Xenopus Heat Shock Factor 1 Is a Nuclear Protein before Heat Stress

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Philippe A. Mercier, Jaroslav Fokса, Nick Ovsenek‡, and J. Timothy Westwood§

From the Department of Zoology, Erindale College, University of Toronto, Mississauga, Ontario L5L 1C6 and the Department of Anatomy and Cell Biology, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E5, Canada

Stress-induced expression of the heat shock (hs) genes in eukaryotes is mediated by a transcription factor known as heat shock factor 1 (HSF1). HSF1 is present in a latent, monomeric form in unstressed metazoan cells and upon exposure to heat or other forms of stress is converted to an “active” trimeric form, which binds the promoters of hs genes and induces their transcription. The conversion of HSF1 to its active form is hypothesized to be a multistep process involving (i) oligomerization of HSF1, plus (ii) additional changes in its physical conformation, (iii) changes in its phosphorylation state, and for some species (iv) translocation from the cytoplasm to the nucleus. Oligomerization of HSF1 appears to be essential for high affinity DNA binding, but it remains unclear whether the other steps occur in all organisms or what their mechanistic roles are. In this study we have examined if heat-induced cytoplasmic-nuclear translocation of HSF1 occurs in Xenopus oocytes. We observed that germinal vesicles (nuclei) that were physically dissected from unshocked Xenopus laevis oocytes contain no HSF1 binding activity. Interestingly, in vitro heat shock treatments of isolated nuclei from unshocked oocytes activated HSF1 binding, indicating that HSF1 must have been present in the unshocked nuclei prior to isolation. Induction of HSF1 binding was not observed in enucleated oocytes. Western blot analysis using an affinity-purified polyclonal antibody made against X. laevis HSF1 showed that HSF1 is present in equal amounts in unshocked and shocked oocytes and isolated nuclei. HSF1 was not detected in enucleated oocytes. These results clearly demonstrate that HSF1 is a nuclear protein in oocytes prior to exposure to stress. In Xenopus oocytes, therefore, HSF1 translocation from the cytoplasm to the nucleus is not part of the multistep process of HSF1 activation. These results also imply that the signals and/or factors involved in HSF1 activation must have their effect in the nuclear compartment.

All organisms respond to elevated temperatures and other forms of “stress” such as inhibitors of oxidative respiration, sulphydryl reagents, certain heavy metals, and the generation of abnormal proteins or oxygen radicals within cells by inducing the transcription of a family of genes known as the heat shock (hs) genes. The products of these genes, the hs proteins (hsp) help cells recover from the effects of the stress and protect it from further trauma (1, 2). In addition, hsp are also present in unstressed cells. The hsp act as protein chaperones or molecular “detergents” during protein synthesis, degradaton, and translocation across membranes during normal and stress conditions (for reviews, see Refs. 3–5).

Transcriptional regulation of the hs genes in eukaryotes is mediated by a preexisting transcription factor known as hs factor (HSF) as well as a DNA element found in the promoters of hs genes known as the hs element (HSE) (for reviews, see Refs. 6 and 7). Different species possess different numbers of HSF genes. Yeast (Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Kluyveromyces lactis) and Drosophila melanogaster appear to have a single HSF gene, whereas most vertebrates and higher plants studied appear to have multiple HSF genes (7). Species with multiple HSFs may not express all genes in each cell type (8), but all cells appear to express the stress-inducible form of HSF, which is generally known as HSF1. The other HSFs are likely involved in responding to other stimuli. For example, HSF2, an HSF found in humans, mice, and chickens, does not appear to be activated by the same stress agents that HSF1 responds to, but is found to be active during embryogenesis, spermatogenesis, and erythroid differentiation (9–11).

Upon exposure to stress, HSF1 undergoes a number of physical changes that are thought to be associated with its activation. In metazoans, HSF1 exists predominantly as an apparent monomer in unshocked cells that is converted to a homotrimer upon exposure to stress (12–15), and it is the trimeric form of HSF which is capable of binding to HSEs with high affinity. In the yeasts S. cerevisiae and K. lactis, HSF1 is trimeric and bound to high affinity HSEs prior to exposure to stress (16) and additional HSEs are occupied upon exposure to stress (17). It has been proposed that stress may cause other physical changes in HSF1 such that certain domains involved in the transcriptional activation of hs genes move from a “buried” to an “exposed” location (7, 18). Hyperphosphorylation of HSF1 has also been associated with its activation. Heat shock results in the hyperphosphorylation of yeast (19), mammalian (15, 20, 21), and fly HSF1 (22). The exact role of HSF1 hyperphosphorylation is still not completely known, but it is not required for activation of DNA binding in vivo (21, 22). In K. lactis, HSF1 hyperphosphorylation appears to play a role in the deactivation of HSF1 (23).

It has been hypothesized that HSF1 activity is regulated in

§ To whom correspondence should be addressed: Dept. of Zoology, Erindale College, University of Toronto, 3359 Mississauga Rd., Mississauga, Ontario L5L 1C6, Canada. Tel.: 905-828-3894; Fax: 905-828-3792; E-mail: twestwoo@credit.erin.utoronto.ca.

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part by its subcellular localization (14, 15). However, there are conflicting reports on localization of HSF1 in metazoans prior to stress. In Drosophila, HSF1 is reported to be a nuclear protein before and after heat stress (12, 24, 25). In mammalian cells, HSF1 has been reported to always be a nuclear protein by some investigators (24, 26), while others have provided evidence that HSF1 is predominantly cytoplasmic prior to stress (14, 15). To help clarify the localization of HSF1 in higher eukaryotes, we have examined the localization of HSF1 in X. laevis oocytes. Using several assays we show that Xenopus HSF1 is a nuclear protein prior to and following exposure to heat stress. This result has important implications in identifying the signaling mechanisms by which heat and other inducers activate HSF1 in vivo.

EXPERIMENTAL PROCEDURES

Tissue Culture

X. laevis A6 cells were grown in Leibovitz's L-15 medium (Life Technologies, Inc.) 2.5 l or more to 10 mM HEPEs, pH 7.6; 5% fetal clone II (HyClone), 5% Cool Calf 2 (Sigma), 20 μg/ml gentamycin (Sigma). Cells were grown at 21 °C in T-75 tissue culture flasks (Starstedt). Heat-shocked cells were prepared by immersion of T-flasks in a circulating water bath at the temperatures indicated in the figure legends.

Oocytes and Germinal Vesicles

Pieces of ovary were surgically removed from mature X. laevis frogs (a gift of Y. Masui, Department of Zoology, University of Toronto, and from Xenopus 1) and the connective tissue was digested with 0.2% collagenase type IV (Sigma) in calcium-free OR-2 (27) or in Steinberg's buffer (28). Stage VI oocytes were selected and were stored in either OR-2 with 1 mM oxaloacetate (29) or Steinberg's buffer. Germinal vesicles (GVs) were prepared either under oil as described by Paine et al. (30) or under Steinberg's buffer. For heat shock experiments, oocytes or germinal vesicles were transferred to 1.5- or 0.5-ml microcentrifuge tubes in their storage solution and then immersed in a circulating water bath at the temperatures indicated in the figure legends. Control samples were handled in the same fashion but kept at room temperature (18–21 °C).

Preparation of Extracts

A6 Cells—Cells were scraped into the medium and pelleted. For whole cell extracts to be used in Western blot analyses, the medium was removed, the pellets resuspended in three volumes of 1 × SDS sample buffer (31) and then sonicated for 10 s at 7 °C with a Heat Systems Ultrasonic sonicator. For EMSA extracts, the medium was removed and the pellets frozen in liquid nitrogen. Pellets were thawed and resuspended in 5 volumes extraction buffer (50 mM Tris–Cl, pH 7.9, 5 mM glycerol, 50 mM KCl, 0.1 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride).

Oocytes and Germinal Vesicles—Oocyte and GV extracts were prepared for Western blot and EMSA as outlined by Karn et al. (32).

EMSA

HSF1 DNA binding activity was analyzed by electrophoretic mobility shift assays. For experiments described in Figs. 1 (A and B) and 2B, binding reactions contained 10 μg of A6 protein extract (as determined by Bradford protein assay; Bio-Rad) or 0.5 of an oocyte or GV, 1 μl of 10 × buffer mix (100 mM HEPEs, pH 7.9, 30% glycerol, w/v) and 1 μl of 10 × bovine serum albumin/nucleotide mix (20 mg/ml bovine serum albumin fraction V, 0.5 μg/ml Escherichia coli DNA, 2 mg/ml RNA, 0.2 mg/ml poly(dN)6, 0.5 μg/ml poly(dI-dC)/poly(dI-dC), 0.5 μM dithiothreitol, and 5% glycerol in a final volume of 20 μl). Reactions were incubated on ice for 20 min, and immediately loaded onto 5% non-denaturing polyacrylamide gels containing 6.7 mM Tris–Cl (pH 7.5), 1 mM EDTA, 3.3 mM sodium acetate. Gels were electrophoresed for 2.5 h at 150 V, dried, and exposed to x-ray film.

Production of Anti-Xenopus HSF1 Antibodies

A HindIII/HindIII fragment from the X. laevis (XI) HSF1 gene in pET3d (a generous gift from A. Wolfe and D. Stump, Laboratory of Molecular Endocrinology, NICHD, NIH, Bethesda, MD; Ref. 34) containing 1036 base pairs of the 3′ terminus of XHSF1 was cloned into the XmnI/HindIII sites of pMal-c2 (New England Biolabs) and transformed into E. coli TB1. E. coli containing either MBP or MBP + XHSF1 constructs were induced to op across, and induced proteins were purified essentially as per manufacturer's instructions (New England Biolabs), and antibodies were produced to MBP + XHSF1 in rabbits by an initial injection of 1.5 mg of purified antigen mixed 1:1 in complete Freund's adjuvant followed by 5 boosts every 3 weeks with 0.75 mg of antigen mixed 1:1 in incomplete Freund's adjuvant. Anti-XHSF1 was affinity-purified following a procedure described by Harlow and Lane (35). Briefly, crude antiserum was passed over Affi-Gel 10 beads (Bio-Rad) coupled to MBP until all the anti-MBP antibodies had been removed (as tested by Western blot analysis). The anti-MBP-depleted serum was passed over an Affi-Gel column coupled with MBP + XHSF1. Bound antibodies were first eluted with low pH buffer followed by high pH buffer (35). The antibodies eluted with low pH buffer contained anti-XHSF1 activity (as tested by EMSA and Western blot analysis) and were used in the subsequent experiments.

Western Blot Analysis

Proteins fractionated by SDS-polyacrylamide gel electrophoresis (8% acrylamide gels) were electroblotted onto nitrocellulose and the blots were blocked with 5% powdered milk in TBST (20 mM Tris-Cl, pH 7.6, 137 mM NaCl, 0.1% Tween 20). Antibodies were diluted in 2% gelatin (Bio-Rad) in TBST (1:1000 for crude anti-MBP-XHSF1, 1:1000 for preimmune, 1:50 for affinity-purified anti-XHSF1, and blots were incubated in the primary antibody for 1 h at room temperature. After being washed with TBST, blots were incubated with secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad)) diluted in 2% gelatin in TBST (1:2000 dilution) for 45 min at room temperature. Blots were then washed in TBST and developed for alkaline phosphatase activity using 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt/nitro blue tetrazolium chloride reagents as per manufacturer's instructions (Life Technologies, Inc.).

Electroelution of HSF-HSE Complexes

The HSF-HSE complex was cut out of an EMSA gel and electroeluted in volatile buffer (50 mM ammonium bicarbonate, 0.1% sodium dodecyl sulfate) using the Bio-Rad model 422 Electro-Eluter. Electroelution was run at 10 mA/sample for 6 h.

RESULTS AND DISCUSSION

The induction of HSF1 in higher eukaryotes to a form that can activate hs gene transcription is thought to be a multistep process involving oligomerization, hyperphosphorylation, and other physical changes of HSF1. Some studies in mammalian cells have provided evidence that HSF1 activation is also accompanied by its translocation from the cytoplasm to the nucleus suggesting that subcellular location could play an important role in the regulation of HSF1 activity (14, 15). However, other studies have shown that mammalian HSF1 is always a nuclear protein (24, 26). To further investigate the question of HSF1 localization, we have used another vertebrate, X. laevis. We have taken advantage of a specialized cell, the oocyte. Mature Xenopus oocytes are unusual in that they are extremely large cells (approximately 1–1.2 mm in diameter) and their nuclei, also known as germinal vesicles, can be physically dis-
shocks producing lower levels of HSE-HSF1 complexes (Fig. 1A). This optimal induction temperature is the same as previously reported for whole oocytes (28). This result clearly demonstrates that HSF1 must have been present in the germinal vesicle prior to heat stress and that HSF1 was induced to maximum binding activity with an in vitro heat shock in the absence of cytoplasm. In vitro activation of HSF1 binding activity has previously been shown to occur in extracts made from unshocked human (36) and Drosophila cells (12, 37) extracts, but generally not with the same efficiency as an in vivo heat shock. When enucleated unshocked oocytes were examined for HSF1 binding activity before and after an in vitro heat shock, none was observed (Fig. 1, B and C), suggesting that the cytoplasmic compartment may not contain HSF1. To determine whether the cytoplasm might contain factor(s) that facilitate the activation/deactivation of HSF1, we added cytoplasmic extract to isolated isolated germinal vesicles and heat-shocked them together in vitro. This combination did not produce any more activated HSF1 than when isolated germinal vesicles were heat-shocked in the absence of cytoplasm (Fig. 1C).

To further examine the localization of HSF1, we made antibodies against a maltose-binding protein-X. laevis HSF1 (MBP-XlHSF1) fusion protein in rabbits (Fig. 2A). Crude and affinity-purified anti-XlHSF1 antisera were added to heat-shocked oocyte and Xenopus A6 cell extracts to determine if they could further retard (i.e. “supershift”) the HSF1-HSE complex in an EMSA assay. EMSA analysis of heat-shocked A6 cells revealed two distinguishable HSF-HSE complexes, whereas heat-shocked oocytes produced a single complex (Fig. 2B). Both the crude and affinity-purified anti-XlHSF1 antibodies supershifted the HSF-HSE complexes, whereas the preimmune sera had no effect on these complexes (Fig. 2B). Unshocked or in vitro heat-shocked germinal vesicles (nuclei) were examined for HSF1 content by Western blot analysis, and a strong cross-reactive band was seen at 78 kDa (Fig. 3A). This band was also observed in unshocked and heat-shocked whole oocytes but not in the cytoplasmic compartment of unshocked or heat-shocked enucleated oocytes (Fig. 3A). This result demonstrates that there is no, or virtually no, HSF1 in the cytoplasm prior to heat shock. Because a few other cross-reactive bands were detected in the oocyte extracts, we decided to further verify that the 78-kDa band was HSF1. HSF1-HSE complexes were excised from EMSA gels, the protein(s) eluted, and subjected to Western blot analysis using the anti-XlHSF1 antibodies. A band of 78 kDa was detected in proteins isolated from oocyte HSF-HSE complexes (Fig. 3B). Although these sizes are all higher than the predicted size of XlHSF1 based on amino acid sequence (49.44 kDa) (34), similar discrepancies have also been shown for Drosophila (38) and human HSF1 (39) and this discrepancy is likely due to the highly asymmetric shape of HSF (13). The 67-kDa HSF1 band we observe is the same size as reported for XIHSF1 translated from in vitro synthesized RNA in reticulocyte lysates or oocytes (34, 40). All of the putative HSF1 bands we observe are lower than a size estimation of 88 kDa made from cross-linked XIHSF1/bromodeoxyuridine-labeled HSE complexes (28). It appears possible that multiple isoforms or separate stress-inducible HSF exist in Xenopus A6 cells. Another possibility is that lower molecular weight bands represent stable degradation products of HSF1. Multiple HSF bands could arise from post-translational modification of a common HSF1 protein, differential splicing of HSF1 message, or the products of separate HSF genes. All three of these

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Figure 2. Production of antibodies that specifically recognize XlHSF1. A, E. coli containing the MBP-XlHSF1 fusion construct described under “Experimental Procedures” were induced to overexpress MBP-XlHSF1 by the addition of isopropyl-1-thio-β-D-galactopyranoside (induction, 0 h and 2 h). After 2 h, the bacteria were isolated and a protein extract was made (soluble extract). This extract was passed over amylose resin twice, and the flow-through was analyzed for the presence of MBP-XlHSF1 (first and second flow through). The eluate was used for antibody production. B, extracts prepared from either control (21 °C) or heat-shocked Xenopus oocytes or A6 cells were subject to electrophoretic mobility shift analysis in the presence of preimmune sera (1:500 dilution), crude anti-MBP-XlHSF1 serum (1:500 dilution), or affinity-purified anti-MBP-XlHSF1 (1:25 dilution). The XlHSF1-antibody complex is labeled XlHSF1 (1:2000), the XlHSF1 antibody complex is labeled HSF + ab, the HSE-HSF1 complex is labeled HSF, and unbound HSE oligonucleotide probe is labeled free probe.

possibilities have been observed in other organisms (20, 41, 42). Further studies will need to be done to clarify the number of stress-inducible HSFs and/or the nature of different HSF1 isoforms.

The Western blotting experiments show that Xenopus HSF1 can be detected in oocytes and A6 cells using a polyclonal antibody made against a fusion protein containing the C-terminal portion of XlHSF1. Previous studies using antibodies against human HSF1 were unsuccessful in detecting the endogenous XlHSF1 in oocyte (34, 40) or Xenopus A6 cell extracts.4

The results presented here clearly show that X. laevis HSF1 is predominantly and perhaps even exclusively, a nuclear protein in mature oocytes before heat shock. Moreover, since inactive HSF1 is found in the nuclear compartment, we conclude nuclear localization is not dependent on post-translational modifications of HSF1 associated with its stress activation. Our results are consistent with previous studies which demonstrated that injection of XlHSF1 mRNA into X. laevis oocytes resulted in accumulation of XlHSF1 in the germline vesicle (nucleus) (34, 40). In those studies, however, nuclear localization may have been a result of the oocytes being unable to regulate the activity of overexpressed HSF1. This interpretation is supported by in vivo footprinting experiments on exogenous hsp70 genes co-injected into oocytes, which showed that at least some of the overexpressed XlHSF1 must be in the trimeric form because it was bound to the HSEs at non-shock (18 °C) temperatures and would therefore be expected to be localized in the nucleus (40).

The detection of HSF1 in the nucleus of oocytes under both stress and non-stress conditions is entirely consistent with other studies showing that HSF1 is predominantly a nuclear protein at all times in Drosophila cells (12, 24, 25, 43) and mammalian cells (24, 26). However, these results are in contrast with other studies, which have proposed that HSF1 is predominantly a cytoplasmic protein in unshocked cells and is translocated to the nucleus after exposure to stress (14, 15). Further studies may be needed to clarify the localization of HSF1 in mammalian cells.

Our finding that HSF1 is a nuclear protein prior to stress in oocytes has important implications for future studies aimed at elucidating the signaling pathways involved in HSF1 activation in response to stress. Previous studies have demonstrated that the endogenous HSF1 present in oocytes is capable of inducing the transcription of exogenous microinjected plasmids containing a hsp70 gene or a hs promoter-reporter gene construct after heat shock (44–46). These studies demonstrate that the endogenous HSF1 in oocytes can be activated to its transcriptionally active conformation once it has been exposed to heat or other forms of stress such as the introduction of abnormal proteins. The fact that XlHSF1 is present in the nucleus prior to stress means that the signals and/or factors, which serve to activate HSF1 and/or keep HSF1 in an inactive state must somehow reach or already be present in the nucleus. Indeed, Mifflin and Cohen (46) have shown that one inducer of the heat shock response, abnormal proteins, can only act as an inducer of hs gene transcription when they are injected directly into the nucleus. One of the central unanswered questions in the activation of hs genes by stress is whether HSF1 senses the various stress stimuli directly or if there may be one or more.

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internal stress signals that serve to activate HSF1. In vitro activation of HSF1 binding in crude extracts (36, 37) and of purified HSF1 (47, 48) suggests that HSF1 perhaps can respond to heat directly. However, the fact the in vitro activation temperature of extracts is several degrees lower than the in vivo temperature of extracts is several degrees lower than the in vitro activation temperature and the fact the overexpression of HSF1 in cells usually results in the constitutive activation of HSF1 binding (15, 38) suggest that there are potentially additional negative regulatory factors in the cell which help keep HSF1 binding inactive state. Furthermore, it is not clear how other inducers, such as abnormal or denatured proteins, can act directly on HSF1. Based on the results of this study, we propose that HSF1 is responding to changing conditions in the nucleus and that whatever mechanisms are involved in HSF1 activation, they must be realized within the nucleus.

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