Reciprocal activation of HSF1 and HSF3 in brain and blood tissues: is redundancy developmentally related?

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The heat-shock response is characterized by induced synthesis of heat-shock proteins (HSPs), the accumulation of which confers cellular thermostolerance (15). The expression of HSPs is primarily regulated at the level of transcription by a family of heat-shock transcription factors (HSFs). Avian cells express two redundant heat-shock responsive factors, HSF1 and HSF3, which differ in their activation kinetics and threshold induction temperature. Unlike the ubiquitous activation of HSF1, the DNA-binding activity of HSF3 is restricted to undifferentiated avian cells and embryonic tissues. Herein, we report a reciprocal activation of HSF1 and HSF3 in vivo. Whereas HSF1 mediates transcriptional activity only in the brain upon severe heat shock, HSF3 is exclusively activated in blood cells upon light, moderate, and severe heat shock, promoting induction of heat-shock genes. Although not activated, HSF1 is expressed in blood cell nuclei in a granular appearance, suggesting regulation of genes other than heat-shock genes. Intrasppec comparison of heat-sensitive and heat-resistant fowl strains indicates that the unique activation pattern of HSF3 in blood tissue is a general phenomenon, not related to thermal history. Taken together, HSF1 and HSF3 mediate transcriptional activity of adult tissues and differentiated cells in a nonredundant manner. Instead, an exclusive, tissue-specific activation is observed, implying that redundancy may be developmentally related. The physiological and developmental implications are discussed.

Heat-shock transcription factor; heat-shock proteins; fowl; temperature regulation

MATERIALS AND METHODS

Real-time in vivo measurements of the heat-shock response. Two different groups of mature chickens, Leghorn and Bedouin (n = 3 in each group), which differ genetically in their resistance to heat, were examined in this setup. The setup was recently described in details (52). Briefly, 20 h before the experiment, a polyethylene (PE) cannula was inserted into the right jugular vein of each chicken (28, 43, 56). Most interestingly, disruption of HSF3 in HSF1-expressing chicken B-lymphoblast cells results in impaired heat-shock response and in loss of thermostolerance (57). In light of the above reported findings, we sought to gain insight into the relative contribution of HSF1 and HSF3 to the transcriptional induction of heat-shock genes in mature tissues and differentiated cells in vivo. For this purpose, we chose brain and blood of a mature hen, two unique tissues that are differentially confronted with thermal challenges. Our results show that in each tissue, only one transcription factor is activated upon heat stress. Whereas HSF1 mediates transcriptional activity in the brain, HSF3 is exclusively activated in blood cells upon moderate and severe heat shock to induce the expression of heat-shock genes.

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was implanted in the brachial vein of mature fowl under local anesthesia, and a 5-cm-long, custom-made PE cannula was implanted dorsal to the rectum and fastened to the skin. Experiments were carried out within a temperature-controlled room (±0.3°C; 12:12 light-dark cycle, lights on at 6:00 AM). The birds had free access to food and water and could freely move in their individual cages. Each experiment started between 8:00 and 9:00 AM at an ambient temperature (T_a) of 24°C (RH = 50 ± 5%), which is within the thermoneutral zone of chickens (5). A copper-constantan thermocouple was introduced into the rectal cannula, located at a predetermined depth of 5 cm and connected to a digital thermometer (±0.1°C). All temperature probes were calibrated against a mercury-in-glass thermometer having accuracy (0.1°C) traceable to the U.S. Bureau of Standards. After ~10 min, body temperature (T_b) stabilized around 41°C (the normal resting T_b for this species), after which blood was remotely sampled through extended tubing. T_a was then elevated to 38°C (RH = 50 ± 5%). Body temperature was followed continuously, and blood samples were taken at each 1°C decrease in T_b down to 41°C. These samples were analyzed for HSF DNA-binding activity, transcriptional and translational activity, and HSF translocation. After each blood sample the cannula was flushed with heparinized saline.

**Protein extraction.** For the purpose of protein extraction, brain tissues were sampled from T_b = 45°C heat-shocked mature hens. The dissected tissues were homogenized in ice-cold buffer, containing 0.1 M NaCl, 20 mM Tris·HCl (pH 7.4), 0.2 mM EDTA, 20% glycerol (vol/vol), 0.5 mM DTT, 15 µg/ml leupeptin, 1 mM PMSF. Samples were centrifuged for 30 min (4°C, 12,000 rpm), and supernatants were collected, frozen in liquid nitrogen, and stored at −70°C. To extract proteins from blood cells, blood samples were washed once with ice-cold PBS and centrifuged for 2 min (4°C, 1,500 rpm). The cells were resuspended in ice-cold TMP buffer [containing 10 mM Tris·HCl (pH 7.4), 1 mM EDTA, 5 mM MgCl_2, 0.5 mM DTT, 15 µg/ml leupeptin, 1 mM PMSF], and were frozen (liquid nitrogen) and thawed (37°C) in four cycles. Samples were centrifuged for 30 min (4°C, 12,000 rpm), and supernatants were collected, frozen in liquid nitrogen, and stored at −70°C. Blood cell nuclear proteins were extracted as previously described by Dyer and Herzog (13).

**SDS PAGE and Western blot analysis.** Blood cell nuclear proteins lysates were boiled in sample application buffer containing 2-mercaptoethanol. Equal amounts of proteins (in each lane) were loaded and separated by SDS-polyacrylamide gel (10%), and transferred onto nitrocellulose membrane (Schleicher&Suell). The membranes were probed with polyclonal anti-HSF1 B (a generous gift from Professor Akira Nakai), followed by appropriate secondary antibodies. The proteins were visualized by enhanced chemiluminescence. Quantification of HSF1 expression was carried out by a densitometry software (TINA).

**EMSA.** Electromobility shift assay was performed as previously described (38). Briefly, equal amounts of cellular proteins (20 µg for brain extraction and 5 µg for blood nuclei proteins) were incubated with a 32P-labeled double-stranded oligonucleotide (5'-CTA-GAAGCTTCTAGAAGCTTCTAG-3'). The protein-bound and free oligonucleotides were electrophoretically separated by 4% native polyacrylamide gels. The gels were dried and autoradiographed.

**Supershift experiments.** The antibody supershift experiments were performed by incubating 2 µl of 1:10 diluted antisera (unless otherwise stated in the figure legend) of anti HSF1γ and anti HSF3 γ (a kind gift from Professor Akira Nakai) in PBS, with 1–2 µl of cell lysates in a total volume of 10 µl. These antibodies recognize the carboxyl-terminal region of each HSF. After incubation on ice for 20 min, a nucleotide probe mixture containing 0.1 ng of 32P-labeled self-complementary ideal heat shock element oligonucleotides and 0.5 µg of poly(dl-dC) (Pharmacia Fine Chemicals) in 10 mM Tris·HCl (pH 7.8) and 1 mM EDTA was added to a final volume of 25 µl. The binding reactions were incubated on ice for 20 min, mixed with 2 µl of a dye solution (0.2% bromphenol blue, 0.2% xylene cyanol, and 50% glycerol), and then separated on 4% native polyacrylamide gels. Gels were run in 0.5×TBE buffer at room temperature for 1.7 h at 140 V, dried, and exposed to X-ray film at −80°C with intensifying screens.

**RNA isolation and Northern blot analysis.** Total RNA was isolated from blood cells by TRI REAGENT-BD (MRC) according to the manufacturer’s instructions. RNA (5 µg) was separated in formaldehyde-agarose gel and transferred onto nylon membrane (Zeta-Probe, Bio-Rad). Chicken HSP90α cDNA (a kind gift from Professor Akira Nakai) was labeled by the extension priming method using [α-32P]dATP. Membrane was hybridized for 16 h at 55°C, washed with 0.1% SDS in 1× SSC at 45°C, 50°C, and 55°C, and exposed to X-ray film (Kodak BioMax MS) at −70°C in the presence of intensifying screen.

**Immunocytochemistry.** Blood samples from control and T_b = 45°C heat-shocked mature hens were prepared as smears. The smears were air dried and fixed with 4% paraformaldehyde (in PBS). After blocking with 10% normal goat serum in PBS, cells were incubated with 1:500 diluted polyclonal antiHSF1 (a kind gift from Professor Akira Nakai). The HSF1 signal was detected by a secondary goat anti-rabbit antibody conjugated to FITC. Confocal reconstructions were made using an MRC-1024, laser confocal scanning microscope (Bio-Rad) with the objective Nikon Plan Apo 603/1.40. DNA was counterstained with 250 ng/ml propidium iodide.

**RESULTS**

**HSF3 is exclusively activated in chicken blood tissue.** We have recently described the in vivo, real-time heat-shock response of fowl from strains that differ in their resistance to heat. Our data revealed a differential DNA-binding kinetics of total HSF in each of the groups that was followed by transcriptional and translational induction of heat-shock genes (52). To elucidate the redundancy in the activation of both, HSF1 and HSF3, in response to elevated temperature, the blood of a mature hen was sampled, at 1°C increments of T_b. We determined the relative levels of HSF1 and HSF3 DNA-binding activity by supershift experiments. At T_b = 45°C, HSF3-bound DNA was entirely supershifted by each antisera concentration, whereas the DNA-binding activity of HSF1 was undetected (Fig. 1A). It has been previously reported that HSF3 is the dominant heat-shock transcription mediator during severe heat-shock conditions. It was thus reasonable to assume that the activation of HSF1 may be abolished at T_b = 45°C, but it is likely to occur at lower body temperatures. To check the activation pattern of both HSFs at moderate heat-shock conditions, we performed supershift experiments of blood sampled at T_b = 43°C, a well-documented temperature for the activation of the ubiquitous factor HSF1. In this experiment, we used the same HSF1 antisera concentrations, but 10-fold more diluted concentrations of HSF3 antisera, to allow the examination of the anti-HSF3 antibody specificity. As seen in Fig. 1B, HSF3 was the exclusive DNA-bound HSF, also upon moderate heat-shock conditions.

**HSF1 and HSF3 DNA-binding states are not affected by intraspecific variations in thermal resistance.** Our previous comparisons of the Leghorn chicken with the desert-origin, genetically heat-resistant Bedouin fowl identified various thermoregulatory mechanisms that contribute to the superiority of the Bedouin fowl with respect to heat resistance (2, 4, 32). This superiority was accompanied by a considerably delayed HSP response, both in relation to time scale and to T_b level (52). In light of the above-mentioned observations, it was intriguing to...
examine the DNA-binding state of both HSFs in heat-sensitive and heat-resistant strains of the same species. Consistent with the findings shown in Fig. 1, HSF3 was solely in a DNA-binding form in both fowl strains, and along the entire range of heat-shock conditions (Tb = 42°C to Tb = 45°C), without any redundant acquisition of HSF1 DNA-binding activity in response to heat shock. However, the amount of expressed HSF1 was not uniform in all samples; its concentration tended to decrease around the peak of heat shock (Tb = 45°C; Tb = 44°C, at recovery). These values were 2 and 3 fold lower, respectively, than the control values) and tended to increase during recovery from heat shock, while Tb was decreasing (Fig. 3A). Despite its expression in blood cell nuclei in nonstress conditions (Fig. 3A), immunohistochemical preparations from control and from heat-shocked cells (Tb = 44°C) indicate that HSF1 translocates from a cytosolic distribution in control to a nuclear granular distribution upon heat shock (Fig. 3B).

Activated HSF1 exclusively mediated transcriptional activity of HSPs in chicken brain tissue. Is the exclusive HSF3 DNA-binding pattern unique to the blood tissue, or may it reflect a general in vivo phenomenon in adult fowl? To address this issue, we conducted supershift experiments in brain extracts from heat-shocked (Tb = 45°C) mature hen, using polyclonal anti-HSF1 and anti-HSF3 antibodies. A reciprocal mode of HSF activation to that of the blood tissue was revealed in the brain, indicating that HSF1 was exclusively in a DNA-binding state (Fig. 4A).

To elucidate whether the DNA-binding state of HSF1 in the brain induces the activation of heat-shock genes, we performed
a Northern blot analysis with the entire chicken HSP90a cDNA as a probe. A burst induction of HSP90 was observed in response to Tb = 45°C heat shock, implying that HSF1 is capable of mediating the transcriptional heat-shock response in the brain in a non-HSF3 redundant manner (Fig. 4B).

DISCUSSION

Stress-induced transcription of heat-shock genes in avian cells is mediated by HSF1 and HSF3 (39). The coexpression and coactivation of these factors have led to the discovery of the HSF functional redundancy (37). Indeed, when undifferentiated avian cells and avian embryonic tissues are subjected to thermal challenges, HSF1 and HSF3 are activated with different kinetic properties and threshold temperatures, to induce transcription of heat-shock genes (28, 43, 56). Surprisingly, as demonstrated in the present study, circulating blood cells lose their functional redundancy and activate only HSF3, also upon light and moderate heat shocks. These results are of developmental and physiological significance, since they imply that during maturation of erythroblasts, HSF1 activity is degenerated. As maturating erythroblasts go through morphological, physiological, and biochemical changes (26), loss of redundancy due to diminished expression of HSF1 may be a reasonable explanation. Supported by the nuclear expression pattern of HSF1 in control and heat-shocked blood cells, it is more likely that this degenerative event has occurred along the pathway that regulates DNA binding activity. Transcriptional induction of heat-shock genes may be a multiple-step process. Under normal conditions of cell growth, the non-DNA binding state of HSF1 is regulated by two distinct mechanisms, involving both negative regulatory domains and constitutive phosphorylation at serine residues (16, 29 –30, 53). To induce transcriptional activity of heat-shock genes, HSF1 must acquire DNA binding activity, preceded by oligomerization and nuclear localization (48). However, the trimer state of HSF1 does not assure binding interactions with the DNA, as interactions of the HSF1 oligomerization domain with regulatory
proteins may negatively affect HSF1 DNA-binding activity (50). Consistent with the findings that DNA binding and transcriptional activities are uncoupled processes (27, 31), HSF1 must be inducibly phosphorylated to promote transcription of heat-shock genes (11, 18).

In our current study, wing blood cell immunohistochemistry shows that apart from its nuclear expression in control and heat-shock conditions, HSF1 translocates from a cytosolic distribution at control conditions to a nuclear granular distribution at \( T_b = 44^\circ C \). Nuclear stress granules have been described as intranuclear sites at which HSF1 is concentrating in response to stress conditions (48). The kinetics of appearance of HSF1 granules parallels the activation of HSF1 and the transient transcriptional activity of heat-shock genes (12). It has been suggested that these nuclear foci are locations at which HSF1 is stored and regulated in its different activation states (22), thereby coordinating the regulation of heat-shock gene expression (23). As HSF1 stress granules are also present in heat-shocked mitotic cells that are devoid of transcription, it is alternatively hypothesized that they are involved in chromatin organization and thus have a structural role in protecting hypersensitive or fragile sites of the genome (23–25). The expression levels of HSF1 in the present study tend to decrease as \( T_b \) reaches its experimental peak and return to control levels upon recovery. This observation is in accordance with a previous study (56), which reported in vitro the movement of HSF1 to the insoluble fraction at high ambient temperature.

Nearly four decades ago, the unique thermoregulatory capacities of the desert-origin Bedouin fowl were described (1–2, 4–8, 32–33). Recently, we have monitored in real-time in vivo the heat-shock response of this heat-resistant fowl strain and showed that its heat resistance superiority was characterized by a considerable delayed activation of HSF, compared with a heat-sensitive, commercial strain (52). In the present study, we demonstrate that HSF3 is solely in a DNA-binding form in both fowl strains and along the entire range of heat-shock conditions and that no redundant activity of HSF1 is taking place. This intraspecific comparison of two evolutionary distinct strains, with respect to thermoregulatory strategies, suggests that the unique activation pattern of HSF3 in the blood tissue is a general phenomenon, unrelated to thermal history. Together with the above mentioned findings, these results imply that loss of HSF1 DNA-binding activity is not caused by decreased levels of HSF1 message, nor because HSF1 becomes insoluble.

In chicken embryonic tissues, including red blood cells and brain tissue, HSF3 was the dominant heat-shock responsive factor in certain developmental stages, whereas in other stages HSF1 and HSF3 showed functional redundancy (28). As opposed to the blood, in which paralleled activation of HSF3 in embryos (28) and adult chicken (present study) is sustained, the present findings in the brain tissue revealed an unexpected loss of HSF3 activation and the introduction of HSF1 as an exclusive heat-shock responsive factor. Chicken HSF3 remains as an inactive dimer, exclusively in the cytoplasm of normally growing cells and is translocated into the nucleus and converted to an active trimer upon heat stress. Its DNA-binding properties, however, are regulated in a cell-specific manner (43). As HSF3 activation in embryonic brain tissue is evident (28) and as HSF3 is expressed in adult chicken brain tissue (present study, data not shown), we believe that the unique regulation of avian brain temperature could provide a reasonable physiological explanation for the non-DNA-binding state of HSF3 in this tissue. Equipped with a unique heat-exchange vasculature, the rete ophthalmicum (35), the avian brain temperature is maintained around 1°C below body temperature in a wide range of ambient temperatures [see review by Arad (3)] and hydration conditions (8, 21, 36). In the context of the present study, this means that brain temperature probably did not exceed 44°C during heat shock. Thus, although HSF3 has been previously shown to be activated at 44°C (56), we cannot exclude that the brain was not confronted with temperatures above the threshold for HSF3 activation. It may alternatively be claimed that as the brain is protected from reaching such high temperatures, it has lost part of its temperature-induced DNA-binding activity to promote transcription of heat-shock genes, during ontogeny. Whereas chicken body temperature that exceeds 45°C is considered harmful and nonreversible, this issue must be solved by a method of acclimation that would allow us to reach a body temperature of 46°C.

In mammals, where HSF1 is believed to be the only factor that mediates heat-shock signals (59), its disruption may not affect adulthood attainment but may result in multiple defective phenotypes, including “maternal effect” mutation (10, 60). Moreover, mammalian HSF1-deficient cells are incapable of stress-induced transcription of heat-shock genes and of acquisition of thermotolerance (34). Compared with the mammalian HSF1, avian HSF1 has little potency to activate heat-shock genes in cells subjected to heat stress (19). It is becoming clearer, however, that similar to the mammalian HSF1, avian HSF1 also possesses thermal-protective traits, independent of heat-shock genes induction (19–20, 41). Also similar to HSF1 in mammalian cells, HSF3 is essential for the heat-shock response in avian cells, and its disruption markedly decreases the induction of heat-shock genes (57). This is further emphasized during chicken embryonic development, where HSF3, which has nearly identical levels among most examined tissues, is the major heat-shock responsive factor (28). These data are in accordance with the “blood picture” but fail to explain the “brain picture” in the present study. The physiological nature of both tissues in respect to thermoregulation may shed light on the reciprocal activation pattern of both HSFs. In marked difference from the brain that “escapes” the actual body temperature [reviewed by Arad (3)], the blood tissue, among its other roles, is responsible for heat transfer from the body core to the surface, and, as such, it is routinely confronted with temperature changes and should have the ability to respond to a broad range of temperatures. This function is shown here to be entirely fulfilled by HSF3. In addition to the biochemical characteristics of HSF1 and HSF3 in regard to the heat-shock response (56), and as HSF3 lacks (so far, as we know) the characteristics of nonstress responsive genes regulator, these physiological challenges make HSF3 a reasonable candidate for taking over the transcriptional mediation of heat-shock genes.

Indeed, HSF3 might be sufficient to activate transcription in chicken blood tissue, as red blood cells of chicken embryos reveal a heat-shock response in the absence of HSF1 (28). As to the functional redundancy observed in nondifferentiated cells such as erythroblasts, these cells are progenitors to circulating cells and are considered as the bank of the differentiated cells. It is thus logical to have them protected from
Physiological and environmental damage by a redundant system. In the brain, loss of HSFs’ functional redundancy during ontogeny may be explained by the different thermal states of the embryo and the mature chicken. The embryo, which is considered ectothermic, undergoes a transition from ectothermy to endothermy, which is completed, at the earliest, by hatching (17). While ectotherms are prone to changes in body temperature in correlation with ambient temperature variations, the internal physiological milieu of endotherms remains relatively stable, despite acute external thermal fluctuations. We have recently shown that embryos are the most heat-sensitive stage in the chicken’s life cycle and are tightly dependent on HSP synthesis for survival. On the other hand, mature hens master additional mechanisms for heat dissipation and T\textsubscript{b} regulation during ontogeny (52). In light of the above discussion, we suggest that functional redundancy of HSFs in the brain is required in the developmental state that is most susceptible to heat stress (i.e., embryos), but may be lost as the individual is capable of maintaining homeostasis (i.e., adult).

It has been previously shown that the temperature set point for HSF activation varies among tissues of a single organism (49). Herein, we present data that may confirm also the existence of a tissue-specific activated HSF.

Taken together, the functional HSFs’ redundancy, previously described in avian embryonic tissues and in undifferentiated cells, may be lost during ontogeny. As a result, an exclusive reciprocal activation of HSF1 and HSF3 is observed in vivo in the brain and in blood tissues, respectively. HSFs’ functional redundancy may be a mechanism unique to undifferentiated embryonic cells and is lost due to physiological alterations that accompany maturation.

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