

Inhibition of Heat Shock Transcription Factor by GR

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The GR is a hormone-activated transcription factor that acts to regulate specific gene expression. In the absence of hormone, the GR and other steroid receptors have been shown to form complexes with several mammalian heat shock proteins. As heat shock proteins are produced by cells as an adaptive response to stress, speculation has existed that communication between the heat shock and glucocorticoid hormone signal pathways must exist. Only recently has evidence to support this hypothesis been reported. In almost all cases, the evidence has been of an ability of heat shock to cause a potentiation of the glucocorticoid hormone response. In this proposal, evidence is now presented that heat shock signaling can, in turn, be regulated by glucocorticoids. In mouse L929 cells stably expressing a chloramphenicol acetyltransferase reporter controlled by the human heat shock protein70 promoter and containing known binding sites for heat shock transcription factor 1 treatment with glucocorticoid agonist (dexamethasone) results in a dose-dependent decrease of stress-induced chloramphenicol acetyltransferase gene expression. In these cells, inhibition of heat shock protein70 promoter activity by dexamethasone was completely blocked by GR antagonist (RU486). Similar treatment of L929 cells stably expressing a chloramphenicol acetyltransferase reporter under the control of the constitutively active SV40 promoter showed no such inhibition by dexamethasone. More importantly, dexamethasone was also found to inhibit heat shock-induced expression of the major heat shock proteins—heat shock proteins70, 90, and 110. Thus, the inhibitory effect of dexamethasone appears to apply to most, if not all, heat shock transcription factor 1-regulated genes. Although dexamethasone did not prevent the DNA-binding function of heat shock-activated heat shock transcription factor 1, it did inhibit a constitutively active mutant of human heat shock transcription factor 1 under nonstress con-

ditions, suggesting that dexamethasone repression of heat shock transcription factor 1 was primarily through an inhibition of heat shock transcription factor 1 transcription enhancement activity. To more accurately characterize the stage of GR signaling responsible for inhibition of heat shock transcription factor 1, a series of Chinese hamster ovary cells containing either no GR, wild-type mouse GR, or single-point mutations of GR were employed. Dexamethasone inhibition of heat shock-induced heat shock transcription factor 1 activity was observed in the presence of wild-type GR, but not in Chinese hamster ovary cells lacking GR, suggesting that signaling cascades other than GR were not involved in this effect of dexamethasone. Consistent with this conclusion was the observation that dexamethasone had no effect on activity of the MAPKs (ERK1, ERK2, or *c-jun* N-terminal kinase), which are known to negatively regulate heat shock transcription factor 1. Dexamethasone inhibition of heat shock transcription factor 1 was not seen in Chinese hamster ovary cells expressing GR defective for DNA-binding function. Moreover, dissociation of GR/Hsp90/Hsp70 complexes was observed in response to hormone for both the wild-type and DNA binding-defective forms of GR, demonstrating that release of Hsp90 or Hsp70 (both of which are known to keep heat shock transcription factor 1 in its inactive state) could be ruled out as a potential mechanism. Thus, it appears that GR-mediated transactivation or transrepression is required for the inhibitory effect of dexamethasone on heat shock transcription factor 1 activity. Taken as a whole, these results provide evidence for a novel mechanism of cross-talk in which signaling by the GR can attenuate the heat shock response in cells through an inhibition of the transcription enhancement activity of HSF1. (*Molecular Endocrinology* 15: 1396-1410, 2001)

THE GR IS A steroid-activated transcription factor involved in physiological responses that serve to protect organisms against stress (1, 2). The heat shock

Abbreviations: CAT, chloramphenicol acetyltransferase; CoA, coenzyme A; Dex, dexamethasone; DTT, dithiothreitol; GRE, glucocorticoid response element; HSE, heat shock element; HSF, heat shock transcription factor; HSP, heat shock protein; JNK, *c-jun* N-terminal kinase; MMTV, mouse mammary tumor virus; nGRE, negative glucocorticoid response element.

response is a well known cellular adaptation to stress that is mediated by heat shock transcription factors (HSFs), principally HSF1 (3, 4). Recently, it has become clear that both GR and HSF1 are maintained in their respective inactive states in the cytoplasm by association with a heat shock protein (HSP)-based chaperone complex containing Hsp70 and Hsp90 (5-10). Because of these similarities, a variety of investigations have tested the notion that the heat shock (HS) and steroid receptor signaling pathways are in-

terrelated. In most of these studies, a dramatic effect of heat shock on the function of steroid receptors has been found. For example, heat shock-induced translocation to the nucleus of unliganded GR has been observed for the endogenous GR of L929 cells (11), mouse GR expressed in Chinese hamster ovary (CHO) cells (12), and human GR expressed in COS cells (13). Partial activation of transcription enhancement activity of hormone-free GR has also been shown in response to heat shock (12). It has also been shown that GR-dependent transrepression of the collagenase promoter, a response involving GR interaction with AP-1, can be induced in the absence of hormone by heat shock treatment in both COS-7 and HeLa cells (13). Similarly, heat shock has been found to mimic the ability of dexamethasone (Dex) to modulate Fc receptor expression in murine macrophages (14).

When heat shock is combined with hormone treatment, dramatic increases in steroid receptor activation have been documented. Combined hormone and stress treatment of T47D breast cancer cells was found to produce a level of PR-mediated transcription activity much higher than that seen in response to hormone alone (15). We have found that heat shock can increase Dex-induced GR-mediated gene expression above that seen with maximal concentrations of hormone (16, 17). Our recent data suggest a role for HSF1 in this response, as specific modulation of HSF1 by quercetin, sodium vanadate, or wortmannin results in a corresponding modulation of the stress potentiation of GR (18, 19).

In contrast to the effects of heat shock on steroid receptor function, evidence for control of the heat shock response by steroids is much less common. Most early attempts to uncover such a relationship tested the effects of steroids on the levels of HSP expression. In our laboratory, we have not seen a change in HSP levels in response to glucocorticoid treatment alone, although we have not measured Dex effects on HSP synthesis under heat shock conditions. Yet, reports of the induction of low molecular weight HSPs in *Drosophila* by the insect steroid hormone α -ecdysterone are available (20), as is a report by Fisher *et al.* (21) in which Dex treatment of CHO cells induces a state of thermotolerance similar to that obtained in response to mild heat shock. In the latter report, however, Dex-induced thermotolerance was not the result of any measurable increase in Hsp70 or Hsp90 protein content. In contrast, Dex treatment of plasmacytoma cells was found to actually prevent induction of Hsp70 in response to heat shock (22).

Recently, Xiao and DeFranco (23) showed that overexpression of GR in COS-1 cells by transient transfection results in the stress-free activation of HSF1. As this response could be mapped to the steroid-binding domain of the GR (also the HSP-binding region), and as this response was abolished when GR was transfected in the presence of Dex, the authors concluded that activation of HSF1 was occurring due to sequestration of HSPs from HSF1 to the newly forming GR/

HSP complexes. As concurrent overexpression of Hsp70 prevented HSF1 activation by GR expression, it was also concluded that Hsp70 was the likely negative regulator of HSF1 sequestered in this way. From our perspective, these observations by Xiao and DeFranco were important evidence that the HSP-based chaperone complexes of intact cells can be readily exchanged between GR and HSF1. In the present study, we have examined the effect of GR signaling on HSF1 by use of cells containing naturally or stably expressed GR. In contrast to transiently expressed GR, we find that hormone activation of GR in these cells results in repression of stress-induced HSF1 activity, by a mechanism that does not involve HSPs released from the GR complex. Instead, repression of HSF1 requires the DNA-binding and the transrepression or transactivation functions of GR. Our data have therefore provided the first direct evidence for cross-talk between GR and HSF1, by demonstrating that GR repression of HSF1 occurs through the genomic actions of naturally expressed receptor—a mechanism that may have important endocrine and therapeutic consequences.

RESULTS

Inhibition of Human Hsp70 Promoter Activity and of Endogenous HSP Expression by Glucocorticoid Agonist

We have stably transfected the GR-containing mouse L929 cell line with a chloramphenicol acetyltransferase (CAT) reporter gene (p2500-CAT) under the control of the human Hsp70 promoter (LHSE cells). Heat shock-induced activation of this promoter has been shown to require binding by HSF1 to consensus heat shock elements (HSEs) present between -65 and -52 bp of the transcription start site (24). As an initial test of the effects of GR signaling on the heat shock response, we performed the experiments of Fig. 1 in which LHSE cells were subjected to a variety of hormone and stress conditions. In Fig. 1A, a Dex-concentration dependence was performed. Maximal inhibition of heat shock-induced Hsp70 promoter activity was observed at approximately $1 \mu\text{M}$ Dex. This concentration of Dex was then used to compare the effects of hormone on two forms of stress. The results show that although heat shock (43 C, 2 h) and chemical shock (200 μM sodium arsenite, 2 h) will activate transcription from the Hsp70 promoter to different levels (Fig. 1B), $1 \mu\text{M}$ Dex caused about the same level of inhibition of CAT gene expression induced by heat or chemical stress (70% and 75%, respectively). In all of these experiments, Dex was added to the cells 4 h before the stress event and was maintained in the media during the 20-h recovery period before assay for CAT. However, similar results have been obtained when Dex is not present during the recovery period, or when Dex is added for only 4 h during recovery (data not shown).

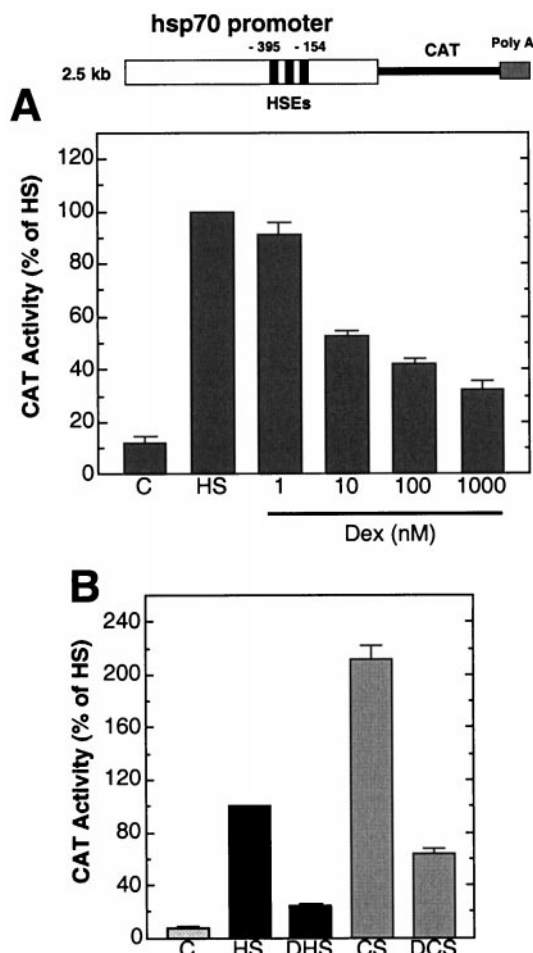


Fig. 1. Dex Inhibits Stress-Induced, HSF1-Mediated CAT Gene Expression in LHSE Cells

A, Mouse L929 cells were stably-transfected with a CAT reporter construct driven by the human Hsp70 promoter to generate LHSE cells. Replicate flasks of LHSE cells were treated with increasing concentrations of Dex, as indicated, followed by heat shock (43 C, 2 h) and recovery for 20 h in the continued presence of hormone. Lysates were prepared and assayed for CAT activity. The results represent means \pm SEM of six independent experiments. B, Replicate flasks of LHSE cells were subjected to the hormone and stress conditions indicated, followed by recovery for 20 h and assay for CAT. The results represent means \pm SEM of 3–6 independent experiments. C, No treatment; HS, 43 C for 2 h; DHS, Dex (1 μ M) for 4 h followed by HS; CS, 200 μ M sodium arsenite, 2 h; DCS, Dex (1 μ M) for 4 h followed by CS.

The specificity of this response was tested in two ways. First, the effect of RU486 antagonist was determined (Fig. 2). The results show that Dex inhibition of heat shock-induced CAT activity in the LHSE cells can be completely blocked by RU486 (Fig. 2A). That RU486 is actually acting as a GR antagonist in these cells was determined by use of L929 cells stably transfected with the GR-responsive mouse mammary tumor virus (MMTV)-CAT reporter (LMCAT cells). In this case, RU486 did not by itself cause activation of GR but did effectively block Dex activation of the receptor

(Fig. 2B). To ensure that Dex inhibition of CAT expression from the Hsp70 promoter was not due to a generalized increase in transcription or to alterations in turnover for CAT mRNA, the effect of Dex was measured on CAT expression controlled by the constitutively active SV40 promoter (LSV2CAT cells). The results of Fig. 3 show that Dex alone or combinations of heat shock, arsenite, and hormone have essentially no effect on CAT enzyme levels in these cells, even after 24 h of treatment with hormone. Thus, by these initial criteria, it appears that inhibition of Hsp70 promoter activity in our system is mediated by agonist-activated GR.

To determine whether the inhibitory effect of hormone was either specific to the Hsp70 promoter or the result of a more general effect on HSP expression, we measured the effects of Dex on the rates of synthesis of the major HSPs. In the experiments of Fig. 4, LHSE cells were subjected to chemical shock using 200 μ M arsenite followed by pulse labeling with [35 S]methionine during a time course of recovery. Replicate flasks were treated with 1 μ M Dex for 4 h before chemical shock, followed by recovery in the continued presence of hormone. As expected, the results show that chemical shock will dramatically increase the rates of synthesis for Hsp70, Hsp90, and Hsp110, especially at the 8 h time point of recovery. These results are consistent with our prior observations, in which maximal induction of HSP synthesis following stress is observed between 8 and 12 h of recovery (18). With respect to "Hsp70," both the so-called constitutive (Hsc70) and inducible (Hsp70) forms of this protein are up-regulated in response to arsenite (Fig. 4A). More importantly, Dex appeared to inhibit the rates of synthesis for all of these HSPs (Fig. 4A)—a result that was confirmed for Hsc/Hsp70 and Hsp90 by densitometric scanning of the autoradiograms (Fig. 4B). Interestingly, the level of inhibition by hormone (at 8 h of recovery) was about the same (50–55%) for both Hsp70 and Hsp90. Taken as a whole, therefore, it appears that the inhibitory effect of Dex is not limited to the Hsp70 promoter but, rather, is due to actions on some factor common to the expression of HSPs in general. As the obvious candidate in this regard is HSF1, we tested this possibility in the following section.

Dex Repression of Heat Shock Signaling through Inhibition of HSF1 Transactivity

In the p2500-CAT reporter used in this study, expression of CAT is controlled by a promoter derived from the inducible form of human Hsp70 (25). The promoter is 2,500 bp in length, of which approximately 700 bp have been sequenced by Voellmy and co-workers (24). Because of the large nature of this promoter, it is highly likely that many *trans*-acting factors in addition to HSF1 bind to this region, some of which may be regulated by stress. Indeed, it has been shown that maximal response to heat shock by this promoter

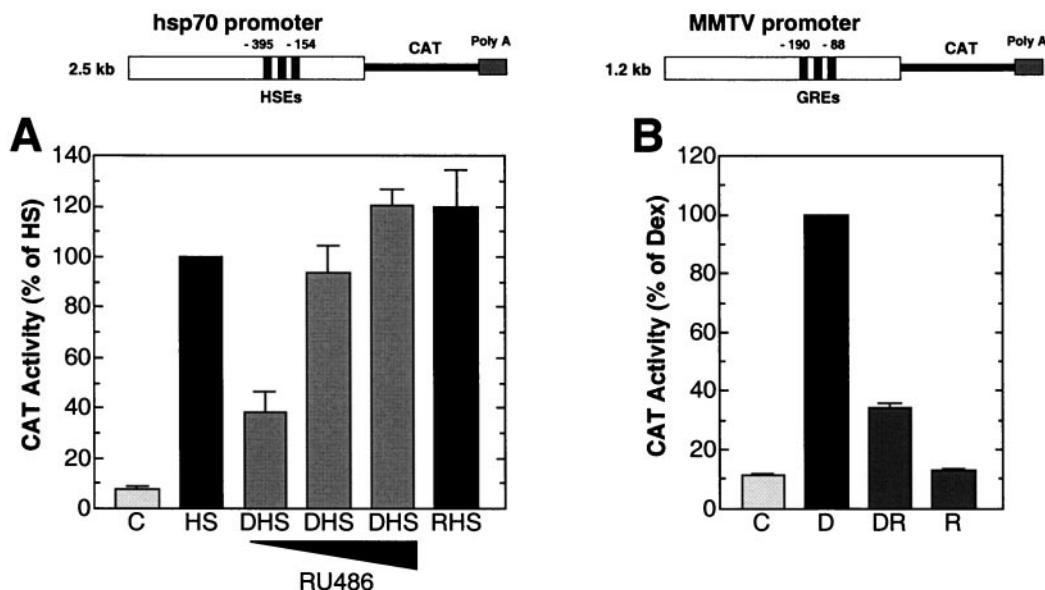


Fig. 2. The GR Antagonist RU486 Blocks Dex-Induced Inhibition of HSF1 Activity in Heat-Shocked LHSE Cells

A, Effects of RU486 on Dex inhibition of HSF1 activity. Replicate flasks of LHSE cells subjected to the indicated hormone and stress treatments were allowed to recover for 20 h in the continued presence of hormone. Lysates were prepared and assayed for CAT activity. The results represent means \pm SEM of three to six independent experiments. C, No treatment; HS, 43 C for 2 h; DHS, Dex (1 μ M) plus 0, 1, or 10 μ M RU486 for 4 h followed by HS; RHS, RU486 (1 μ M) for 4 h followed by HS. **B**, Effects of RU486 on Dex-induced GR-mediated CAT gene expression. L929 cells were stably transfected with an MMTV-CAT reporter to generate LMCAT cells. Replicate flasks of LMCAT were subjected to the indicated hormone treatments. Lysates were prepared and assayed for CAT activity. The results represent means \pm SEM of six independent experiments. C, No treatment; D, Dex (1 μ M) for 20 h; DR, Dex (1 μ M) and RU486 (10 μ M) for 20 h; R, RU486 (10 μ M) for 20 h.

requires more than merely binding by HSF1 (26). For these reasons, it was reasonable to speculate that Dex inhibition of stress-induced p2500-CAT activity was due to an effect of Dex on a transcription factor(s) other than HSF1. To discriminate between these two possibilities, we sought a means by which HSF1 transactivity could be measured in isolation. This was achieved through use of a constitutively active mutant of human HSF1 (hHSF1-E189) developed by Voellmy and co-workers (27). hHSF1-E189 was generated by a single-amino acid substitution at residue 189 (Fig. 5A), which resides in one of three hydrophobic LZ domains thought to be required for maintaining the monomeric state of HSF1 and for interaction with HSP chaperones. Mutation of this residue has therefore resulted in a form of HSF1 that cannot be chaperoned and which, by default, is converted into active HSF1 trimers under nonstress conditions (27, 28).

To test hHSF1-E189 in our system, we placed the cDNA for hHSF1-E189 under the control of a tetracycline-inducible vector (pBI, CLONTECH Laboratories, Inc., Palo, Alto, CA). This vector was cotransfected into the p2500-CAT-containing LHSE cells, along with the pUHD172-1hygro vector expressing the "reverse tet" transcriptional activator and hygromycin resistance genes, as originally developed by Bujard and co-workers (29). After selection with hygromycin antibiotic, the stably transfected LHSE-E189 cell line was established. In an initial test of these cells, hHSF1-

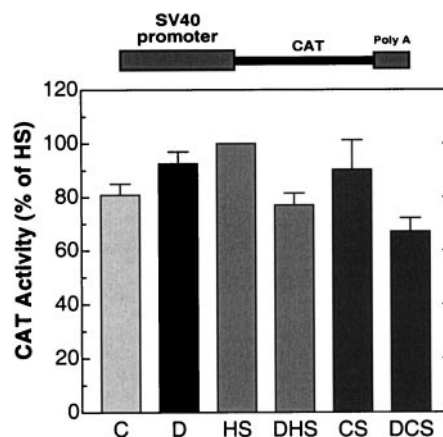


Fig. 3. Effects of Dex and Stress on CAT Gene Expression Controlled by the SV40 Promoter

Mouse L929 cells were stably transfected with a CAT reporter construct driven by the constitutively active SV40 promoter to generate LSV2 cells. Replicate flasks of LSV2 cells were subjected to a variety of hormone and stress conditions and were allowed to recover for 20 h in the continued presence of hormone until lysates were prepared and assayed for CAT activity. The results represent means \pm SEM of nine independent experiments. C, No treatment; D, Dex (1 μ M) for 24 h; HS, 43 C, 2 h; DHS, Dex (1 μ M) for 4 h, followed by HS; CS, 200 μ M sodium arsenite, 2 h; DCS, Dex (1 μ M) for 4 h followed by CS.

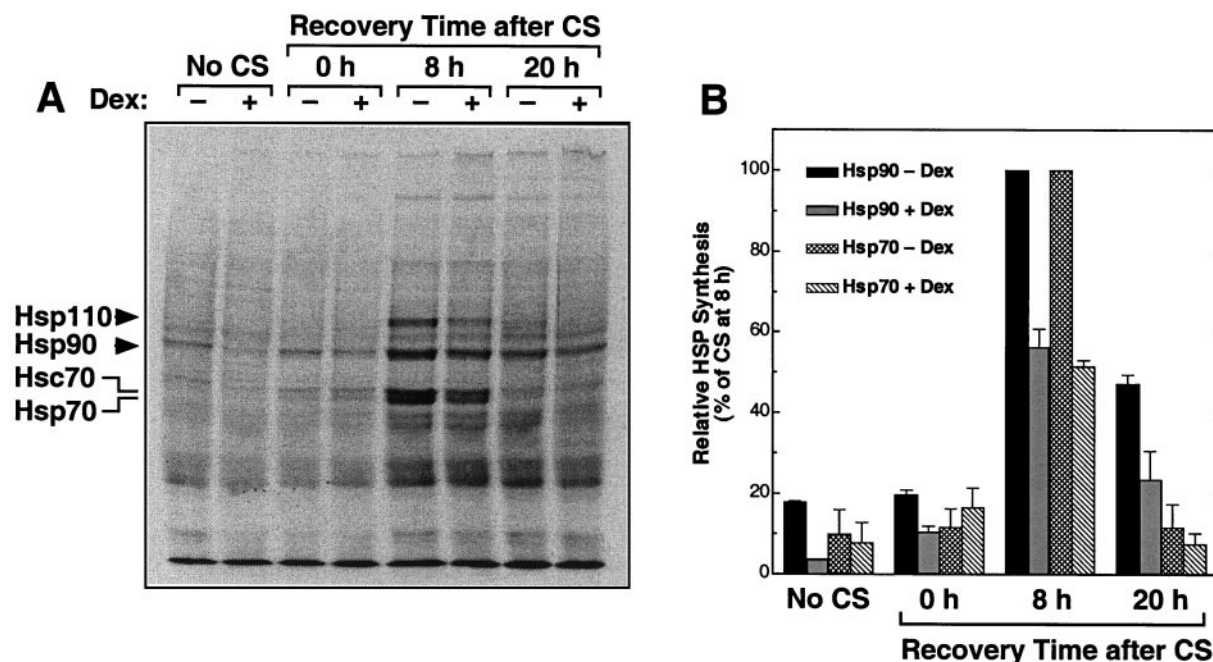


Fig. 4. Dex Inhibits Heat Shock-Induced Expression of the Major HSPs—Hsp70, Hsp90, and Hsp110

A, LHSE cells were subjected to chemical shock (CS) using 200 μ M sodium arsenite (2 h) followed by washing and recovery. Duplicate flasks were incubated with 1 μ M Dex for 4 h before chemical shock, followed by recovery in the continued presence of Dex. At the indicated time intervals during recovery, cells were pulse labeled with [35 S]methionine for 45 min, and whole cell extracts (equal protein) were analyzed by gel electrophoresis and autoradiography. B, Quantitation of relative expression levels for Hsp70 (Hsc70 and Hsp70) and Hsp90 was performed by densitometric scanning of the autoradiograms. The results represent means \pm SEM of two independent experiments.

E189 expression in response to doxycycline antibiotic was measured by Western blotting using an antibody specific to human HSF1 (Fig. 5B). The results show a large increase in hHSF1-E189 protein following 20 h of doxycycline treatment. Measurement of CAT activity following doxycycline treatment showed an approximate 5-fold increase compared with vehicle-treated controls (Fig. 5D), indicating that the expressed hHSF1-E189 can indeed stimulate Hsp70 promoter activity in nonstressed cells. More importantly, concurrent treatment of these cells with doxycycline and Dex showed a large decrease in CAT activity relative to doxycycline alone (65% inhibition) with no effect on the levels of hHSF1-E189 expression (Fig. 5C)—demonstrating that Dex hormone can inhibit the intrinsic activity of hHSF1-E189 to the same degree as that seen for endogenous HSF1 (70%; see Fig. 1). As parallel experiments in LHSE cells (p2500-CAT only) showed no effect of doxycycline on the endogenous, wild-type HSF1 (data not shown), it is clear that the inhibition seen in the LHSE-E189 cells is due solely to an effect of hormone on mutant HSF1. Based on these results, we conclude that the inhibitory effect of Dex on Hsp70 promoter activity under stress conditions is most likely not mediated by unknown stress-activated transcription factors acting on this promoter. Rather, it appears to be an effect on the intrinsic activity of HSF1 alone.

To test whether the repression of HSF1 activity by Dex was due to an inhibition of HSF1 binding to DNA, we performed EMSA assays using a synthetic, 32 P-labeled oligonucleotide containing multiple, consensus HSEs (Fig. 6). In response to heat shock alone, activation of HSF1 DNA-binding function can clearly be seen. Interestingly, pretreatment of cells with 1 μ M Dex before heat shock had no effect on this function, even after 24 h of hormone pretreatment. It appears, therefore, that the inhibitory effect of Dex cannot be explained on this basis. Moreover, it can also be concluded that all earlier stages in the HSF1 signal pathway are also not targets for the actions of hormone. Thus, the inhibitory effect of Dex on HSF1 activation is most likely due to an effect on the transcription enhancement function of this factor—a process that, at present, is poorly understood.

In work by our laboratory, we have shown that induction of MAPKs (ERK1 and ERK2) will lead to an inactivation of HSF1 activity in stressed cells as measured by the p2500-CAT reporter (19), an observation that is consistent with other reports demonstrating negative regulation of HSF1 by MAPK family members (30, 31). In our prior work, activation of ERK1/2 was achieved by treatment of cells with sodium vanadate, a known tyrosine phosphatase inhibitor, resulting in the attenuation of the transcriptional enhancement activity of HSF1 (19). Based on these observations, we

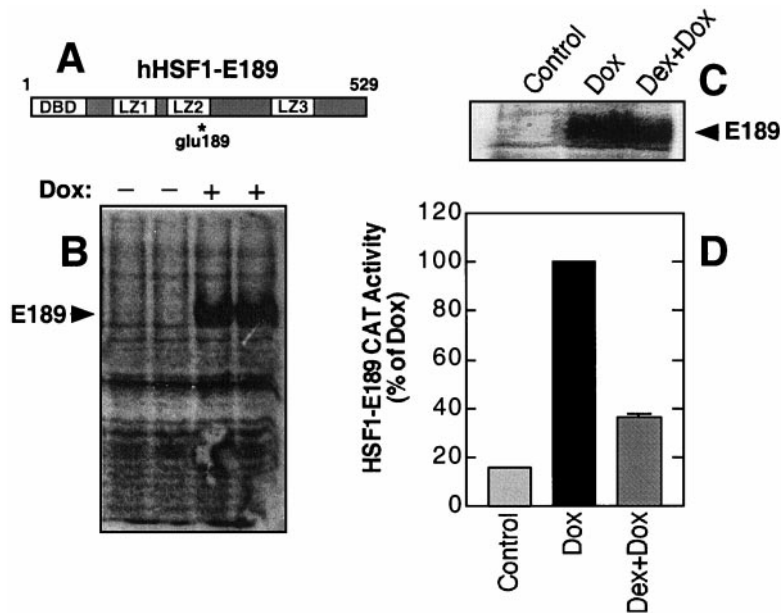


Fig. 5. The Constitutively Active E189 Mutant of Human HSF1 Is Inhibited by Dex

A, Domain structure of hHSF1-E189. The 529-amino acid sequence of human HSF1 contains a conserved DNA-binding domain (DBD) at the amino terminus, as well as three conserved hydrophobic domains (LZ1–LZ3) responsible for maintaining the monomeric, inactive state of HSF1. In E189, a single point mutation at amino acid 189 within LZ2 results in HSF1, which undergoes trimerization and activation under nonstress conditions. **B**, LHSE cells containing the p2500-CAT reporter were stably transfected with a tetracycline-inducible construct controlling expression of hHSF1-E189 to generate LHSE-E189 cells, as described in *Materials and Methods*. Replicate flasks of LHSE-E189 cells were treated with or without doxycycline (Dox) antibiotic (10 $\mu\text{g}/\text{ml}$ for 20 h), followed by Western blotting of whole cell extracts using an antibody to human HSF1 as probe. **C** and **D**, Replicate flasks of LHSE-E189 cells were treated with doxycycline or doxycycline and Dex, as indicated, followed by assay for E189 protein by Western blotting (panel **C**) or CAT activity (panel **D**). The results are representative of three independent experiments (panel **C**) or depict the means \pm SEM of three independent experiments (panel **D**). **C**, Control; **D**ox, 10 $\mu\text{g}/\text{ml}$ doxycycline for 20 h; **D**ex+**D**ox, 1 μM dexamethasone + 10 $\mu\text{g}/\text{ml}$ doxycycline for 20 h.

reasoned that the inhibitory effect of Dex on HSF1 could result from an effect of hormone to increase ERK1/2 activity. We therefore tested the effect of Dex on these kinases by use of an antibody specific to the active, phosphorylated forms of ERK1/2 (Fig. 7A). As expected, the results show activation of ERK1/2 by sodium vanadate. However, ERK1/2 activity was affected neither by short- nor long-term treatment of cells with Dex. Because more recent reports (32–34) have also demonstrated a similar inhibition of HSF1 by *c-jun* N-terminal kinase (JNK), we also tested the effects of *in vivo* Dex treatment on the activity of this MAPK family member (Fig. 7B). The results show activation of JNK activity by vanadate treatment but no such activation by hormone. Based on these results, it appears that targeting of these members of the MAPK family cannot explain the actions of hormone on HSF1.

Inhibition of HSF1 Requires GR Transactivity

Our results to this point have provided evidence that hormonal inhibition of HSF1 requires agonist-bound receptor. As a first step to determining the exact stage of GR signaling responsible for cross-talk with HSF1, we reasoned that this inhibition could occur through one of two general stages: 1) hormone-induced re-

lease of GR-associated HSPs (Hsp70 and Hsp90), which negatively regulate HSF1 (dissociation model), or 2) inhibition of HSF1 through GR-mediated transactivation or transrepression (genomic model). To discriminate between these alternatives, we have used a series of Chinese hamster ovary (CHO) cell lines that stably express either no GR (CHOd cells), wild-type mouse GR (WCL2 cells), DNA-binding-defective GR (NB cells), or hormone-binding-defective GR (NA cells). These cells were originally developed by Ringold and co-workers (35) and were further characterized by us (36). For the present work, we stably transfected these cells with the p2500-CAT reporter to generate CHSE, WHSE, NBHSE, and NAHSE cells, respectively. The various properties of the receptors expressed in these cells, and in the LHSE cell line described above, can be seen in Fig. 8. Panel A of this figure shows the relative amounts of GR protein present in each cell line, along with relative values for hormone-binding and Dex-induced transactivation functions. The results show that wild-type GR (WHSE cells) can bind hormone and activate transcription, while the GR of NAHSE cells cannot effectively perform either function, as would be expected of receptor with a functional mutation in the hormone-binding

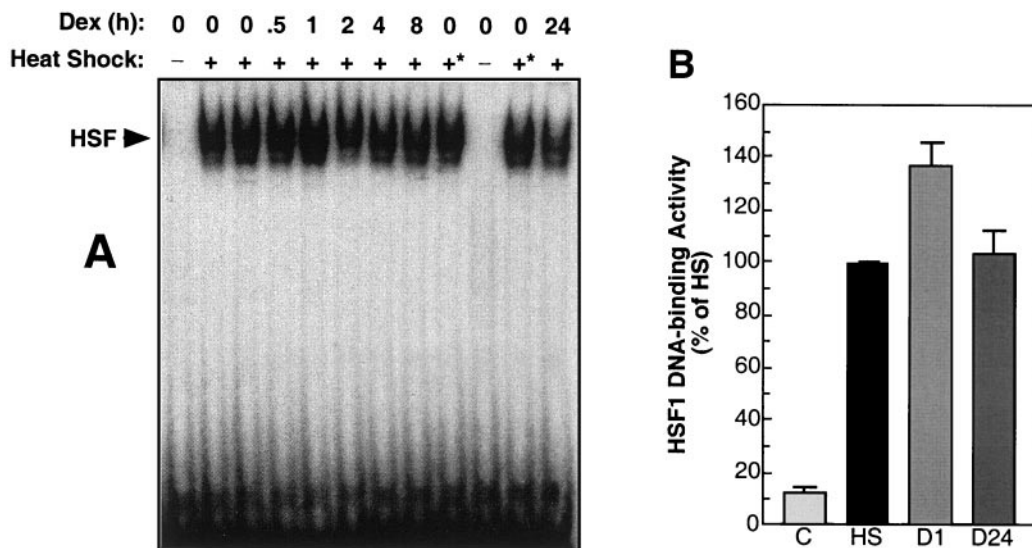


Fig. 6. Dex Activation of Wild-Type GR Has No Effect on HSF1 DNA-Binding Function

A, WHSE cells were subjected to a time course of treatment with Dex, as indicated, followed by heat shock (43 C, 2 h). Cells were harvested immediately after stress and EMSAs for HSF1 were performed, as described in *Materials and Methods*. *, Lanes marked with *asterisk* represent extracts from cells allowed to culture an additional 8 or 24 h *without* Dex and then subjected to heat shock, to account for any effect of prolonged culture on HSF1 DNA-binding activity. B, Quantitation of HSF1 DNA-binding function was performed by densitometric scanning of DNA-bound HSF1 and normalization to the HS-only control. The results represent means \pm SEM of 12 experiments. C, Control; HS, 43 C for 2 h; D1, 1 μ M Dex for 1 h followed by HS; D24, 1 μ M Dex for 24 h followed by HS.

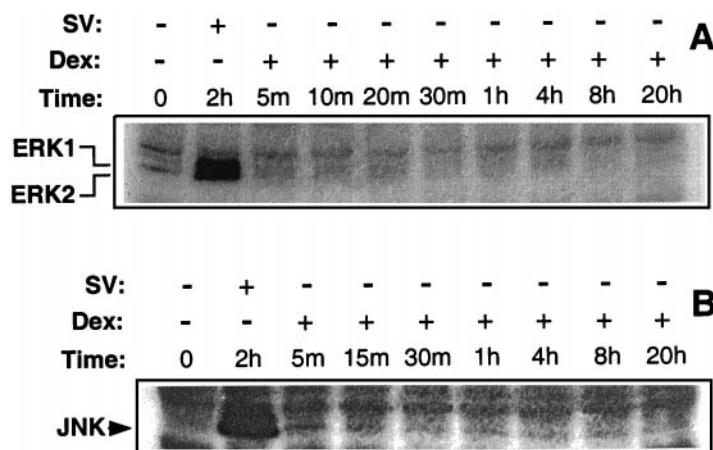


Fig. 7. Dex Treatment Does Not Activate the MAPKs ERK1/2 or JNK

Replicate flasks of WHSE cells were treated with 1 μ M Dex for increasing amounts of time, as indicated. Cytosolic fractions were prepared and aliquots containing equal protein were resolved by SDS-PAGE and immunoblotting with the SC-7383 antibody recognizing the active forms of ERK1 and ERK2 (panel A) or the SC-6254 antibody against active JNK (panel B). As positive controls, flasks of WHSE cells were treated with the tyrosine phosphatase inhibitor sodium vanadate (SV; 200 μ M) for 2 h to activate ERK or JNK. In prior studies, this level of ERK activation by vanadate has been shown to cause 70% inhibition of HSF1 activity (19).

pocket. The GR of NBHSE cells showed the highest level of hormone-binding function, in keeping with its greater level of expression, but, as expected, this mutant GR did not exhibit appreciable transactivation function. Analysis of untransformed GR/HSP complexes in the WHSE and NBHSE cells (Fig. 7B) showed association of both Hsp90 and Hsp70 to the GR, a result that is in agreement with prior observations (36).

A similar pattern of GR binding to Hsp90 and Hsp70 was seen for the GR of NAHSE cells (data not shown); while the GR of LHSE cells was found to associate only with Hsp90 (data not shown), as previously documented for GR present in the parental L929 cells (36).

To use the above cells to discriminate between the dissociation and genomic models, we first measured the effects of stress and hormone treatment on CAT

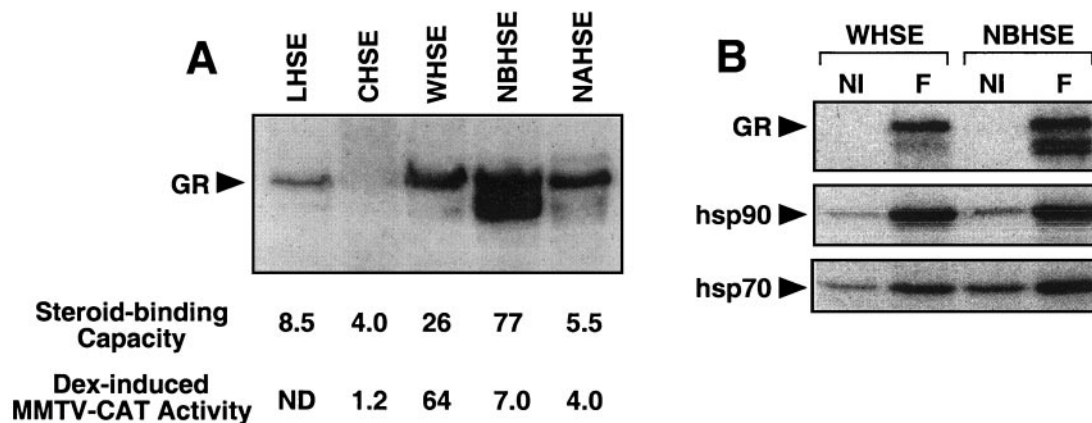


Fig. 8. Properties of Wild-Type and Mutant Mouse GR Expressed in L929 and CHO Cells

A, Relative levels of GR protein, steroid-binding capacity, and transactivation function. LHSE cells were generated as previously described. CHO cells stably transfected with the same HSF1-responsive CAT reporter and expressing no GR (CHSE), wild-type GR (WHSE), DNA-binding-defective GR (NBHSE), or hormone-binding-defective GR (NAHSE) were generated as described in *Materials and Methods*. Western blot analysis of GR from each cell line was performed using the BuGR2 monoclonal antibody as probe on aliquots of cytosol containing equal protein (400 μ g). Steroid-binding capacities (dpm/ μ g protein) were measured by incubation of cytosols with 50 nM [3 H]triamcinolone acetonide, as previously described (55). To measure Dex-induced MMTV-CAT activity, the parental CHO, WCL2, NB, and NA cell lines were transiently cotransfected with MMTV-CAT and β -galactosidase reporters, followed by treatment with or without 1 μ M Dex for 24 h. CAT assays were performed, and the disintegrations per min were first corrected for transfection efficiency based on galactosidase activity. This was followed by normalization to basal activities for each cell line to yield fold-induction values. ND, Not determined. **Panel B,** Western blot analysis of GR/HSP complexes. Aliquots of cytosol from WHSE and NBHSE cells were immunoadsorbed with the FiGR monoclonal antibody against GR (F) or with nonimmune mouse IgG (NI). Samples were resolved by SDS-PAGE and immunoblotted using BuGR2, anti-Hsp90, and anti-Hsp70 antibodies as probes.

gene expression (Fig. 9). In the WHSE cells, we observed a strong inhibitory effect of Dex on heat shock-induced CAT gene expression (80%) that could be completely blocked by RU486 antagonist (Fig. 9B). In contrast, no inhibitory effect of Dex was seen in the CHSE cells (Fig. 9A). Based on this comparison, we can now eliminate all signal pathways that do not involve GR as mechanisms to explain this inhibitory property of hormone. There was no inhibitory effect of Dex on HSF1 activity in the NAHSE cells, as was expected of a receptor in which hormone cannot activate any step in its signal pathway (Fig. 9C). More interesting were the results obtained in the NBHSE cells (Fig. 9D). In this case, Dex had little or no effect on heat shock-induced HSF1 activity, demonstrating that DNA-binding function is required for GR-mediated repression of HSF1. In addition, these data also provide evidence against the dissociation model, since the NB GR is perfectly capable of binding hormone (see Fig. 8), which would presumably lead to transformation of the GR/HSP complex. Thus, it is likely that hormone-induced release of Hsp70 and/or Hsp90 cannot account for the inhibitory effect observed with the wild-type receptor (WHSE cells). However, it has yet to be shown that hormone-induced transformation of the NB GR can indeed take place. Although the NB GR is a single-point mutation in the DNA-binding domain (35), and not in the region of GR responsible for Hsp90 interaction, it remained remotely possible that this substitution could alter the ability of hormone to cause release of this HSP. For

these reasons, we performed the experiments of Fig. 10 to directly measure NB GR transformation under *in vivo* conditions. The results demonstrate that the NB GR is found in the cytosolic fraction in the absence of hormone as a complex with both Hsp90 and Hsp70 (Fig. 10A). However, in response to hormone treatment, a large shift of NB GR to the nuclear pellet fraction can be seen that coincides with a decrease in the amount of receptor-associated Hsp90 and Hsp70. To corroborate these results, we have analyzed GR/HSP transformation in LHSE cells in response to both Dex and RU486 (Fig. 10B). The results show that both Dex and RU486 will cause nuclear translocation of GR and dissociation of the GR/Hsp90 complex, demonstrating that RU486 is an antagonist solely at the level of GR transactivation function. As RU486 treatment of these cells does not inhibit HSF1 activity (Fig. 2), it can be concluded that dissociation of the GR/HSP complex cannot be the mechanism by which HSF1 inhibition is achieved. Taken as a whole, our results are consistent with a model in which GR-mediated inhibition of HSF1 is a “nuclear” event, requiring the DNA-binding function of the receptor and, most likely, transcription enhancement activity by the GR.

DISCUSSION

Using cells stably transfected with the human Hsp70 promoter, we have provided evidence for negative

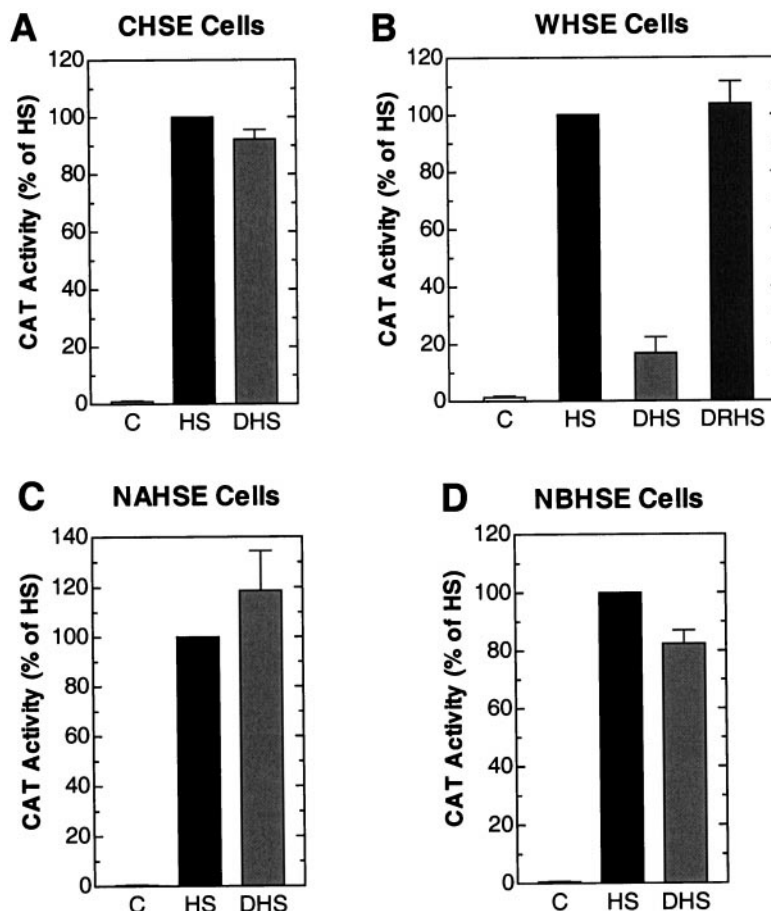


Fig. 9. Dex Inhibition of HSF1 Requires GR DNA-Binding Function

Replicate flasks of CHO cells stably transfected with the HSF1-responsive CAT reporter and expressing no GR (CHSE), wild-type GR (WHSE), nonhormone-binding GR (NAHSE), and non-DNA-binding GR (NBHSE) were subjected to the indicated hormone and heat shock conditions. After recovery for 20 h, lysates were prepared and assayed for CAT activity. These results represent means \pm SEM of six independent experiments. C, No treatment; HS, 43 C, 2 h; DHS, Dex (1 μ M) for 4 h followed by HS; DRHS, Dex (1 μ M) plus RU486 (10 μ M) for 4 h followed by HS.

regulation of HSF1 by the agonist-activated GR. Dex treatment of these cells resulted in a dose-dependent inhibition of Hsp70 promoter activity as induced by heat or chemical shock (Fig. 1). Inhibition of Hsp70 promoter activity by GR was shown to be at the level of HSF1 transcriptional activity based on the fact that Dex treatment of cells caused inhibition of a constitutively active form of HSF1 under nonstress conditions (Fig. 5) and that heat shock-induced binding of HSF1 to DNA was not prevented by Dex (Fig. 6). Moreover, the inhibitory effect of Dex on HSF1-mediated transactivation was not limited to the Hsp70 promoter, as hormone treatment before heat shock reduced the rates of synthesis of several endogenous HSPs, including Hsp70 (both constitutive and inducible forms), Hsp90, and Hsp110 (Fig. 4).

As mentioned above, we initially postulated that antagonism of HSF1 by GR could result from one of two overall stages of GR signaling: 1) hormone-induced release of Hsp70 and/or Hsp90 from the GR complex, or 2) genomic actions on the part of GR. Before our

studies, evidence to support the dissociation model could be found. First and foremost was the existence of strong evidence for an Hsp70/Hsp90-based chaperone complex that serves to keep HSF1 in an inactive state (6–9). In addition, Xiao and DeFranco (23) had recently shown that transient overexpression of GR in COS-1 cells caused stress-free activation of HSF1, by a mechanism involving temporary loss of Hsp70 from inactive HSF1 complexes to newly forming GR/HSP complexes. Thus, if HSPs could move from HSF1 to GR, why not from GR to HSF1? However, it is clear from the present results that this mechanism of action is not the means by which hormone-activated GR is causing repression of HSF1, as hormone-induced dissociation of the GR/HSP complex was observed under conditions in which there was no inhibition of Hsp70 promoter activity (Figs. 2, 8, and 9). Instead, we propose that inhibition of HSF1 by GR is through a genomic mechanism of action. Evidence to support this conclusion is as follows: 1) inhibition of HSF1 requires GR DNA-binding function (Fig. 9D); and 2)

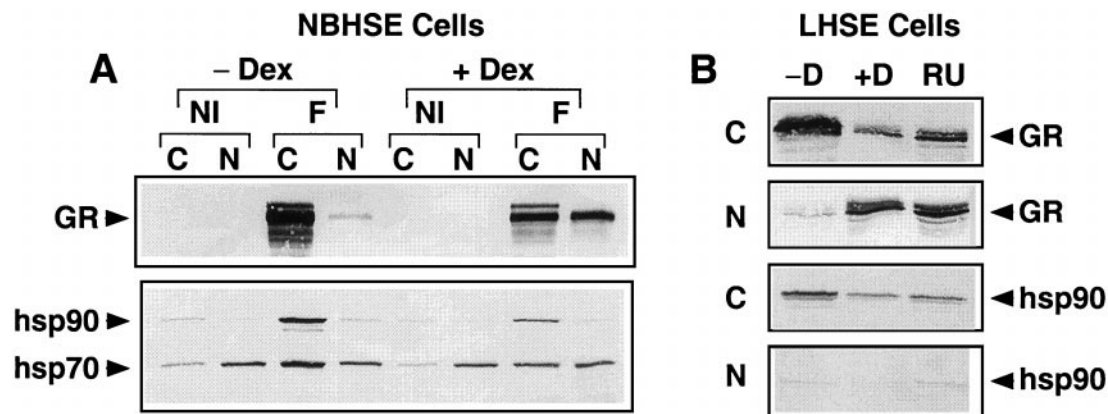


Fig. 10. Dex- and RU486-Induced Dissociation of GR/HSP Complexes in NBHSE and LHSE Cells

Panel A, Replicate flasks (175 cm²) of NBHSE cells were treated with vehicle or 1 μ M Dex for 2 h followed by Dounce homogenization and preparation of cytosolic (C) and nuclear pellet (N) fractions, as described in *Materials and Methods*. Fractions were immunoabsorbed with the FiGR monoclonal antibody against GR (F) or with nonimmune mouse IgG (NI). Each sample was split in half followed by SDS-PAGE and immunoblotting using BuGR2 (*upper blot*), or anti-Hsp90 and anti-Hsp70 antibodies (*lower blot*), as probes. Panel B, Replicate flasks (175 cm²) of LHSE cells were treated with vehicle, 1 μ M Dex, or 1 μ M RU486 for 2 h followed by preparation of cytosolic (C) and nuclear (N) fractions. Fractions were immunoabsorbed with FiGR antibody against GR, followed by immunoblotting with BuGR2 (*upper blots*) or antibody to Hsp90 (*lower blots*).

inhibition of HSF1 is blocked by RU486, which acts as a GR antagonist, not by preventing transformation of the GR/HSP complex (Fig. 10), but by preventing GR transactivation (Fig. 2).

Although enhancement of transcription is the most commonly accepted genomic effect of GR, a variety of other genomic mechanisms exist that could explain antagonism of HSF1 by GR. These include repression of gene expression by GR bound to so-called negative glucocorticoid response elements (nGREs); direct or indirect inactivation by GR of stress-activated, *trans*-acting factors, such as HSF1 itself or factors cooperatively binding to the Hsp70 promoter; or competition for a common coactivator that mediates both HSF1 and GR transactivation. With respect to the common coactivator model, data from our laboratory and others suggest that this mechanism is not likely to be operating in our system, as reciprocal inhibition of GR by HSF1 does not seem to occur. Rather, activity of both GR (16) and PR (15) have been shown to greatly increase in cells subjected to heat shock and other forms of stress—in a manner that appears to require intrinsic HSF1 activity (18, 19). In fact, under the same conditions of hormone treatment and heat shock used in the present study, response of GR in the L929 cells can be increased several fold by the stress event, as measured by a CAT reporter gene driven by a minimal GRE promoter (16). It is conceivable, however, that competition between GR and HSF1 for a common coregulator may not be reciprocal, and for this reason further studies into this potential mechanism are warranted. Such studies would require some indication of coregulators that mediate HSF1 signaling. Yet, to our knowledge, no such observations have been reported.

The present data also suggest that a mechanism by which GR binds or otherwise inactivates stress-

induced transcription factors, including HSF1, is not likely to be operating. First, Dex was found to inhibit the constitutive activity of the HSF1-E189 mutant (Fig. 5). As this inhibition occurred under nonstress conditions, it is clear that the GR cannot be repressing other stress-activated transcription factors; even though several candidates exist (e.g. AP-1, CCAAT binding factor) for which cognate binding elements have been found within the human Hsp70 promoter (our personal observation). Second, results of the EMSA experiments (Fig. 6) showed no effect of Dex on the amounts or relative sizes of the HSF1/DNA complexes, suggesting that GR is neither preventing the HSF1 DNA-binding event nor participating in it. Yet, it could be argued that, if the putative GR/HSF1 interaction is relatively weak, GR that is tethered to the HSF1/DNA complex could dissociate during electrophoresis and would thus go undetected. Conceivably, our data with the NB mutant (Fig. 9) could be taken as evidence that the GR DNA-binding domain may be the site for interaction with HSF1 or that a mutation in this domain alters a distal HSF1-interaction surface. Although this mechanism remains a possibility, our data with RU486 do not support it. In this case, RU486 was found to effectively block Dex-induced inhibition of HSF1 (Figs. 2 and 9), while at the same time causing translocation of the GR and tight binding to nuclei (Fig. 10). Thus, the RU486-bound GR appears to have a fully-functional DNA-binding domain but is incapable of HSF1 inhibition.

Based on the above, we propose that GR-mediated inhibition of HSF1 can occur either through the transrepression or transactivation functions of the agonist-bound receptor (Fig. 11). Transrepression by the GR is typically thought to occur by direct binding of GR to nGREs in the promoter region of genes. With this in

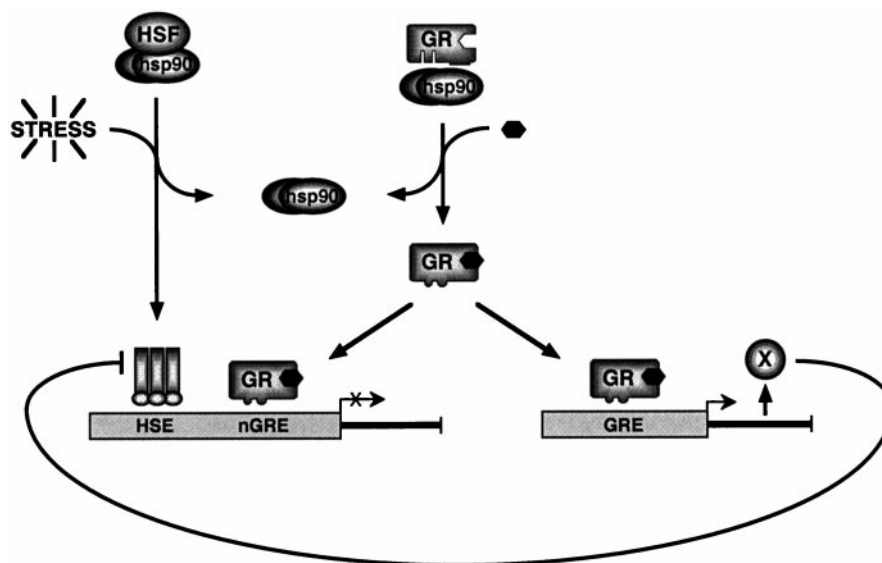


Fig. 11. Model for Repression of HSF1 Transactivation by Agonist-Activated GR

We have shown that glucocorticoid agonist (Dex) but not antagonist (RU486) results in the inhibition of HSF1 transactivity following stress. This response is not the result of HSPs (e.g. Hsp90) released from the untransformed GR complex. Rather, it is a process requiring the DNA-binding function of GR. Based on these results, we propose that hormone-activated GR can inhibit the transcriptional activity of HSF1 by one of two processes: 1) by direct binding of GR to nGREs present in the hsp70 promoter, or 2) by the actions of a GR-induced gene product (X) that directly or indirectly inhibits the activity of DNA-bound HSF1 (see *Discussion* for further details).

mind, we have screened the published sequence (729 bp) of the human Hsp70 promoter used in this study and have found several consensus or near-consensus nGRE sequences known to be sites of GR transrepression in a variety of genes (37–40). By these criteria, therefore, the actions of GR as a repressor remain a plausible mechanism. Further experimentation to test this potential mechanism will require mutagenesis/deletion studies of the Hsp70 promoter or use of a minimal promoter comprised of synthetic HSE elements.

Our data are also consistent with a model in which GR transactivation function is responsible for inhibition of HSF1. As depicted in Fig. 11, inhibition of HSF1 could result from the production of a GR-regulated gene product (X) that directly or indirectly affects the activity of DNA-bound HSF1. Yet, at present, there is no obvious, Dex-induced gene that could serve this role. However, a variety of proteins known to act as negative regulators of HSF1 have been identified. These include: heat shock factor binding protein 1 (41), DNA-dependent protein kinase (19, 42) and MAPK members ERK1 and 2 (30, 31); as well as Hsp90, Hsp70, and Cyp40 (6, 7, 9). Of these, Hsp90, Hsp70, and Cyp40 are thought to principally act by chaperoning HSF1 into its inactive state in the cytoplasm. As such, the HSPs and Cyp40 would not likely act on DNA-bound HSF1, although reports do exist that Hsp70 will cause release of HSF1 from DNA (43). Moreover, no reports exist demonstrating glucocorticoid-induced expression of Hsp90, Hsp70, or Cyp40, and, in our laboratory, no such effect of Dex has been observed on overall cellular levels of Hsp90 and Hsp70

(our unpublished observations). In contrast, heat shock factor binding protein 1, DNA protein kinase, and ERK1/2 fit the criteria of being able to act on the DNA-bound form of HSF1. Thus, any effect of glucocorticoid hormones on amounts or activities of these factors would do much to explain the actions of hormone on HSF1. Indeed, evidence for a rapid activation of MAPK in MCF-7 cells by 17 β -estradiol has recently been reported (44). For these reasons, we have tested for an effect of Dex on two members of the MAPK family. The results show that Dex treatment of cells for up to 24 h does not increase activity of ERK1/2 or of JNK, as measured by an antibody specific to the phosphorylated forms of these kinases (Fig. 7). While ruling out a fast-acting, nongenomic effect of Dex on ERK1/2 and JNK signaling in our system, this result does not bring us any closer to identifying the GR-regulated gene product(s) responsible for the actions of hormone on heat shock signaling. Future approaches to solve this problem may require the use of screening methods, such as DNA arrays, as a way by which to identify one or more genes potentially involved in this response.

A working hypothesis of our laboratory is that heat shock and glucocorticoid hormone responses are coordinated to ensure survival of cells following stress. We base this hypothesis on the following observations. First, it is clear that HSPs can protect cells through their ability to act as chaperones that prevent denaturation of proteins in response to stress (45). Second, it has been long known that glucocorticoids are required for physiological adaptation to stress. In

this case, stress (e.g. infection, surgery, trauma), acting through the hypothalamus-pituitary axis, will lead to increased secretion of cortisol from the adrenal [see Munck and colleagues (1, 46) for excellent reviews of this topic]. For these reasons, we have not found it surprising that heat shock leads to increased activation of the GR, and that these results have recently been corroborated for GR-regulated genes in certain tissues (13, 14).

With this in mind, how do we explain our current results, in which glucocorticoid hormone inhibits the heat shock response? One possible answer, of course, is that the inhibition of HSF1 we have observed here is not limited to glucocorticoids, but, rather, can also be achieved in response to other classes of steroid hormones. In this case, the physiological relevance of steroid inhibition of the heat shock response is less clear. On the other hand, should the inhibitory effect of steroids on HSF1 be unique to glucocorticoids, or to a limited set of related hormones, then an attractive hypothesis would be that the heat shock response is fast acting compared with that of the GR, in which more time is needed for serum cortisol levels to rise in response to a stress event. Once cortisol has risen, however, one role of glucocorticoids may be to attenuate the heat shock response, perhaps to prevent overstimulation by this pathway. In this sense, glucocorticoid actions on the heat shock response may be analogous to their antiinflammatory properties in the lymph system, in which damage by prolonged or excessive inflammation is effectively mitigated by these hormones.

MATERIALS AND METHODS

Materials

[³H]Triamcinolone acetonide (42.8 Ci/mmol), [³H]acetate (10.3 μ Ci/mmol), [³⁵S]methionine ("Tanslabel"; 1,175 Ci/mmol), and [¹²⁵I]conjugates of goat antimouse IgG (11.8 μ Ci/ μ g) and goat antirabbit IgG (9.0 μ Ci/ μ g) were obtained from ICN Biochemicals, Inc. (Cleveland, OH). Sodium vanadate, ATP, dimethylsulfoxide, sodium arsenite, Dex, G418 (geneticin) antibiotic, hygromycin, acetyl coenzyme A (CoA) synthetase, acetyl CoA, Tris, HEPES, EDTA, protein A-Sepharose, DMEM-powdered medium, and horseradish peroxidase conjugates of goat antimouse and goat antirabbit IgG were from Sigma (St. Louis, MO). Autoradiography enhancer (Amplify) was obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). The steroid antagonist RU486 was obtained from Rousset-Uclaf (Paris, France). Iron-supplemented newborn calf serum was from HyClone Laboratories, Inc. (Logan, UT); Immobilon polyvinylidene fluoride membranes were obtained from Millipore Corp. (Bedford, MA). GenePorter transfection reagent was obtained from Gene Therapy Systems, Inc. (San Diego, CA). The SC-7383 monoclonal antibody against phosphorylated (active) ERK and the SC-6254 antibody against phosphorylated (active) JNK were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The BuGR2 monoclonal antibody against GR (47) was purchased from Affinity BioReagents, Inc. (Golden, CO); FiGR monoclonal antibody against GR (48) was a gift from Jack Bodwell (Dartmouth Medical School, Hanover, NH). The SPA-

901 antibody against human HSF1 and the SPA-820 antibody against human Hsp70 were purchased from StressGen Biotechnologies (Victoria, British Columbia, Canada). Monoclonal antibody against Hsp90 was obtained from Transduction Laboratories, Inc. (Lexington, KY).

In the p2500-CAT reporter used in this study, expression of CAT is controlled by the human Hsp70 promoter containing consensus HSEs known to be activated by the binding of HSF1 (25). The pMMTV-CAT plasmid contains the complete mouse mammary tumor virus (MMTV)-long terminal repeat promoter (MMTV-LTR) upstream of CAT (49). Hormonally driven expression of CAT by this reporter is controlled by GREs residing within the long terminal repeat region (50). The pBI-EGFP vector was obtained from CLONTECH Laboratories, Inc. In this vector, tetracycline-induced expression is controlled by a tetracycline response element and two minimal cytomegalovirus promoters in opposite orientations. The pUHD172-1hygro vector (29) expressing the "reverse tet" transactivator and hygromycin resistance genes was obtained from Hermann Bujard (Universitat Heidelberg, Heidelberg, Germany). The cDNA for the E189 mutant of human HSF1 (27) was the generous gift of Richard Voellmy (University of Miami, Coral Gables, FL).

Transfection of Cell Lines

The LHSE and LMCAT2 cell lines were established as previously described (16, 18). Briefly, mouse L929 cells were cotransfected with pSV2neo and a 2-fold excess of p2500-CAT (LHSECAT cells) or pMMTV-CAT (LMCAT2 cells) using lipofectin as carrier. This was followed by selection for stably transfected, cloned cell lines using G418 (Geneticin) antibiotic at 0.4 mg/ml. Once established, both cell lines were grown in an atmosphere of 5% CO₂ at 37 C in DMEM containing 0.2 mg/ml G418 and 10% iron-supplemented NCS.

The tetracycline-inducible LHSE-E189 cells were made by stably transfecting LHSE cells with the pUHD172-1hygro plasmid and a 7-fold excess of pBI-E189 plasmid, followed by selection and cloning using 0.4 μ g/ml hygromycin. The pBI-E189 construct was made by excising the cDNA for the constitutively-active hHSF1-E189 mutant from the pGEM-E189 vector originally developed by Voellmy and co-workers (27). This cDNA was then inserted into the multiple cloning site of the pBI-EGFP vector (CLONTECH Laboratories, Inc.).

The CHSE, WHSE, NBHSE, and NAHSE cells used in this study were generated by cotransfecting p2500-CAT and pSV2neo plasmids into CHO cells that contain either no GR (CHOd cells), wild-type mouse GR (WCL2 cells), DNA-binding defective mouse GR (NB cells), or hormone-binding defective mouse GR (NA cells), respectively. This was followed by selection of cloned-resistant cell lines using G418 antibiotic. The GR-expressing CHO cells were originally developed by Gordon Ringold and co-workers (35) using methotrexate-based selection and amplification. The various properties of these GRs have been further characterized by us (36) and others.

Stress and Hormone Treatment of Cell Lines

For all experiments, the NCS was stripped of endogenous steroids by extraction with dextran-coated charcoal. Most stress experiments were performed on cells that were at or near confluence, although similar results were obtained with subconfluent cultures. Heat shock treatment was achieved by shifting replicate flasks to a second 5% CO₂ incubator set at 43 C. Typical duration of heat shock treatment was 2 h. Cells were also subjected to chemical shock by addition of 200 μ M sodium arsenite to the medium. In the chemical shock experiments, the arsenite-treated and nontreated cells were incubated at 37 C for 2 h and were then washed with DMEM and allowed to recover.

CAT Assay

Measurement of CAT enzyme activity was performed according to the method of Nordeen *et al.* (51) with minor modifications. In this assay, a reaction mixture containing acetyl CoA synthetase, [³H]sodium acetate, CoA, and ATP was briefly preincubated to enzymatically generate labeled acetyl CoA from CoA and labeled acetate. Acetylation of chloramphenicol was then initiated by adding cell lysate containing CAT enzyme. The reaction was stopped by extraction with cold benzene, and 75% of the organic phase was counted. Cell lysates were prepared by sequential freezing and thawing in 0.25 M Tris, 5 mM EDTA (pH 7.5), and centrifugation at 14,000 × *g*. Aliquots of lysate containing equal protein content were added to the enzymatic reaction mixtures. As the GRE- and HSE-containing promoters employed in this study have distinct basal and inducible activities, all data are represented as percent of control, maximum, or the equivalent. In this way, the relative inhibitory or stimulatory effects of each treatment can be readily seen.

Analysis of HSP Synthesis by Labeling with [³⁵S]Methionine

In the experiment of Fig. 4, LHSE cells were shocked by incubation with 200 μM sodium arsenite in the presence or absence of 1 μM Dex. At indicated intervals during recovery, the cells were pulse labeled with [³⁵S]methionine for 45 min by removing the medium and replacing it with methionine-free medium containing 10% dialyzed calf serum and [³⁵S]methionine at a final concentration of 10 μCi/ml. All subsequent steps were carried out on ice (0–4 C). Cells were washed three times by pelleting and resuspension in HBSS, followed by three cycles of freezing and thawing in 0.25 M Tris, 5 mM EDTA (pH 7.5) and centrifugation at 100,000 × *g*. The protein content of these whole cell extracts were determined by the BCA procedure of Pierce Chemical Co. (Rockford, IL), and the extracts were used immediately or were frozen at –80 C. Samples were resolved by electrophoresis in 7% polyacrylamide SDS gels as described by Laemmli (52). Following impregnation of the gels with an autoradiography enhancer (Amplify), the gels were dried under vacuum and mild heating (70 C) and exposed to Kodak XAR-5 x-ray film (Eastman Kodak Co., Rochester, NY) with an intensifying screen at –80 C. The relative amounts of newly synthesized hsp70 and hsp90 were calculated by densitometric scanning using Molecular Analyst software (Bio-Rad Laboratories, Inc., Hercules, CA).

Lysate Preparation, Immune Purification of GR Complexes, and Western Blotting

In the experiments of Figs. 4 and 6, whole cell extracts were prepared by freeze/thaw in WCE buffer [20 mM HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM pregnant mare's serum, and 0.5 mM dithiothreitol (DTT), pH 7.9] and centrifugation at 14,000 × *g* for 30 min. In the experiments of Fig. 8, cytosols were prepared by Dounce A homogenization of cells in hypotonic buffer (10 mM HEPES, 3 mM EDTA, 10 mM sodium molybdate, pH 7.4), followed by centrifugation at 14,000 × *g*. In the experiments of Fig. 10, cells were fractionated into cytosolic and nuclear portions by Dounce A homogenization in hypotonic buffer, followed by centrifugation at 1,000 × *g*. The cytosolic fractions were saved and the nuclear pellets were washed two times by resuspension and pelleting in hypotonic buffer. Hypotonic buffer containing 0.5 M NaCl was then added to the pellet fractions and incubated on ice with occasional vortexing for 1 h. After salt extraction, the nuclear pellets were centrifuged at 14,000 × *g* and the supernatants saved. For the experiments of Figs. 7 and 9, BuGR2 anti-GR monoclonal antibody (1.5 μg) was added to the regular cytosols, or to the cytosolic

and nuclear fractions, and each sample was then adsorbed in batch to protein A-Sepharose, followed by washing with TEG buffer (10 mM *N*-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 1 mM EDTA, 10% glycerol, 50 mM NaCl, 10 mM sodium molybdate, pH 7.6) and elution with 2× SDS sample buffer.

All samples were resolved by electrophoresis in 7% polyacrylamide SDS gels, followed by transfer to Immobilon polyvinylidene fluoride membranes. The relative amounts of hHSF1-E189, ERK 1/2, GR, or GR-associated HSPs were determined via a Western blotting technique previously described (53), employing primary antibody and both peroxidase- and [¹²⁵I]-conjugated counter antibodies. After color development, the blots were exposed to Kodak XAR-5 film with an intensifying screen at –70 C.

HSF1 EMSA

EMSA for HSF1 were performed according to the protocol of Mosser *et al.* (54), with minor modifications. Briefly, single 75-cm² flasks of LHSECAT cells were subjected to the indicated stress and hormone conditions. Cells were harvested, centrifuged, and rapidly frozen at –80 C. The frozen pellets were resuspended in WCE buffer (20 mM HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM pregnant mare's serum, and 0.5 mM DTT, pH 7.9) and centrifuged at 100,000 × *g* for 10 min. The supernatants were either stored at –80 C or used immediately. EMSA assays were performed by mixing 10 μg of whole cell extract with 0.1 ng (50,000 cpm) of [³²P]-labeled HSE oligonucleotide (5'-GAT,CTC,GGC,TGG,AAT,ATT,CCC,GAC, CTG,GCA,GC-C,GA-3') and 1.0 μg of poly (dl-dC) in 10 mM Tris (pH 7.8), 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 5% glycerol in a final volume of 25 μl. For competition experiments, the binding reactions contained 0.1 ng of the [³²P]HSE and a 100-fold molar excess of unlabeled HSE. Reactions were incubated at 25 C for 30 min and loaded onto 4% polyacrylamide gels in 0.5× Tris-borate-EDTA. The gels were run at room temperature for 1.5 h at 150 V and were exposed to Kodak XAR-5 film with an intensifying screen at –80 C. The relative amounts of probe-bound HSF1 were then measured by densitometric scanning of the film using the Bio-Rad Laboratories, Inc. Molecular Analyst system.

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