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Modulation of tolerance by mutant heat shock transcription factors

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Abstract It ought to be possible to recruit normal cellular defenses to mitigate ischemia/reperfusion damage and to reduce toxicity of chemotherapeutic drugs. Stress-preconditioned cells acquire a tolerant state characterized by increased resistance to such insults. This state is widely believed to be mediated, partially, by heat shock proteins (Hsps). Indirect evidence suggests that stress-induced Hsp expression is controlled by heat shock transcription factor 1 (Hsf1), which factor may therefore represent a preferred target for therapeutic modulation of tolerance. In support, positively acting (Hsf1(+)) and negatively acting (Hsf1(–)) mutants of Hsf1 were identified. Inhibition of endogenous Hsf1 activity by Hsf1(–) prevents stress-induced Hsp synthesis and development of tolerance. Hsf1(+) drastically enhances expression of major Hsps in the absence of stress and induces tolerance against heat, simulated ischemia and toxicity by cyclophosphamide. Where compared, tolerance induced was slightly better than that produced by heat preconditioning. Thus, development of the tolerant state is dependent on increased levels of the cohort of Hsps induced by stress preconditioning, and Hsf1 can induce accumulation of a typical set of Hsps, which proteins are alone capable of providing tolerance at a similar level as heat preconditioning. These findings make Hsf1 a preferred target for pharmacological intervention to deliberately induce tolerance.

INTRODUCTION

When cultured cells are preconditioned by exposure to a moderate heat shock or to sublethal doses of certain chemicals, they acquire a certain level of resistance to heat or chemicals (Hahn and Li 1982; Parsell and Lindquist 1993 and references cited therein). The state that preconditioned cells are in has been labeled the tolerant state or phenotype or, when resistance to heat is discussed, the thermotolerant state. Interestingly, cells preconditioned by heat shock become not only tolerant to more severe heat but also to chemicals such as arsenite, ethanol and diamide (Kampinga et al 1995), a phenomenon referred to as cross-tolerance. Heat preconditioning also protects cells against simulated ischemia (Mestril et al 1994). Tolerance has been traditionally quantified either as increased resistance of cells to stress killing or as increased rate of recovery of protein synthesis from stress-induced translational arrest. The acquisition of the tolerant state correlates with increased levels of heat shock proteins (Hsps), and it is therefore believed that Hsps are the main mediators of tolerance (Parsell and Lindquist 1993).

A related phenomenon has been discovered in experiments involving entire mammalian organs or animals. Heat preconditioning of animals was observed to induce beneficial effects during ischemia of the heart that can be detected as marked limitation of infarct size (Donelly et al 1992; Currie et al 1993; Marber et al 1993; Kaeffer et al 1995). In a rabbit model of infrarenal aorta occlusion, heat preconditioning completely prevented ischemic damage to the spinal cord, whereas a majority of untreated animals showed signs of paralysis one day after surgery (Lena et al 1996). Survival of acute skin flaps of rats was shown to be improved significantly by heat precondtioning (Koenig et al 1992). In a gerbil model, a

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single hyperthermic treatment 18 h before ischemia or repeated treatments at 18-h intervals were found to protect partially or completely against neuronal death (Kitigawa et al 1991). Pre-exposure of rats to either heat or sodium arsenite had a substantial protective effect against sepsis in a model involving cecal ligation and perforation (Ribeiro et al 1994). Similar effects were observed when sepsis was mimicked by administration of lipopolysaccharide (Hotchkiss et al 1993).

Evidence for an involvement of Hsps, particularly of the Hsp70 family, in protection against ischemia/reperfusion damage was obtained from experiments with transgenic mice, showing that overexpression of a rat or human *hsp70* gene partially protected the heart against ischemia/reperfusion injury (Marber et al 1995; Plumier et al 1995). At the cell culture level, transfection with genes encoding Hsp70 or, in some cases, other Hsps increased the resistance of cells against thermal stress (Liu et al 1992; Lavoie et al 1993; Mailhos et al 1994; Li et al 1995). Overexpression from transfected genes of Hsp70 in rat heart myogenic cells protected them against simulated ischemia (Mestril et al 1994).

Promoters of stress-inducible *hsp* genes contain at least one copy of a so-called heat shock element (HSE) that is required for heat-inducible gene expression (Voellmy 1994). A protein factor, heat shock transcription factor (Hsf), that binds the HSE motif was identified in cell extracts. Three mammalian genes encoding different Hsf, named Hsf1, Hsf2 and Hsf4, were isolated (Rabindran et al 1991; Sarge et al 1991; Schuetz et al 1991; Nakai et al 1997). Hsf1 is a constitutively expressed factor that resides as a non-DNA-binding monomer or heterooligomer in the cytoplasm (Baler et al 1993; Sarge et al 1993). When cells are exposed to heat or certain chemical stressors, Hsf1 homotrimerizes, acquires the ability to bind to HSE sequences and translocates to the nucleus (Baler et al 1993; Sarge et al 1993). In a further reaction, the factor acquires transcriptional competence (Zuo et al 1995). Antibody supershift experiments suggested that most or all HSE DNA-binding activity in extracts from mammalian cells exposed to heat or chemical stress originates from Hsf1 (Baler et al 1993; Sarge et al 1993). Systematic mutational analyses of human Hsf1 produced two types of mutants that were used in the present work (Zuo et al 1995). One type of mutant, referred to below as positively acting Hsf1 mutant, is deregulated as shown by its ability to activate a reporter gene controlled by the promoter of the highly inducible, minor *hsp70B* gene in the absence of stress. The second type of mutant, referred to below as negatively acting Hsf1 mutant, lacks transactivation domains. It has wild-type HSE DNA-binding activity, but is non-functional.

MATERIALS AND METHODS

Cell culture, plasmids, transfections, viral vectors and FACS

Human HeLa cells, HeLa-CAT cells (Baler et al 1992) containing copies of a human *hsp70B* promoter-driven chloramphenicol acetyltransferase (CAT) gene or 293 cells were cultured in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37°C. Human HepG2 cells were cultured in Earle's salt-based MEM supplemented with 10% FBS and 1 mM sodium pyruvate.

Expression plasmids were derived from pcDNA1 or pcDNA3 (Invitrogen Corp., Carlsbad, CA, USA) by insertion of cDNA genes for human Hsf1 (Hsf1(wt)), $LexA_{87}$–human $Hsf1_{79}$ (LexA-Hsf1(wt)), human Hsf1d202–316 (Hsf1(+)), $LexA_{87}$–human $Hsf1_{79}$d202–316 (LexA-Hsf1(+)), human Hsf1d453–523 (Hsf1(–)), and bacterial beta-galactosidase as described previously (Zuo et al 1994, 1995). Construction of reporter plasmid LexA-CAT containing a CAT gene under the control of a minimal promoter supplemented with multiple LexA binding sites was also reported previously (Zuo et al 1995). Plasmid EGFP-C1 (referred to herein as EGFP), containing a coding sequence for enhanced green fluorescence protein, was obtained from Clontech Laboratories, Inc. (Palo Alto, CA, USA). Cells (> 70% confluent cultures) were transfected by Lipofectamine™ (Gibco BRL, Life Technologies, Grand Island, NY, USA), using the procedure suggested by the manufacturer.

Recombinant adenovirus vectors were generated by co-transfection of human 293 cells with plasmid AC CMV pLpA SR(–) containing or not containing human Hsf1d202–316 (Zuo et al 1994, 1995) and the infectious plasmid JM17 according to the protocol of Graham and Prevec (1991). Viral plaques were isolated, expanded and viral DNA was analyzed by restriction enzyme digestion. Human cell (HeLa-CAT or HepG2) cultures (less than 70% confluent) were infected at a multiplicity of infection (MOI) of 5–10 PFU/cell.

For fluorescence-activated cell sorting, cells from cultures transfected or co-transfected with EGFP-expressing construct were removed from tissue culture dishes by treatment with 0.04% EDTA in phosphate-buffered saline (PBS) and were resuspended in PBS. Cell suspensions were then applied to a FACStar Plus (Becton-Dickinson, San Jose, CA, USA) machine. Excitation wavelength was 488 nm, and detection wavelengths were 650 nm (long pass) for propidium iodide and 520 nm (narrow band) for detection of EGFP fluorescence. For analyses of cell cycle distribution, cells were fixed for at least 18 h with 70% ethanol and stained with 50 µg/ml propidium iodide (Molecular Probes, Eugene, OR, USA).

Cell survival/killing and detachment assays, and simulated ischemia

After heat treatment, cultures were incubated overnight at 37°C. When Trypan blue exclusion was used as the criterion for cell survival, cells (unattached and attached) were harvested, and trypan blue-excluding cells were counted in a hemocytometer. In some experiments, cell killing was estimated by the lactate dehyrogenase (LDH) release assay, using the LDH Assay Kit from Sigma Corp. (St Louis, MO, USA). To quantify detachment from the substratum, cultures were washed three times with prewarmed DMEM, and numbers of cells remaining on dishes were determined using a hemocytometer. In the experiment shown in Figure 1E, cells were fixed in 2% formaldehyde and 0.2% glutaraldehyde in PBS and stained with X-Gal Reagent (Invitrogen Corp., Carlsbad, CA, USA). Ischemic conditions were simulated as described previously (Mestril et al 1994). Briefly, cells were placed in slightly hypotonic HBSS (1.3 mM $CaCl_2$, 5 mM KCl, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 69 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4) without glucose or serum, and made hypoxic for 4–6 h at 37°C. Control cells were left under normoxic conditions for the same period of time. Hypoxia was achieved by using an air-tight jar from which oxygen was removed by displacement with argon. After 10 min of gas exchange, the oxygen concentration was < 0.2%. Hypoxia was maintained using the oxygen-consuming GasPak System from BBL Microbiology Systems (Cockeysville, MD, USA).

Translational recovery experiments

In a typical experiment, parallel HeLa-CAT cultures in 60 mm dishes (more than 70% confluent) were transfected with 0.75 μg of EGFP construct and 2.25 μg of Hsf1(+) or Hsf1(–) construct and 15 μl Lipofectamine™. Before exposing the cultures to heat shock about 36 h post-transfection, cells were replated (to achieve about 70% confluence). Note that in some experiments (such as that shown in Fig. 3C) Hsf1(+) was delivered by infection with an adenoviral vector rather than by transfection. Cultures were exposed to a heat shock and then returned to 37°C. Rates of protein synthesis before, during, and at different times after a subsequent, more severe heat shock were determined by pulse-labeling of cells with ^{3}H-leucine. For labeling, cultures were washed two times with prewarmed PBS, and 1 ml of labeling medium (leucine-free DMEM with 2% FBS and 65 μCi of ^{3}H-leucine (179 Ci/mmole)) was added. Cultures were incubated for 30 min at 37°C, washed two times with PBS, and cells scraped off, collected and lysed in buffer C (20 mM Hepes, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 25% glycerol). Aliquots of lysates were used for protein determinations by the Bradford method (Bradford 1976) and for precipitation with 10% trichloroacetic acid to measure incorporation of radiolabel into proteins. Relative rates of protein synthesis were calculated as cpm/μg protein. In some experiments, aliquots were also compared by SDS-PAGE and fluorography.

Western blots

Cells were scraped off dishes and lysed in buffer C. Aliquots containing equal amounts of cell protein as well as prestained molecular weight markers were applied to 8.5% SDS-PAGE gels. Following electrophoresis, proteins were transferred to Immobilon-P Transfer Membranes (Millipore Corp., Bedford, MA, USA), and membranes were blocked by incubation with TBS (25 mM Tris-HCl, pH 8.0, 0.136 M NaCl, 2.7 mM KCl) with 2% non-fat dry milk. Appropriate sections of membranes were incubated with anti-HSF1 polyclonal antibody (Baler et al 1993), anti-Hsp antibodies (all from StressGen Biotechnologies Corp., Victoria, BC, Canada) or anti-actin antibody (Amersham, Arlington Heights, IL, USA). Following three washes with TBS, membranes were reblocked and incubated with second antibody (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham). After several washes in TBS, membranes were developed using the ECL Western Blot Detection Kit (Amersham).

Other assays

CAT assays were performed using the chromatographic assay of Gorman et al (1982). DNA replication was assessed by a standard ^{3}H-thymidine incorporation assay. In most experiments, including all western blot assays, standardization was based on protein content. All protein determinations were made using the Bradford method. In a few experiments involving sorted cells, standardization was based on cell numbers. All experiments were repeated at least twice, and, in most cases, each data point was derived from assays of 3–5 parallel samples.

RESULTS

Hsf1(–) prevents stress-induced Hsp expression and the establishment of a tolerant state

Two previously characterized Hsf1 deletions were selected as potential positively and negatively acting factors. Hsf1d202–316, referred to below as Hsf1(+), expressed from transfected genes is constitutively trimeric and DNA binding and is capable of transactivating an *hsp70B* promoter-driven reporter gene in the absence of stress (Zuo et al 1995). Hsf1d453–523, referred to below as Hsf1(–), is also DNA-binding but

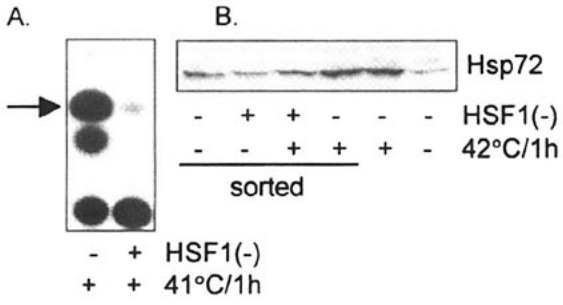


Fig. 1 (A) Inhibition of *hsp70B*-CAT expression in HeLa-CAT cells by Hsf1(–). HeLa-CAT cells were co-transfected with EGFP and Hsf1(–) DNAs. One day later, cells were heat-treated, incubated for an additional day and then separated by FACS into EGFP-positive (Hsf1(–)-positive) and negative (Hsf1(–)-negative) cells. Chromatographic CAT assays of the two populations are shown. The arrow points to the major acetylated product of ^{14}C-chloramphenicol. (B) Hsf1(–) blocks induction of Hsp72. A similar experiment as that described in (A), except that cells were harvested 5 h after heat shock, was analyzed by anti-Hsp72 Western blot. (C) Hsf1(–) abrogates the protective effect of heat preconditioning. Cells transfected with EGFP DNA alone or with EGFP and Hsf1(–) DNAs were subjected to a 41°C/1 h heat shock 36 h post transfection, allowed to recover for 3 h and exposed to a 44°C/30 min heat shock. Cultures were pulse-labeled with ^{3}H-leucine either before or during the latter heat shock or after 1 h of recovery. * Rates of translation of Hsf1(–)-expressing cells calculated by taking into account a cytometrical estimate of transfection efficiency of 53% (percentage of cells expressing EGFP). (D) Hsf1(–) inhibits and Hsf1(+) enhances translational recovery subsequent to a 42°C/1 h heat shock administered 36 h after transfection. Rates of protein synthesis were estimated as before. (E) Hsf1(–) enhances heat killing. Cells were transfected with a beta-galactosidase construct or co-transfected with the beta-galactosidase construct and an HSF1(–) construct. Thirty-six hours later, cultures (2×10^5 cells/60 mm dish) were either heat-treated at 48°C for 10 min or not heat-treated, incubated for another day and cells stained for beta-galactosidase activity. Average numbers of stained cells were determined by scoring five identically sized areas in each dish. Not-heat-treated cultures of cells transfected with or without Hsf1(–) had similar numbers of stained cells (not shown).

C. Recovery of translation after heat shock of heat-preconditioned cells

Plasmids	Relative Incorporation of ^{3}H-Leucine			
	Before HS	During 2nd HS (44°C/30min)	Recovery 1h	Recovery* 1h
	Experiment 1:			
None	100*	7 +/-1	39 +/-13	
EGFP	96 +/-4	9 +/-4	**33 +/-2**	**33**
EGFP/ HSF1(-)	104 +/-3	4 +/-0	**18 +/-3**	**5**
	Experiment 2:			
None	100*	4 +/-1	60 +/-3	
EGFP	104 +/-1	4 +/-1	**60 +/-3**	**60**
EGFP/ HSF1(-)	98 +/-9	4 +/-1	**41 +/-3**	**24**

D. Recovery of translation after heat shock

Plasmids	Relative Incorporation of ^{3}H-Leucine			
	Before HS	During HS 42°C/1h	Recovery 2h	Recovery* 2h
None	100*	2 +/-0	63 +/-6	
EGFP	105 +/-6	2 +/-0	**63 +/-4**	**63**
EGFP/ HSF1(+)	97 +/-9	2 +/-0	**93 +/-13**	**100**
EGFP/ HSF1(-)	103 +/-10	2 +/-0	**44 +/-6**	**27**

E. Cell survival after heat-shock (48°C/10min)

Plasmids	β-Galactosidase-positive Cells
β-Galactosidase	39.4 +/- 10.8
β-Galactosidase/HSF1(-)	24.9 +/- 6.9

lacks functional transcription activation domains (Zuo et al 1995). Genes for the mutant factors were delivered to cells either by infection with an adenoviral vector (MOI of 5–10) or by Lipofectamine™ transfection. In some experiments, transfection mixtures also included a gene encoding a highly fluorescent green fluorescent protein (EGFP), and when transfection of most cells was deemed important, EGFP-fluorescing cells isolated by fluorescence-activated cell sorting (FACS) were employed.

To find out whether Hsf1(–) could function as a negatively acting factor, HeLa-CAT cells containing integrated copies of an *hsp70B*-chloramphenicol acetyltransferase (CAT) gene were co-transfected with an Hsf1(–)-and an EFGP-expressing construct. One day later, cells were subjected to a 41°C/1 h heat shock and incubated overnight at 37°C. CAT assays on FACS-isolated populations of cells containing or not containing Hsf1(–) showed that Hsf1(–) severely inhibited heat-induced reporter expression (Fig. 1A). The factor also greatly reduced heat-induced Hsp accumulation, as demonstrated by anti-Hsp72 Western blot (Fig. 1B). To determine whether elevated Hsp levels induced by heat preconditioning are required for development of tolerance, measured as an increased rate of recovery from heat-induced translational arrest, cultures co-transfected with EGFP- and Hsf1(–)-expressing constructs or transfected with EGFP-expressing construct only (control cultures) were mildly heat-preconditioned (41°C/1 h) and, 3 h later, subjected to a 44°C/30 min (severe) heat shock. To allow for recovery, cultures were then incubated at 37°C for various lengths of time. Rates of protein synthesis were assessed by pulse-labeling cells with ^{3}H-leucine (Fig. 1C). Results from two independent experiments are shown. In the first experiment, the rate of protein synthesis dropped to below 10% during heat shock and, in preconditioned control cells, recovered to about 33% one hour after heat shock. In cultures that had been transfected with the Hsf1(–) construct, the rate of protein synthesis after the same recovery period was

only 18%. In the second experiment, the rate of translation recovered to 60% in cells lacking Hsf1(–), but only to 41% in cells expressing Hsf1(–). In the absence of preconditioning, the rate of translation after a 1-h recovery period was below 5% (not shown). These results demonstrated that recovery from translational arrest was substantially reduced in Hsf1(–)-expressing cells compared with control cells. Because of the large numbers of cells needed in this type of experiment (and the subsequent experiment), FACS isolation of successfully transfected cells was omitted. To remove the contribution of cells that had not received the Hsf1(–) construct to the rates of protein synthesis determined with cultures transfected with the Hsf1(–) construct, transfection efficiency was determined cytometrically and was found to be about 53%. Subtraction of the contribution of untransfected cells revealed corrected rates of protein synthesis for Hsf1(–)-expressing cells 1 h after heat shock of about 5% and 24%, respectively, for the two experiments.

To learn whether Hsp accumulation induced by a stressful event is protective during the same event, EGFP/Hsf1(–)- and EGFP-transfected (control) cultures were heat-shocked (42°C/1 h), and recovery from translational arrest was compared 2 h after heat shock (Fig. 1D). EGFP-transfected cells recovered significantly better than EGFP/Hsf1(–)-co-transfected cells. After correction for the presence of untransfected cells was made, the rate of protein synthesis in Hsf1(–) -containing cells was found to be only 27% of the rate of not-heat-treated cells, whereas the rate in control cells was restored to about 63% at the end of the 2-h recovery period. To also assess the effect of expression of Hsf1(–) on stress-induced cell killing, cultures were transfected with a beta-galactosidase gene or co-transfected with the beta-galactosidase gene and the Hsf1(–) construct (Fig. 1E). Transfected cultures were subjected to a severe heat shock (48°C/10 min; about 75% cell survival after one day of postshock incubation) or left untreated, and beta-galactosidase-positive cells were counted the following day. Untreated, singly and doubly transfected cultures contained similar numbers of beta-galactosidase-expressing cells. In the heat shocked cultures, the presence of Hsf1(–) increased cell killing by about 60% ($P < 0.05$). Thus, the increase in Hsp levels occurring as a consequence of a stressful event has a significant, immediate protective effect.

Hsf1(+) mediates drastic accumulation of major Hsps and the development of a tolerant state

The above experiments demonstrated that elevated levels of Hsps induced by preconditioning are critical to the establishment of the tolerant state. If Hsps are the only or at least the main mediators of the tolerant state, deliberate induction of accumulation of Hsps should result in the same or a similar state. To find out whether Hsf1(+) is capable of enhancing the activity of endogenous *hsp* genes, and, if it is, whether this results in substantial accumulation of Hsps, parallel HeLa-CAT cultures were infected with an Hsf1(+)-expressing adenoviral construct (Ade-Hsf1(+)), an empty adenoviral vector (Ade) or left uninfected. After incubation for different lengths of time, cells were harvested, and extracts analyzed by Western blot (Fig. 2A). Readily detectable levels of Hsf1(+) accumulated 24 and 36 h after infection. Increased levels of Hsp90 and Hsp72 were evident 15 h, and of all Hsps tested 24 h after infection with Ade-Hsf1(+) but not with Ade. Comparable results were obtained with several other human cell lines, including a HepG2 line (not shown). To confirm that Hsf1(+) enhances *hsp* gene expression directly rather than through activation of the normal stress signal pathway and endogenous Hsf1, the HSE DNA-binding domains of wild-type factor Hsf1(wt) and of mutant factor Hsf1(+) were replaced with that of LexA repressor. Of the resulting chimeras, only LexA-Hsf1(+) but not LexA-Hsf1(wt) activated a CAT reporter gene under the control of a minimal promoter supplemented with LexA binding sites in the absence of stress (Fig. 2B, left panel). Neither chimera enhanced expression of the *hsp70B*-CAT gene (Fig. 2B, right panel). Thus, the chimera containing the mutant Hsf1(+) sequence and, by extension, Hsf1(+) itself are active transcription factors capable of enhancing expression of endogenous *hsp* genes and causing accumulation of Hsps. This conclusion is further supported by a recent observation made with cell lines inducibly expressing Hsf1(+) that transfected *hsp70B*-CAT reporter gene is significantly activated by Hsf1(+) present at lower concentration than endogenous Hsf1 (not shown).

To find out whether accumulation of Hsps mediated by Hsf1(+) is protective, i.e. induces a tolerant state, HeLa-CAT cells were infected with Ade-Hsf1(+) or Ade and, 1 day later, were exposed to a 49°C/20min extreme heat shock (Fig. 3A). After 1 day of post incubation, cell survival was assessed by the Trypan blue exclusion assay. Whereas survival of control cells was below 40%, virtually all Hsf1(+)-containing cells survived the insult. In similar experiments, adherence to the substratum was followed as a measure of cytoskeletal integrity. Cells were exposed to a 47°C/20min treatment and then incubated at 37°C for an additional day, after which time 85% of control cells but only 24% of Hsf1(+)-containing cells had detached from the tissue culture surface (Fig. 3B). To test for recovery from translational arrest following heat shock, cells infected with Ade-Hsf1(+) or Ade were subjected to a 44°C/30 min heat shock and then allowed to recover at 37°C (Fig. 3C). Protein synthesis dropped to about 10% of the normal rate during heat shock but, after a 1-h recovery period, returned to pre-shock level in

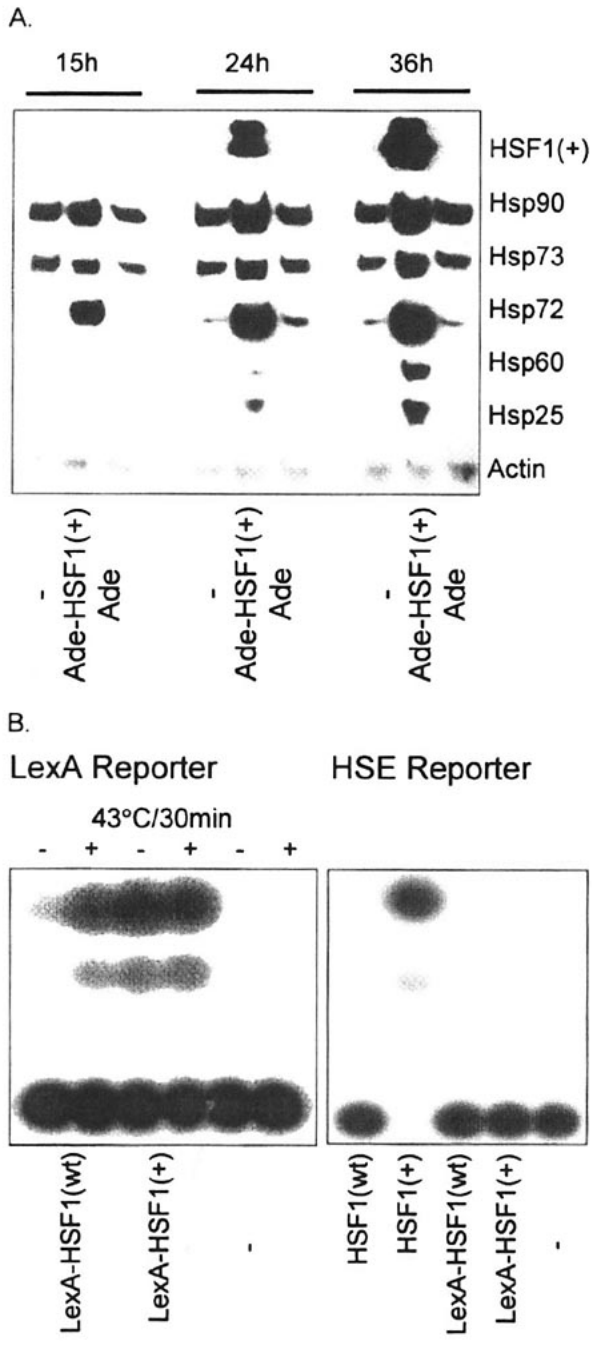


Fig. 2 (A) Hsf1(+) drastically enhances expression of a set of *hsp* genes. Parallel HeLa-CAT cultures were left untreated, or infected with Ade-Hsf1(+) or Ade. At the times indicated, cultures were harvested, lysed, and protein analyzed by Western blot using antibodies against Hsf1, different Hsps and actin. Strips of membranes developed with antibodies against the indicated proteins are shown. (B) Hsf1(+) is a constitutively active transcription factor. On the left, CAT assays are shown of HeLa cells co-transfected with a LexA-CAT reporter gene and the constructs indicated at the bottom of the panel. Where applicable, heat treatment was 1 day after transfection, and all cultures were harvested 1 day after heat treatment. On the right, CAT activities are from HeLa-CAT cells transfected with the constructs indicated below the panel.

Hsf1(+)-expressing but not control cells. Thus, increased accumulation of Hsps mediated by Hsf1(+) effectively protected cells against heat stress. See Figure 1D for analogous experiments in which cells were transfected rather than infected with Hsf1(+) genes. Hsf1(+)-expressing cells were also partially protected against medically relevant stresses such as toxicity from cyclophosphamide, a chemotherapy drug with known hepatotoxicity, and simulated ischemia. Exposure (24 h) of human hepatocyte-derived HspG2 cells to cyclophosphamide (20mM) caused substantial mortality (38%) that was reduced by 55% by the presence of Hsf1(+) (Fig. 3D). When mortality was assessed by the lactate dehydrogenase (LDH) leakage assay, expression of Hsf1(+) resulted in a 43% reduction that compared well with the 25% reduction observed for heat preconditioning (Salminen et al 1996). Hsf1(+) also decreased the mortality of HepG2 cells subjected to simulated ischemia by more than 50% (heat preconditioning had a 40% effect) (Fig. 3E). Thus, an increase in Hsp levels mediated by Hsf1(+), i.e. by deregulation of Hsf1 activity, is generally cytoprotective.

Consequences of drastic overexpression of Hsps

To test for effects on cell proliferation, HeLa-CAT cells were transfected with EGFP construct alone or co-transfected with EGFP and Hsf1(+) constructs. In one type of experiment, Hsf1(+)-expressing and not-expressing cells were isolated by FACS and replated (on day 1), and their proliferation was followed between days 2 and 4 (Fig. 4A). Results indicated that elevated Hsp levels severely inhibited cell proliferation. Similar results were obtained from another type of experiment, in which cells were not sorted, and numbers of transfected cells were determined from total cell numbers and cytometric assessments of the fraction of cells expressing EGFP (not shown). The latter experiment also revealed that cells proliferated between days one and two, i.e. that proliferation only stopped after day 2. When, in the experiment shown in Figure 4A, cells from the last time point (day 4) were analyzed for DNA content, numbers of Hsf1(+)-expressing cells in S and G2/M phases were found to be somewhat elevated (Fig. 4B). Cytometric analysis showed that the average cell size had not changed appreciably. Thus, increased Hsp levels dramatically slow the rate of progression through the cell cycle, without arresting cells in a particular position in the cycle. Inhibition of progression appeared to be somewhat less severe between G1 and S, with the result that, with time, there was some accumulation of cells in S and G2/M phases. DNA replication was assessed (on day 2) by ^{3}H-thymidine incorporation and was found to be reduced 4- to 5-fold in Hsf1(+)-expressing cells compared to control cells (Fig. 4C). Protein synthesis was examined by pulse-labeling

A. Cell survival after heat shock (49°C/20min)

Vector	Treatment	Cell Survival (%)
None	None	100
None	Heat shock	39 +/-3
Ade-HSF1(+)	Heat shock	98 +/-3
Ade	Heat shock	33 +/-8

B. Cell attachment to substratum after heat shock (47°C/20min)

Vector	Treatment	Cells Attached(%)
None	None	100
None	Heat shock	15 +/-2
Ade-HSF1(+)	Heat shock	76 +/-2

C. Recovery of translation after heat shock (44°C/30 min)

Vector	Relative Incorporation of ^{3}H-Leucine		
	Before HS	During HS	Recovery (1 h)
None	100	9	14
Ade-HSF1(+)	105	12	98
Ade	119	8	9

D. Mortality from exposure to cyclophosphamide

Vector	Cyclophos.	Relative LDH Release	Mortality
Ade	None	21	13
Ade	20mM	100 +/-24	51 +/-1
Ade-HSF1(+)	None	25	13
Ade-HSF1(+)	20mM	70 +/-18	30 +/-1

E. Mortality from simulated ischemia

Vector	Pretreatment	Relative LDH Release
None	None	100 +/-11
None	Heat shock	60 +/-0
Ade-HSF1(+)	None	47 +/-2
Ade	None	94 +/-8

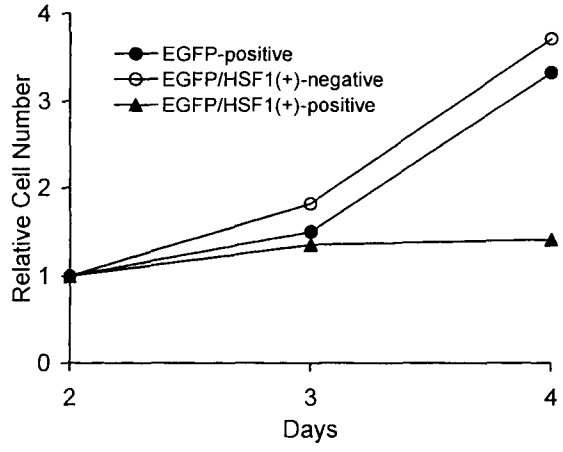
Fig. 3 Hsf1(+) protects cells against killing by extreme heat (A) and detachment from the substratum (B) as well as against the cytotoxic effects of cyclophosphamide (D) and against simulated ischemia (E). In the experiment shown in (C), the protective effect of Hsf1(+) was assessed by a recovery of translation experiment (see Fig. 1 for experimental details). Experiments in (A), (B) and (C) were performed with HeLa-CAT cells, and those in (D) and (E) with HepG2 cells. LDH: lactate dehydrogenase.

cells with ^{3}H-leucine followed by TCA precipitation of labeled proteins. The data shown in Figure 4C are from day 4. Similar data were obtained on day 2 (not shown). Thus, elevated Hsp levels did not affect the overall rate of protein synthesis. Although this was less apparent at early time points, analysis by SDS-PAGE and fluorography showed that, at day 4, synthesis of non-Hsp proteins was significantly reduced (not shown). To obtain an estimate of the transcriptional (RNA polymerase II) capability of cells with elevated Hsp levels, cells were infected with Ade-Hsf1(+) or Ade and, 1 day later, were transfected with the EGFP construct (Fig. 4D). Cytometric quantitation indicated that cells with elevated Hsp levels were capable of substantial EGFP expression, although absolute levels were somewhat reduced (by about 45%) compared with control cells. In summary, the tolerant state induced by drastic overexpression of Hsps is characterized by normal translation and substantial transcription activity. However, DNA replication and cell cycle progression are compromised, possibly as a consequence of reduced synthesis (and, possibly, enhanced degradation; see Discussion) of labile proteins regulating the latter processes. Apparently, the tolerant state can be maintained for several days.

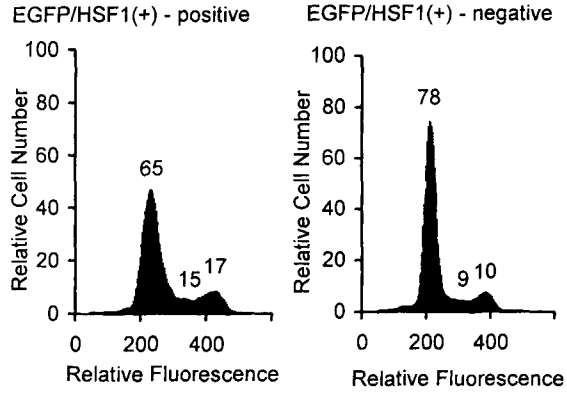
DISCUSSION

Expression of Hsf1(–) prevents heat activation of an *hsp70B* reporter gene and of endogenous *hsp* genes such as that encoding inducible Hsp72. Hsf1(–) is constitutively trimeric and has specific DNA-binding activity but, owing to the absence of transcription activation domains, lacks transactivation ability (Zuo et al 1995). Presumably, the mutant factor binds to HSE sequences in *hsp* promoters, preventing them from being bound by heat-activated endogenous Hsf1. Experiments in which Hsf1(–)- and control-transfected cells were preconditioned by mild heat treatment, and recovery of protein synthesis after a second, more severe heat stress was followed to assess the level of tolerance induced by the preconditioning treatment revealed that Hsf1(–) severely inhibited the development of tolerance. Furthermore, protein synthesis in cells expressing Hsf1(–) recovered more slowly after heat stress than that in cells not expressing the mutant factor. Analogous observations were made when mortality due to heat stress was analyzed. These findings strongly suggest that upregulation of the cohort of Hsps that occurs during stress preconditioning is critically important to the development of tolerance. The data further suggest that, under certain conditions, synthesis of Hsps induced by a stressful event may provide some measure of immediate protection, extending the realm of possible applications of the protective response beyond situations in which the response is activated preemptively. While not directly relevant to mammalian systems, observations made recently by Jedlicka et al (1997) with *Drosophila* flies expressing a heat-sensitive version of the organism's single Hsf species are in agreement with our findings. The authors showed that, at the restrictive

A.



B.



C. Rates of protein and DNA synthesis in HSF1(+)-expressing cells

Constructs	Relative Incorporation of	
	^{3}H-leucine (%)	^{3}H-thymidine (%)
None	100	100
EGFP/HSF1(+), sorted as EGFP-negative	93	100
EGFP/HSF1(+), sorted as EGFP-positive	93	23

D. RNA polymerase II transcription in HSF1(+)-expressing cells

Vector/Construct	Transfection Efficiency (%)	Mean Relative Fluorescence
None/EGFP	39 +/- 5	1403 +/- 145
Ade/EGFP	46 +/- 1	1525 +/- 1
Ade-HSF1(+)/EGFP	42 +/- 0	848 +/- 27

Fig. 4 (A) Inhibition of cell proliferation by Hsf1(+). Cells were transfected with EGFP construct alone or co-transfected with EGFP and Hsf1(+) constructs. One day later cells were sorted (based on EGFP fluorescence) and replated at similar densities (day 1). Cell numbers were determined on each of the following 3 days. (B) Sorted cells (day 4) were stained with propidium iodide, and DNA contents were assessed cytometrically. Relative numbers of cells in G1, S and G2/M phases are indicated in the histograms. (C) Incorporation of ^{3}H-leucine and ^{3}H-thymidine by sorted cells was determined as described in Methods. The ^{3}H-leucine incorporation data are from day-4 cells, and the ^{3}H-thymidine incorporation data from day-2 cells. (D) Untreated cells, or cells infected with Ade-Hsf1(+) or Ade were transfected with the EGFP construct 1 day after infection. One day after transfection, the cells were analyzed cytometrically (EGFP fluorescence). Transfection efficiency was determined as the fraction of cells expressing EGFP.

temperature, heat pretreatment of flies did not result in Hsp accumulation and acquisition of a thermotolerant state.

Hsf1(+) enhances expression of a set of major endogenous *hsp* genes in the absence of stress. Expression of Hsf1(wt) from transfected genes results neither in the activation of the *hsp70B* reporter gene (Zuo et al 1995) nor in enhanced activity of endogenous *hsp* genes (data not shown). Thus, Hsf1(+) appears to function as an activated transcription factor capable of generally stimulating *hsp* gene activity. However, since endogenous Hsf1 and Hsf1(+) act on the same target sequences, it was conceivable that expression of Hsf1(+) somehow activated the endogenous factor, and that it was the endogenous factor that interacted with and stimulated *hsp* gene expression. For the following reasons, the latter possibility was considered to be unlikely. First, to distinguish transcriptional activities of endogenous Hsf1 and transfected Hsf1(+), the HSE DNA-binding domain of Hsf1(+) was replaced with that of bacterial repressor LexA. The resulting chimeric factor, LexA-Hsf1(+), binds to LexA sites but not to HSE sequences and activates a reporter gene controlled by a minimal promoter containing LexA binding sites but not the *hsp70B* reporter gene. Conversely, the LexA reporter gene is not activated by either the endogenous Hsf1 or by Hsf1(+). Thus, LexA-Hsf1(+) and, consequently, Hsf1(+) are active transcription factors. Second, Hsf1(+) is binding to HSE DNA with similar affinity as activated endogenous Hsf1 and is capable of being translocated to the nucleus (Zuo et al 1995). Thus, there is no indication that Hsf1(+) has a non-native conformation and could activate *hsp* genes by virtue of

being a non-native protein. Furthermore, as is typical for specific transcription factors, endogenous Hsf1 is present at very low concentrations. Hsf1(+) expressed at a concentration below that of endogenous Hsf1 is capable of activating the *hsp70B* reporter gene in the absence of stress. In contrast, it is known that activation of *hsp* genes or endogenous Hsf1 by non-native proteins only occurs when the concentration of these proteins reaches levels of 1–2% of total cellular protein (Lepock et al 1993). Finally, if Hsf1(+) were not an active transcription factor, it would be expected to affect *hsp* gene expression similarly as Hsf1(–), i.e. it should compete with activated, endogenous Hsf1 for binding *hsp* promoters and suppress *hsp* gene expression. It is therefore concluded that Hsf1(+) directly activates endogenous *hsp* genes.

The finding that Hsps accumulate to substantially elevated levels in cells expressing Hsf1(+) provides compelling direct evidence that Hsf1 can activate many of the major endogenous *hsp* genes, supporting the notion that this factor is generally responsible for the stress regulation of non-constitutive *hsp* genes. Hsp expression is known to be regulated at both transcriptional and post-transcriptional levels. Heat stress was shown to enhance the stability of certain Hsp mRNAs and of mediating their selective nuclear export and translation (DiDomenico et al 1982a, 1982b; Saavedra et al 1996). Without knowledge of the relative importance of transcriptional versus post-transcriptional regulation of Hsp expression, it remained uncertain whether activation of Hsf1 in the absence of stress would result in substantial expression of Hsps and in development of a tolerant state. Our experiments with Hsf1(+) demonstrated that post-transcriptional events do not play a sufficiently dominant role to prevent massive accumulation of Hsps in unstressed cells having deregulated Hsf1 activity. Thus, Hsf1 is a reasonable target for developing therapeutic approaches based on providing elevated Hsp levels.

Regarding the question of whether increased accumulation of the entire cohort of Hsps protects cells from stress-induced damage, our experiments with Hsf1(+) clearly showed that this is the case. Expression of Hsf1(+) and the consequential overexpression of all Hsps that can be regulated by the factor protect cells effectively against different types of stress, namely heat, toxicity of cyclophosphamide and simulated ischemia, i.e. a state of general tolerance is induced. Note that resistance to cyclophosphamide toxicity and to ischemia were chosen as indicators of tolerance in view of potential medical applications. Cancer chemotherapy is plagued by toxic side-effects, and cyclophosphamide with its known hepatotoxicity was chosen as an example of cancer chemotherapeutic drugs. Although often too mild to detect, ischemia and reperfusion damage may occur in the course of any kind of surgical procedure, and damage can be severe in major surgery. It seems likely that our results obtained from cell culture experiments will translate to the organ and organism levels based on the many parallels observed previously. For example, tolerance of cultured heart cells to simulated ischemia can be enhanced by heat preconditioning and by overexpression of Hsp70 from transfected genes (Mestril et al 1994). Hearts are partially protected against ischemia/reperfusion damage in heat-preconditioned animals or in animals expressing an *hsp70* transgene in the myocardium (Donelly et al 1992; Currie et al 1993; Marber et al 1993, 1995; Kaeffer et al 1995; Plumier et al 1995). Heat-preconditioning of hepatocyte-derived HspG2 cells enhances their tolerance to the hepatotoxicant bromobenzene (Salminen et al 1996). Mice heat-preconditioned by administration of a dose of amphetamine are partially protected from liver damage by bromobenzene (Salminen et al 1997). It appears therefore reasonable to propose that induction of a state of general tolerance, be it by means of novel drugs targeting Hsf1 or, in some situations, perhaps even by Hsf1(+) gene or protein delivery, should be widely useful to mitigate forseeable ischemia/reperfusion damage or toxicity from planned medical interventions.

Expression of Hsf1(+) can be maintained for an extended period of time (i.e. for at least 4 days) without any negative effects on cell viability. The tolerant state induced is characterized by a normal rate of protein synthesis and a nearly normal rate of RNA polymerase II transcription. In addition, we observed drastically reduced rates of cell cycle progression and DNA replication. When cells are subjected to a proteotoxic insult such as heat stress, chemical stress, ischemia/reperfusion, etc., many different proteins and enzymes may be rendered nonfunctional, including those involved in DNA replication, with the result that the rate and fidelity of DNA replication may be negatively affected. Programmed inhibition of replication and cell cycle progression may serve to prevent genetic damage. Even after days of continued protein synthesis in the absence of cell division, the size of Hsf1(+)-expressing, tolerant cells does not increase significantly. This implies, and preliminary assays of rates of proteolysis appear to support this conclusion (Vilaboa et al, unpublished data), that tolerant cells turn over proteins at substantially increased rates. Such increased protein turnover should accelerate clearance of nonnative proteins and should therefore be highly beneficial to cells experiencing a proteotoxic insult. However, it is important to note that, in our experiments, Hsf1(+) was expressed from a strong, constitutively active promoter. Consequently, Hsps were continuously expressed at near maximal rates in Hsf1(+)-containing cells and accumulated to drastically elevated levels after 1 or 2 days of synthesis. Thus, as our

experiments were not designed to provide dose response information, they could not reveal whether inhibition of proliferation and acquisition of the tolerant state occur at similar Hsp concentrations, i.e. whether inhibition of cell proliferation is an important aspect of the tolerance phenotype. The relationship between tolerance and inhibition of proliferation may be probed by using an inducible promoter system to control Hsf1(+) expression and, consequentially, levels of Hsp overexpression.

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