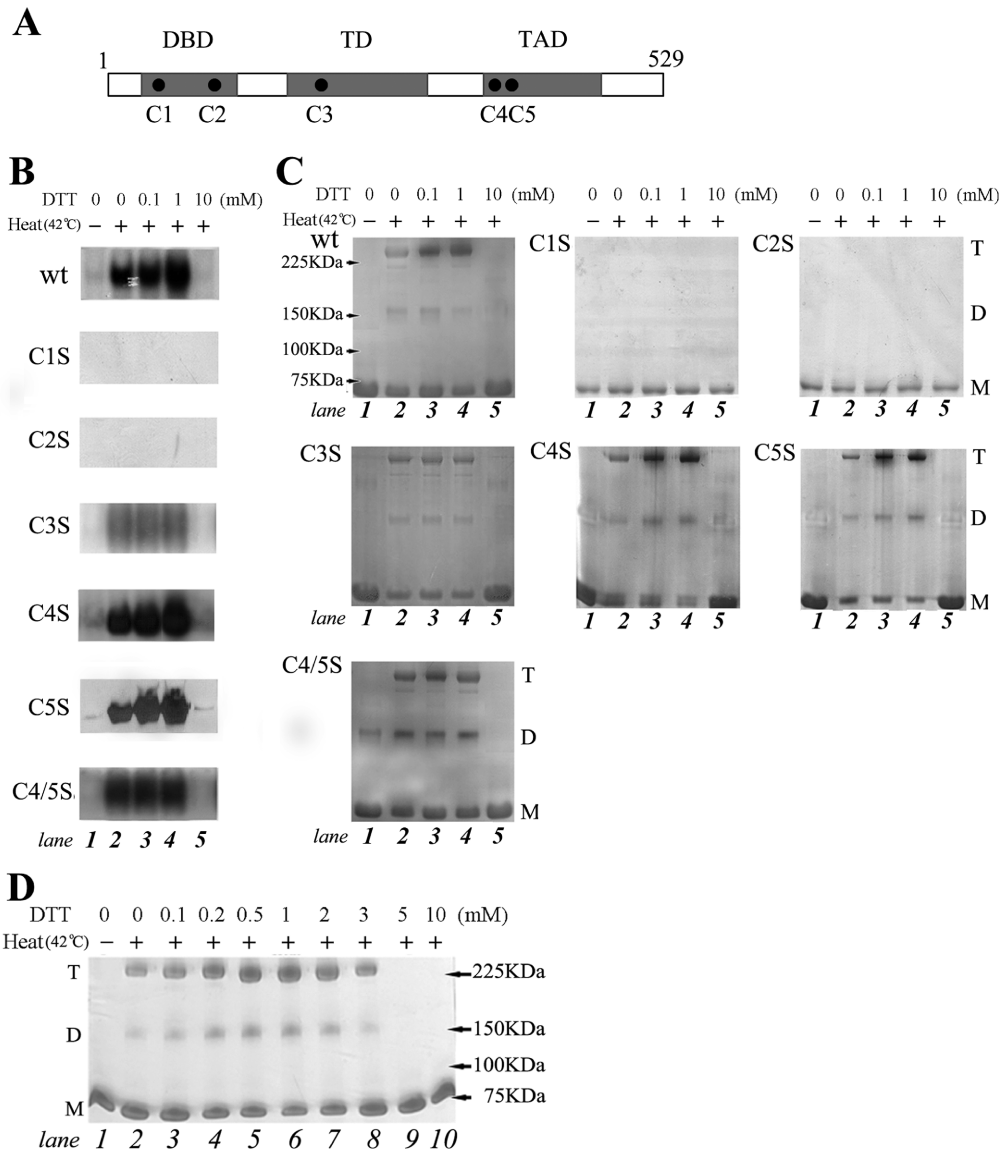

FIGURE 1: Effects of the reducing agent (DTT) on the in vitro DNA binding and trimerization of human HSF1 (hHSF1) and its cysteine mutants. (A) Diagrammatic illustration of the placement of cysteine residues in human HSF1. C1−C5 represent the locations of cysteine residues 36, 103, 153, 373, and 378, respectively. DBD is the DNA-binding domain, TD the trimerization domain, and TAD the transcriptional activation domain. (B) hHSF1 and its cysteine mutants were pretreated with DTT (10 min at 4 °C) and then heat-activated (30 min at 42 °C). DNA binding activities were measured with an EMSA using ^{32}P-labeled HSE: lane 1, proteins treated for 30 min at 4 °C; lane 2, proteins treated for 30 min at 42 °C; and lanes 3−5, proteins treated with DTT and then heat-activated (30 min at 42 °C). (C) Samples were treated with DTT, as described above, and then analyzed by SDS−PAGE. For the intermolecular assays, hHSF1 monomers (M), dimers (D), and trimers (T) are shown on the right: lane 1, proteins treated for 30 min at 4 °C; lane 2, proteins heat-activated for 30 min at 42 °C; and lanes 3−5, samples treated with DTT (0.1, 1, and 10 mM, respectively) and then heat-activated. wt is wild-type hHSF1. (D) Wild-type hHSF1 was pretreated with increasing amounts of DTTs. The procedures used were those described for panel C and for the in vitro trimerization assays described in Materials and Methods.

there was no reducing agent [dithiothreitol (DTT) or β-mercaptoethanol] in the 5× electrophoresis sample buffer. Panels B and C of Figure 1 show that at low temperatures, wild-type hHSF1 lacked DNA binding or trimerization activity (wt, lanes 1), whereas these activities were greatly stimulated at a high (42 °C) temperature (wt, lanes 2). Moreover, an increase in the level of DNA binding and trimer formation of wild-type hHSF1 was observed when 0.1 and 1 mM DTT were added to wild-type hHSF1 (wt, lanes 3 and 4). Trimer formation of hHSF1 was detected by SDS−PAGE in the absence of DTT (wt, lanes 2), which suggests that intermolecular noncovalent bonds were disrupted but that intermolecular covalent bonds like the disulfide bonds (SS bonds) persisted. Moreover, these heat-induced DNA binding and trimerization activities of hHSF1 were reversed with excess DTT (10 mM) (Figure 1B,C, wt, lanes 5), which suggests that this covalent bond could be cut by DTT, and this result provides further evidence of the existence of redox-dependent intermolecular SS bonds.

To investigate the roles of SS bonds, five cysteine residues (Figure 1A) in hHSF1 were replaced with serine using PCR-mediated mutagenesis, and then, cysteine mutants (C1S−C5S single and C4/5S double mutants) of hHSF1 were prepared as described in Materials and Methods. Panels B and C of Figure 1 show that C1S and C2S single mutants of hHSF1 lacked the heat-induced trimerization and DNA binding activities (C1S and C2S in lanes 1 and 2, respectively). In contrast, C3S, C4S, C5S single and C4/5S double mutants

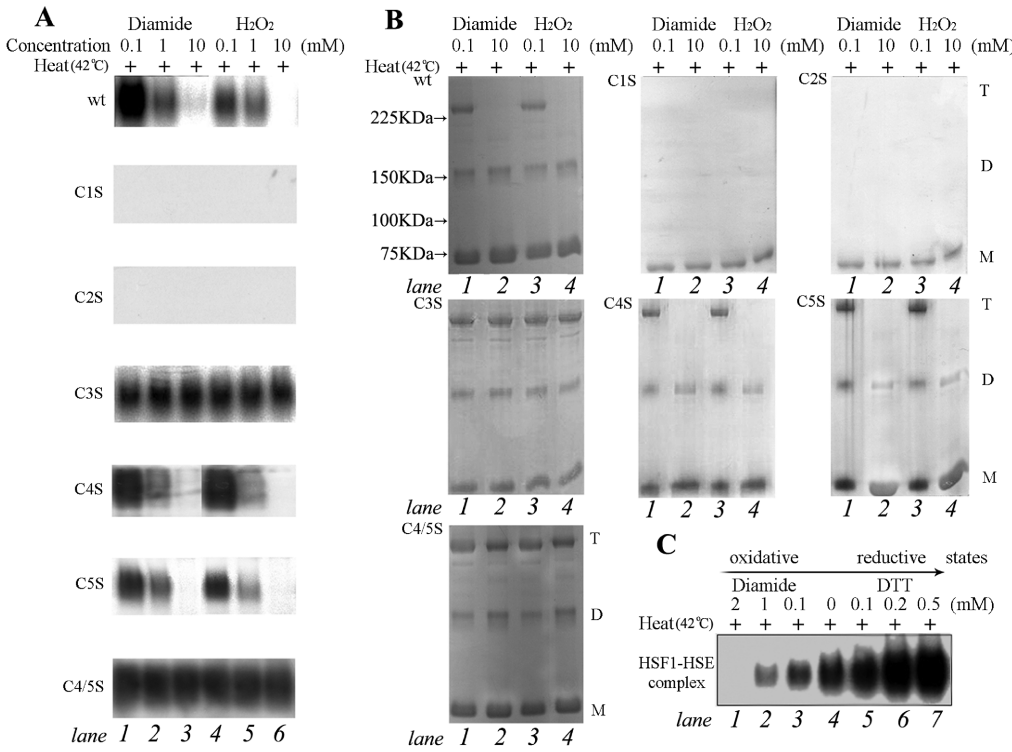

FIGURE 2: Effects of oxidation on the in vitro DNA binding and trimerization of hHSF1 and its mutants. (A) hHSF1 was treated in the presence of oxidative reagents (diamide and H_2O_2, for 10 min at 4 °C) and then heat-activated (42 °C for 30 min). DNA binding activities were measured by an EMSA: lanes 1−3, samples treated with diamide at 0.1, 1, and 10 mM, respectively; and lanes 4−6, samples treated with H_2O_2 at 0.1, 1, and 10 mM, respectively. (B) Samples were pretreated with diamide or H_2O_2 and then subjected to trimerization assays, as described in Materials and Methods, and then analyzed by SDS−PAGE. hHSF1 monomers (M), dimers (D), and trimers (T) are shown at the right: lanes 1 and 2, samples treated with 0.1 and 10 mM diamide, respectively; and lanes 3 and 4, samples treated with 0.1 and 10 mM H_2O_2, respectively. (C) HSE binding activity of hHSF1 under mild redox conditions. hHSF1 solutions were pretreated with diamide or DTT and then heat-activated, as described in Figures 1B and 3A. DNA binding activities of hHSF1 were measured with an EMSA.

of hHSF1 could be heat-activated, similar to wild-type hHSF1 (lanes 2 on the wt, C3S, C4S, C5S, and C4/5S). These results indicate that C1 and C2, but not C3, C4, or C5, participate in the heat-induced trimerization, and this covalent trimerization of hHSF1 is required for DNA binding. In summary, heat shock causes the C1 and C2 residues to form intermolecular SS-I bonds, and the formation of SS-I bonds causes the covalent trimerization and DNA binding of hHSF1.

Here we note that in a previous study (*29*), Manalo et al. reported a contrary conclusion according to their experiments, in which C1 and C2 variants (including C1T/C2Y variants, which corresponded to substitution of the corresponding residues in HSF2 and C1S/C2S variants) exhibited wild-type-like DNA binding and trimerization. Thus, they asserted that C1 and C2 residues have no effect on the redox sensitivity of human HSF1. However after that, Ahn and Thiele (*30*) preformed a series of convincing experiments with mouse HSF1 (a very highly similar homologue of human HSF1) and found that C1 and C2 residues are essential for HSF1 activity, which supports our opinion.

Trimerization of hHSF1 was analyzed in the presence of increasing concentrations of DTT (Figure 1D), and it was found that the level of heat-induced hHSF1 covalent trimerization was markedly reduced by adding DTT at concentrations higher than 2 mM (lanes 8−10). When treated with 5−10 mM DTT, hHSF1 lost its trimerization ability (lanes 9 and 10). The variance of hHSF1 trimerization by DTT suggests that participation of C1 and C2 in intermolecular SS-I bond formation is redox-dependent, because the intermolecular SS-I bond might be reduced by DTT at concentrations higher than 2 mM, thereby inactivating hHSF1.

Intramolecular Redox-Sensitive SS-II Bond Formation Prevents Heat-Induced hHSF1 Activation. To examine the effects of oxidative stress on heat-induced hHSF1 trimerization and HSE binding, hHSF1 and its cysteine mutant proteins were incubated in vitro with different concentrations of oxidizing agents (diamide or hydrogen peroxide) and then heat-activated. Panels A and B of Figure 2 show that the addition of oxidizing agents decreased the extent of heat-induced wild-type hHSF1−HSE complex formation and trimerization, respectively, in a dose-dependent manner (wt, lanes 1−4). However, C1S and C2S mutants of hHSF1 showed no DNA binding or trimerization activity (C1S and C2S, lanes 1−4), further demonstrating the importance of the intermolecular SS-I bond. Importantly, C3S single and C4/5S double mutants, but not C4S and C5S single mutants, prevented oxidation-induced loss of hHSF1 DNA binding and their abilities to trimerize (C3−C4/5S, lanes 1−4). These results strongly suggest that although the intermolecular SS-I bond is essential for hHSF1 trimer formation and DNA binding, another SS bond (intramolecular SS-II bond between cysteine residues C3 and C4/C5) also participates in heat-induced hHSF1 activation. However, redox-sensitive disulfide bonds may play two quite different roles in hHSF1 activation, for although SS bond formation appears to be essential for

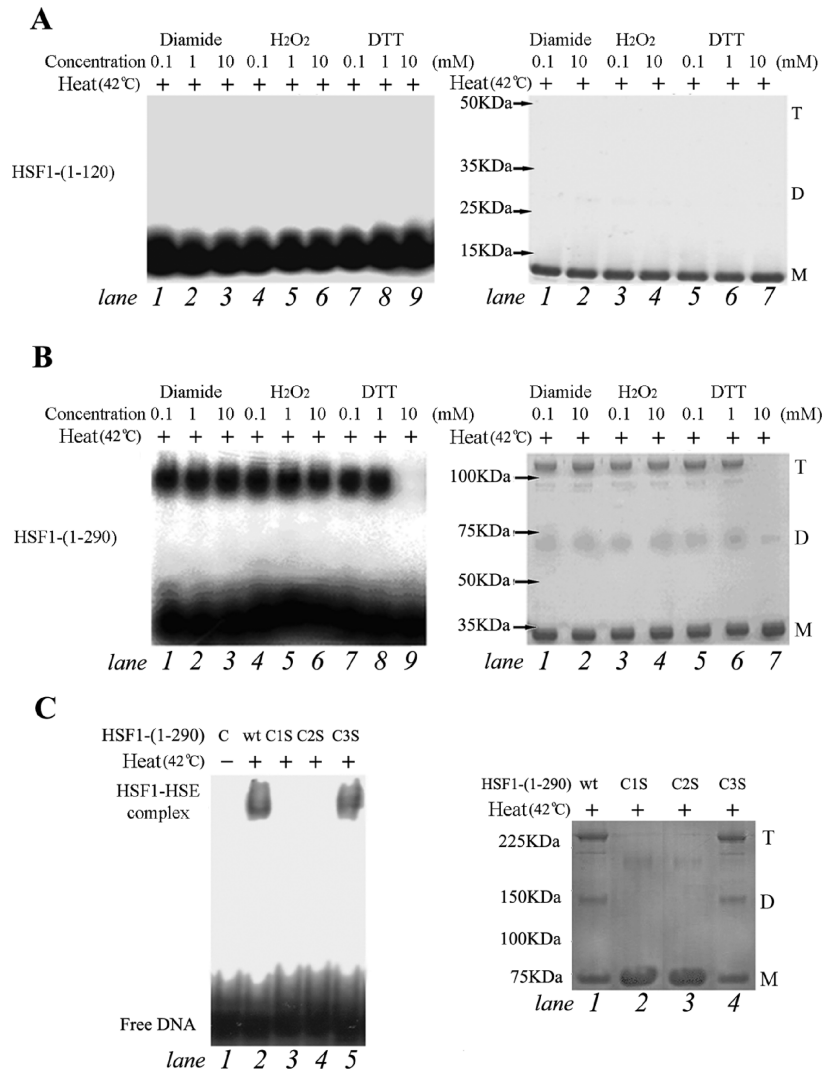

FIGURE 3: Trimerization domain of hHSF1 required for intermolecular SS-I bond formation. (A and B) DNA binding and SS-I bond formation of deletion mutants hHSF1-(1−120) and hHSF1-(1−290) were analyzed by an EMSA and by using intermolecular SS bond assays, using the experimental procedures described in the legends of Figures 1 and 2. (C) Heat shock-induced DNA binding and trimerization activities of deletion mutant hHSF1-(1−290) and its cysteine mutants (C1S, C2S, and C3S single mutants). The experimental procedure was the same as that described for panels B and C of Figure 1. For intermolecular SS bond assays, hHSF1 monomers (M), dimers (D), and trimers (T) are shown at the right.

hHSF1 activation under reductive conditions, it also prevented hHSF1 activation under oxidative conditions.

When trimerization was analyzed after treatment of wild-type hHSF1 with increasing concentrations of DTT (Figure 1D), heat-induced trimerization was found to be prevented by DTT concentrations higher than 2 mM (lanes 8−10), but to occur in a dose-dependent manner when DTT was administered at concentrations of less than 2 mM (lanes 2−6), demonstrating that in vitro heat-induced hHSF1 trimerization is redox-dependent and that DTT treatment has contrary effects on this process. These results suggested that SS-I and SS-II bonds had different redox sensitivities. The intermolecular SS-I bond is highly resistant to DTT, whereas the intramolecular SS-II bond is by comparison redox-sensitive. An increase in the level of hHSF1 trimerization and DNA binding by DTT at <2 mM was also observed for C4S and C5S single mutants like wild-type hHSF1 (Figure 1B,C, wt, C4S, C5S, lanes 3 and 4), but not for C3S single or C4/5S double mutants (C3S and C4/5S, lanes 3 and 4), which is attributed to the ability of C3 to form a disulfide bond with either C4 or C5 (*29*). These results suggest that the SS-II bond between C3 and C4/C5 is disrupted by DTT at <2 mM and that this enhances SS-I bond-mediated hHSF1 trimerization and DNA binding. In other words, SS-II bond formation may have an inhibitory effect on heat-induced hHSF1 activation.

Interestingly, Ahn and Thiele (*30*) observed that a low concentration (200 μM) of peroxide (H_2O_2) could activate mouse HSF1 (a very highly similar homologue of human HSF1) without heat treatment (Figure 1A of ref *30*). This observation seems to contrast with our results (Figure 2A,B), where H_2O_2 treatment inhibits HSF1 heat activation. However, also in this case (Figure 1A of ref *30*), H_2O_2-treated HSF1 showed a relatively lower level of trimerization activity than the heat shock-treated protein. For these results, we asked if H_2O_2 plays the differential roles in HSF1 activation. Although H_2O_2, as a peroxide stress, could activate HSF1, it also could inhibit HSF1 activation as an oxidizer. Maybe HSF1 exists as two forms in a neutral environment: some could be activated by H_2O_2, while others could not. For this hypothesis, we examined the heat-induced DNA binding activity of hHSF1 in a range of redox states. Figure 2C shows

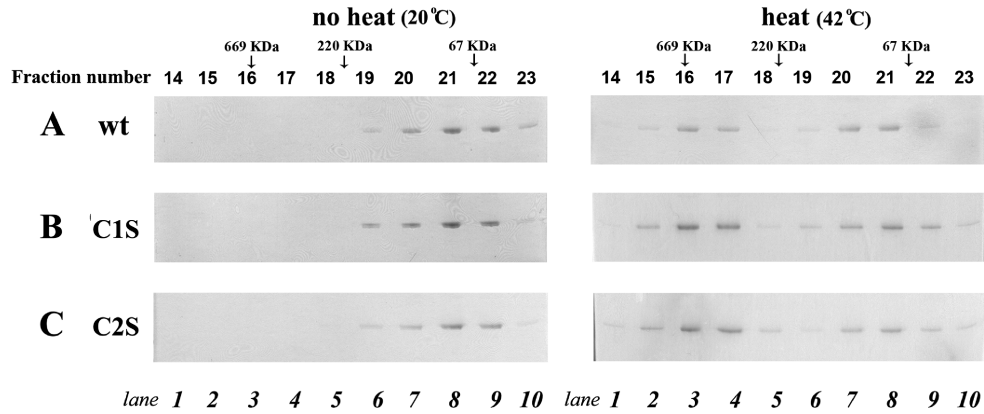

FIGURE 4: (A-C) Gel filtration assays of wild-type hHSF1 and its cysteine mutants (C1S and C2S single mutants of hHSF1). Samples were pretreated at 25 °C (no heat) or 42 °C (heat) for 30 min and then injected into a Superdex 200 (10/300 GL) column (GE Healthcare). All experiments were preformed at room temperature (20 °C) at a gel filtration column flow rate of 0.4 mL/min. Fractions were mixed with 5× sample buffer containing 3.5 mM β-mercaptoethanol and analyzed by SDS−PAGE and by Western blotting.

that the DNA binding activity of hHSF1 gradually increased with increase in DTT concentration, which result strongly suggests that in a neutral environment, some hHSF1 monomers contain SS-II bonds. Moreover, mild redox changes might induce the formation or disruption of this bond. Thus, the SS-II bonds in hHSF1 monomers appear to exist in equilibrium (regulated by the environmental redox state) with the dithiols under normal conditions.

The Trimerization Domain Is Required for Intermolecular SS-I Bond-Mediated hHSF1 DNA Binding. To further test the effects of the two SS bonds on hHSF1 activation, we generated two deletion hHSF1 mutants: hHSF1-(1−120) (amino acids 1−120, a region that contains the DNA-binding domain of hHSF1, which contains the C1 and C2 residues) and hHSF1-(1−290) (amino acids 1−290, a region that contains the DNA-binding and trimerization domains of hHSF1, which contains residues C1, C2, and C3). We found that hHSF1-(1−120) did not exhibit SS-I bond-mediated covalent trimerization or HSE binding upon treatment after heat shock (Figure 3A). These results suggest that although the DNA-binding domain is important for C1/C2 participant HSE binding and trimerization, a domain (trimerization domain) other than the DNA-binding domain also regulates this hHSF1 activation. In fact, hHSF1-(1−290) could form trimer and bind HSE in response to heat shock (Figure 3B). Thus, the trimerization domain appears to play an essential role in SS-I bond-mediated covalent trimerization and HSE binding in response to heat shock. We further generated the C1S, C2S, and C3S single mutants of the hHSF1-(1−290) deletion mutant and found that C1S and C2S, but not C3S, single mutants of this deletion mutant prevented heat-induced HSE binding (Figure 3C), which further confirmed the positive role of the SS-I bond in heat-induced HSF1 activation. In addition, deletion mutant hHSF1-(1−290) did not exhibit oxidation-induced DNA binding loss, and DTT (at <2 mM) increased the level of hHSF1 trimerization and HSE binding, thus confirming the negative role of SS-II bond formation in SS-I bond-mediated hHSF1 activation.

A previous study (*17*) by Zuo et al. demonstrated that intermolecular noncovalent hydrophobic (leucine zippers in the trimerization domain of HSF1) interactions enabled HSF1 trimerization. However, our trimerization experiments (Figure 1C, wt) showed that SS-I bond formation also resulted in hHSF1 trimerization. To verify the relationship between hydrophobic interactions and the SS-I bond with respect to hHSF1 activation, we examined the oligomerization patterns of wild-type hHSF1 and of its cysteine mutants by gel filtration chromatography using a Superdex 200 10/300 GL column. The results (Figure 4A) showed that the wild-type hHSF1 monomer stayed predominantly in fractions 20−22 at 20 °C (wt, no heat, lanes 7−9), while a complex (molecular mass of >200 kDa) was eluted in fractions 15−17 after heat activation at 42 °C (wt, heat, lanes 2−4). Moreover, C1S and C2S single mutants behaved in the same manner as wild-type hHSF1 in gel filtration assays (Figure 4B,C). These results meant that like wild-type hHSF1, the C1S and C2S single mutants could form oligomers and that the mutations of the C1 and C2 residues did not impact this oligomerization. However, as shown in Figure 1C, C1S and C2S single mutants could not form a SS-I bond (C1S and C2S, lanes 1 and 2), like wild-type hHSF1 (wt, lanes 1 and 2). In summary, these results suggest that C1S and C2S single mutants oligomerize by intermolecular hydrophobic noncovalent interactions, whereas wild-type hHSF1 trimer formation includes both intermolecular hydrophobic interactions and SS-I bond formation. Heat stimuli could induce the intermolecular noncovalent hydrophobic interaction-mediated (iHI-m) oligomerization of hHSF1, but intermolecular SS-I bond formation appears to be essential for DNA binding. This iHI-m noncovalent oligomer formation provides a rationale for intermolecular SS-I bond formation, and thus, iHI-m noncovalent oligomerization appears to be a prerequisite of intermolecular SS-I covalent bond formation.

DISCUSSION

In cells and tissues, homeostasis requires a mildly reducing environment. Moreover, the biological states of cells change in concert with changes in the cellular redox environment. Many transcription factors are sensitive to cellular redox changes, and thus, changes in the cellular redox environment can initiate signaling cascades (*40*). Previous studies (*29, 30*) have shown two SS bonds in mammalian HSF1, but their roles in the regulation of HSF1 activation remain unclear. In this study, we discovered an interesting mechanism (Figure 5) in which

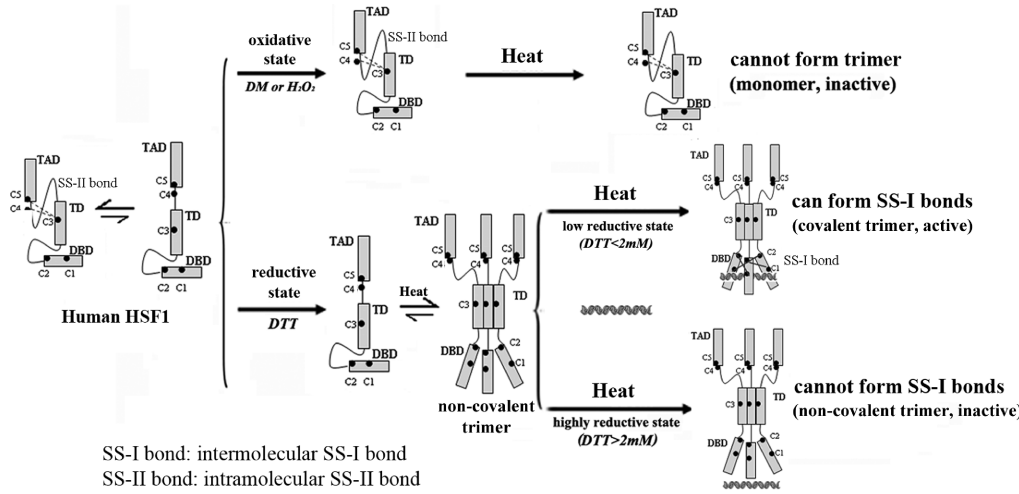

FIGURE 5: Schematic illustration of dual regulation imposed by SS-I and SS-II bonds in hHSF1. In a neutral environment, the SS-II bond in the hHSF1 monomer appears to exist in equilibrium with the dithiol. However, under oxidizing conditions, intramolecular SS-II bond formation is favored and prevents hHSF1 covalent trimer formation and hHSF1 DNA binding, whereas under reducing conditions, hHSF1 monomers self-assemble to form noncovalent homotrimers. Under mild reducing conditions, heat shock induces the formation of three intermolecular SS-I bonds, which result in the acquisition of HSE binding activity. However, under strongly reducing conditions, DTT inhibition prevents the formation of heat-induced SS-I bonds, and thus, hHSF1 has no DNA binding activity. DBD is the DNA-binding domain, TD the trimerization domain, TAD the transcriptional activation domain, and DM diamide.

two distinct types of SS bonds regulate hHSF1 DNA binding activity. The acquisition of HSE binding activity by hHSF1 was found to require SS-I bond formation, whereas SS-II bond formation was found to impede iHI-m noncovalent trimerization and thus inhibit HSF1 activation. Importantly, these two SS bonds were found to have different DTT sensitivities; i.e., the intramolecular SS-II bond was more sensitive than the intermolecular SS-I bond in this respect (Figure 1D). These different DTT sensitivities reveal that intracellular redox environment changes could efficiently regulate the DNA binding of hHSF1.

Previous studies have shown that the coiled-coil domain (*17*), the loop domain (*33*), and the linker domain (*41*) of HSF1 can regulate stress-induced trimerization and DNA binding in vivo and in vitro. However, our trimerization experiments (Figure 1C,D) show that hHSF1 trimerization requires the formation of intermolecular covalent bonds that are influenced by DTT, which suggests that they are intermolecular SS bonds. Experiments using C1S and C2S single mutants showed that the C1 and C2 residues participate in the formation of this bond (Figures 1 and 2, C1S and C2S). Cysteine residues C1 and C2 are evolutionarily conserved in mammals HSF1 (humans and mice) (*31, 42*), which demonstrates that they are required for hHSF1 function. In the hHSF1 homotrimer, C1 residues from one monomer may associate with C2 residues from another monomer to form an intermolecular SS-I bond. Thus, three SS-I bonds could be formed in the hHSF1 trimer (Figure 5). Three intermolecular SS-I bonds would greatly enhance hHSF1 trimer stabilization in the presence of different stresses. Our intermolecular SS bond assays by SDS−PAGE (Figure 1D) also revealed that SS-I bond formation is retarded only under strongly reducing conditions (lanes 9 and 10), which suggests that under physiological conditions, the intracellular redox environment cannot prevent SS-I bond formation. These features illustrate the importance of the SS-I bond for hHSF1 activation. Actually, a similar conclusion was deduced in the mouse homologue by Ahn and Thiele (*30*). However, our further experiments on deletion mutants hHSF1-(1−120) and hHSF1-(1−290) (Figure 3A,B) showed not only that the C1 and C2 residues play a critical role in hHSF1 activation (trimerization and DNA binding) but also that the trimerization domain participates in this activation. Moreover, gel filtration assays showed that the wild type and the C1S and C2S single mutants of hHSF1 can form noncovalent oligomers via an intermolecular hydrophobic (leucine zippers) interaction (Figure 4A−C). However, the C1S and C2S single mutants of hHSF1 did not show covalent trimerization and DNA binding (Figure 1B,C). These results strongly suggest that intermolecular hydrophobic interactions facilitate hHSF1 trimerization, but that intermolecular SS-I bond formation is required for DNA binding (Figure 5).

Many in vitro studies have demonstrated that various environmental stresses can induce hHSF1 to form homotrimers and gain DNA binding activity (*1–3*). In mouse embryonic fibroblast cells, a failure to form a SS-I bond, due to point mutations at C1 and C2 (both were replaced with serine residues), directly impacted stress-induced DNA binding activity and subsequently inhibited the expressions of heat shock proteins (*30*), which suggests that redox-resistant SS-I bond formation is a critical step in hHSF1 activation in vivo, which contrasts with the deactivating effect of the redox-sensitive SS-II bond. In the mammalian system, HSF1 is maintained as a latent monomer at normal temperatures (e.g., 37 °C in humans and 38 °C in mice) (*2, 4*). Intramolecular hydrophobic interactions (*17*) are believed to predominantly repress hHSF1 activation in vivo. However, in an in vitro experiment (*33*), purified mouse HSF1 was found to be able to form a homotrimer at only 32 °C. In addition, Clos et al. (*43*) found that hHSF1 expressed in *Drosophila* cells remained in the monomeric state at 22 °C and was activated at 37 °C. These results suggest, although weak hydrophobic interactions can repress HSF activation, that there exists another negative regulation factor. In panels A and B of Figure 3, intramolecular SS-II bond formation

inhibited hHSF1 trimer formation and DNA binding even at 42 °C, which suggests that intramolecular SS-II bond formation can repress hHSF1 activation. Actually, similar results were published in previous study by Manalo et al. (*29*). However, our further experiments (Figures 1D and 2C) suggest that the formation and disruption of SS-II bond can be regulated in a mild redox environment. In mammalian systems, the actions of many transcription factors are complemented by repressors and activators that ensure the efficient regulation of transcriptional activities. In the case of HSF1, some partner species, such as HSPs (*8*–*14*), PLK1 (*43*), HSC70 (*22*), and ASC-2 (*21*), have been found to participate in HSF1 activation. In addition, molecular chaperones, like HSP90, have been reported to be essential for HSF1 activation and regeneration in vivo (*13*), but the detailed regulatory mechanisms remained unknown. One possible mechanism is that some partner species, such as HSP70 or HSP90, interact with hHSF1 to repress its activation by promoting SS-II bond formation, and that following exposure to stress, disruption of this interaction directly results in intramolecular SS-II bond cleavage. A second possibility is that extracellular stresses induce changes in the intracellular redox environment which disrupt the SS-II bond. Recently, it was reported that the in vivo overexpression of HSP27 by oxidative stress elevated the intracellular level of glutathione (a common in vivo reducing agent) in mouse liver and consequently activated mouse HSF1 to promote the survival of cells exposed to oxidative stress (*44*). This observation indicates when cells are exposed to extracellular oxidative environmental stress in the form of excessive amounts of intracellular glutathione, but within physiological limits, that mouse HSF1 is activated via SS-II bond disruption, but that the formation of the redox-resistant intermolecular SS-I bond is unaffected.

CONCLUSION

Our findings present a unique model in which two distinct types of SS bonds regulate hHSF1 activation. Specifically, the formations of SS-I and SS-II bonds were found to promote and inhibit hHSF1 activation, respectively. Moreover, redox changes in the intracellular environment could effectively regulate this mechanism, since these two bonds have different DTT sensitivities. In particular, our results show that the intermolecular hydrophobic interaction-mediated noncovalent trimerization is a precondition of SS-I bond formation and that SS-II bond formation inhibits this trimerization. We hope that the elucidation of this interesting mechanism promotes the understanding of hHSF1 activation in response to stress.

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