

Triptolide, an Inhibitor of the Human Heat Shock Response That Enhances Stress-induced Cell Death^{*[5]}

Received for publication, November 8, 2005, and in revised form, February 3, 2006 Published, JBC Papers in Press, February 9, 2006, DOI 10.1074/jbc.M512044200

Sandy D. Westerheide¹, Tiara L. A. Kawahara^{1,2}, Kai Orton, and Richard I. Morimoto³

From the Department of Biochemistry, Molecular Biology, and Cell Biology, Rice Institute for Biomedical Research, Northwestern University, Evanston, Illinois 60208

Molecular chaperones, inducible by heat shock and a variety of other stresses, have critical roles in protein homeostasis, balancing cell stress with adaptation, survival, and cell death mechanisms. In transformed cells and tumors, chaperones are frequently overexpressed, with constitutive activation of the heat shock transcription factor HSF1 implicated in tumor formation. Here, we describe the activity of triptolide, a diterpene triepoxide from the plant *Tripterygium wilfordii*, as an inhibitor of the human heat shock response. Triptolide treatment of human tissue culture cells prevented the inducible expression of heat shock genes, shown by suppression of an *HSP70* promoter-reporter construct and by suppression of endogenous *HSP70* gene expression. Upon examining the steps in the HSF1 activation pathway, we found that triptolide abrogates the transactivation function of HSF1 without interfering in the early events of trimer formation, hyperphosphorylation, and DNA binding. The ability of triptolide to inhibit the heat shock response renders these cells sensitive to stress-induced cell death, which may be of great relevance to cancer treatments.

Elevated levels of heat shock protein expression have been identified as a biomarker for many cancers and are correlated with poor prognosis (reviewed in Ref. 1). Molecular chaperones have diverse roles in the regulation of signal transduction and in numerous aspects of cell growth and cell death. At the level of specific chaperones, HSP90, HSP70, and HSP27 have each been implicated in promoting cancer. HSP90 regulates the activities of many signaling molecules and kinases important in cancer, including NF- κ B, p53, Raf1, Akt, and steroid aporeceptors (reviewed in Ref. 2). In addition, HSP90, HSP70, and HSP27 have been shown to be anti-apoptotic (reviewed in Ref. 3). Although they serve to prevent stressed primary cells from initiating cell death, in transformed cells the elevated levels of these chaperones may interfere with the proper regulation of caspase activities.

Several cancer strategies have focused on the inhibition of individual chaperones. For instance, inhibition of HSP90 by the fungal antibiotic radicicol, the benzoquinone ansamycin geldanamycin, and the geldanamycin-related analog 17-allylamino-17-demethoxygeldanamycin (17-AAG), which has been shown to potently reduce tumor growth, is cur-

rently in clinical trials (4). Depletion of HSP70 levels by antisense RNAs causes cell death in transformed cell lines, but not in nontransformed cells, and inhibits tumorigenesis in xenograft models (5, 6). Similar anti-cancer effects have also been observed with antisense RNA against *HSP27* (7).

The regulation of heat shock gene expression is complex and involves a multitude of promoter elements that confer responses to specific signals and growth factors (8–12). Common to all heat shock-induced genes are heat shock elements, promoter sites that have high affinity for binding to a family of heat shock transcription factors (HSFs).⁴ HSF1 is well characterized and is essential for the heat shock response (reviewed in Ref. 13). Upon induction by diverse stimuli, HSF1 trimerizes and binds to the promoters of target genes. HSF1 must be hyperphosphorylated to become transcriptionally activated and induce a rapid and coordinate increase in molecular chaperone levels. Elevated levels of chaperones allow for recognition and processing of misfolded polypeptides that appear in stressed cells and promote recovery from protein damage. In addition to the many known activators of the heat shock response, including elevated temperature, heavy metals, oxidants, and a variety of small molecules (reviewed in Ref. 14), various disease states are also correlated with altered molecular chaperone expression levels, including inflammation, ischemia, tissue wounding and repair, cancer, and neurodegenerative diseases. Therefore, the levels of chaperones and the relative proportions of individual chaperones within chaperone networks may be critical in determining the state of the cell.

The recent evidence that HSF1 levels and activities are up-regulated in certain cancers (15–17) suggests that it may be more effective to target multiple chaperones at once instead of targeting individual chaperones separately. A logical direction, therefore, would be at the level of pharmacological control of heat shock gene expression. Although many classes of inducers of the heat shock response are known, only a small number of inhibitors have been identified. These include quercetin, a flavonoid found in apples, onions, and black tea, which was shown to decrease HSF1 activity and HSP70 expression (18–22), and KNK437, a benzylidene lactam compound that reduces HSP70 expression by an unknown mechanism (23). Triptolide, a diterpene triepoxide derived from the Chinese plant *Tripterygium wilfordii*, was identified in our laboratory as part of a small molecule screen for regulators of human heat shock gene transcription. Triptolide is a biologically active molecule, as several groups have studied its ability to inhibit cell growth, induce apoptosis, and suppress NF- κ B and activator protein-1 (AP-1) transcriptional activation (24–26). However, triptolide is not a general inhibitor of transcription, as it has been previously shown that triptolide does not inhibit the cytomegalovirus-luciferase or SV40-luciferase reporters and that triptolide does not inhibit DNA damage-induced

^{*} This work was supported by grants from NIGMS, National Institutes of Health and the Daniel F. and Ada L. Rice Foundation (to R. I. M.), National Institutes of Health Training Grant T32 CA70085 in Signal Transduction and Cancer (to S. D. W.), an undergraduate research fellowship (to T. L. A. K.), and a National Science Foundation predoctoral fellowship (to K. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental material.

¹ These authors contributed equally to this work.

² Current address: Cancer Biology Graduate Program, Stanford Medical School, Stanford, CA 94305.

³ To whom correspondence should be addressed. Tel.: 847-491-3340; Fax: 847-491-4461; E-mail: r-morimoto@northwestern.edu.

⁴ The abbreviations used are: HSF, heat shock factor; HSP, heat shock protein; EMSA, electrophoretic mobility shift assay; RT, reverse transcription; TBP, TATA-binding protein.

GADD45 and *MAP4* RNA levels (26, 27). Here, we show that triptolide is a potent inhibitor of the transcriptional activity of HSF1 and thus suppresses expression of heat shock genes and sensitizes cells to stress-induced apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture and Drug Treatment Conditions—Human HeLa, stable *HSP70.1* promoter-luciferase reporter HeLa, and 293T cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were maintained at 5% CO₂. The stable cell line was prepared as previously reported (28). Triptolide, obtained from Calbiochem, was dissolved in dimethyl sulfate at a concentration of 10 mM and added to cells at the indicated concentrations. Heat shock was administered by submerging cells in a pre-warmed circulating 42 °C water bath for the indicated times.

Plasmids, Transfection, and Luciferase Assays—The GAL4-HSF1 fusion construct contains GAL4 residues 1–147 and HSF1 residues 124–503 (29). For transient transfection experiments, 293T cells were transfected using Polyfect (Qiagen, Valencia, CA) according to manufacturer's protocol. For experiments with the stable *HSP70.1* promoter-luciferase reporter HeLa cell line, cells were plated to 1×10^4 cells/well in 96-well plates 24 h prior to transfection. Luciferase assays were performed using the Bright-Glo assay system (Promega, Madison, WI) according to manufacturer's instructions.

Electrophoretic Gel Mobility Shift Assays—Electrophoretic mobility shift analysis was executed as described previously (30) using a ³²P-labeled probe containing the proximal heat shock element from the human *HSP70.1* gene promoter.

RNA Extraction and Reverse Transcription-PCR—Total RNA was isolated using TRIzol reagent (Invitrogen) and treated with DNase. Analysis of RNA was performed by reverse transcription-PCR (RT-PCR). The *HSP70* primers were: 5'-AGAGCCGAGCCGACAGAG-3' (forward) and 5'-CACCTTGCCGTGTTGGAA-3' (reverse). The *18S* rRNA primers were: 5'-CGTCTGCCCTATCAACTTTCG-3' (forward) and 5'-TGCCTTCCTTGGATGTGGTAG-3' (reverse). PCR reactions were carried out for 25 cycles.

Western Blot Analysis—Western blot analysis was performed using the Odyssey system (Li-COR, Lincoln, NE). The HSF1 r2 antibody (31) was used at a 1:5,000 dilution.

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation reactions were performed as described previously (28). HeLa S3 cells exposed to the indicated treatments were cross-linked with 1% formaldehyde, sonicated, and immunoprecipitated with 10 μl of HSF1 r2 antibody at 4 °C overnight. PCR analysis was performed on DNA isolated from the immunoprecipitated protein using primers specific to the *HSP70.1* promoter. The primers used were 5'-GGCGAAACCCCTGGAATATTCCTCGA-3' (forward) and 5'-AGCCTTGGGACAACGGGAG-3' (reverse).

Cytoprotection Analysis—HeLa cells, HSF1 wild-type mouse embryonic fibroblasts and HSF1 null mouse embryonic fibroblasts were pretreated as indicated, washed, and allowed to recover for 5 h prior to a 45 °C heat shock for 40 min (HeLa cells) or 20 min (mouse embryonic fibroblasts). The cells were then assayed for the percentage of cell death by trypan blue uptake 24 h after the 45 °C heat shock treatment.

RESULTS

Triptolide Inhibits Activation of the Heat Shock Response—Triptolide (Fig. 1) was shown to inhibit the heat shock response in a HeLa cell line stably expressing a human *HSP70* promoter-luciferase reporter (Fig.

1B) that allows for efficient and reproducible screening for small molecule regulators (28). Using this system, a 1-h heat shock at 42 °C followed by 8 h of recovery prior to luciferase analysis was found to provide optimal reporter activity (data not shown). To test the effect of triptolide on induction of the *HSP70* promoter, HeLa *HSP70-luc* cells were incubated with triptolide at increasing concentrations from 10 to 100 nM delivered into the tissue culture medium for 1 h prior to heat shock (Fig. 1C). Although triptolide on its own did not activate the reporter (Fig. 1C, lane 2), triptolide treatment prior to heat shock was shown to suppress activation of the *HSP70* reporter in a dose-dependent manner, with 100 nM triptolide pretreatment resulting in an ~80% inhibition (compare lane 3 with lane 6). Thus, triptolide effectively blocks heat shock induction of the *HSP70* promoter-luciferase reporter.

We next tested the effect of triptolide in HeLa cells on the inducible expression of the endogenous *HSP70* gene using the same pretreatment and heat shock regimen. To measure *HSP70* mRNA levels, the treated cells were immediately harvested following heat shock, and *HSP70* mRNA was quantified by RT-PCR (Fig. 1D). Consistent with the results obtained using the *HSP70* promoter-reporter construct, triptolide pretreatment completely suppressed the expression of *HSP70* mRNA in a concentration-dependent manner, with 100 nM triptolide reducing the level of *HSP70* mRNA below that of untreated cells (Fig. 1D, compare lanes 1 and 2 with lane 5).

To see whether the effect of triptolide is conserved across species, we also tested for inhibition of heat shock-induced *Hsp70* levels in mouse embryonic fibroblast cells (Fig. 1E). Triptolide was equally effective as an inhibitor in this system as well. Thus, triptolide pretreatment can effectively abrogate induction of *HSP70* mRNA accumulation by heat shock in both human and mouse cell lines.

We next addressed whether triptolide had general effects on other inducible transcription systems. ZnSO₄ treatment induces multiple stress responsive genes, including in heat shock protein genes in an HSF1-dependent manner and in metallothionein genes in a metal response element-binding transcription factor-1 (MTF-1)-dependent manner (32). This affords the opportunity to address the specificity of triptolide on both inducible systems at the same time. Thus, we tested the ability of triptolide to inhibit ZnSO₄-induced *HSP70* mRNA versus metallothionein IIA (*MTIIA*) mRNA. HeLa cells were pretreated with or without 100 nM of triptolide for 1 h followed by treatment with 100 μM ZnSO₄ for 2 h. RNA was then harvested, and the levels of *HSP70* and *MTIIA* mRNA were tested by RT-PCR. Our results clearly show that triptolide only affects the metal induction of *HSP70*, with no effects on inducible *MTIIA* expression levels (supplemental Fig. 1). Therefore, triptolide shows specificity toward HSF1 signaling versus MTF-1 signaling.

Triptolide Does Not Affect HSF1 DNA Binding and Hyperphosphorylation—To identify the step in the heat shock response affected by triptolide, we next focused on the steps involved in the stress-induced activation of HSF1, including formation of DNA-binding trimers and hyperphosphorylation. We first performed electrophoretic gel mobility shift assays (EMSA) to explore whether the inhibitory effect of triptolide is at the level of HSF1 DNA binding. Cells treated either with a 42 °C heat shock for various times or with 100 nM triptolide prior to a 42 °C heat shock were harvested and assayed by EMSA using a ³²P-labeled probe containing the proximal heat shock element from the human *HSP70* gene promoter (Fig. 2A). Low levels of HSF1 DNA binding activity are detected in control, unstressed cells (Fig. 2A, lane 1), and upon heat shock there is a strong activation of HSF1 DNA binding activity due to HSF1 trimer formation (Fig. 2A, lanes 2–6). The levels of heat shock-induced HSF1 binding were not affected by triptolide pretreatment (Fig.

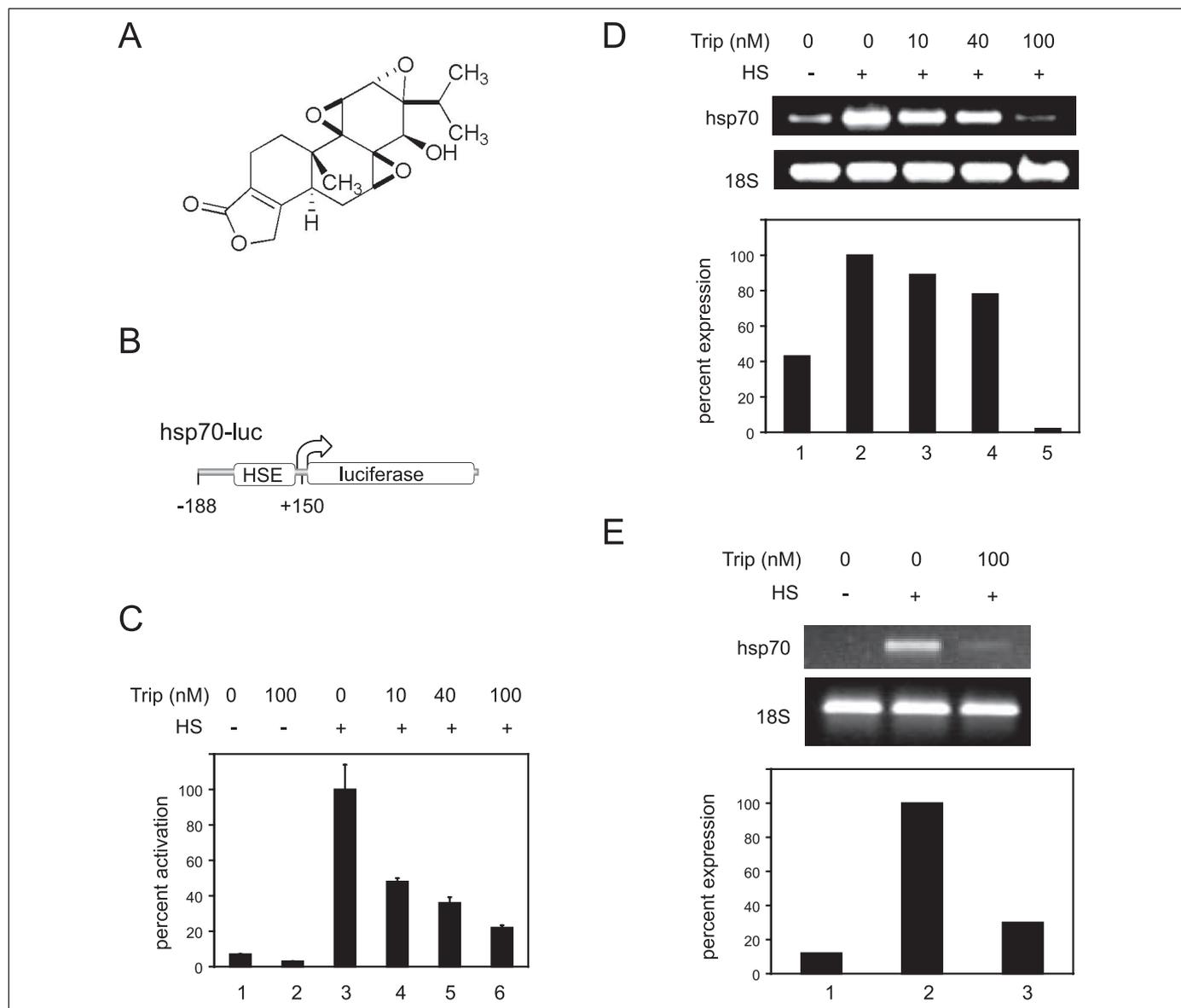


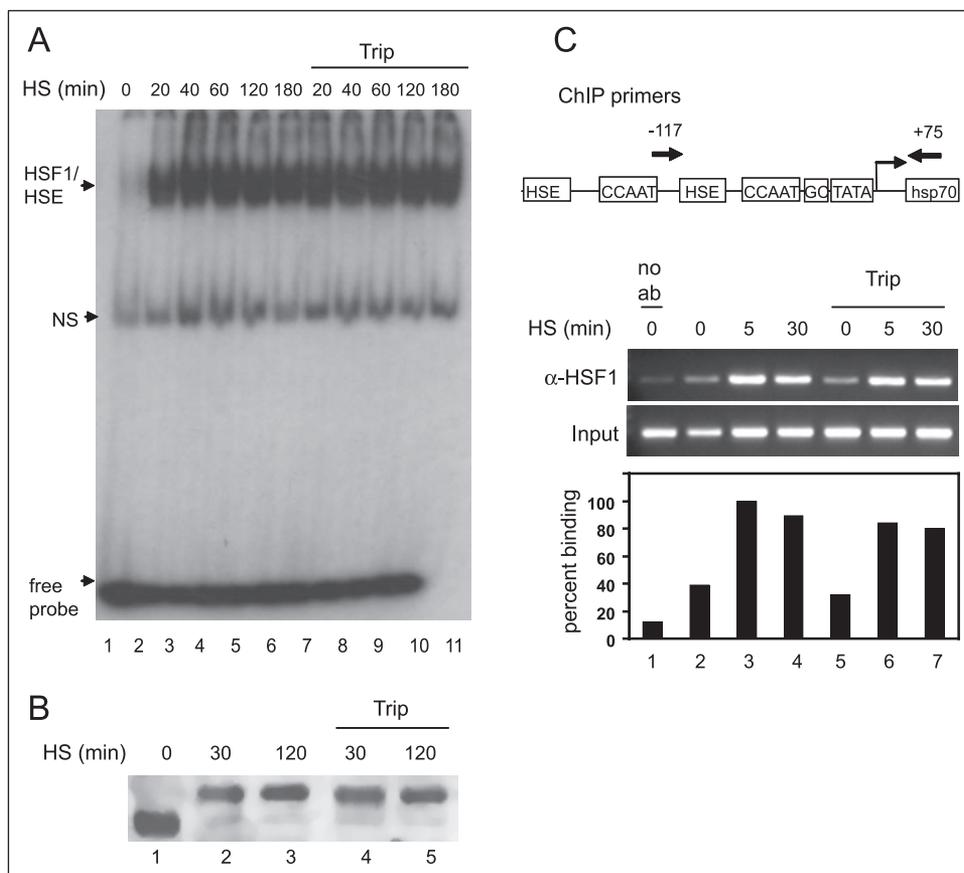
FIGURE 1. Triptolide inhibits the heat shock response. *A*, the structure of triptolide, a diterpene triepoxide derived from the Chinese plant *T. wilfordii*. *B*, diagram of *HSP70-luc*, an *HSP70.1* promoter reporter stably integrated into HeLa cells. *HSE*, heat shock element. *C*, triptolide inhibits *HSP70* promoter transcriptional activity in HeLa cells stably expressing the *HSP70-luc* construct. Cells were sequentially treated with the indicated concentrations of triptolide (*Trip*) followed by a 42 °C heat shock (*HS*) for 1 h. Luciferase assays were carried out 8 h after heat shock. The experiments were performed in triplicate, and the standard error is shown. *D*, triptolide inhibits heat shock-induced *HSP70* mRNA expression in HeLa cells. Cells were pretreated with the indicated concentrations of triptolide for 1 h followed by a 1-h 42 °C heat shock. RNA levels for *HSP70* and *18S* were determined by RT-PCR (top panels), and the relative intensities of the bands relative to *18S* were quantified (bottom panel). *E*, triptolide inhibits heat shock-induced *HSP70* mRNA expression in mouse embryonic fibroblast cells. Cells were pretreated with or without 100 nM triptolide for 1 h followed by a 1-h 42 °C heat shock. RNA levels for *HSP70* and *18S* were determined by RT-PCR (top panels) and the relative intensities of the bands relative to *18S* were quantified (bottom panel).

2A, lanes 7–11), revealing that the triptolide effect on the heat shock response is not at the level of inhibition of HSF1 DNA binding.

We next investigated another well established step in activation of HSF1 corresponding to stress-induced hyperphosphorylation, which can be detected readily by electrophoretic retardation on SDS-PAGE and Western blot analysis (33). HeLa cells exposed to either heat shock or sequential triptolide followed by heat shock regimens were harvested and assayed for the shift in HSF1 mobility indicative of hyperphosphorylation. As shown in Fig. 2B, the HSF1 induced in triptolide-treated cells exhibited the same retarded mobility on SDS-PAGE (compare lanes 2 and 3 with lanes 4 and 5), suggesting that hyperphosphorylation of HSF1 was unaffected by triptolide. From these results, we can conclude that triptolide does not disrupt acquisition of DNA binding and hyperphosphorylation in the early steps of HSF1 activation.

Having demonstrated that triptolide did not interfere with the appearance of the DNA binding-competent state of HSF1, we next assessed whether activated HSF1 was in the nucleus and bound to the endogenous *HSP70* promoter. Whereas the EMSA assay is an *in vitro* assay that detects only total available amounts of a DNA binding-competent transcription factor using a DNA binding assay, chromatin immunoprecipitation assays detect the *in vivo* recruitment of transcription factors to promoter sequences. Chromatin immunoprecipitation using an HSF1 antibody was performed on cells treated with either heat shock or triptolide pretreatment followed by heat shock. The samples were then analyzed by PCR with primers specific for the *HSP70* promoter to determine the relative amounts of HSF1 bound (Fig. 2C). These results show the increased binding of heat shock-induced HSF1 to the *HSP70* promoter (Fig. 2C, lanes 3 and 4) and show that triptolide

FIGURE 2. Triptolide does not inhibit the formation of the HSF1/heat shock element (HSE) DNA-binding complex or HSF1 hyperphosphorylation. *A*, triptolide does not inhibit HSF1 DNA binding. Whole cell extracts from HeLa cells were sequentially treated with or without 100 nM triptolide (*Trip*) for 1 h followed by a 42 °C heat shock (*HS*) for the indicated periods of time and then were analyzed via EMSA analysis. The HSF1/HSE complex is indicated as well as a non-specific band (*NS*). *B*, triptolide does not inhibit hyperphosphorylation of HSF1. Western analysis of whole cell extracts harvested from HeLa cells treated with heat shock alone (*lanes 2 and 3*) or sequentially treated with 100 nM triptolide followed by a 42 °C heat shock (*lanes 4 and 5*) for the indicated time intervals reveal that triptolide does not inhibit HSF1 hyperphosphorylation. *C*, chromatin immunoprecipitation (*ChIP*) experiments show that triptolide does not inhibit induction of HSF1 binding to the *HSP70* promoter *in vivo*. The primer positions on the *HSP70.1* promoter are indicated in the top panel. Cells were treated with heat shock alone (*lanes 3 and 4*) or with 100 nM triptolide for 1 h followed by heat shock (*lanes 6 and 7*). A no antibody control (*no ab*) is shown in *lane 1*. Quantification of the band intensities compared with input is shown in the bottom panel.



does not inhibit the *in vivo* recruitment of HSF1 to the *HSP70* promoter (Fig. 2C, lanes 6 and 7). Taken together with the previous results, these data confirm that triptolide does not inhibit HSF1 trimer formation, hyperphosphorylation of HSF1, or translocation and binding of HSF1 in the nucleus to the endogenous *HSP70* gene promoter.

Triptolide Inhibits the Transactivation Domain of HSF1—Because triptolide inhibits HSF1 activation without altering its DNA binding activity or subsequent hyperphosphorylation, we extended our analysis to examine the effect of triptolide on the transactivation function of DNA-bound HSF1. We transiently cotransfected 293T cells with plasmids encoding a GAL4-HSF1 chimera and GAL4-luciferase reporter. In the GAL4-HSF1 chimera, the DNA binding domain of HSF1 is replaced by the DNA binding domain of GAL4, allowing the analysis of regulatory events downstream of HSF1 DNA binding. The GAL4-HSF1 fusion system has previously been utilized in our laboratory to delineate the C-terminal transactivation domain of HSF1 and the negative regulatory domain (29). 293T cells, employed in this assay because of their high transfection efficiency, respond in a similar fashion to heat shock as HeLa cells (data not shown). Transfected cells were subjected to either heat shock alone or sequential exposure to triptolide followed by heat shock and were assayed for luciferase activity (Fig. 3). Triptolide incubation prior to heat shock results in an 11-fold inhibition of heat shock-induced activity (Fig. 3, compare lanes 3 and 4). From these results, we conclude that the inhibitory effect of triptolide is at the level of transcription by interfering with the proper activity of the C-terminal transactivation domain of HSF1.

Triptolide Is a Reversible Inhibitor of the Heat Shock Response—An irreversible inhibitor of the heat shock response is likely to be harmful to a cell or organism. Permanent effects could occur if the transcriptionally arrested HSF1 persists in an inert complex bound to the endogenous

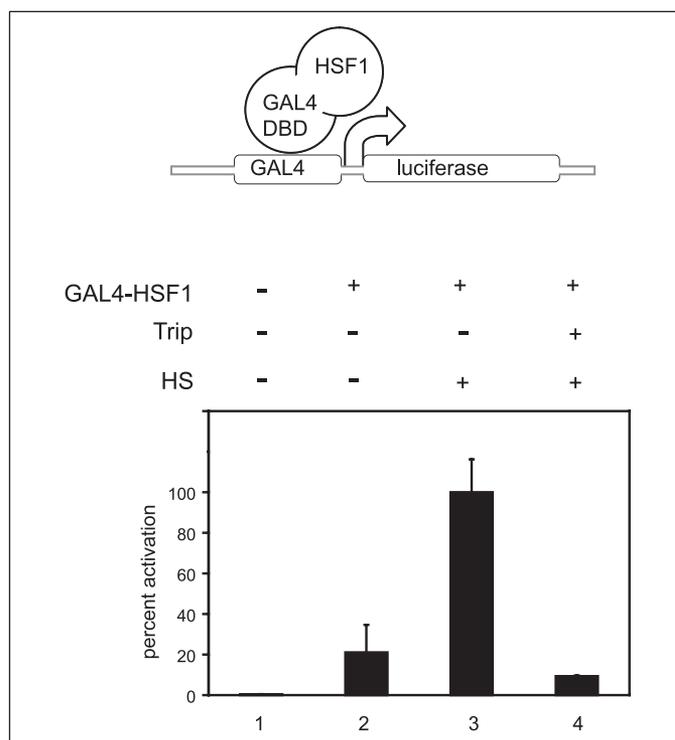


FIGURE 3. Triptolide inhibits the transactivation function of HSF1. 293T cells were transfected with a GAL4-luciferase reporter and a GAL4-HSF1 fusion construct in which the HSF1 DNA binding domain was replaced by the GAL4 DNA binding domain (DBD). 24 h after transfection, cells were treated sequentially with 100 nM triptolide (*Trip*) for 1 h followed by a 42 °C heat shock (*HS*) for 1 h. Luciferase assays were performed 8 h later. Data are shown as percent of activation, with heat shock alone normalized to 100%. The experiments were performed in triplicate, and the standard error is shown.

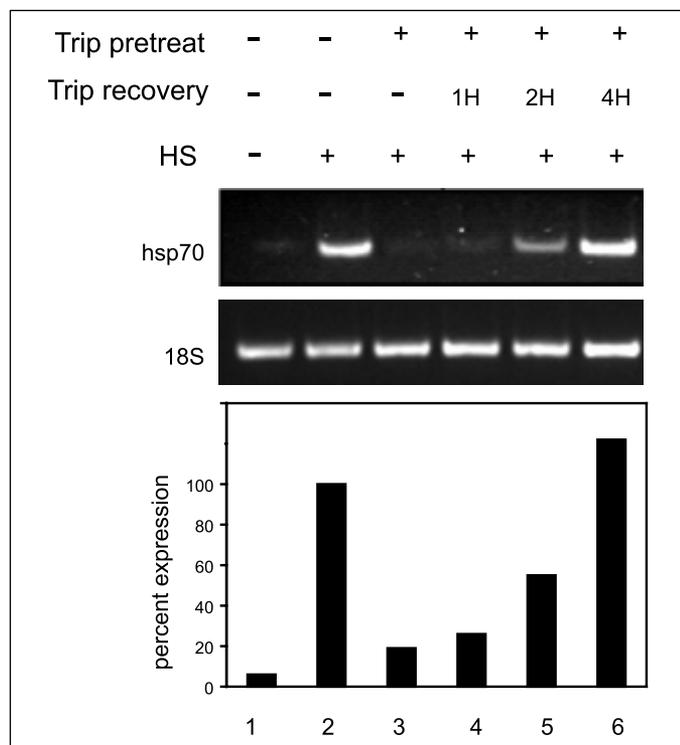


FIGURE 4. **Triptolide can inhibit HSP70 mRNA expression reversibly.** The indicated samples were pretreated with 100 nM triptolide (*Trip pretreat*) for 1 h prior to triptolide removal and incubation at 37 °C with fresh medium for various recovery time intervals (0, 1, 2, and 4 h) before subjection to a 1-h 42 °C heat shock (*HS*). For all experiments, RNA levels were determined by RT-PCR for *HSP70* and *18S*. The bottom panel shows quantitation of the bands relative to *18S*.

HSP70 promoter or if triptolide is sequestered in the nucleus of treated cells. Alternatively, if the compound is degraded or readily released from the cell, the effect of triptolide could be transient. This latter possibility would predict that the heat shock response should be restored upon removal of triptolide from the medium. To test whether the effect of triptolide is reversible, HeLa cells were treated with 100 nM triptolide for 1 h, after which the cells were washed of the triptolide-containing medium and replaced with fresh medium for periods ranging from 0 to 4 h, subjected to heat shock, and harvested for analysis of *HSP70* mRNA levels (Fig. 4). Using RT-PCR to measure the levels of *HSP70* mRNA, we showed that triptolide is not an irreversible inhibitor. A period of at least 4 h following triptolide treatment is necessary for the complete restoration of the heat shock response (Fig. 4, compare lanes 2 and 6). From these results, we conclude that the inhibitory effect of triptolide, while at the level of functionality of the active HSF1 complex at the *HSP70* promoter, does not generate an inert, irreversible complex. Either this complex dissociates, or the inhibitory effect of triptolide itself is dynamic. Triptolide treatment, therefore, can be precisely controlled both for the level and duration of inhibition of the heat shock response.

Triptolide Enhances Stress-induced Cell Death—A well established cellular phenomenon associated with the induction of the heat shock response is cytoprotection, a process in which stressed cells can acquire an enhanced capacity to survive normally lethal stress exposures (28, 34–37). The ability of triptolide to block the cytoprotective benefits of a 42 °C heat shock administered prior to a lethal 45 °C challenge condition was investigated in HeLa cells (Fig. 5A). Similar to what has been reported previously (28), treatment of HeLa cells with a 45 °C heat shock for 40 min resulted in 75% cell death (Fig. 5A, lane 3), whereas pretreatment of the cells with a 1-h heat shock at 42 °C prior to the lethal 45 °C challenge condition reduces cell death to 39% (Fig. 5A, lane 4). Triptol-

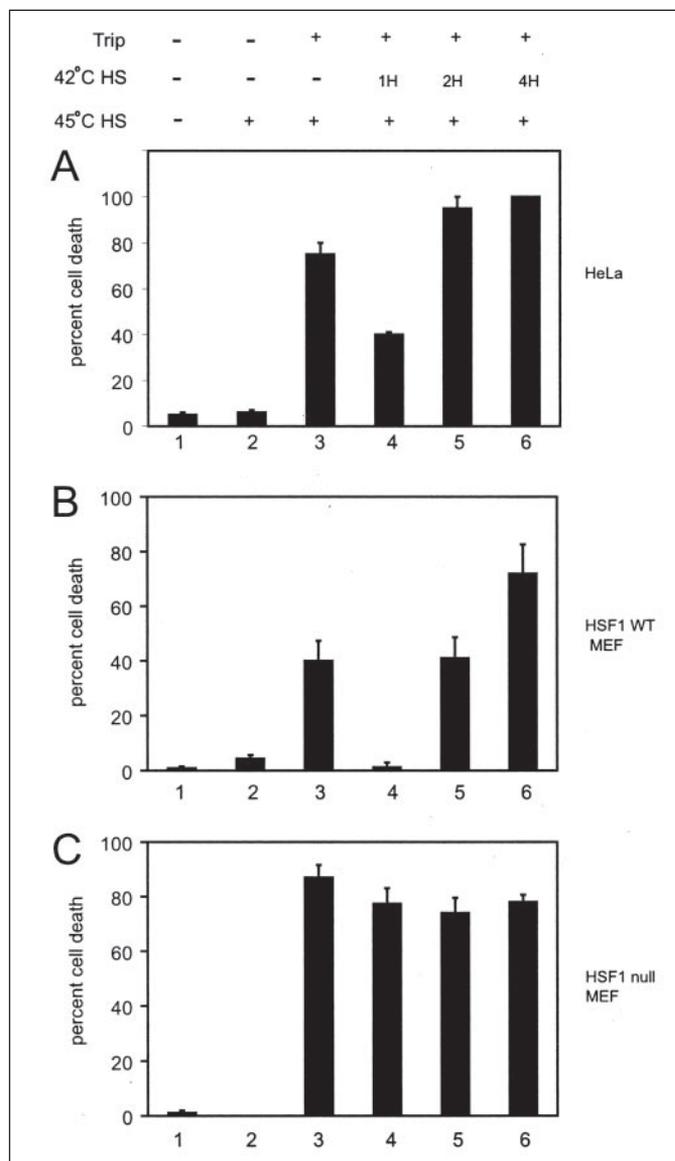
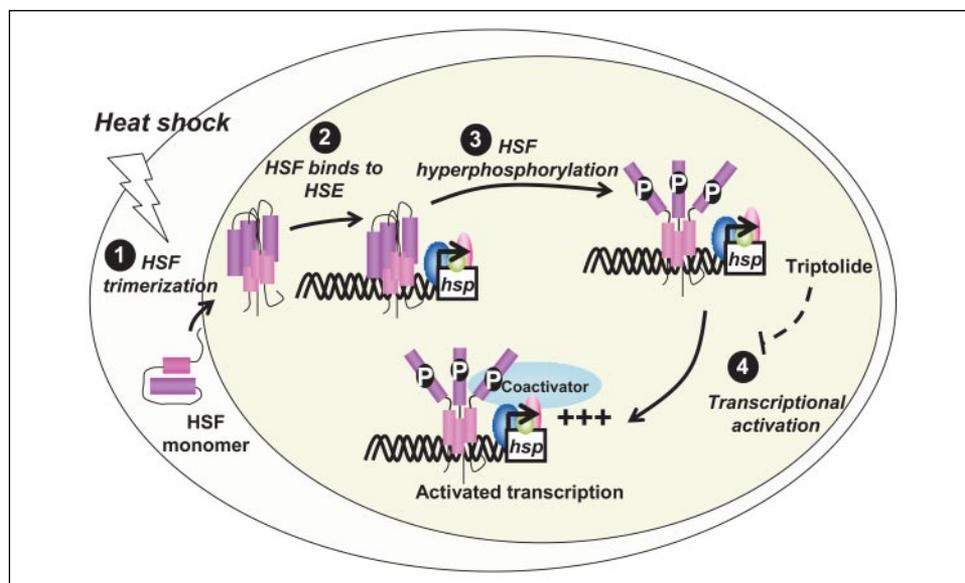


FIGURE 5. **Triptolide inhibits cytoprotection and enhances stress-induced cell death.** HeLa cells (A), HSF1 wild-type mouse embryonic fibroblasts (*HSF1 WT MEF*) (B), or HSF1 null embryonic fibroblasts (C) were pretreated with or without 100 nM triptolide (*Trip*) for 1 h as indicated. Cells were then treated with or without a 1-h 42 °C cytoprotective heat shock (*HS*). After removal of the triptolide and a 5-h recovery at 37 °C, cells were treated with a 45 °C heat shock for 40 min (HeLa cells) or for 20 min (mouse embryonic fibroblasts). The cells were then assayed for the percentage of cell death by trypan blue uptake 24 h after the 45 °C heat shock treatment. The experiments were performed in triplicate, and the standard error is shown.

ide treatment alone is neither cytoprotective nor causes cell death (Fig. 5A, lane 2). However, triptolide pretreatment prior to the cytoprotective 42 °C heat shock regimen completely blocks cytoprotection and instead results in enhanced cell death (Fig. 5A, lane 5). In fact, pretreatment with triptolide alone followed by exposure to 45 °C resulted in 100% cell death (Fig. 5A, lane 6) compared with 75% cell death when cells are directly challenged with 45 °C in the absence of any triptolide treatment (Fig. 5A, lane 3).

HSF1 is required for cells to exhibit the cytoprotective benefits of a prior heat shock (38). To determine whether the effects of triptolide on blocking cytoprotection depend on HSF1, we performed cytoprotection experiments utilizing HSF1 wild-type versus null mouse embryonic fibroblasts (Fig. 5, B and C). As expected, a prior heat shock provides cytoprotection in the wild-type cells but not in the HSF1 null cells.

FIGURE 6. **Model for the inhibition of the heat shock response by triptolide.** HSF1 exists as an inert monomer and attains its transcriptional activity in a stepwise manner upon exposure to heat shock. In *step 1*, HSF1 accumulates in the nucleus and oligomerizes into a DNA-binding-competent trimer. In *step 2*, trimeric HSF1 binds to HSE sequences present in the promoters of target genes. In *step 3*, HSF1 becomes hyperphosphorylated (P). In *step 4*, the hyperphosphorylated HSF1 attains complete transcriptional activity resulting in the induction of heat shock gene transcription. Triptolide inhibits the heat shock response at the level of HSF1 transactivation.



Triptolide was only able to prevent cytoprotection in the wild-type cells, as no cytoprotection was observed in the HSF1 null cells. Additionally, triptolide was able to enhance cell death induced by a lethal heat shock in the wild-type cells but not in the HSF1 null cells, indicating a dependence on HSF1 for this effect. Taken together, these results show that triptolide not only effectively prevents the molecular events that result in cytoprotection, but also sensitizes cells to enhanced levels of stress-induced cell death in an HSF1-dependent mechanism.

DISCUSSION

Triptolide inhibits induction of the heat shock response by blocking a step in the activation of HSF1, which, in turn, prevents the induction of molecular chaperones and thus sensitizes cells to stress-induced cell death. Our results demonstrate that triptolide affects a step in the heat shock transcriptional response after trimerization of HSF1, nuclear localization, and binding to the promoter of the endogenous *HSP70* gene but prior to induction of *HSP70* mRNA levels (Fig. 6). We suggest that a likely target of triptolide action is in the assembly of functional transcriptionally active HSF1 complexes on the *HSP70* promoter. As one requirement of a transcriptionally active promoter is the recruitment of TBP, and HSF and TBP are known to interact (39), we tested whether triptolide could disrupt TBP recruitment. However, chromatin immunoprecipitation analysis shows that the association of TBP with the *HSP70* promoter is not blocked by triptolide.⁵ Therefore, the transcriptional step that triptolide inhibits may occur post transcription factor occupancy.

Triptolide is derived from *T. wilfordii*, the same family of plants from which celastrol was identified as a potent inducer of the heat shock response (28). It is tempting to speculate that these small molecule regulators of the heat shock response could have physiological relevance in the regulation of the plant heat shock response as well. From a mechanistic perspective, triptolide is a new chemical genetic tool that may prove useful for further stepwise dissection of the heat shock transcriptional response. In this regard, triptolide complements the activity of another plant natural product, sodium salicylate. Sodium salicylate was shown previously to induce HSF1 DNA binding and occupancy but not hyperphosphorylation (40, 41). However, the HSF1 complexes induced by sodium salicylate were still "on pathway" in the HSF1 cycle and could

become hyperphosphorylated by exposure to a subsequent heat shock to acquire full activity upon subsequent stress treatment. The reversible inhibition of the heat shock response by triptolide suggests that the HSF1 complexes bound to the endogenous *HSP70* promoter, although nonfunctional, are nevertheless dynamic as heat shock gene transcription recovers upon removal of triptolide.

Triptolide or other functionally related small molecules that act to inhibit the heat shock response may have promise as a novel class of disease therapeutics. An increasing number of disease states are associated with elevated expression of molecular chaperones and the chronic up-regulation of the heat shock response; consequently it follows that these disease states may benefit from small molecule inhibitors of chaperone expression. In the case of cancer, for instance, up-regulation of molecular chaperones is associated with oncogenesis. Experimental evidence supports the hypothesis that the induction of heat shock proteins is required for cells to survive the transformation process. For example, cells derived from mice deficient in HSF1 are insensitive to the oncogene *H-ras*, whereas wild-type cells are readily transformed (4). Because both molecular chaperones and HSF1 are essential in protecting cells against proteotoxicity and have important roles in aging, a key aspect in disease therapy will be an ability to modulate the heat shock response transiently rather than to achieve permanent inhibition. The reversibility of triptolide on suppression of the heat shock response, therefore, could represent a particularly advantageous characteristic of this molecule.

Perhaps the most intriguing aspect of triptolide is the ability to enhance stress-induced cell death. Not only was triptolide effective in blocking the cytoprotection achieved by the transient 42 °C heat shock, but triptolide pretreatment enhanced the lethality of the 45 °C treatment. The sensitization to cell death may be because of the effects of triptolide on other cellular processes, as previous laboratories have demonstrated that triptolide can modulate the transcription factors NF- κ B and AP-1 (24–26). However, because HeLa cells have a low basal level of HSF1 activity, it is also possible that this effect of triptolide occurs through the inhibition of basal expression levels of heat shock genes. Using HSF1 wild-type and null mouse embryonic fibroblasts, we show that the increase in heat-induced cell death in response to triptolide is HSF1-dependent. In summary, our results suggest a role for triptolide in combination with other cancer therapies because of its role in the inhibition of molecular chaperone expression and cytoprotection.

⁵ S. D. Westerheide and R. I. Morimoto, unpublished data.

Acknowledgments—We thank Ivor Benjamin for the HSF1 wild-type and null mouse embryonic fibroblasts and James West for a critical reading of the manuscript.

REFERENCES

1. Ciocca, D. R., and Calderwood, S. K. (2005) *Cell Stress Chaperones* **10**, 86–103
2. Pratt, W. B., and Toft, D. O. (2003) *Exp. Biol. Med. (Maywood)* **228**, 111–133
3. Mosser, D. D., and Morimoto, R. I. (2004) *Oncogene* **23**, 2907–2918
4. Whitesell, L., Bagatell, R., and Falsey, R. (2003) *Curr. Cancer Drug Targets* **3**, 349–358
5. Nylandsted, J., Rohde, M., Brand, K., Bastholm, L., Elling, F., and Jaattela, M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 7871–7876
6. Nylandsted, J., Wick, W., Hirt, U. A., Brand, K., Rohde, M., Leist, M., Weller, M., and Jaattela, M. (2002) *Cancer Res.* **62**, 7139–7142
7. Rocchi, P., So, A., Kojima, S., Signaevsky, M., Beraldi, E., Fazli, L., Hurtado-Coll, A., Yamanaka, K., and Gleave, M. (2004) *Cancer Res.* **64**, 6595–6602
8. Wu, B. J., Kingston, R. E., and Morimoto, R. I. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 629–633
9. Wu, B. J., Williams, G. T., and Morimoto, R. I. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 2203–2207
10. Morgan, W. D., Williams, G. T., Morimoto, R. I., Greene, J., Kingston, R. E., and Tjian, R. (1987) *Mol. Cell. Biol.* **7**, 1129–1138
11. Williams, G. T., McClanahan, T. K., and Morimoto, R. I. (1989) *Mol. Cell. Biol.* **9**, 2574–2587
12. Williams, G. T., and Morimoto, R. I. (1990) *Mol. Cell. Biol.* **10**, 3125–3136
13. Pirkkala, L., Nykanen, P., and Sistonen, L. (2001) *FASEB J.* **15**, 1118–1131
14. Westerheide, S. D., and Morimoto, R. I. (2005) *J. Biol. Chem.* **280**, 33097–33100
15. Hoang, A. T., Huang, J., Rudra-Ganguly, N., Zheng, J., Powell, W. C., Rabindran, S. K., Wu, C., and Roy-Burman, P. (2000) *Am. J. Pathol.* **156**, 857–864
16. Khaleque, M. A., Bharti, A., Sawyer, D., Gong, J., Benjamin, I. J., Stevenson, M. A., and Calderwood, S. K. (2005) *Oncogene* **24**, 6564–6573
17. Tang, D., Khaleque, M. A., Jones, E. L., Theriault, J. R., Li, C., Wong, W. H., Stevenson, M. A., and Calderwood, S. K. (2005) *Cell Stress Chaperones* **10**, 46–58
18. Hosokawa, N., Hirayoshi, K., Nakai, A., Hosokawa, Y., Marui, N., Yoshida, M., Sakai, T., Nishino, H., Aoike, A., Kawai, K., and Nagata, K. (1990) *Cell Struct. Funct.* **15**, 393–401
19. Hosokawa, N., Hirayoshi, K., Kudo, H., Takechi, H., Aoike, A., Kawai, K., and Nagata, K. (1992) *Mol. Cell. Biol.* **12**, 3490–3498
20. Nagai, N., Nakai, A., and Nagata, K. (1995) *Biochem. Biophys. Res. Commun.* **208**, 1099–1105
21. Elia, G., Amici, C., Rossi, A., and Santoro, M. G. (1996) *Cancer Res.* **56**, 210–217
22. Hansen, R. K., Oesterreich, S., Lemieux, P., Sarge, K. D., and Fuqua, S. A. (1997) *Biochem. Biophys. Res. Commun.* **239**, 851–856
23. Yokota, S., Kitahara, M., and Nagata, K. (2000) *Cancer Res.* **60**, 2942–2948
24. Lee, K. Y., Chang, W., Qiu, D., Kao, P. N., and Rosen, G. D. (1999) *J. Biol. Chem.* **274**, 13451–13455
25. Qiu, D., Zhao, G., Aoki, Y., Shi, L., Uyei, A., Nazarian, S., Ng, J. C., and Kao, P. N. (1999) *J. Biol. Chem.* **274**, 13443–13450
26. Jiang, X. H., Wong, B. C., Lin, M. C., Zhu, G. H., Kung, H. F., Jiang, S. H., Yang, D., and Lam, S. K. (2001) *Oncogene* **20**, 8009–8018
27. Chang, W. T., Kang, J. J., Lee, K. Y., Wei, K., Anderson, E., Gotmare, S., Ross, J. A., and Rosen, G. D. (2001) *J. Biol. Chem.* **276**, 2221–2227
28. Westerheide, S. D., Bosman, J. D., Mbadugha, B. N., Kawahara, T. L., Matsumoto, G., Kim, S., Gu, W., Devlin, J. P., Silverman, R. B., and Morimoto, R. I. (2004) *J. Biol. Chem.* **279**, 56053–56060
29. Shi, Y., Kroeger, P. E., and Morimoto, R. I. (1995) *Mol. Cell. Biol.* **15**, 4309–4318
30. Mosser, D. D., Theodorakis, N. G., and Morimoto, R. I. (1988) *Mol. Cell. Biol.* **8**, 4736–4744
31. Sarge, K. D., Murphy, S. P., and Morimoto, R. I. (1993) *Mol. Cell. Biol.* **13**, 1392–1407
32. Westin, G., and Schaffner, W. (1988) *EMBO J.* **7**, 3763–3770
33. Holmberg, C. I., Tran, S. E., Eriksson, J. E., and Sistonen, L. (2002) *Trends Biochem. Sci.* **27**, 619–627
34. Solomon, J. M., Rossi, J. M., Golic, K., McGarry, T., and Lindquist, S. (1991) *New Biol.* **3**, 1106–1120
35. Elia, G., De Marco, A., Rossi, A., and Santoro, M. G. (1996) *J. Biol. Chem.* **271**, 16111–16118
36. Wissing, D., and Jaattela, M. (1996) *Int. J. Hyperthermia* **12**, 125–138
37. Mosser, D. D., Caron, A. W., Bourget, L., Denis-Larose, C., and Massie, B. (1997) *Mol. Cell. Biol.* **17**, 5317–5327
38. McMillan, D. R., Xiao, X., Shao, L., Graves, K., and Benjamin, I. J. (1998) *J. Biol. Chem.* **273**, 7523–7528
39. Mason, P. B., Jr., and Lis, J. T. (1997) *J. Biol. Chem.* **272**, 33227–33233
40. Jurivich, D. A., Sistonen, L., Kroes, R. A., and Morimoto, R. I. (1992) *Science* **255**, 1243–1245
41. Cotto, J. J., Kline, M., and Morimoto, R. I. (1996) *J. Biol. Chem.* **271**, 3355–3358