A nuclear localization signal is essential for stress-induced dimer-to-trimer transition of heat shock transcription factor 3

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Running title: NLS is necessary for HSF3 trimerization

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The abbreviations used are: HSF, heat shock transcription factor; Hsp, heat shock protein; NLS, nuclear localization signal; HR, hydrophobic heptad repeat; GFP, green fluorescence protein; GST, glutathione S-transferase; PBS, phosphate-buffered saline.
Summary

Expression of heat shock genes is regulated mainly at the level of transcription by heat shock transcription factors (HSFs). In avian, HSF1 and HSF3 are maintained in a cryptic monomer and dimer form, respectively, in the cytoplasm in the absence of stress. Upon heat stress, they undergo conformational change associated with the formation of a trimer and nuclear translocation. In this study, we identified regions that are necessary for the dimer-to-trimer transition of chicken HSF3 (cHSF3) upon stress conditions. One of these regions coincides with a functional nuclear localization signal (NLS) of cHSF3 that was recognized by a nuclear transport receptor importin α. Point mutations of basic amino acids in the NLS inhibit both nuclear translocation and trimer formation of cHSF3. These results demonstrate that the NLS acts positively on the trimer formation of cHSF3 upon stress conditions.
Introduction

Cells respond to various kinds of physiological signals and environmental stresses through the expression of programmed sets of genes. These inducible systems of gene expression are regulated by transcription factors that bind to specific DNA sequences. Many transcription factors that govern inducible gene expression remain in the cytoplasm in a cryptic form and in response to signaling they are transported to the nucleus where they bind to and activate specific sets of genes. One of the examples of inducible gene expression is heat shock gene expression induced by heat stress (1). The expression of eukaryotic heat shock genes is controlled by heat shock transcription factors (HSFs) that bind to heat shock element (HSE) located in the promoter regions of all heat shock genes. In mammals, HSF1 is activated by heat stress and induces expression of heat shock genes (2 - 4). HSF1 not only protects cells subjected to stresses by inducing a set of heat shock genes, but also is involved in normal development (5, 6). Furthermore, HSF1 induces apoptosis of spermatocytes subjected to thermal stress (7).

In the absence of stress, HSF1 is mostly located in the cytoplasm in an inactive monomeric form. HSF1 is activated in roughly two steps (1, 8, 9). First, HSF1 is translocated to the nucleus, a process that is associated with a conversion to a trimer form that can bind to HSE with high affinity. Second, it acquires transcriptional activity probably by exposing a carboxyl-terminal activation domain (10 - 12). All members of the HSF gene family share common structural features including a conserved DNA binding domain which exhibits a winged helix-turn-helix motif (13, 14), an extended heptad repeat of hydrophobic amino
acids (HR-A/B) involved in trimerization (15, 16). With the exception of HSFs in budding yeasts and mammalian HSF4, HSFs also have another carboxyl-terminal hydrophobic repeat (HR-C) (17, 18). Although a consensus has yet to be reached (19, 20), HR-C is suggested to maintain HSF in an inactive state by interacting intramolecularly with the HR-A/B (21 - 23). HSF1 is also negatively regulated in trans by molecular chaperone complexes (24 - 27). Sequestration of molecular chaperones to denatured substrates by heat stress is speculated to lead to the activation of HSF1. The existence of a bipartite type of NLS was suggested in HSF2 and these sequences are conserved among HSFs (28).

Avian cells respond to heat stress by activating HSF3 as well as HSF1. Chicken HSF3 (cHSF3) stays as an inactive dimer exclusively in the cytoplasm in normally growing cells, and is translocated into the nucleus and converted to an active trimer upon heat stress (29). Activation of cHSF3 exhibits a delayed response as compared to the relatively rapid activation of HSF1, and occurs predominantly upon exposure to extremely high temperatures (30, 31). Fundamentally, however, the activation mechanism of cHSF3 may be similar to that of HSF1 (32). Analysis of cHSF3-deficient B lymphocytes showed that cHSF3 is essential for the burst activation of heat shock genes even in the presence of cHSF1 (33). cHSF3 is also specifically activated by the c-myb proto-oncogene product through a direct interaction, and may involve in cell growth and development (34). Thus, cHSF3 has significant roles in activating heat shock genes in response to heat stress and physiological stress.

Studies using various experimental systems have attempted to identify cis-acting domains that regulate the monomer-to-trimer transition of HSF1. In
addition to the core hydrophobic heptad repeat domains (HR-A/B and HR-C domains), several other domains play roles in maintaining HSF1 in an inactive form (35, 36). However, no domain has been identified to participate positively in activating HSFs. In this study, we analyzed the dimer-to-trimer transition of a series of deletion mutants of chicken HSF3 and found domains that are necessary for trimer formation. One of them coincides with the NLS of cHSF3. We suggest a novel regulatory mechanism of HSF regulation.

Experimental Procedures

Plasmid construction.

Chicken HSF3 expression vector pCMV-cHSF3 was constructed by ligating a 2,665-bp EcoRI fragment of pCHSF3-14a into the vector pCMV/Blue (Pharmingen). All of the expression vectors of cHSF3 with deletions or point mutations were created by using QuickChange site-directed mutagenesis kit (Stratagene) and pCMV-cHSF3 plasmid as a template. To confirm mutations, sequencing reactions were performed by using ALFExpress AutoRead sequencing kit (AmershamPharmacia), ALFExpress dATP labeling mix, and each synthetic oligonucleotide. Sequences were analyzed by using an ALFExpress sequencer (AmershamPharmacia).

To construct expression vectors pGFP-aa(79-110), pGFP-aa(111-139), and pGFP-aa(202-224), each cDNA fragment was modified by PCR to introduce an EcoRI site and ligated into pEGFP-C1 (which is an expression vector of a tandem repeat GFP (a gift from Y. Eguchi, Osaka Univ.). To examine sequences, sequence reactions were performed by using a pEGFP-C1 primer; 5’-CATGGTCCTGCTGGA
GTTCGTG-3’.

To construct pGEX2T-NLS vector, a cDNA fragment corresponding to amino acids 202 to 224 of cHSF3 was modified by PCR to introduce a Sma I site and ligated into pGEX2T plasmid vector (AmershamPharmacia). pGEX2T-NLS6, pGEX2T-NLS7, and pGEX2T-NLS8 were created by using QuickChange site-directed mutagenesis kit (Stratagene) and pGEX2T-NLS vector as a template. To confirm mutations, sequence reactions were performed by using a pGEX2T primer; 5’-GTATTGAAGCTATCCAC-3’.

Cell culture, transfection and screening

DT40 cells were maintained as described previously (33). For generating cells expressing a mutant cHSF3, 10⁷ cHSF3-null cells (33) suspended in 0.7 ml of PBS were mixed with 0.1 ml of DNA solution which contained a linear pCMV-cHSF3 mutant plasmid vector (25 µg) with Xho I and a linear pZeoSV2(+) (3 µg) (Invitrogen) with Xho I. After keeping on ice for 10 min, cells were electroporated with a Gene Pulser apparatus (Bio Rad) at 550 V and 25 µF, and then incubated on ice for 10 min. After suspension in 20 ml of fresh medium, cells were divided into two 96-well plates and incubated for 20 h. Medium containing zeocine (Invitrogen) was then added to a final concentration of 300 µg/ml. After 10-14 days, drug-resistant clones were expanded and whole cell extracts were prepared as described previously (30). Aliquots containing equal amount of protein were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and then blotted with
αHSF3γ antiserum as described previously (29). The level of cHSF3 protein in each clone was compared with that of endogenous cHSF3 in wild type DT40 cells and clones expressing less cHSF3 protein than wild type cells were analyzed further. To examine the oligomer form of cHSF3 mutant-2 (amino acids 392-467 was deleted), antiserum αHSF3β hat recognizes the whole cHSF3 molecule was used (data not shown).

**Gel filtration analysis**

Size fractionation of cell extracts was performed as described previously (29). Briefly, whole cell extracts (200 µl containing 500 to 1000 µg protein) from each cell were applied to a Superdex 200 HR column for fast protein liquid chromatography (AmershamPharmacia). The samples were eluted at 0.3 ml/min with a buffer containing 1 % glycerol, 20 mM Tris-HCl (pH 7.9), 200 mM KCl, and 1.5 mM MgCl2. The fractions (0.5 ml) were precipitated with trichloroacetic acid, washed with acetone, dried, suspended in gel sample buffer and analyzed by SDS-PAGE and western blot.

**Immunostaining**

Cells were washed with PBS and stuck to a slide glass by using a centrifugal cell collector (Tomy, Tokyo, Japan) according to the manufacturer’s instructions. They were then fixed with 4 % paraformaldehyde. After blocking with 1 % bovine serum albumin (BSA; fraction V) in PBS at room temperature for 1 h, cells were incubated with a 1:300 dilution of antiserum (αHSF3γ) in 1 % BSA/PBS 10 % normal goat
serum in PBS, and then with FITC-conjugated goat anti-rabbit IgG antibody (1: 200 dilution) (Cappel). The cells were washed with PBS and mounted in 80 % glycerol in 10 mM Tris-HCl (pH 7.5). Immuno-labeled cells were visualized by using fluorescence microscopy (Axiophot, Zeiss).

**Analysis of GFP-fusion proteins**

QT6 quail fibroblasts were maintained at 37°C in 5% CO₂ in Dulbecco’s modified Eagle’s medium containing 5 % fetal calf serum, 2 % chicken serum and 2 % tryptose phosphate broth. Cells were cultured on glass coverslips for 24 h, and were transfected with each expression vector by the CaPO₄ method. After 14 h in transfection salts, the plates were washed three times with PBS, fed with fresh medium, and incubated further for 24 h. Then the cells were fixed with 4 % paraformaldehyde for 30 min at 4°C. The coverslips were washed and mounted in 80 % glycerol in 10 mM Tris-HCl (pH 7.5) on coverglasses. Photograph was taken by using by fluorescence microscopy (Axiophot, Zeiss).

To examine the level of GFP-fusion proteins, cells were lysed in NP40 lysis buffer consisting of 150 mM NaCl, 1% NP-40, 50 mM Tris (pH 8.0), 1 mM PMSF, 1μg/ml leupeptin, and 1μg/ml pepstatin. Western blot analysis was then performed using anti-GFP polyclonal antibody (Clontech).

**Pull-down analysis**

HeLa cells were maintained in Dulbecco’s modified Eagle’s medium containing 10 % fetal calf serum and these extracts were prepared in NP-40 lysis buffer.
Cultures of *E. coli* (strain JM109) transformed with pGEX2T, pGEX2T-NLS, pGEX2T-NLS6, pGEX2T-NLS7, and pGEX2T-NLS8 were treated with 0.4 mM IPTG for 3 hr. The cells were washed and suspended in PBS containing 1 mM PMSF, 1µg/ml leupeptin, and 1µg/ml pepstatin. After sonication, soluble fractions were mixed with glutathione-sepharose 4B beads (AmershamPharmacia) on ice for 1h. After being washed with PBS, beads (bed volume of 20 µl) were mixed with HeLa cell extracts on ice for 2 h. After being washed with PBS, the beads were added to SDS-sample buffer (20 µl) and boiled at 95°C for 3 min. Proteins bound to the beads were analyzed by western blot by using mouse monoclonal antibody against importin α (anti-Rch-1, Transduction Laboratories) and peroxidase-conjugated goat anti-mouse IgG antibody (Cappel) as a second antibody. The signals were visualized using an enhanced chemiluminescence system (AmershamPharmacia).

**Results**

**cHSF3 reintroduced into cHSF3-deficient cells is correctly regulated**

We transfected cHSF3 expression vector into DT40 cells in which *HSF3* gene was disrupted (33), and isolated lines stably expressing cHSF3 under the control of CMV promoter. The heat shock response was restored in these cells (33). Unexpectedly, cHSF3 was observed as two bands of molecular sizes 65 kDa and 85 kDa. The mobility of the lower band is identical to that of a translated product from cHSF3 transcript in rabbit reticulocyte lysate, whereas the mobility of the upper band is identical to that of endogenous cHSF3 in cells (Fig. 1A). These results suggest that
cHSF3 polypeptide is modified post-translationally (see Discussion). We examined the oligomer form of the reintroduced cHSF3 by gel filtration analysis. The 85kDa form of the reintroduced cHSF3 was eluted at the dimer position, fractions 21 to 24, and after heat shock at 45°C for 30 min it was eluted at the trimer position, fractions 18 to 20 (Fig. 1B, d-f, upper arrows), just like endogenous cHSF3 (Fig. 1B, a-c) (29). In contrast, the 65 kDa form was eluted at a monomer position (Fig. 1B, d, a lower arrow). After heat shock, its elution peak was observed a little at a trimer position and disappeared from the soluble fraction at higher temperatures such as 45°C (Fig. 1B, f). These results suggest that a dimer-to-trimer transition of the 85 kDa form of reintroduced cHSF3 is correctly regulated just like endogenous cHSF3 irrespective of the presence of the 65 kDa form. In this study, we examined the oligomer form of only the 85 kDa form.

Identification of deletion mutants of cHSF3 that remained dimer even after heat shock

We generated cells stably expressing a series of deletion mutants of cHSF3 (Fig. 2A and B). Because negative regulation of HSF1 activation was shown to be limited (3, 19, 21) and was shared with negative regulation of cHSF3 activation (32, 33), we analyzed cells which expressed less cHSF3 mutant protein than endogenous cHSF3 protein in wild type DT40 cells (Fig. 2B). cHSF3 mutants lacking an amino-terminus (see lines Z10, Z11, and Z12) or carboxyl terminus (line Z2, data not shown, and see Materials and Methods) were not modified. The central region including HR-B was also necessary for the modification (see line Z8). We analyzed the oligomer forms of all of the cHSF3 mutants in control and heat shocked cells by
Previous works analyzing the monomer-to-trimer transition of mammalian and Drosophila HSF1 identified two hydrophobic heptad repeats those maintain HSF1 in a monomer state (21-23). We reported a similar observation, that a cHSF3 mutant lacking carboxyl-terminal region containing the hydrophobic heptad repeat (HR-C) exhibited a DNA binding form in vitro in rabbit reticulocyte lysate (37). We extended these works and showed that a mutant lacking a hydrophobic heptad repeat at the carboxyl-terminus formed a trimer in the absence of heat shock (Fig. 3A, line Z3). Furthermore, substitution of isoleucine at amino acid 371 or leucine at amino acid 375 in the HR-C with glutamic acid made cHSF3 constitutively a trimer (data not shown). Although there is controversy about the role of HR-C (19, 20), our data conclusively showed that this structure plays a role in maintaining cHSF3 in an inactive oligomeric state.

Previous studies have identified some other regions of HSF1 than the HR-C which contribute to the suppression of HSF1 trimerization (21, 35, 36). However, there is no evidence that suggests existence of domain acting positively on the HSF1 trimerization. Surprisingly, we found that two deletion mutants of cHSF3, cHSF3 mutant-6 and mutant-14, remained as dimer even after cells were heat-shocked at 45°C for 30 min. Regions necessary for cHSF3 trimerization include one containing a putative nuclear localization signal (NLS) downstream of the trimerization domain (formerly denoted as NLS2 in human HSF2, see reference 28) (Fig. 3B, see line Z6) and a carboxyl-terminal region of the DNA binding domain (which corresponds to a linker region of hHSF1, see reference 36) (Fig. 3C, see line Z14). The significance of the latter region will be discussed elsewhere.
The lack of trimer formation of cHSF3 mutant-6 and mutant-14 was observed not only when cells were heat shocked, but also when cells were treated with sodium arsenite, hydrogen peroxide, high concentration of NaCl (osmotic shock), proline analogue azetidine, prostaglandin A1, and proteasome inhibitor lactacystin (data not shown). These results suggest that stress signals are not transmitted to cHSF3 without the putative NLS.

The functional NLS of cHSF3

To examine whether the putative NLS, which is necessary for the trimer transition of cHSF3, is functional, we analyzed first the subcellular localization of cHSF3 mutants. Endogenous and reintroduced wild type cHSF3 changed their localization from the cytoplasm to the nucleus after heat shock (Fig. 4, lines wt and Z1). In contrast, the deletion mutant of the putative NLS did not move to the nucleus after heat shock (line Z6). The cHSF3 mutant-14 did not translocate to the nucleus after heat shock (Fig. 4, line Z14), nor did a mutant-15, which lacks another putative NLS between the DNA binding domain and oligomerization domain (formerly denoted as NLS1 in human HSF2) (28), moved to the nucleus (Line Z15). Other cHSF3 mutants did translocate to the nucleus after heat shock (Fig. 4). Staining patterns of cHSF3 and cHSF3 mutants in the nucleus of heat-shocked cells were same.

We next examined whether each of the three regions necessary for nuclear translocation of cHSF3 could transport GFP when fused to one of these polypeptides. We expressed GFP-NLS fusion protein as well as GFP fused to another putative nuclear localization sequence (amino acid 111-139) or GFP fused
to another region (amino acid 79-110) which is also necessary for trimer formation (Fig. 5A, B, and C). GFP-NLS was accumulated in the nucleus (Fig. 5B, c), whereas GFP, GFP-aa(79-110), and GFP-aa(111-139) located mostly in the cytoplasm (Fig. 5B, a, b, and d).

We further examined whether the NLS of cHSF3 was recognized by a nuclear import receptor. The NLS of cHSF3 (amino acids 202-224) was fused to carboxyl-terminus of GST (GST-NLS) and a GST-NLS column was generated. After mixing with HeLa cell extract, complexes bound to GST-NLS were examined (Fig. 5D). Since importin α is known to bind to the typical bipartite NLS among various kinds of nuclear import receptors, we performed immunoblotting with antibody against importin α. It was revealed that importin α efficiently bound to the NLS of cHSF3 (Fig. 5D, lane 3). These results indicate that the NLS (amino acid 202-224) in cHSF3 is a classical NLS.

The NLS is essential for dimer-to-trimer transition

To further clarify which amino acids are responsible for both trimer formation and nuclear translation, we created a series of point mutations and examined their oligomer formation and subcellular localization before and after heat shock. The NLS in cHSF3 could be a bipartite nuclear localization sequence consisting of four charged amino acids (204KRKR207) and one charged amino acid (219K) spaced by eleven amino acids (Fig. 6A). Clusters of charged amino acids are known to be necessary and sufficient for nuclear localization. Therefore, we substituted one or two of charged amino acids into alanine in the NLS sequences (Fig. 6A).
Substitution of a single amino acid did not affect the heat shock-induced nuclear translocation (Fig. 6A, mutants NLS1 to NLS5, data not shown). cHSF3 could still move to the nucleus after heat shock, although less efficiently, by the substitution of two amino acids including K (amino acid 204) in a cluster of charged amino acids and R (amino acids 196 or 222) (Fig. 6A and B, mutants NLS7 and NLS9). The nuclear translocation was clearly blocked when two K (amino acids 204 and 219) were substituted (Fig. 6A and B, mutant NLS8). Deletion of the KRKR sequence (amino acid 204-207) also prevented nuclear translocation (Fig. 6A and B, mutant NLS6). These results suggested that KRKR (amino acid 204-207) and K (amino acid 219) are essential amino acids for nuclear translocation of cHSF3. These amino acids necessary for nuclear translocation in the NLS of cHSF3 were consistent with those of one of the putative NLS of human HSF2 (NLS2, see reference 28). We next examined the ability of mutated NLS to bind to importin α. Although the amount of importin α coprecipitated with GST-NLS7 was greatly reduced compared with that coprecipitated with GST-NLS, it was above the background level (Fig. 5D, lanes 2 and 4). In contrast, neither GST-NLS8 nor GST-NLS-6 bound to importin α at all (Fig. 5D, lanes 5 and 6). These results showed that the abilities of NLS mutants to bind to importin α are well correlated with nuclear localization (Fig. 6).

We then examined the oligomeric form of these cHSF3 mutants. cHSF3 mutant NLS6 which lacked a cluster of charged amino acids KRKR remained a dimer after heat shock, and more than half of the mutant NLS8, which did not move to the nucleus, eluted at dimer fractions (fractions 21 to 23) after heat shock at 45°C for 30 min (fractions 18 to 20) (Fig. 6C). In contrast, NLS7 was eluted mostly...
at the trimer position (Fig. 6C), and effects of other point mutations in the NLS sequences on the trimer formation were undetectable (Fig. 6A and data not shown). These results indicated that mutations that block nuclear translocation also prevent trimer formation. The basic amino acids in the NLS are conserved among all of the vertebrate HSFs (Fig. 6D).

Discussion

Our mutational analysis of cHSF3 provided unexpected results. We identified two regions that are essential for trimer formation of cHSF3 upon heat stress. One of these regions is coincident with a functional NLS that is a classical bipartite type and is recognized by a nuclear import receptor importin α. Point mutations of the basic amino acids in the NLS inhibit both stress-induced trimer formation and nuclear translocation of cHSF3.

Among vertebrate HSFs, HSF1 and HSF3 respond to environmental stresses such as heat stress and change subcellular localization. In the absence of stress, HSF1 and HSF2 is located both in the cytoplasm and the nucleus (2, 3, 28, 35), HSF3 is mostly in the cytoplasm (29), and HSF4 is in the nucleus (18, 38). Upon heat stress, HSF1 and HSF3 accumulate in the nucleus, where they bind to DNA and activate heat shock gene expression. Thus, HSF1 and HSF3 are strictly inactivated by unique mechanisms in the absence of stress. The first step of HSF1 and HSF3 activation is the conversion to a trimer that is associated with nuclear translocation. The trimer formation of HSF1 is shown to be partly under the negative control of chaperone complexes (24, 25, 27), and the sequestration of Hsps
under condition of stress may alleviate the negative regulation and lead to the activation of HSF1. Negative regulation of cHSF3 should share this mechanism because depletion of cHSF3 resulted in increased negative regulation to cHSF1 activation (32, 33). In this study, we suggest another mechanism that controls the first step of cHSF3 activation under stress conditions. Our results indicate that release from the negative regulator is not sufficient for the cHSF3 activation.

How does the NLS act positively on trimer formation of cHSF3? One possibility is that the NLS may affect the structure of the trimerization domain (HR-A/B) which is located just upstream of the NLS. The HR-A/B is thought to interact with another hydrophobic heptad repeat HR-C on unstressed condition, and the interaction should be dissociated on stress conditions. Mutation of basic amino acids in the NLS may alter the structure of the HR-A/B and inhibit the stress-induced dissociation of the two hydrophobic heptad repeats. Another possibility is that positively acting factors may be necessary for trimer formation of cHSF3. These factors can interact to cHSF3 through the NLS. Nuclear import receptor is a candidate for a positively acting factor because they bind to the NLS of cHSF3 and could induce conformational change of cHSF3. Conformational change induced by binding of nuclear transport receptor is shown in the yeast transcriptional activator GAL4 (39). It may possible that nuclear transport receptor affects an oligomeric form of cHSF3 in addition to transport it to the nucleus.

We are aware of some discrepancies in the roles of the NLS and HR-C. The HR-C of human and Drosophila HSF1 were first shown to maintain HSF1 as an inactive monomer in the absence of stress, as examined by overexpression of mutant proteins in cells (21). This result was confirmed by experiments conducted
in Xenopus oocytes (22) and in vitro in rabbit reticulocyte lysate (23). However, analysis of cells, in which the expression of mutant HSF1 was from integrated promoter, showed that HR-C is not necessary for maintaining Drosophila HSF1 in an inactive state (19). It is hard to compare these results as the experiments were not performed under physiological conditions. Our experimental system has the advantage that cHSF3 gene-disrupted cells stably expressing cHSF3 mutants are used. Regulation of cHSF3 mutants was monitored in nearly physiological conditions. Our results conclusively indicated that HR-C is necessary for maintaining cHSF3 in an inactive state (Fig. 3A, and data of point mutations were not shown), suggesting that the HR-C of human and Drosophila HSF1 play a similar role. HR-C is expected to prevent trimer formation through HR-A/B by binding intramolecularly to HR-A/B (21 - 23). Heat stress may cause dissociation of the interaction and induce homo-trimerization of HR-A/B.

cHSF3 exists as a dimer in normally growing cells. This dimer state is necessary for cHSF3 to be correctly converted to a trimer after heat shock. The unmodified form of cHSF3 (65 kDa) stayed a monomer and was converted to incorrectly aggregated after heat shock (Fig. 1). To understand the molecular mechanism of cHSF3 regulation, it is necessary to identify the nature of post-translational modification. We have tried to reveal the modification by examining phosphorylation, glycosylation and covalent modifications in other polypeptides. Unfortunately, we could not identify the cHSF3 modification. The mobility of 85 kDa cHSF3 was not affected by phosphatase treatment of cell lysates (data not shown). cHSF3 did not bind to a concanavalin A column nor wheat germ agglutinin column (data not shown). cHSF3 was not conjugated by ubiquitin-like
proteins such as ubiquitin, NEDD-8, sentrin-1 (Sumo-1), sentrin-2 or sentrin-3 (Dr. T. Kamitani, personal communication). As the 65 kDa cHSF3 is not degradation product (Fig. 1A), 85 kDa cHSF3 must be post-translationally modified by unknown mechanism.

Trimer formation of cHSF3 is shown to be specifically regulated by factors which is involved in cell proliferation, differentiation, and death. A proto-oncogene product c-Myb induces trimer formation of cHSF3 through binding to DNA binding domain of cHSF3 (34). Tumor suppressor protein p53 also bind to cHSF3 and inhibit c-Myb-induced trimer formation of cHSF3 by competing c-Myb binding to cHSF3 (40). It is possible that positively acting factors such as c-Myb may regulate trimer formation of cHSF3 more generally on stress conditions.

Acknowledgments - We are grateful to N. Imamoto and R. I. Morimoto for critical discussion, Y. Eguchi for plasmid, T. Kamitani for analysis of HSF3 modification, and Y. Kawazoe and M. Tanabe for technical assistance.

This work was supported by Grants-in-Aids from the Ministry of Education, Science and Culture of Japan for scientific research and for scientific research on priority areas, by Core Research for Evolutional Science and Technology (CREST), the Japan Science and Technology Corporation (JST), and by Uehara Memorial Foundation.

References


**Figure Legends**

**Fig. 1.** Reintroduction of cHSF3 into HSF3-deficient DT40 cells. (A) Comparison of apparent molecular sizes of cHSF3 synthesized in vitro in a rabbit reticulocyte lysate (lane 1), cHSF3 in HSF3-deficient DT40 cells in which a cHSF3 expression vector was stably introduced (lane 2), and cHSF3 in wild type DT40 cells (lane 3). In wild type cells, most of the cHSF3 was detected as a 85 kDa band with a small amount also observed as a 65 kDa band. In contrast, the molecular size of cHSF3 synthesized in vitro was 65 kDa. cHSF3 in cHSF3-reintroduced cells was observed as two bands of 65 kDa and 85 kDa. (B) A dimer-to-trimer transition of 85 kDa-cHSF3 (upper arrows) was induced by heat shock independent of 65 kDa-cHSF3 (lower arrows). Wild type DT40 cells (a-c) and cHSF3-reintroduced cells (d-f) were heat shocked at the indicated temperature for 30 min. Whole cell extracts were prepared and were size-fractionated by gel filtration using a Superdex 200 HR column. The amount of cHSF3 protein in each fraction was estimated by western blotting. The elution fractions of monomer and trimer forms of cHSF1 and a dimer form of endogenous cHSF3 are indicated at the bottom. The 65 kDa-cHSF3 was eluted at a monomer position at a control temperature. The approximate elution positions of protein standards are indicated on the top: 669 kDa, thyroglobulin; 440 kDa, ferritin; 158 kDa, aldolase; 69 kDa, bovine serum albumin. A position of void volume is indicated by v.

**Fig. 2.** Structure and expression of cHSF3 mutants. (A) Schematic representation of cHSF3 deletions containing a wild type (number 1) and deletion mutants...
(numbers 2 to 15). Positions of the deleted amino acids are indicated in each mutants. Functional domains are indicated as DBD, DNA-binding domain; HR, heptad repeat of hydrophobic amino acids. (B) Expression of mutant cHSF3 proteins in HSF3-deficient DT40 cells. Each expression vector was stably transfected into cells and whole cell extracts from control and heat shocked (43°C for 30 min) cells were prepared. Aliquots containing same amounts of protein (40 µg) were subjected to SDS-PAGE, followed by western blotting using an antiserum αHSF3γ. The protein levels of cHSF3 mutants were below that of endogenous cHSF3 in wild type DT40 cells. The cell lines expressing cHSF3 mutants are denoted as Z1 to Z15 according to the serial number of cHSF3 mutants shown above.

**Fig. 3.** Oligomeric states of cHSF3 mutants. Whole cell extracts were prepared from control cells (37°C) or heat shocked cells (43°C for 30 min). Proteins were size-fractionated and western blotting was performed using an antiserum _HSF3_ as described in figure 1. Deletion mutants of hydrophobic heptad repeats (A), internal regions between HR-A/B and HR-C (B), and DNA binding domain (C) are shown. 85 kDa-cHSF3 is indicated by an open arrow, and 65 kDa-cHSF3 by a closed arrow.

**Fig. 4.** Indirect immunofluorescence analysis of cHSF3 mutants. Control (37°C for 30 min) and heat shocked (43°C for 30 min) cells expressing cHSF3 mutants (lines Z1, Z3, Z4, Z6, Z7, Z14, and Z15) were fixed in 4% paraformaldehyde, permeabilized with 0.2% Nonidet P-40 in PBS, and stained with an antiserum αHSF3γ as a first
antibody and FITC-conjugated goat anti-rabbit IgG (Cappel) as a second antibody.

**Fig. 5.** One of the two regions necessary for heat-induced trimer formation acts as a nuclear localization signal. (A) The positions and sequences of the regions necessary for heat-induced trimer formation of cHSF3. Underlined amino acid sequences are clusters of basic amino acids in candidate bipartite nuclear localization signals. (B) QT6 cells were transfected transiently with an expression vector of each polypeptide fused to the C-terminal end of a tandemly repeated GFP. At 24 h after transfection, cells were fixed with 4% paraformaldehyde and fluorescence was examined under the Axiophot microscope. a, GFP only; b, GFP-aa(79-110); c, GFP-aa(111-139); d, GFP-aa(202-224). (C) At 24 h after transfection, cells were harvested and cell extracts were prepared with lysis buffer containing 1% Nonidet P-40. Aliquots containing same amounts of protein were subjected to SDS-PAGE, followed by western blotting using anti-GFP antibody (Clontech). Fused proteins were expressed at similar levels. (D) The NLS of cHSF3 or mutant NLSs (amino acids 202-224) were fused to the carboxyl terminus of GST. GST, GST-NLS, and GST-mutant NLSs were stained with Coomassie Brilliant Blue (lower panel). After the mixing of each GST-fusion protein with HeLa cell extracts, proteins bound to these fusion proteins were analyzed by western blotting using antibody specific for importin α (upper panel).

**Fig. 6.** Trimer formation and nuclear translocation of cHSF3 was inhibited by mutations in the nuclear localization signal. (A) Summary of the mutations and
subcellular localization of cHSF3. Substitutions of a single basic amino acid exhibited no effect on the localization. Heat induced nuclear translocation was inhibited by deletion of four basic amino acids (NLS6) or substitution of two basic amino acids located in two clusters (NLS8). C, cytoplasm; N, nucleus. (B) Cells stably expressing cHSF3 mutants analyzed by indirect immunofluorescence microscopy. Representative staining is shown. (C) Oligomeric forms of NLS6, NLS7, and NLS8. NLS6 did not form a trimer after heat shock. Trimer formation of NLS8 was also partially inhibited, whereas that of NLS7 was not inhibited. (D) Alignment of the NLS of cHSF3 (amino acids 202-224) with corresponding regions of other vertebrate HSFs. Identical amino acids are indicated by bold letters.
A

in vitro
HSF3 in HSF3-/-

85kDa
65kDa

1 2 3 4

B

v 669 440 158 69

15 16 17 18 19 20 21 22 23 24 25 26 27 28 29

37
41
45

wt

37

HSF3 in HSF3-/-

41
45

trimer dimer monomer
**A**

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DBD | HR-A/B | HR-C

**B**

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WT | Z10 | Z11 | Z12 | Z13 | Z14 | Z15 |
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**A**

HR-A/B, C

**B**

internal region between HR-A/B and HR-C

**C**

DBD
A nuclear localization signal is essential for stress-induced dimer-to-trimer transition of heat shock transcription factor 3

Akira Nakai and Terumi Ishikawa

J. Biol. Chem. published online August 14, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M005302200

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