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Repression of Heat Shock Transcription Factor HSF1 Activation by HSP90 (HSP90 Complex) that Forms a Stress-Sensitive Complex with HSF1

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Summary

Heat shock and other proteotoxic stresses cause accumulation of nonnative proteins that trigger activation of heat shock protein (Hsp) genes. A chaperone/Hsp functioning as repressor of heat shock transcription factor (HSF) could make activation of *hsp* genes dependent on protein unfolding. In a novel in vitro system, in which human HSF1 can be activated by nonnative protein, heat, and geldanamycin, addition of Hsp90 inhibits activation. Reduction of the level of Hsp90 but not of Hsp/c70, Hop, Hip, p23, CyP40, or Hsp40 dramatically activates HSF1. In vivo, geldanamycin activates HSF1 under conditions in which it is an Hsp90-specific reagent. Hsp90-containing HSF1 complex is present in the unstressed cell and dissociates during stress. We conclude that Hsp90, by itself and/or associated with multichaperone complexes, is a major repressor of HSF1.

Introduction

Transcription of heat shock protein (Hsp) genes and, consequently, synthesis of Hsps are enhanced upon exposure of cells to a proteotoxic stress, including heat shock. In vertebrate species, this transcriptional enhancement is due to the activation of heat shock transcription factor 1 (HSF1), one of four related factors capable of specifically binding heat shock element (HSE) sequences present in *hsp* promoters. In extract from unstressed cells, HSF1 is found predominantly as a monomeric polypeptide that lacks specific DNA-binding activity. A small fraction of HSF1 appears in a binary complex with Hsp/c70. When cells are stressed, HSF1 homotrimerizes, acquires DNA-binding activity, translocates from the cytoplasm to the nucleus, is hyperphosphorylated, and becomes transcriptionally competent (Morimoto et al., 1996; Voellmy, 1996; Nakai et al., 1997).

The mechanism of stress activation of HSF1 is not yet understood. However, previous work provided two important clues. First, stress induces protein unfolding, which results in accumulation of nonnative protein. Nonnative protein appears to be the common proximal inducer of HSF1 activity and enhanced *hsp* gene expression in the stressed cell (Kelley and Schlesinger, 1978; Hightower, 1980; Ananthan et al., 1986). Second, an Hsp, possibly Hsp/c70, may play a role in retaining HSF1 in the inactive state in the absence of stress and/or in returning the factor to this state following a stressful event (Lindquist, 1980; DiDomenico et al., 1982; Abravaya et al., 1991; Baler et al., 1992, 1996; Mosser et al., 1993). It was proposed that Hsp/c70 may function as a repressor of HSF1 activation. Nonnative proteins accumulating during stress may compete with HSF1 for binding chaperone Hsp/c70, and unbound HSF1 may homotrimerize and acquire transcriptional competence (Craig and Gross, 1991; Abravaya et al., 1992; Baler et al., 1992, 1996). However, this role of Hsp/c70 has remained tentative, and it appears equally possible that another chaperone(s) or chaperone complex(es) may be the repressor of HSF1. Hsp90, Hsp40, CyP40, FKBPs, and p23 are all known to have chaperoning activity (Bose et al., 1996; Freeman et al., 1996). Hsp90 participates in the formation of multichaperone complexes that bind target proteins such as steroid receptors (Catelli et al., 1985; Sanchez et al., 1985; Schuh et al., 1985; Pratt, 1993; Smith and Toft, 1993; Nair et al., 1996) and several protein kinases. One such multichaperone complex, referred to herein as Hop complex, includes Hsp90, Hsp/c70, and Hop and transiently associates with Hip. Another group of multichaperone complexes are the p23 complexes that contain Hsp90, p23, and a third protein such as CyP40 or an FKBP (for reviews see Smith and Toft, 1993; Frydman and Hoehfeld, 1997).

Interestingly, Hsp90 affinity chromatography experiments suggested that Hsp90 can interact with HSF1 (Nadeau et al., 1993), and reconstitution assays using recombinant (trimeric) human HSF1 showed that Hsp40, Hsp90, and Hsp90 multichaperone complex constituents Hsp/c70, Hop, Hip, p23, CyP40, and FKBPs have a propensity for associating with HSF1 (Nair et al., 1996; Voellmy, 1996, and unpublished data). Furthermore, certain benzoquinone ansamycins such as geldanamycin and herbimycin A specifically bind Hsp90 (Whitesell et al., 1994) and disrupt p23 complexes in vitro and in vivo (Smith et al., 1995; Whitesell and Cook, 1996). The same compounds also activate HSF1 in vivo (Hedge et al., 1995; Zou et al., 1998). This observation raises the possibility that Hsp90 may participate in the negative regulation of HSF1. However, since geldanamycin and herbimycin A are also potent redox cyclers and may cause oxidative protein denaturation (Zou et al., 1998), it has remained uncertain whether their activating effect is directly related to their ability to bind Hsp90.

In this paper we investigate whether Hsp90 or another constituent of Hsp90-containing multichaperone complexes is a repressor of HSF1 activation. Chaperones are generally present at high levels in the unstressed cell. Hsp90 is the most abundant chaperone, constituting about 1%–2% of total protein (Jakob and Buchner, 1994). Thus, it would be difficult to substantially overexpress chaperones, particularly Hsp90. In addition, chaperones tend to be long-lived proteins, and inhibition of their expression would only slowly reduce their concentration. Associated chaperones generally serve to stabilize their target proteins (Whitesell and Cook, 1996; Whitesell et al., 1997) and, as has been shown in the

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case of steroid receptors, maintain them in an inactive but activation-competent conformation (Picard et al., 1990; Scherrer et al., 1992; Nathan and Lindquist, 1995). By analogy, a slow decline in the concentration of relevant chaperones may destabilize inactive HSF1. In contrast, chaperones may become rapidly unavailable for binding HSF1 during stress, resulting in a sharp increase in the concentration of unbound HSF1. Because of the strong concentration dependence of the HSF1 trimerization reaction (Larson et al., 1988), factor trimerization may be favored over inactivation/degradation in this situation. To avoid these predictable difficulties of in vivo approaches to identify HSF1 repressor, we developed an in vitro system that recapitulates the initial step of HSF1 activation in vivo.

Results

An In Vitro System that Reproduces the Initial Step of HSF1 Activation

A lysate system (activation lysate) was established that reproduces the earliest step in HSF1 activation in vivo, which is the conversion of inactive, nontrimeric factor to trimeric factor capable of binding HSE DNA. The criteria chosen for validation of the system were induction by heat shock, the classical inducer of Hsp expression, by denatured protein, the common proximal inducer of Hsp expression during stress, and by the Hsp90-binding compound geldanamycin.

Activation lysate, prepared as described in Experimental Procedures, was exposed for 5 min to different temperatures between 0°C and 45°C (Figure 1A). HSE DNA-binding activity was assessed by electrophoretic mobility shift assay (EMSA), using a radiolabeled HSE oligonucleotide probe. Note that our gels typically resolved two or more distinct HSE DNA-HSF complexes. HSE DNA-binding activity increased gradually with temperatures up to 41°C and more sharply between 41°C and 43°C. The temperature at which the latter, more discrete increase occurred varied somewhat between experiments (between 37°C and 41°C). Cells sense the severity of a heat stress, which is a function of both temperature and duration of exposure (DiDomenico et al., 1982). As in vivo, HSE DNA-binding activity of activation lysate increased with exposure time at all but the highest temperatures (Figure 1B: exposure at 27°C). Thus, the in vitro system is capable of sensing heat stress, although DNA-binding activity increases with temperature more gradually in vitro than in vivo. The latter difference may be explained, in part, by the observation (see below) that HSF1 activation is a reversible process in vivo but not in vitro.

The heat-activated factor binds the HSE DNA probe but not an unrelated oligonucleotide probe (Figure 1C). Furthermore, a 25-fold excess of unlabeled HSE DNA suppressed binding to the HSE probe (in the presence of a 500-fold excess of nonspecific DNA). Thus, heat treatment induced the DNA-binding activity of an HSF that was identified as HSF1 by HSF1 antibody supershift (Figure 1C). As in vivo (Zimarino and Wu, 1987), heat activation of HSF1 in vitro was independent of protein synthesis (Figure 1D).

HSF1 regulation was expected to be least stable and, therefore, most readily influenced by changes in experimental parameters under threshold activation conditions. Therefore, activation lysate was routinely incubated at 27°C–29°C for 60 min (threshold heat shock), which resulted in a minor but clearly detectable increase in HSF1 DNA-binding activity. Where appropriate (indicated in figures and legends), reactions incubated for 60 min at room temperature (basal level of activation) or 37°C (heat shock condition inducing substantial DNA-binding activity) were analyzed for comparison. Relative DNA-binding activities induced by the three conditions are shown in Figure 1E. Relative amounts of HSF1 trimers formed were estimated by native anti-HSF1 blot (Figure 1F).

Since processes involving chaperones are generally ATP-dependent, activation lysate was routinely preincubated with an ATP-regenerating system (see Experimental Procedures). That activation of HSF1 is, in fact, dependent on the presence of ATP was confirmed by the experiment in Figure 1G.

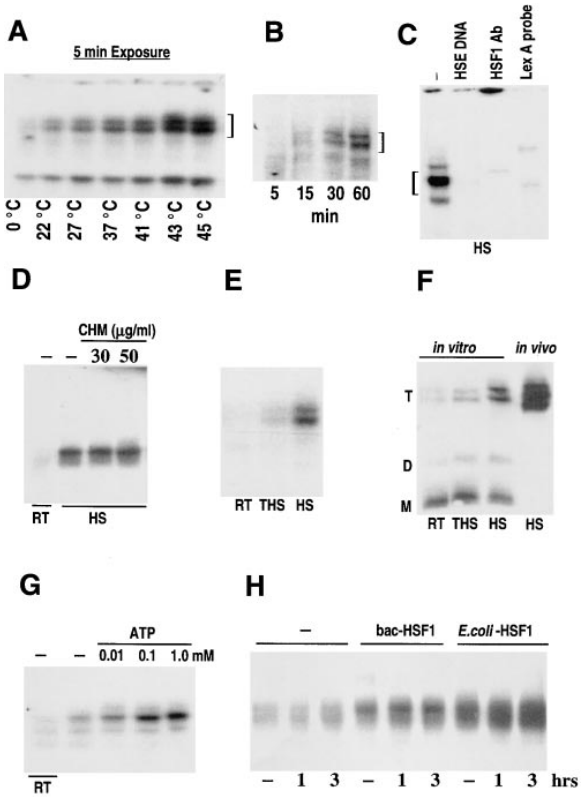

Figure 1. Activation of HSF1 In Vitro by Heat Shock

(A–E, G, and H) EMSA of aliquots of activation lysate. RT, room temperature; THS, threshold heat shock; HS, heat shock; CHM, cycloheximide; bac-HSF1, baculovirus-expressed HSF1; *E. coli*-HSF1, bacterially expressed HSF1. In (C) EMSA used either a radiolabeled LexA binding site-containing probe (LexA probe) or an HSE DNA probe (all other lanes). HSE DNA, 25-fold excess of unlabeled HSE probe; HSF1 Ab, HSF1 antibody. In (G) reactions were with activation lysate lacking ATP-regenerating system.

(F) Native anti-HSF1 blot of activation lysate. In vivo: extract from HeLa cells exposed to 42°C for 45 min. M, HSF1 monomers; D, HSF1-Hsp/c70 heterodimers; T, HSF1 trimers. Note that different panels show results from independent assays involving independent exposures of gels.

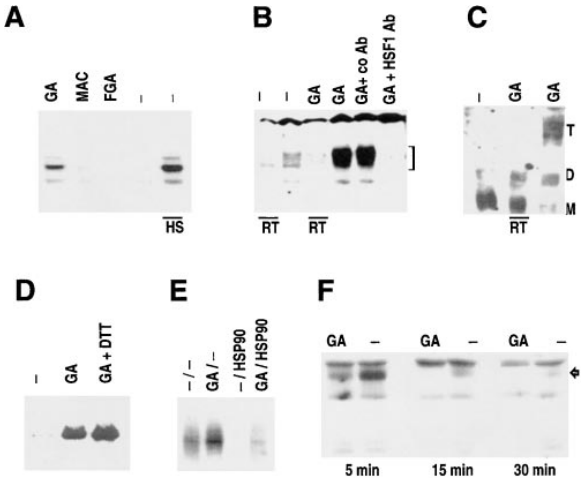

Figure 2. Activation of HSF1 by Geldanamycin

(A,B,D, and E) EMSA of activation lysate. GA, geldanamycin; MAC, macbecin II; FGA, 1,9-formyl geldanamycin; co Ab, rabbit preimmune serum; DTT, 10 mM dithiothreitol; Hsp90, 2 mg/ml Hsp90.

(C) Native anti-HSF1 blot of activation lysate.

(F) Anti-HSF1 Western blot of lysate incubated with chymotrypsin. Full-length HSF1 is indicated by arrow. See Figure 1 for other details.

Activation of HSF could result from either an increased rate of HSF trimerization or a reduced rate of trimer dissociation, or both. To find out which reaction was affected by heat shock, we tested whether dissociation of HSF1 trimers could occur in activation lysate. Trimeric recombinant HSF1 was added to activation lysate in amounts approximately comparable to endogenous HSF1 (estimated from EMSA of recombinant HSF1 and heat-treated activation lysate). Reactions were incubated for up to 3 hr at room temperature (Figure 1H) or threshold heat shock temperature (data not shown). EMSA revealed no decrease in DNA-binding activity and, therefore, in the concentration of recombinant HSF1 trimers. Similar results were obtained (data not shown) when lysate was first heat-shocked and then incubated at room temperature or threshold heat shock temperature. Thus, HSF1 trimers are not efficiently disassembled by activation lysate as used here. Activation of HSF1 appears to result exclusively from an increased rate of factor trimerization.

Activation of HSF1 In Vitro by Geldanamycin and Denatured Proteins

Addition to activation lysate of geldanamycin but not of analogues macbecin II and 1,9-formyl geldanamycin drastically enhanced HSE DNA-binding activity (Figure 2A). That this increase in DNA-binding activity reflected activation of HSF1 was documented by HSF1 antibody supershift (Figure 2B) and native anti-HSF1 blot (Figure 2C). Activation was not inhibited by strongly reducing conditions (Figure 2D). Thus, activation of HSF1 by geldanamycin in vitro most likely results from a reduced activity of Hsp90 and not from oxidative protein damage.

Geldanamycin activated HSF1 at threshold heat shock temperature but not at room temperature (Figures 2B and 2C), even though the compound is known to bind effectively to Hsp90 and to disrupt p23 complexes at the latter temperature (Whitesell et al., 1994; Smith et al., 1995). A similar observation was made for activation of HSF1 by denatured proteins (discussed below). These findings may reflect the temperature dependence of the HSF1 trimerization reaction. Use was made of this property of the in vitro system to further probe the notion that HSF1 activation by geldanamycin occurred as a consequence of the compound's interaction with Hsp90. Activation lysate was incubated with geldanamycin for 20 min at room temperature, which was expected to result in binding of the compound to Hsp90 and latent activation of HSF1 (Figure 2E). To allow trimerization to occur (here measured as DNA-binding activity), lysate was then subjected to threshold heat shock. Addition of purified Hsp90 immediately prior to the latter incubation greatly reduced the extent of HSF1 activation by geldanamycin (Hsp90 also suppressed the low-level activation occurring in the absence of geldanamycin, but see below for a more extensive analysis of this aspect). That trimerization does not occur at room temperature was also exploited in an experiment demonstrating that geldanamycin greatly increases the protease sensitivity of inactive HSF1 (Figure 2F; see Experimental Procedures for details of protocol). Taken together, the results of the latter two experiments suggest that inactive HSF1 is bound by Hsp90 and that this association prevents and its interruption promotes factor trimerization. Note that in the above experiments geldanamycin was used at 50 μg/ml to produce maximal effects. Activation of HSF1 was detected at concentrations as low as 2 μg/ml.

To find out whether HSF1 could be activated by nonnative proteins in our in vitro system, chemically denatured bovine serum albumin (CM-BSA) and activation lysate (CM-extract) were prepared by denaturation in urea and reductive carboxymethylation (Ananthan et al., 1986). Addition of these preparations of denatured protein to activation lysate (at 0.4 mg/ml) strongly stimulated HSE DNA-binding activity, whereas comparable amounts of native activation lysate, BSA, or ovalbumin had no effect (Figure 3A). This increased DNA-binding activity was due to HSF1 (Figures 3B and 3C). To rule out that the observed activation of HSF1 was caused by residual urea or other chemicals, a preparation of CM-BSA was diluted severalfold and reconcentrated using a Centricon 30 Microconcentrator. The volume of the pass-through fraction was also reduced to the original volume of the protein preparation by lyophilization. Reconcentrated protein fraction but not pass-through fraction greatly stimulated DNA-binding activity (Figure 3D). It is interesting to note that the relative concentration of denatured protein at which activation of HSF1 occurs in vitro (0.5%–1.5% of total protein) is similar to that previously estimated to be required to trigger Hsp expression in vivo (1%–2%; Lepock et al., 1993).

To learn whether HSF1 activation was mediated by soluble, nonnative proteins or by protein aggregates, a preparation of CM-BSA was subjected to a 30 min centrifugation at 100,000 $\times$ g. About 50% of protein was found in the pellet fraction. Similar amounts of protein from the pellet and soluble fractions were incubated with activation lysate. Only soluble, nonnative proteins caused significant activation of HSF1 DNA-binding activity (Figure 3D).

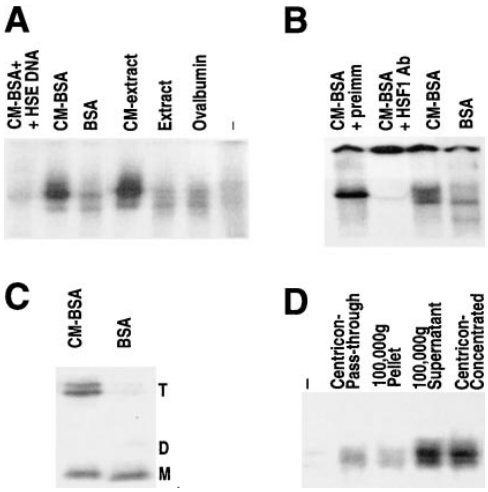

Figure 3. Activation of HSF1 by Nonnative Proteins

(A,B, and D) EMSA.

(C) Native anti-HSF1 blot. BSA, bovine serum albumin; CM-BSA, denatured, carboxymethylated BSA; Extract, activation lysate; CM-extract, denatured, carboxymethylated lysate. All protein fractions were added at final concentrations of 0.4 mg/ml. See Figure 1 for other details.

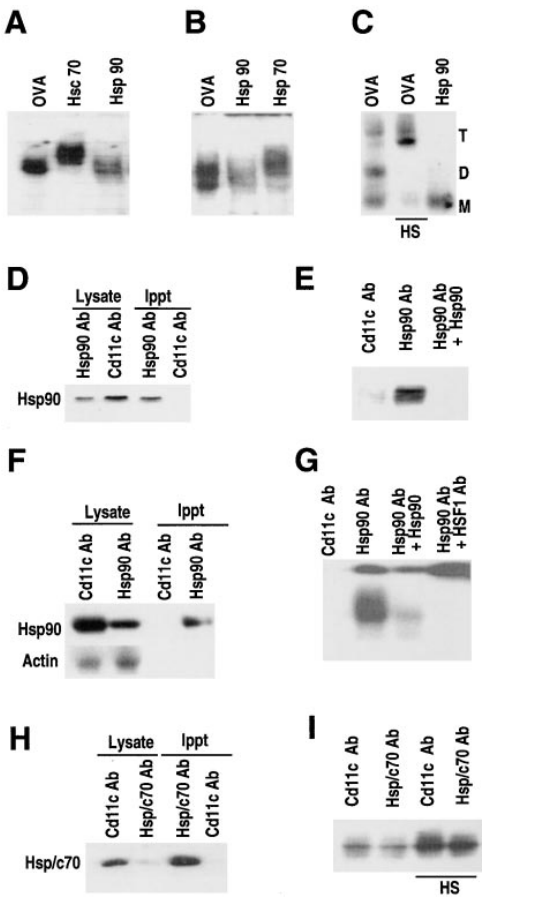

Figure 4. Effects of Increased or Reduced Levels of Hsp90 and Hsp/c70 on HSF1 Activation

(A and B) EMSA of lysate incubated in the presence of 2 mg/ml ovalbumin (OVA), Hsc70, Hsp70, or Hsp90.

(C) Native anti-HSF1 blot.

(D and E) First Hsp90 depletion experiment. (D) Anti-Hsp90 Western blot. (E) EMSA of antibody-treated lysates. Hsp90 Ab + Hsp90: 1 mg/ml purified Hsp90 (2 mg/ml in the second experiment) added to Hsp90-depleted lysate prior to threshold heat shock.

(F and G) Second Hsp90 depletion experiment. (F) Anti-Hsp90 and anti-actin Western blot. (G) EMSA.

(H and I) Hsp/c70 depletion experiment. (H) Anti-Hsp/c70 Western blot. (I) EMSA.

See text and Figure 1 for other details.

Hsp90 Retains HSF1 in the Inactive, Nontrimeric State

To test whether chaperones Hsp90 and Hsp/c70 could suppress heat activation of HSF1, purified chaperones were added (at 2 mg/ml in the experiments shown) to activation lysate, and reactions were exposed to threshold heat shock. Control reactions included ovalbumin instead of a chaperone. Hsp90 severely inhibited induction of HSF1 DNA-binding activity (Figures 4A and 4B). Inhibition by Hsc70 and Hsp70 was weak and variable. As reported previously (Abravaya et al., 1992; Baler et al., 1992), DNA-HSF1 complexes in reactions containing added Hsp/c70 are retarded because of binding of Hsp/c70 to trimeric HSF1. Addition of Hsp90 to activation lysate prevented trimerization of HSF1 as well as resulted in the abolishment of HSF1-Hsp/c70 heterodimers (Figure 4C). This observation most likely reflects the formation of Hsp90-containing HSF1 complex at the expense of HSF1 trimers and HSF1-Hsp/c70 complex. That no new signal corresponding to Hsp90-containing HSF1 complex appeared may be explained (see below) by the instability of this complex in buffers containing salt at moderate to high concentrations (such as the buffers used in native electrophoresis).

If Hsp90 plays a significant, nonredundant role in retaining HSF1 in the inactive state, immunodepletion of Hsp90 from activation lysate would be expected to result in factor activation. Results from two immunodepletion experiments are presented in Figures 4D–4G. These experiments were selected from nine independent experiments to show maximal and minimal activating effects observed. Parallel reactions were incubated on ice for 4 hr with immobilized Hsp90 antibody AC88 and isotype-matched control antibody Cd11c, respectively. Note that antibody AC88 is known to prefer free over complexed Hsp90. At the end of reactions, antibodies were removed by centrifugation. Antibody additions resulted in a slight dilution (typically less than 2-fold) of reactions. Because the HSF1 trimerization reaction is highly concentration-dependent, Hsp90 and control antibody-treated lysates were reconcentrated to the original protein concentration by lyophilization. Incubation with Hsp90 antibody reduced the concentration of Hsp90 by about 80% (Figures 4D and 4F). Similar aliquots of Hsp90 and control antibody-treated lysates were exposed to threshold heat shock and then assayed by EMSA (Figures 4E and 4G). On average, Hsp90 antibody-treated lysate exhibited about 10-fold higher HSE DNA-binding activity than control antibody-treated lysate. Induced DNA-binding activity in Hsp90 antibody-treated lysate was supershifted quantitatively by anti-HSF1 antibody (Figure 4G). Deregulation of HSF1 DNA-binding

activity could be prevented by addition of purified Hsp90 (at 1 mg/ml in Figure 4E and 2 mg/ml in Figure 4G) to Hsp90 antibody-treated lysate prior to exposure to threshold heat shock. We conclude from these results that Hsp90 plays a nonredundant role in keeping HSF1 in the inactive, nontrimeric state, that is, in repressing HSF1 activation. The results also imply that the interaction between inactive HSF1 and Hsp90 is dynamic. In additional experiments (data not shown) in which Hsp90 and control antibody-treated lysates were mixed at different ratios prior to incubation, we observed that a 40%–50% reduction of the level of Hsp90 was sufficient to cause detectable activation of HSF1.

HSF1 Is Not Activated upon Depletion of Constituents of Hsp90-Containing Multichaperone Complexes Other than Hsp90

In light of the dramatic activating effect of Hsp90 depletion, it appeared unlikely that Hsp/c70 could function as an independent HSF1 repressor of similar importance as Hsp90. However, Hsp/c70 is also a constituent of Hop complex. This and other Hsp90-containing multichaperone complexes exist in dynamic equilibria with their respective constituents. If Hsp90 repressed HSF1 activation only in the context of Hop complex, depletion of any essential constituent of this complex (i.e., Hsp90, Hop, and Hsp/c70) should result in dissociation of the complex and in deregulation of HSF1 activity. To test this possibility, activation lysate was incubated with monoclonal antibody BB70 that recognizes both Hsc70 and Hsp70 or a control monoclonal antibody (Cd11c) and was reconcentrated as before. Depletion of Hsp/c70 was virtually complete (Figure 4H). EMSA of Hsp/c70-depleted and mock-depleted lysates exposed to threshold heat shock revealed that removal of Hsp/c70 did not deregulate HSF1 activity (Figure 4I). Exposure to higher temperature (heat shock condition) induced similarly elevated levels of HSF1 DNA-binding activity in the two lysates (Figure 4I). Thus, Hsp/c70- and, by extension, Hsp90-containing Hop complexes appear not to be independent repressors of HSF1 activation. Similar immunodepletion experiments were also carried out using antibodies against Hop and Hop complex–associated protein Hip. Again, even though the concentrations of Hop and Hip could be reduced to low levels (Figures 5B and 5D), no significant activation of HSF1 occurred (Figures 5A and 5D). Immunodepletion (Figures 5B and 5C) of two constituents of p23 complexes, p23 and CyP40, similarly failed to activate HSF1 (Figures 5A and 5C). Thus, neither Hop nor p23 complexes, nor their individual constituents (other than Hsp90), play nonredundant roles in repressing HSF1 activity. Analogous observations were also made for Hsp40 (Figures 5A and 5B).

Hsp90-Containing HSF1 Complex Forms in the Cell and Dissociates Rapidly during Stress

As discussed before, the interaction between HSF1 and Hsp90 appears to be dynamic. In agreement with this notion, we were consistently unable to immunoprecipitate an Hsp90-containing HSF1 complex from activation

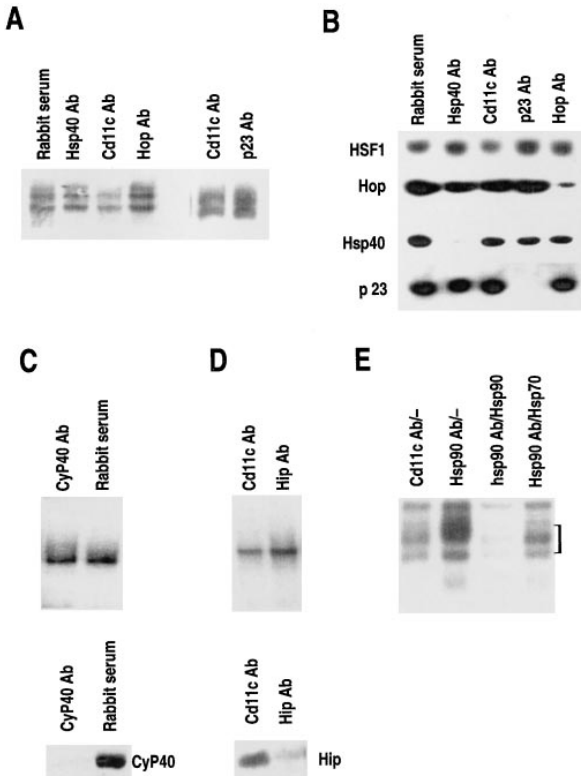

Figure 5. Immunodepletion of Different Constituents of Hsp90-Containing Multichaperone Complexes

(A-D) Immunodepletion with antibodies against Hop, Hip, p23, CyP40, and Hsp40. (A) and top parts of (C) and (D): EMSA. (B) and bottom parts of (C) and (D): Western blots detecting the proteins indicated on the side.

(E) Suppression of HSF1 activation in Hsp90-antibody-treated lysate by Hsp70 (2 mg/ml) and Hsp90 (2 mg/ml) added prior to incubation under threshold heat shock conditions. Rabbit serum served as control for Hsp40, CyP40, and HSF1 antibodies, and mouse monoclonal antibody Cd11c for Hop, Hip, and p23 antibodies.

lysate. Steroid receptor complexes, which are similarly dynamic, were previously found to be reasonably stable in extract prepared in buffer containing little salt and lacking detergent (Sanchez et al., 1990). Using similar conditions, we recently succeeded in coimmunoprecipitating HSF1 from extract of unstressed cells using an Hsp90 antibody (data not shown). To obtain evidence that the mechanism of HSF1 repression delineated by the above in vitro experiments can operate in the cell, we wished to demonstrate that Hsp90-containing (inactive) HSF1 complex is formed in the cell. In the experiment shown in Figure 6A, unstressed HeLa cell cultures were exposed to the chemical cross-linker dithiobis(succinimidyl propionate) for 5 min. As indicated by trypan blue staining, the integrity of cells was preserved under the conditions used. Cross-linking reagent was rapidly removed, cells were harvested, and extract was prepared. Hsp90-containing complexes were pulled down under stringent conditions using Hsp90 antibody 9D2 (or, in experiments not shown, Hsp90 antibody H9010 provided by David Toft). After reversal of cross-linking, immunoprecipitated proteins were analyzed by anti-HSF1 and anti-Hsp90 blot. Results showed that HSF1 was

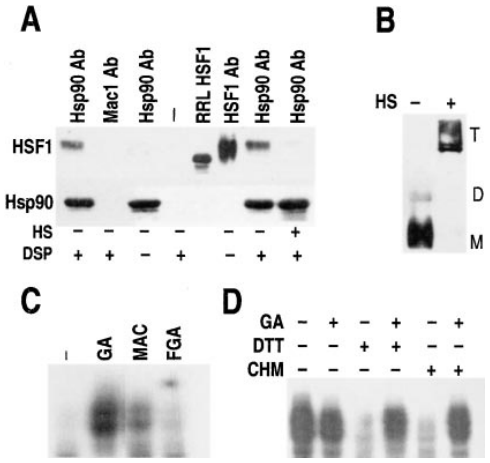

Figure 6. Hsp90-Containing HSF1 Complex in the Unstressed Cell, and Activation of HSF1 by Benzoquinone Ansamycins In Vivo

(A) Immunoprecipitation of in situ-cross-linked Hsp90-containing HSF1 complex. After reversal of cross-linking, immunoprecipitated protein was analyzed by anti-HSF1 and anti-Hsp90 Western blot. On top: antibodies used in immunoprecipitation; RRL HSF1: ^{35}S-methionine-labeled, underphosphorylated HSF1 synthesized in vitro. On the bottom: HS: 5 min/45°C heat shock; DSP: cross-linker dithiobis(succinimidyl propionate).

(B) Native anti-HSF1 blot of extracts from unstressed or heat-shocked (5 min/45°C) cells.

(C) EMSA of extracts from cells exposed for 90 min to the compounds (at 1 μg/ml) indicated.

(D) EMSA of extracts from cells exposed as indicated on top. Final concentrations: DTT, 1 mM; GA, 1 μg/ml; CHM, 50 μg/ml. See previous figures for other details.

present in immunoprecipitated complexes. Control immunoprecipitations in which the Hsp90 antibody was substituted by an isotype-matched control antibody, or omitted, failed to recover HSF1. Furthermore, HSF1 could not be coimmunoprecipitated from extract of naive cells. These data indicate that Hsp90-containing HSF1 complex is formed in the cell. That HSF1 was quantitatively present in the inactive, nontrimeric form in the unstressed cells (Figure 6B) strongly suggests that the complex includes inactive HSF1. To find out whether HSF1 is released from the complex during stress, relative amounts of complex were compared in unstressed cells and cells exposed to a 5 min 45°C heat shock (Figure 6A). The complex was virtually absent in the heat-treated cells, and essentially all HSF1 was in the trimeric form (Figure 6B). Thus, Hsp90-containing HSF1 complex dissociates during stress, and complex dissociation and HSF1 trimerization are closely correlated events.

HSF1 Activation by Benzoquinone Ansamycins In Vivo

Susceptibility of a process to benzoquinone ansamycins such as geldanamycin is generally accepted as strong evidence for an involvement of Hsp90 in the process. However, since the compounds can also act as redox cyclers and may cause oxidative protein unfolding, they may not reliably indicate the participation of Hsp90 in a mechanism such as the heat shock response that is triggered by nonnative protein. To address this concern, activation of HSF1 by different benzoquinone ansamycins was compared in vivo and in vitro. Geldanamycin but not macbecin II and 1,9-formyl geldanamycin was found to activate HSF1 in vitro under strongly reducing conditions (Figures 2A and 2D). The experiment in Figure 6C revealed that the relative potencies (for HSF1 activation) of the three compounds are similar in vivo and in vitro, suggesting that they act in vivo as they do in vitro. To demonstrate that geldanamycin can activate HSF1 in vivo under conditions in which oxidation of the intracellular environment cannot occur, cells were preincubated for 2 hr in the presence of 1 mM DTT (conditions adapted from Huang et al., 1994). At this relatively high concentration of reducing agent, protein synthesis is severely inhibited (data not shown). Inhibition of protein synthesis is known to substantially raise the threshold for stress activation of HSF1, presumably because of an increased availability of chaperones for processes other than protein synthesis (Baler et al., 1992). To compensate for this change in setpoint, cells were shifted to 42°C at the end of the preincubation period. As shown in Figure 6D, preincubation with DTT and, reproducing earlier observations, with cycloheximide prevented activation of HSF1 DNA-binding activity at 42°C. Exposure (for 45 min) of preincubated cells to geldanamycin strongly induced DNA-binding activity. Thus, geldanamycin can activate HSF1 in a reducing intracellular environment. Protein synthesis is not required for this activation.

Discussion

Our evidence for a role of Hsp90 in the repression of HSF1 activation is threefold. First, immunodepletion of Hsp90 results in drastic enhancement of HSF1 activity upon incubation of depleted lysate under threshold activation conditions. Purified Hsp90 suppresses this deregulation of HSF1 activity (Figures 4D–4G). While these results were obtained from experiments that monitored deregulation of HSF1 activity at 27°C–29°C (threshold heat shock), analogous immunodepletion experiments confirmed that Hsp90 can also function as HSF1 repressor at 37°C, the normal temperature of the human cell (data not shown). The latter experiments were carried out with 2-fold diluted activation lysate. This dilution reduced the rate of HSF1 activation at 37°C to about that typically observed at 27°C–29°C. Second, addition of Hsp90 to the in vitro system suppresses activation of HSF1 by threshold heat shock (Figures 4A and 4B), geldanamycin (Figure 2E), and chemically denatured BSA (not shown). Finally, Hsp90-specific compound geldanamycin activates HSF1 in vitro as well as in vivo under strongly reducing conditions in which the compound cannot cause protein oxidation (Figures 2D and 6D).

Several independent lines of experimentation support our conclusion that Hsp90 forms a complex with inactive HSF1 and is released from this complex during stress. First, assays of protease sensitivity of HSF1 in activation lysate suggested that inactive HSF1 is bound by a factor that can be released by geldanamycin (Figure 2F). Since geldanamycin is a specific Hsp90-binding compound, this factor is likely to be identical with Hsp90 or Hsp90-containing chaperone complex(es). Second, a typically

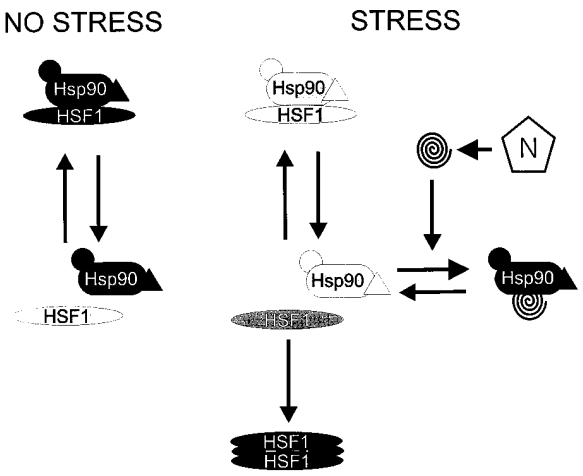

Figure 7. Hsp90 Model of Regulation of HSF1

Repressor is represented here as an Hsp90-containing complex. Differences in grayscale indicate differences in relative concentrations of proteins/complexes.

minor fraction of inactive HSF1 is associated with Hsp/c70 in activation lysate. Addition of Hsp90 displaces Hsp/c70 from HSF1 (Figure 4C). This is likely to reflect the formation of an Hsp90-containing HSF1 complex at the expense of HSF1-Hsp/c70 complex. Recently, stabilizing conditions were identified under which Hsp90-containing HSF1 complex can be immunoprecipitated (data not shown). Finally, Hsp90-containing HSF1 complex can be stabilized in the cell by cross-linking. Upon heat treatment of cells, the complex dissociates rapidly, and HSF1 trimers form (Figures 6A and 6B).

Hsp90-containing HSF1 complex is dynamic. As discussed before, the complex is normally unstable and can only be isolated under conditions known to stabilize Hsp90 interactions. Furthermore, that immunodepletion of Hsp90 results in HSF1 activation implies that HSF1-bound Hsp90 is in rapid equilibrium with unbound Hsp90. Our findings are summarized in the following model of regulation of HSF1 activation (Figure 7). In the unstressed cell, most HSF1 molecules are present in an Hsp90-containing complex(es) (which may further include other chaperones and associating proteins as indicated in the figure). HSF1 complex(es) continuously dissociates and reassembles. Upon stress, nonnative proteins accumulate and compete with HSF1 for binding to Hsp90 or Hsp90 multichaperone complex(es). As a result, the cycle of HSF1 complex assembly and dissociation is interrupted, and unbound HSF1 polypeptide accumulates. In this situation, both because of the increased concentration of unbound HSF1 and because the competing pathway (reassembly of HSF1 complex[es]) is effectively blocked, HSF1 trimerization is strongly favored.

We are uncertain as to whether Hsp90 can function as a repressor of HSF1 by itself or only in the context of multichaperone complexes (i.e., Hop or a p23 complex). Immunodepletion of constituents of Hop and p23 complexes other than Hsp90 does not activate HSF1, suggesting that none of these complexes plays a nonredundant role in repressing HSF1 activation. However, a substantial fraction of Hsp90 is present in multichaperone complexes, and steroid receptors appear only to be activatable when associated with a multichaperone complex (Hutchison et al., 1995; Smith et al., 1995). Future work will need to examine whether Hop and p23 complexes can interchangeably function as HSF1 repressors. Alternatively, Hsp90 may repress HSF1 activation in the context of a yet undiscovered multichaperone complex.

Two observations appear to be inconsistent with a role of Hsp70 as a major HSF1 repressor. First, virtually quantitative depletion of both Hsp70 and Hsc70 from activation lysate fails to cause deregulation of HSF1 activity. Second, reduction of the level of Hsp90 massively activates HSF1. Thus, there appears to be little opportunity for Hsp/c70 to function as an independent HSF1 repressor of consequence. Still, a small fraction of inactive HSF1 is found in a binary complex with Hsp/c70 in cell extracts (Rabindran et al., 1994; Baler et al., 1996; see also Nunes and Calderwood, 1995). The assembly of mature Hsp90-containing steroid receptor complex has been portrayed as an ordered process initiated by binding of Hsp70 to receptor (Smith, 1993; Smith et al., 1995). By analogy, we suggest that formation of HSF1-Hsp/c70 complex may be a required initial step leading to the formation of an Hsp90-containing HSF1 complex, from which HSF1 can be activated. Conceivably, HSF1 may be prevented from trimerizing as soon as it associates with Hsp/c70. To test this notion in a situation in which mature HSF1 complex does not form efficiently and a large fraction of inactive HSF1 should be available for binding Hsp/c70, an experiment was performed using Hsp90-depleted lysate (Figure 5E). Addition of Hsp70 to this lysate prevented activation, albeit less efficiently than back addition of Hsp90. Thus, it does appear that HSF1 associated with Hsp/c70 is unavailable for trimerization. This result may provide for an explanation for the observation that overexpression of Hsp70 renders cells somewhat refractory to heat activation of HSF1 DNA-binding or transcriptional activity (Mosser et al., 1993; Baler et al., 1996; but see also Rabindran et al., 1994) as well as for analogous findings made by Xiao and DeFranco (1997). Note, however, that the latter findings may be explained differently.

Experimental Procedures

Preparation of In Vitro Activation System

Adherent human HeLa cells were grown to 80% confluence in 150 mm dishes in Dulbecco's modified Eagle medium (DMEM [high glucose]) supplemented with 10% fetal calf serum and antibiotics (penicillin and streptomycin, both at 100 IU/ml) at 37°C and under 5% CO_2. Cells were scraped off 30 culture dishes in a minimal volume of DMEM at room temperature, combined, and collected by centrifugation (5 min at 750 × g). Cell pellet was cooled on ice and washed twice with ice-cold reaction buffer (10 mM HEPES [pH 7.5], 0.13 M NaCl, 5 mM KCl, 10 mM $MgCl_2$, 0.1 mM dithiothreitol [DTT], 0.3 mM PMSF). Washed cell pellet was transferred to a loose fitting Dounce homogenizer, and cells were homogenized by 30 strokes. Lysate was subjected to three successive centrifugations, each for 10 min at 10,000 × g. Supernatant solution (about 300 μl) was aliquoted, quick-frozen in liquid nitrogen, and stored at −70°C. Lysates contained 25–40 mg/ml protein as estimated by Dc Protein Assay (Biorad Laboratories, Melville, NY).

Activation In Vitro

Lysate was thawed on ice and supplemented with ATP-regenerating system (10 mM creatine phosphate, 50 μg/ml creatine phosphokinase) from 100× stock solutions of creatine phosphate and creatine

phosphokinase. Lysate containing ATP-regenerating system is referred to herein as activation lysate. Typically, reactions containing 9 μl of activation lysate were preincubated for 20 min at room temperature. After additions (1–2 μl), reactions were incubated in a waterbath for 1 hr at 27°C–29°C (threshold activation/heat shock temperature). Subsequent to incubation, reactions were cooled in ice water and kept on ice. In most experiments, aliquots of 1 μl were tested by EMSA or native anti-HSF1 blot.

Preparation of Hsps for Use in In Vitro Activation Assays

Sephadex G25 spin columns (Boehringer Mannheim Corp., Indianapolis, IN) were blocked and equilibrated in 0.4× reaction buffer containing 2 mg/ml bovine serum albumin (BSA). Purified bovine Hsp90, bovine Hsc70, and recombinant human Hsp70 (obtained in lyophilized form from StressGen Biotechnologies Corp., Victoria, BC) were reconstituted in water to concentrations of 2 mg/ml. Aliquots of 50 μl were added to preequilibrated spin columns, which were centrifuged at 1,000 × g for 2 min at room temperature. Eluates (about 50 μl) were concentrated by lyophilization to 5 μl, resulting in Hsp stock solutions with concentrations close to 20 mg/ml in 4× reaction buffer. These stocks were diluted 10- to 20-fold into activation reactions, which increased the buffer concentration in reactions to maximally 1.4× reaction buffer. Preliminary experiments demonstrated that activatability of HSF1 was maintained at buffer concentrations as high as 2.0×.

Immunodepletion Experiments

For Hsp90 immunodepletion, protein A agarose beads (50 μl packed beads) were preincubated with reaction buffer containing 2 mg/ml of BSA. Half of the beads were then reacted for 4 hr at 4°C with 200 μl of reaction buffer containing 20 μg of monoclonal Hsp90 antibody AC88 (Riehl et al., 1985), and the other half with a similar amount of control antibody, which was a monoclonal lymphocyte function antigen 1 antibody (Cd11c). In vitro activation lysate (50 μl) was incubated with the loaded antibody beads for 4 hr at 4°C. After removal of antibody beads, lysate was concentrated about 2-fold by lyophilization (to restore original protein concentration). Protein concentration was verified by Protein Assay, and adjustment was made as necessary by addition of a small volume of water. The same protocol was used for immunodepletion of other chaperones and associating proteins. When rabbit sera were used (CyP40, Hsp40, preimmune), 25 μl protein A (packed) beads were loaded with 5 μl of serum. Antibodies: F5 (Hop; Smith et al., 1993), JJ5 (p23; Johnson et al., 1994), BB70 (Hsp/c70; Smith et al., 1993), 2G6 (Hip; Prapapanich et al., 1996), Hsp40 antibody (polyclonal; Hattori et al., 1993), and CyP40 antibody (polyclonal; PA3–022). AC88 and BB70 were from StressGen Biotechnologies Corp., Cd11c from Boehringer Mannheim Corp., PA3–022 from Affinity Bioreagents, Inc., Golden, CO, and actin antibody from Amersham Corp., Arlington Heights, IL.

Cross-linking In Situ

Cultures of human HeLa cells were grown as described before. Heat treatment was by incubation in a 45°C water bath. Cultures (in 150 mm dishes) were washed twice with phosphate-buffered saline (PBS) and then incubated for 5 min at room temperature with PBS (8 ml) containing 2 mM dithiobis(succinimidyl propionate) in DMSO or DMSO only. Cross-linker was quenched by addition of 10 mM glycine. Cells were washed once with PBS and then harvested and lysed by incubation for 15 min on ice with RIPA buffer (50 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid, and protease inhibitors ["Complete"; Boehringer Mannheim]). After centrifugation for 10 min at 16,000 × g, protein concentrations of lysates were determined by Dc Protein Assay (Bio-Rad Laboratories) and equalized. Immunoprecipitations were performed with 500–600 μl of lysates (2–4 mg/ml protein). Lysates were first incubated in the cold with 30 μg of monoclonal antibody and then with 50 μl of a slurry of protein G–agarose conjugate. Immunoprecipitates were collected by centrifugation and washed extensively with RIPA buffer. Washed immunoprecipitates were taken up in SDS-PAGE sample buffer (containing reducing agent) and boiled for 5 min. The latter step reverses cross-linking. In the experiments shown, monoclonal Hsp90 antibody 9D2 (Lai et al., 1984) and isotype-matched control monoclonal antibody Mac 1 (Boehringer Mannheim) were used for immunoprecipitation, and the rabbit anti-HSF1 antibody described by Rabindran et al. (1991) for Western blot detection of HSF1. Both the HSF1 antibody and 9D2 were obtained from StressGen Biotechnologies Corp.

Protease Sensitivity Assay

Activation lysate was preincubated at room temperature for 15–20 min with or without geldanamycin. One microliter of a solution of chymotrypsin (62.5 μg/ml) in reaction buffer was then added to 10 μl of activation lysate, which was then incubated further at room temperature. Aliquots of 1–2 μl were removed at intervals into 9 μl of SDS-PAGE sample buffer and immediately boiled for 5 min. Samples were analyzed by anti-HSF1 Western blot.

Recombinant HSF1

For preparation of bacterially expressed HSF1, a full-length human HSF1-coding sequence (Baler et al., 1993) was subcloned into vector pGEX-2T (Pharmacia, Piscataway, NJ). Glutathione-HSF1 fusion protein was expressed in *E. coli* BL21 and purified on glutathione beads (Glutathione Sepharose 4B, from Pharmacia). Bound fusion protein was treated with bovine thrombin (Pharmacia) to release full-length HSF1. For preparation of baculovirus-expressed HSF1, a DNA sequence encoding amino-terminally FLAG-tagged HSF1 (Nair et al., 1996) was transferred to baculovirus shuttle vector VL1393 (Pharmingen, San Diego, CA), and recombinant baculovirus was isolated and stock prepared by a standard procedure (O'Reilly et al., 1992). FLAG-tagged HSF1 was expressed in insect SF9 cells, purified on anti-FLAG M2 antibody affinity matrix (IBI, New Haven, CT), and released by FLAG peptide (IBI). FLAG peptide was subsequently removed. Amounts and purity of HSF1 preparations were estimated by SDS-PAGE and Coomassie blue staining.

Electrophoretic Mobility Shift Assays

Reactions were conducted essentially as described in Zuo et al., 1994. For HSE and LexA oligonucleotide probes see Baler et al., 1993, and Zuo et al., 1994, respectively. Samples tested were either activation lysate or, in the case of in vivo experiments, extract prepared by repeated freezing and thawing of cells in high-salt buffer essentially as described in Baler et al., 1993. For HSF1 supershift experiments, 0.5 μl of anti-HSF1 serum (Baler et al., 1993) or preimmune rabbit serum were added to 10 μl binding reactions at the end of incubations. Reactions were analyzed by electrophoresis on 4.5% PAGE gels in TGE buffer (40 mM Tris-base, 400 mM glycine, 2 mM EDTA). Gels were dried and exposed for autoradiography.

Native Anti-HSF1 Blots and Western Blots

For native PAGE, typically 1 μl of activation lysate or, in the case of in vivo experiments, cell extract prepared as described above was added to 10 μl of EMSA buffer, and the solution was applied to a limiting pore size gel in TGE buffer. Electrophoresis, blotting, and immunodetection were performed as described previously (Baler et al., 1993; Zuo et al., 1994). Immunoblots of SDS-PAGE gels were prepared analogously. For all HSF1 blots, except those in Figure 6, and antibody supershift assays the polyclonal HSF1 antibody characterized by Baler et al. (1993) was used. In addition to HSF1 (75–80 kDa), this antibody recognizes two other HeLa proteins with subunit MW of 110 kDa and 57 kDa, respectively, in Western blots. Note that the latter proteins are not seen by the antibody in their native conformations (Baler et al., 1993).

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