Targeted Disruption of Heat Shock Transcription Factor 1 Abolishes Thermotolerance and Protection against Heat-inducible Apoptosis*

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D. Randy McMillan‡, Xianzhong Xiao‡, Lei Shao‡, Kathy Graves‡, and Ivor J. Benjamin¶

From the Departments of Internal Medicine and Biochemistry, Molecular Cardiology Research Laboratories, The University of Texas Southwestern Medical Center, Dallas, Texas 75235-8573

Heat shock transcription factor 1 (HSF1) is a member of the vertebrate HSF family that regulates stress-inducible synthesis of heat shock proteins (HSPs). Although the synthesis of the constitutively expressed and inducible members of the heat shock family of stress proteins correlates with increased cellular protection, their relative contributions in acquired cellular resistance or “thermotolerance” in mammalian cells is presently unknown. We report here that constitutive expression of multiple HSPs in cultured embryonic cells was unaffected by disruption of the murine HSF1 gene. In contrast, thermotolerance was not attainable in hsf1−/−cells, and this response was required for protection against heat-induced apoptosis. We conclude that 1) constitutive and inducibly expressed HSPs exhibit distinct physiological functions for cellular maintenance and adaptation, respectively, and 2) other mammalian HSFs or distinct evolutionarily conserved stress response pathways do not compensate for HSF1 in the physiological response to heat shock.

Heat shock transcription factors (HSFs)1 regulate stress-inducible synthesis of HSPs during development, growth, and adaptation (1–3). This response protects the ischemic heart (4–6) and promotes tumor cell survival (7, 8), thus indicating the clinical importance of this regulatory pathway. During unstressed conditions, constitutively expressed stress proteins may function as molecular chaperones to facilitate the synthesis, folding, or translocation of nascent polypeptides and the translocation or repair of existing polypeptides (9–11). Similar chaperone functions have been proposed, but not established, for inducible HSPs during cellular adaptation such as thermotolerance (12, 13).

Up-regulation of stress protein expression, within minutes after exposure to noxious stimuli, is accomplished through mechanisms that involve both transcriptional activation and preferential translation (2, 14). Physiological stresses induce monomers of metazoan HSFs to: 1) oligomerize into trimers that bind DNA with high affinity, 2) translocate into the nucleus, and 3) activate transcription of target stress protein genes (reviewed in Ref. 3). HSF1 is the major stress-inducible transactivator of the heat shock response (15); in contrast, HSF2 has been proposed to regulate “nonstress” HSP gene expression during early development stages and spermatogenesis (16–19).

To date, genetic studies indicate pleiotropic functions of the single copy HSF gene in Saccharomyces cerevisiae and Drosophila. Yeast HSF expression is essential for cell viability during unstressed conditions (20–22), a property that may be related to regulation of basal HSP gene expression (23). Interestingly, the Drosophila HSF protein is not essential for general growth or viability, but is required for larval development, oogenesis, and survival at extreme stress conditions (24). In vertebrates, multiple HSFs have been identified in chicks, plants, mice, and humans (16, 25–27). We hypothesize that members of the mammalian HSF family exert redundant and/or complementary functions during growth, development, and physiological adaptation.

To better determine the physiological functions of mammalian HSF1, mouse embryonic fibroblasts lacking the HSF1 gene were derived, and their phenotype was characterized at the genetic, biochemical, and functional levels during physiological adaptation. We report here that mammalian HSF1 is required for inducible HSP expression and acquired thermotolerance, a function that is consistent with similar requirements for the single-copy homolog in lower eukaryotes (28) (24) but is not compensated for by other members of the HSF family. Our findings also provide the first direct evidence that constitutively expressed and inducible members of the HSF family exhibit distinguishable functional roles in physiological maintenance and adaptation.

EXPERIMENTAL PROCEDURES

Targeting Vector and Deletion Studies—Overlapping clones spanning a 13.5-kb Xbal genomic fragment were isolated from a 129Sv library. An HSF1 targeting vector was constructed by deleting a 1.8-kb NheI/SaiI genomic fragment, corresponding to approximately half the DNA binding domain plus leucine zippers 1–3 and replacing it with a 2.0-kb NEO expression cassette (kindly provided by P. Soriano); the herpes simplex virus thymidine kinase expression cassette was inserted at the 5′-end for negative selection (29). The vector was linearized and electroporated into ES cells (129/Sv, KG1 passage 4). Double resistant clones (G418/FIAU) were screened for homologous recombination by Southern analysis with the indicated probe. Approximately 5% of the 150 clones screened contained the legitimate recombination event.

Generation of hsf1−/−Mice and Isolation of Embryonic Fibroblasts—Cells from recombinant clones were microinjected into BALB/c blastocysts and implanted into pseudopregnant BALB/c mice. The resulting high percentage chimeric males were bred to BALB/c females for germ line transmission of the mutation into the F1 generation. Embryos of F1 heterozygous crosses were harvested at E14.5 and eviscerated and the remaining tissue dissociated into individual cells with trypsin. We screened four litters at 14.5 days and obtained 31 embryos with the following genotypes: 5 wild-type (16%), 18 heterozygote (58%), and 8...
homozygote (26%). The cells could be maintained in culture for at least seven passages; all studies were performed with primary cells between the third and fifth passages.

**Electrophoretic Mobility Shift Assay**—Aliquots (10 μg) of whole cell extracts, prepared as described previously (30), from control and heat-shocked cells were mixed in a binding reaction with a labeled double-stranded heat shock element (HSE) oligonucleotide probe (AATTCGAAACCCCTGGAATATTCCCGACCTGGCAGC and its complementary strand). The reaction was then subjected to nondenaturing polyacrylamide gel electrophoresis and autoradiography.

**Characterization of the Heat Shock Response**—100-mm plates of logarithmically growing embryonic cells were sealed, immersed in a water bath (43 °C for 60 min) and allowed to recover at 37 °C for the indicated times before extraction of total RNA. Total RNA was subjected to Northern analysis, using the following cDNA probes: murine HSF1 and HSF2 (16), rat Hsp70i (31), human Hsc70, human Hsp27, murine Hsp60, and murine BiP (kindly provided by M. Gething). Sample loading was normalized by hybridization with a 24-mer oligonucleotide specific for murine 18 S rRNA. Recovery after heat shock was extended for six hours before cell extracts were extracted for protein analysis. Whole cell protein extracts were subjected to Western analysis with the following antibodies: anti-human Hsp70i (polyclonal, provided by R. Tanguay), anti-murine Hsc70 (monoclonal), anti-murine Hsp25 (monoclonal), anti-murine Hsp60, anti-murine GRP78 (anti-25, -60, and -78, StressGen Biotechnologies Corp.), anti-HSF1 (polyclonal), anti-HSF1 (monoclonal), and anti-HSF2 (polyclonal) antibodies were kindly provided by R. Morimoto of Northwestern University.

**Thermotolerance**—To induce thermotolerance, 25-cm² flasks of logarithmically growing embryonic cells were sealed, immersed in a water bath (43 °C for 60 min) and allowed to recover at 37 °C for 6 h. Lethal heat stress (LS) (45 °C for 40 min) was followed by recovery (37 °C for 40 h). Cell viability and the level of apoptosis was determined with annexin V and propidium iodide (Apoptosis Detection Kit, R&D Systems) on a FACscan (Becton-Dickinson). Cell survival rates of heat-shocked cell populations were calculated as a percentage of normal control. TUNEL assays were performed with an in situ cell detection kit (Boehringer Mannheim).

**Statistics**—Differences in group means were compared by Student’s t test. A p value, 0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

Targeted disruption of the HSF1 allele at the 5’-end was performed in 129Sv embryonic stem cells using homologous recombination (29). A 1.8-kb SphI/XbaI genomic probe, 3’ of the integrated targeting vector, hybridizes to a wild-type ~13.5-kb XbaI fragment and a recombinant 5.5-kb XbaI fragment. B, Southern blot analysis of XbaI-digested, genomic DNA (15 μg) isolated from HSF1 wild-type (+/+), HSF1 heterozygous (+/-), and HSF1 null (-/-) mice. Arrows indicate the ~13.5-kb wild-type band and the 5.5-kb recombinant band resulting from integration of the targeting vector at the HSF1 locus. C, Northern blot analysis of RNA isolated from HSF1 wild-type (+/+), HSF1 heterozygote (+/-), and HSF1 null (-/-) embryonic cells. Total RNA (20 μg) was probed for HSF1, HSF2, and 18 S rRNA. The positions of the wild-type and mutant transcripts relative to the RNA standards are as indicated.

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2 X. Xiao and I. J. Benjamin, unpublished results.
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Fig. 2. Analysis for the presence and activity of the HSF1 protein. A, Western blot analysis of whole cell extracts from cells subject to heat stress. Total cellular protein was extracted from control (C) and heat-shocked cells (43 °C for 60 min) (H). Detergent-soluble extracts (20 μg) were reduced, denatured in the presence of SDS, and probed with a polyclonal HSF1 antibody. The positions of HSF1 and the lower mobility phosphorylated (P) species seen upon heat shock are indicated (arrows). B, electrophoretic mobility shift assay. Protein samples from HSF1+/+ and HSF1−/− cells were subjected to the following treatments: lane 1, no cellular protein; lanes 2 and 8, control; lanes 3 and 9, heat shock; lanes 4 and 10, heat shock plus the addition of a 50-fold molar excess of unlabeled oligonucleotide probe in the binding reaction; lanes 5 and 11, heat shock + the addition of preimmune serum; lanes 6 and 12, heat shock plus the addition of anti-HSF1 antibody in the binding reaction; lanes 7 and 13, heat shock plus the addition of anti-HSF2 antibody in the binding reaction.

recent studies of the Drosophila hsf mutation on larval development (24). Further analysis of the effect of the null mutation on embryogenesis and development in animals will appear in detail elsewhere.3 The present report focuses on analysis of the effect of the hsf1 mutation in cultured primary embryonic fibroblasts derived from wild- type, heterozygote and null embryos at day 14.5 (E14.5) of gestation (Fig. 1B and see “Experimental Procedures”).

Northern blot analysis showed HSF1 mRNA expression was dependent on HSF1 gene dosage (Fig. 1C) as the heterozygous cells produced ~50% as much mRNA as wild-type cells. In addition, a truncated HSF1-specific transcript of ~1.8 kb, corresponding to the mutant allele, was detected in heterozygous cells and consequently was the only HSF1-specific transcript detected in null cells (Fig. 1C). Similar levels of HSF2 mRNA were expressed in all three genotypes (Fig. 1C), suggesting the HSF1 and HSF2 genes are independently regulated.

We used an anti-HSF1 polyclonal antibody to detect the HSF1 protein, which was expressed in a dose-dependent manner as was the mRNA, again with the level in heterozygous cells ~50% of the wild-type cells (Fig. 2). Consistent with previous results (32–34) HSF1 is hyperphosphorylated following heat stress in wild-type and heterozygous cells (Fig. 2A, lanes 2 and 4, respectively). No immunoreactive HSF1 protein of any molecular weight was detected in cell extracts from null cells (Fig. 2A, lanes 5 and 6), strongly suggesting that the truncated hsf1 mRNA is either nonfunctional or an expressed hsf1 mutant protein is below the limit of our detection.

We performed further biochemical studies on cells exposed to heat shock (43 °C for 60 min) to determine the effect of the mutation on the DNA binding activity of HSF1. As expected, electrophoretic mobility shift assay detected HSF1-specific DNA binding activities in wild-type (Fig. 2B, lanes 3 and 4) but not in null cells (Fig. 2B, lanes 8 and 9). The inability to detect any additional retarded bands in extracts from null cells suggested the truncated HSF1-specific transcript in these cells is functionally silent. As expected, mobility shift studies of extracts from heat-shocked cells incubated with an anti-HSF1 monoclonal antibody showed further retardation of the HSF1-specific protein band in the wild-type but not in the null cells (Fig. 2B, lanes 8–13). Mutations of hsf1 homolog in yeast and Drosophila that retained DNA binding activities were still incapable of transcription activation (24, 35). These data provide direct evidence that other members of the mammalian Hsf family (e.g. HSF2–4) are unable to compensate for the absence of HSF1 binding activity during heat shock, thereby confirming the specificity of the functional HSF1 protein for the HSE under physiological conditions (36, 37).

We next determined the effects of HSF1 inactivation on the basal and stress-inducible expression of representative series of HSPs that reside in separate intracellular compartments. Heat shock (43 °C for 60 min) substantially increased the strictly inducible Hsp70 transcript to equivalent levels in wild-type and heterozygous cells but not in null cells (Fig. 3A). Likewise, heat-induced expression of the cytosolic Hsc70, Hsp27, and mitochondrial Hsp60 was observed in both wild-type and heterozygous cells. No induction of Hsp70, Hsc70, Hsp60, and Hsp27 was observed in null cells similarly treated (Fig. 3A). As expected, expression of endoplasmic reticulum BiP/GRP78 was induced to equivalent degrees in all cell types after heat shock and recovery (compare Fig. 3A, lane 4, 8, and 12). This result is consistent with the lack of an HSE in the mammalian BiP promoter (38) and the distinct signaling and regulatory mechanisms of the unfolded protein response pathway (39), independent of HSF1. Additionally, this result illustrates the specificity of the HSF1 deletion, that only the primary heat shock response is affected and subsequent cellular responses, secondary to the initial heat stress, are left intact. Constitutive levels of Hsc70, Hsp60, BiP/GRP78, and Hsp27 mRNA (Fig. 3A, lanes 1, 5, and 9) and protein expression (Fig. 3B, lanes 1, 3, and 5) were unaltered in all cells, indicating that basal expression of these genes is independent of HSF1 expression. This is consistent with a mutation in Drosophila, effectively deleting the HSF protein in which constitutive HSP expression was similarly unaffected (24).

To determine the functional significance of HSF1 inactivation, we assessed the cellular survival of primary cells after heat stress. However, particular growth characteristics such as terminal differentiation and senescence of primary cells upon prolonged culture, as well as the inability to grow at high dilution, precluded the use of a clonogenic assay as a quantitative measurement of cell survival.4 To circumvent this problem, we quantitatively assessed cellular survival using flow cytometric analysis. All cell types were preconditioned with a sublethal heat challenge (43 °C for 30 min), and the cells were allowed to recover for 6 h before exposure to a subsequent lethal heat challenge (45 °C for 40 min). Exposure of unpre-
conditioned (naive) cells to severe heat stress showed similar survival rates regardless of genotype, suggesting a physiological role for unaltered constitutive HSP expression or other stress response pathways (Fig. 4A). However, thermal preconditioning of wild-type and heterozygous cells produced a significant level of thermotolerance that protected them from a subsequent thermal stress (40% survival compared with 90% survival in naive cells, p < 0.01). In contrast, naive and preconditioned null cells showed identical amounts of cell death upon lethal challenge, indicating that thermotolerance is independent of HSF1 gene dosage, but is unattainable in the absence of HSF1. The development of thermotolerance in cultured embryonic cells (Fig. 4A) was analyzed using FACS analysis of a total of five independent experiments. The percent viable cells of each population was calculated in comparison with the viable cells of the normal control for each experiment. Error bars represent 1 S.D. Differences in group means were compared by Student’s t test. A p value < 0.05 (*) was considered statistically significant. Symbols: ■, normal control (NC); □, PC; □, LS; ■, PC + LS (n = 5; p < 0.01). B, a typical log fluorescence intensity versus cell number dot plot of annexin V-fluorescein isothiocyanate and propidium iodide treated HSF1 (1/1) and HSF1 (1/2) cells. FACS analysis, gated for 10⁴ events, was performed on cells after the following treatments: top panels, normal control (NC); upper middle panels, preconditioning treatment (PC) 43 °C for 30 min; lower middle panels, lethal heat shock (LS) 45 °C for 40 min alone; bottom panels, preconditioning treatment plus lethal heat shock (PC + LS). The percentage of apoptotic cells was estimated from the upper and lower right-hand quadrants of each panel.
sence of functional HSF1 protein (Fig. 4A). This result confirms the conservation of eukaryotic HSF function for thermotolerance in yeast (28), Drosophila (24), and mammals. Moreover, this study provides the first direct evidence that members of the mammalian HSF family are functionally distinct.

Based on the results of flow cytometry with fluorescein-conjugated human annexin V and propidium iodide, we attributed the predominant route of cell death of null cells to be heat-induced apoptosis (>80%), (Fig. 4B). These findings were confirmed visually by confocal microscopy of a TUNEL assay. Propidium iodide was used for nuclear staining, while DNA strand breaks were detected by terminal deoxynucleotidyl transferase incorporation of biotin-labeled dCTP and decoration with streptavidin-labeled fluorescein isothiocyanate (Fig. 5).

Distinct regulatory mechanisms, which inhibit programmed cell death, or apoptosis, have been shown recently to increase thermotolerance-induced cell survival, although the interdependence of these pathways is less well understood (40). The proto-oncogene, c-myc, potentiates heat shock-induced apoptosis (41, 42); in contrast, Bcl-2 overexpression augments thermotolerance-induced cellular survival (40). In addition, expression of some HSPs such as the tissue-specific expression of Hsp70-2 prevents apoptosis in spermatocytes (43). Neither related members of the vertebrate HSF family nor putative redundant pathways can compensate and render cells thermotolerant to heat-induced apoptosis in cells lacking a functional HSF1 protein. Additionally, yeast Hsp104 has been shown to facilitate resolubilization of insoluble protein aggregates during stress (44), whether similar mechanisms of cytoprotection are shared by other members of the HSP family is presently unknown. This work provides a genetic model to examine potential interdependent relationships between stress-inducible expression of mammalian heat shock proteins and mechanisms involved in cell survival and/or cell death pathways.

While the Drosophila HSF and Mus musculus HSF1 proteins are dispensable for cell growth and viability, the Drosophila HSF protein is required for oogenesis and early larval development (24). This finding is of particular significance to ongoing work in our laboratory, with respect to fertility of the female null animals, as oocytes from these animals would be devoid of maternally derived HSF1 protein. Because the Drosophila HSF protein appears to serve nonidentical functions in larval development and oogenesis, it has been proposed that additional targets of HSF protein could be non-hsp genes (24). Ongoing studies with the hsf1 knockout animals will facilitate a clearer understanding of the pleiotropic functions of the mouse HSF1 protein in mammalian reproduction, development, and physiological adaptation. In addition, the functions of the mouse HSF2 protein in mammalian cell growth and differentiation can now be addressed in the absence of a “classical” heat shock response.

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