Active HSF1 Significantly Suppresses Polyglutamine Aggregate Formation in Cellular and Mouse Models*

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Poliglutamine diseases are inherited neurodegenerative diseases characterized by misfolding and aggregation of proteins possessing expanded polyglutamine repeats. As overexpression of some heat shock protein (Hsp) suppresses polyglutamine aggregates and cell death, it is assumed that combined overexpression of Hsps will suppress that more effectively. Here, we examined the impact of active forms of heat shock transcription factor 1 (HSF1), which induces a set of Hsps, on polyglutamine inclusion formation and disease progression. We found that active HSF1 suppressed polyglutamine inclusion formation more significantly than any combination of Hsps in culture cells, possibly by regulating expression of unknown genes, as well as major Hsps. We crossed R6/2 Huntington disease mice with transgenic mice expressing an active HSF1 (HSF1Tg). Analysis of the skeletal muscle revealed that the polyglutamine inclusion formation and its weight loss were improved in R6/2/HSF1Tg mice. Unexpectedly, the life span of R6/2/HSF1Tg mice was significantly improved, although active HSF1 is not expressed in the brain. These results indicated that active HSF1 has a strong inhibitory effect on polyglutamine aggregate formation in vitro and in vivo.

Polyglutamine expansion is a major cause of inherited neurodegenerative diseases called polyglutamine diseases. Eight polyglutamine diseases have been identified, including Huntington disease, spinobulbar muscular atrophy, dentatorubral pallidoluysian atrophy (DRPLA), and five forms of spinocerebellar ataxia (SCAs). Aggregates or inclusion bodies of polyglutamine proteins within the nucleus, or in the cytoplasm of neuronal cells in some Huntington disease patients, are a prominent pathological hallmark of most polyglutamine diseases (1–3). The formation of polyglutamine protein inclusions mostly correlates with an increased susceptibility to cell death (4, 5), with some exceptions (6, 7). Actually, the early stages of aggregates during inclusion body formation are highly toxic to cells (8). Although inclusion body formation may be a cellular response to cope with the toxic effects of the aggregates (9), suppression of aggregates should be beneficial to cells and could delay the progression of polyglutamine diseases (10).

The inclusion bodies in polyglutamine disease contain heat shock proteins, as well as components of the ubiquitin-proteasome pathway (11). Heat shock proteins act as molecular chaperones that control folding of proteins (12). There is a lot of evidence indicating that Hsp70 and/or its co-chaperone Hsp40 inhibit inclusion body formation and cell death in cultured cells (11, 13–18), in yeast (19), in worms (20), in Drosophila (6, 21, 22), and in mice (7, 23). Furthermore, Hsp27 (24), Hsp110 (25), and yeast Hsp104 (26), which belong to diverse Hsp families, also suppress inclusion body formation and cell death. As one variety of Hsps acts coordinately to assist the folding of proteins (12), it might be better to express a set of Hsps to inhibit aggregate formation (27, 28).

The induction of Hsp expression on stress conditions such as heat shock is regulated by heat shock transcription factor 1 (HSF1), which binds to heat shock elements (HSE) located on the upstream region of all Hsp genes (29). A pre-existing monomeric HSF1 is converted to a trimer that can bind to HSE when cells are heat-shocked. We previously generated an HSF1 mutant that up-regulates expression of a set of Hsps in the absence of stress in culture cells and generated transgenic mice expressing its mutant in tissues such as the heart and testes but not in the brain (30). This overexpression causes infertility in male mice but is not toxic to culture cells and to several other organs such as the heart. It was reported that overexpression of HSF1 could suppress inclusion formation of a polyglutamine protein in culture cells (31). However, we do not know how strongly HSF1 can suppress aggregate formation and whether it can act in vivo in a mouse model. In fact, overexpression of Hsp70 has little effect on disease progression in some mouse models of polyglutamine disease (32–34). By generating active HSF1 mutants, we examined the impact of active HSF1 on polyglutamine aggregate formation and disease progression by using a cellular model of inclusion formation of a polyglutamine protein derived from human DRPLA (35) and Huntington disease R6/2 model mice (36). Our results clearly showed that active HSF1 inhibits inclusion body formation much more efficiently than any Hsp or any combination of Hsps. Furthermore, we showed that expression of an active HSF1 in nonneural tissues markedly prolonged the life span of Huntington disease R6/2 mice.

**MATERIALS AND METHODS**

**Construction**—A plasmid pHβ-hHSF1ΔRD was generated as described previously (30). A 1.3-kb HindIII fragment of pHβ-hHSF1ΔRD was inserted into a vector pcDNA3.1(+) (Invitrogen). Using this plasmid (pcDNA3.1/hHSF1ΔRD) as a template, leucine at amino acid 395 was substituted with glutamic acid using a QuikChange site-directed mutagenesis kit (Stratagene), generating a plasmid, pcDNA3.1/hHSF1ΔRDT. To confirm the mutation, sequencing reactions were performed with an ALFexpress AutoRead sequencing kit.
and sequences were analyzed with an ALFexpress sequencer (Amersham Biosciences). cDNAs for FLAG-tagged hHSF1, hHSF1ΔRD, and hHSF1ΔRDT at the N terminus were created by PCR, and each cDNA was inserted into a pcDNA3.1(+) vector at HindIII and EcoRI sites.

An hHsp70 expression vector, pcDNA3.1-hHsp70, was constructed by inserting a BamHI/XhoI fragment of pBK-CMV-HSP72 (a gift from Dr. H. Itoh, Akita University) (37) into the pcDNA3.1(+) vector. hHsp40 expression vector, pcDNA3.1-hHsp40, was constructed by inserting an Apal/EcoRI fragment of phHsp40 (a gift from Dr. K. Ohtsuka, Chubu University) (37). mHsp110 cDNA (a gift from Dr. Hatayama, Kyoto Pharmaceutical University) (25) was amplified by PCR with a 5′-primer containing a Kozak sequence (GCCACC) in front of a start codon and a 3′-primer and was inserted into the pcDNA3.1(+) vector at EcoRI and BamHI sites (pcDNA3.1-kozak-mHsp110).

**Cell Culture and Generation of HeLa Cells Stably Expressing hHSF1 or Hsp—**HeLa and HEK293 cells were maintained at 37 °C in 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum. PC12 cells were maintained in DMEM containing 5% bovine serum. PC12 cells were maintained in DMEM containing 5% bovine serum and 5% horse serum. Each expression plasmid was transfected into HeLa cells using a calcium phosphate method. At 4 h after transfection, the cells were treated for 3 min with 15% glycerol in an HBS buffer (140 mM NaCl, 25 mM HEPES, and 1.4 mM Na2HPO4). At 24 h after transfection, the cells were incubated in medium containing 1.5 mg/ml G418 disulfate (Nacalai Tesque, Kyoto, Japan). Clones expressing hHSF1, its mutant, or Hsp were selected by Western blot analysis as described below. HeLa cells stably expressing mHsp27 were generated previously (39).

**Western Blot Analysis—**Cell extracts were prepared in lysis buffer containing 1.0% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. Western blot analysis was performed as described previously (40). Antibodies used were anti-HSF1 (aCHSF1x and aHSF1g) (40, 41), for Hsp110 (aHSF110a) (41), for Hsp90 (aHSF90d) (42), for human Hsp27 (aHsp27a), which was generated by immunizing rabbit with recombinant hHsp27, and for rat Hsp27 (a gift from Dr. K. Kato and H. Itoh, Aichi Human Service Center) (43) and mouse monoclonal antibodies for Hsp70 (W27, Santa Cruz Biototechnology), FLAG (M2, Sigma), GFP (GF200, Nacalai Tesque), and β-actin (AC-15, Sigma). To detect Hsp40, we generated antiseraum αHsp40α by immunizing rabbits with human Hsp40 (full-length) as described previously (41).

To analyze protein levels in the mouse tissues, tissues were homogenized in a Nonidet P-40 lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, 50 mM Tris (pH 8.0), 8.0 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml pepstatin A, and 1 mM dithiothreitol) by Polytron (Kinematica, Inc., OH). After centrifugation at 15,000 × g for 10 min, supernatants were removed. Equal mounts of protein (100 µg) were loaded on SDS-PAGE and subjected to Western blot analysis.

**Gel Shift Assay—**HEK293 cells were transfected with expression vectors for FLAG-tagged wild-type and mutant hHSF1 by using a calcium phosphate method, and cells were harvested after 24 h. Cells were frozen at −80 °C and then suspended in buffer C containing 20 mM HEPES (pH 7.9), 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 µM dithiothreitol, 1 µg/ml leupeptin, and 1 µg/ml pepstatin A. After centrifugation at 100,000 × g for 5 min at 4 °C, the supernatants were frozen in liquid nitrogen and stored at −80 °C. Aliquots containing 10 µg of proteins were subjected to gel shift assay using a 32P-labeled ideal HSE-oligonucleotide as a probe (40).

**Ectopic Expression of GFP-Polyglutamine Protein and Estimation of Inclusion Formation—**The expression vectors for Q19-GFP and Q81-GFP were kind gifts from Drs. W. J. Strittmatter, J. R. Burke, and Y. Nagai (Duke University Medical Center). In these plasmids, 19 or 81 CAG repeats of DRPLA cDNA were inserted into an expression vector, pEGFP-N1 (Clontech) (35). Each expression vector (2 µg) was mixed with DMEM containing nonessential amino acids (100 µl) and Plus reagent (20 µl) (Invitrogen) for 15 min. The mixture was combined with DMEM (50 µl) containing Lipofectin (0.5 µl) (Invitrogen) for 15 min and then added to a 35-mm dish containing 800 µl of DMEM. After the cells were incubated for 8 h, 2 ml of DMEM containing 15% fetal bovine serum was added to the dish. The medium was changed to DMEM containing 10% fetal bovine serum at 8 h after transfection. The formation of inclusion bodies was observed by detecting GFP fluorescence, and the cell morphology was examined by phase contrast under Axiovert 200 microscopy (Zeiss). The numbers of cells expressing GFP fusion protein and cells containing inclusion bodies were counted.

To analyze solubility of Q81-GFP fusion protein in HeLa cells, cells were lysed on ice for 10 min in lysis buffer (400 µl) containing 40 mM HEPES (pH 7.5), 50 mM KCl, 1% Triton X-100, 2 mM dithiothreitol, 50 mM β-glycerophosphate, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, and 5 µg/ml pepstatin (44). After centrifugation at 15,000 rpm for 10 min, supernatants were removed. Pellets were suspended in 1× SDS sample buffer (400 µl) and were sonicated. Twenty microliters of the supernatant and pellet fractions were subjected to SDS-polyacrylamide electrophoresis and Western blot analysis.

**Generation of Adenoviruses Expressing an Active HSF1 and Hsps—**An Nhel/KpnI fragment of pcDNA3.1/hHSF1ΔRDT was inserted into pShuttle vector (Clontech). A viral DNA, pAd-hHSF1ΔRDT, was generated according to the manufacturer’s instructions (Clontech). Ad-LacZ was similarly generated by using pShuttle-LacZ (Clontech). cDNA for mHsp110 was amplified by PCR using a 5′-primer containing NotI and Kozak (GCCACC) sequences and a 3′-primer containing EcoRV sequence and was inserted into a pShuttle-CMV vector (Stratagene) at NotI and EcoRV sites. cDNA for hHsp70 was amplified by PCR using a 5′-primer containing HindIII and Kozak sequences and a 3′-primer containing Xhol sequence and was inserted into the pShuttle-CMV vector at the same sites. A NotI/HindIII fragment of phHsp40 (38) was inserted into the pShuttle-CMV vector at the same sites. A NotI/Nhel fragment of pcDNA3.1neo-mHsp27 (39) was inserted into the pShuttle-CMV vector. Viral DNAs containing cDNAs for Hsps were generated according to the manufacturer’s instructions for the AdEasy adenoviral vector system (Stratagene). Viruses were infected into HEK293 cells, and the virus particles were enriched by CsCl gradient centrifugation and stored at −80 °C until use. Titers of virus stocks were 2–10 × 10^8 pfu/ml.

**Adenovirus Infection—**HeLa cells plated in 60-mm dishes containing 4 ml of medium were infected with each adenovirus at a titer of 3 × 10^5 pfu/ml. More than 80% of HeLa cells were infected with Ad-LacZ at a titer of 2 × 10^5 pfu/ml judged by β-galactosidase staining. Abnormal morphology of HeLa cells was observed when they were infected with an adenovirus at a titer of 3 × 10^5 pfu/ml. At 24 h after infection of viruses, an expression vector for GFP-polyglutamine protein was transfected and maintained for 24–48 h. The numbers of cells expressing GFP fusion protein and cells containing inclusion bodies were counted, and these cells were harvested for the analysis of Hsps and GFP fusion protein expression by Western blot. To examine the effects of virus infection on the inclusion formation in PC12 cells, cells were infected with each adenovirus at a titer of 1.2 × 10^5 pfu/ml.
HSF1 Suppresses Polyglutamine Aggregation

Maintenance and Crossing of Transgenic Mice—A transgenic mouse line R6/2 (Jackson codes B6CBA-TgN(Hdixon1)62Gpb) was obtained from Jackson Laboratory (Bar Harbor, ME). To maintain the line, ovari- ries of R6/2 female mice were transplanted to (CBA × 57BL/6) F1 females, and ovarian-transplanted females were mated with (CBA × 57BL/6) F1 males. Genotyping was performed as described previously (36). Transgenic mice expressing hHSF1RD (HSF1RD mice) (30) were maintained by crossing HSF1RD females with (CBA × 57BL/6) F1 males. To generate double transgenic mice, HSF1RD females were crossed with R6/2 males.

Histopathology and Estimation of Polyglutamine Inclusion Formation in Tissues—Mice were sacrificed by cervical dislocation, and the quadriceps, heart, and brain were dissected and immediately frozen in iso- pentane and stored at −80 °C. Sections of 5-μm thickness were cut using a CM1900 cryostat (Leica). Immunohistochemistry was performed essentially as described previously (30). Antibodies used were goat IgG against Huntingtonin (N-18, Santa Cruz Biotechnology) (1: 100 dilution in 3% dried milk) and fluorescein isothiocyanate-conjugated rabbit anti-goat IgG (Jackson) (1: 50 dilution in 3% dried milk). The sections were mounted in VECTASHIELD with 4', 6-diamidino-2-phenylindole (Vector Laboratories) and examined by Axioplan 2 microscope (Zeiss). The numbers of Huntingtonin-positive inclusions were counted in 500 nuclei, and the percentages of nuclei possessing the inclusions were calculated. All experimental protocols were reviewed by the Committee for Ethics on Animal Experiments of Yamaguchi University School of Medicine.

To distinguish type I and type II fibers, histochemical reactions for myosin ATPase were performed at a series of pH (45). The cross-sectional area (CSA) was estimated by using the NIH Image program.

Electron Microscopy—For the transmission electron microscopic study, the specimens were fixed in 4% paraformaldehyde containing 0.25% glutaraldehyde and 4.5% sucrose, postfixed in 1% osmium tetrox- ide, dehydrated through passage in a series of graded ethanol, and embedded in Epon. Ultrathin sections obtained from the embedded blocks were stained with uranyl acetate and lead citrate and were exam- ined with a Hitachi H-7000 electron microscope (46).

Statistical Analysis—Significant values were determined by analyzing data with the Mann-Whitney's U test using StatView version 4.5 for Macintosh (Abacus Concepts, Berkley, CA). A level of p < 0.05 was considered significant.

RESULTS

Active Forms of HSF1—To examine the effects of HSF1-mediated gene activation in cells expressing the pathological length of polygluta- mine proteins, it is necessary to introduce into cells an HSF1 mutant that has a stronger potential to activate target genes. HSF1 stays in an inactive monomer in the absence of stress and is activated through two steps: trimer formation and the acquisition of transcriptional activity. Previous studies showed the potential to activate heat shock genes of an HSF1 mutant, hHSF1RD, lacking the regulatory domain that masks the activation domain (30, 31, 47). However, a high expression level was required for hHSF1RD to form a trimer that could bind to DNA (30). It was shown that a leucine zipper-like motif of HSF1 near the C terminus suppresses trimer formation (48). Here, we substituted leucine at amino acid 396 in the suppression domain of the trimerization in hHSF1RD with glutamic acid (hHSF1RDT) (Fig. 1A). To compare protein levels of HSF1 mutants, a FLAG polypeptide was tagged at the N terminus of HSF1 and its mutants. These HSF1 mutants were tran- siently expressed in HEK293 cells, and the HSE binding activities were estimated. This clearly showed that hHSF1RD bound to HSE much more strongly than hHSF1RDT in HEK293 cells (Fig. 1B). To examine the potential to activate heat shock genes, we generated HeLa cells stably expressing similar levels of HSF1 mutants and estimated protein levels of Hsps. Hsp levels were increased in cells expressing both hHSF1RD (HeLa/HSF1RD cells) and hHSF1RDT (HeLa/ HSF1RDT cells), but the levels were much higher in HeLa/ HSF1RDT cells (Fig. 1C). Growth rates and proportions of cells in each stage of the cell cycle in cells expressing an active HSF1 were similar to those in parental cells (data not shown) (30).

An Active HSF1 Suppresses Polyglutamine Inclusions More Efficiently than Any Heat Shock Protein in Cells—To study the formation of inclusion bodies in culture cells, we transiently transfected polyglutamine-GFP fusion proteins into HeLa cells. When a nonpathologic length glu- tamine 19 from human DRPLA fused to GFP (Q19-GFP) was expressed under the control of a CMV promoter (35), diffuse GFP fluorescence was observed in both the cytoplasm and the nuclei of cells (data not shown). These cells, expressing Q19-GFP (30–40% of total cells), looked similar to cells that did not express the fusion protein by mor- phology (data not shown). In marked contrast, when a pathologic length polyglutamine 81 fused to GFP (Q81-GFP) was expressed, distinct inclusions were observed in about 25% of GFP-positive cells (data not shown). Phase contrast examination showed that among cells having inclusions, many (12 and 56% of cells at 24 and 36 h after transfection, respectively) were round and were detached from the dish, indicating the detrimental effects of the inclusion formation. We transfected the expression vector for Q81-GFP into HeLa, HeLa/HSF1, HeLa/ HSF1RD, and HeLa/HSF1RDT cells and examined the inclusions. The numbers of inclusions were low in HeLa/HSF1, HeLa/HSF1RD, and HeLa/HSF1RDT cells and were lowest in HeLa/HSF1RDT cells (50.7 and 42.9% of the number in HeLa cells at 24 and 36 h, respectively) (Fig. 2, A and B). We next examined insoluble fractions of Q81-GFP and found that the level of insoluble Q81-GFP was lowest in HeLa/ HSF1RDT cells (6.9 and 20.9% of the number in HeLa cells at 24 and 36 h, respectively) (Fig. 2C). To compare the inhibitory effect of an active HSF1 with that of each Hsp on inclusion formation, we generated HeLa cells stably expressing substantial levels of Hsp27, Hsp40, Hsp70, and Hsp110 (Fig. 2D). The numbers of inclusions decreased by about 25% in some of these cells. In marked contrast, the number of inclusions was lowest in HeLa/HSF1RDT cells (about 50% of the number in HeLa cells). These data indicated that overexpression of HSF1RDT suppresses the polyglutamine aggregates more efficiently than overexpression of any of the major Hsp5 in cells.

Inhibitory Effects of an Active HSF1 on Inclusion Formation Are Mediated through Regulation of Unknown Genes, as Well as Major Heat Shock Genes—To examine the effects of combined overexpression of various Hsps, we generated adenovirus vectors expressing HSF1RDT and Hsps. We determined that in HeLa cells, the inhibitory effect of Ad-HSF1RDT reaches a peak at a concentration of 6 × 10⁵ pfu/ml in a culture medium, whereas expression of major Hsps was highest at a concentration of 3 × 10⁵ pfu/ml (Fig. 3A). We found that combined overexpression of Hsp70 and Hsp40 did not inhibit the inclusion for- mation more efficiently than overexpression of Hsp70 or Hsp40 alone (Fig. 3B). Furthermore, combined overexpression of any Hsp did not inhibit the inclusion formation more than overexpression of HSF1RDT in either HeLa or PC12 cells. As there are many reports showing the combined effects of Hsp70 and Hsp40 on protein aggrega- gates, we carefully titrated expression levels of Hsp70 and Hsp40. We did not observe any additive effect of Hsp70 and Hsp40 on the suppres-
tion of Q81-GFP inclusion formation in the cells (Fig. 3C). These results suggested that the inhibitory effect of HSF1\(\Delta\)RDT on inclusion formation is not only mediated through the major Hsps but also through other Hsps or unidentified gene products.

To confirm this hypothesis, we generated HeLa cells stably expressing chicken HSF1 (cHSF1). cHSF1 activates constitutive expression of genes, including some heat shock genes, but does not activate heat shock genes in response to heat shock (49). Overexpression of cHSF1 in HeLa cells did not induce major Hsps (Fig. 3D). Nevertheless, cHSF1 did inhibit inclusion formation as strongly as hHSF1. Furthermore, a mutant human HSF1 (HSF1R71G) (49), which does not bind to DNA, had no effect on inclusion formation (Fig. 3D), indicating that HSF1 suppresses inclusion formation through regulation of gene expression. These results suggested that HSF1 has more beneficial effects on the inhibition of Q81-GFP inclusion formation than we expected.

An Active HSF1 Suppresses Inclusion Formation and Nonspecific Injury in Muscle Tissues—Overexpression of Hsp70 and Hsp40, which efficiently suppressed aggregation of a mutant ataxin-7 in a cellular model, did not inhibit the same aggregation in a mouse model (34). Furthermore, overexpression of Hsp70 in R6/2 Huntington model mice, which expressed exon 1 of the Huntington disease gene with an expanded CAG repeat, had only modest effects on aggregate formation and did not affect neurological phenotypes and survival (32, 33). Therefore, we crossed transgenic mice overexpressing hHSF1\(\Delta\)RD (HSF1Tg mice) (30) with R6/2 Huntington model mice (1, 36) and examined the effects of a hHSF1 on disease progression. As an active HSF1 is expressed in the skeletal muscle, as well as in the heart and testes (Fig. 4A), and the R6/2 mice show severe muscle atrophy with polyglutamine inclusions (50, 51), we first examined the skeletal muscle. We found that the weight of the quadriceps in 66-day-old R6/2 mice (233 ± 4.7 mg) was markedly lower as compared with wild-type mice (316 ± 4.0 mg), whereas the weight was partially restored in R6/2/HSF1 mice (262 ± 7.7 mg) (Fig. 4B). CSAs of type I and type II fibers of quadriceps in R6/2 mice were decreased by 18 and 10%, respectively (Fig. 4C). Notably, CSAs in both fibers were markedly restored in R6/2/HSF1 mice. Electron microscopy showed nuclear invagination (data not shown) and
membrane disintegration (Fig. 4D). Poorly osmophilic granular (1–15 nm in diameter) and filamentous (8–10 nm) intranuclear inclusions were characteristic. Furthermore, streaming of Z-band materials was noted in many myocytes in R6/2 mice. In R6/2/HSF1 mice, however, all of the above findings were modest, and myofilaments were well preserved (Fig. 4D). These results indicated that an active HSF1 suppresses nonspecific injury in the skeletal muscle.

Immunohistochemical analysis of the Huntington mice showed that the average number of inclusion-positive nuclei in 52-, 66-, and 87-day-old R6/2-HSF1Tg mice was decreased by 59, 65, and 79% of that of R6/2 mice, respectively (Fig. 5A). As decreased levels of Hsps might be deleterious to cells in R6/2 mice (33), we analyzed the expression of Hsps. Levels of Hsp110 and Hsp27 in the skeletal muscle and heart markedly decreased in 66-day-old R6/2 mice, whereas these were restored in R6/2-HSF1Tg mice (Fig. 5B). These results indicated that an active HSF1 efficiently inhibits inclusion formation in the skeletal muscle.

Increased Life Span in Huntington Model Mice Expressing an Active HSF1—R6/2 mice show severe weight loss from around 7 weeks of age and exhibit neurological phenotypes such as the claspng phenotype (1, 36). We examined the body weight and claspng phenotype in R6/2-HSF1Tg mice. The weight loss was not significantly improved in R6/2-HSF1Tg mice (Fig. 6A), and the paw-clasping phenotype when suspended by the tail was similarly observed (data not shown). Unexpectedly, the life span was significantly longer in R6/2-HSF1Tg mice, resulting in a mean survival of 122 days as compared with 107 days in R6/2 mice (Fig. 6B).

To confirm that neuronal degeneration was not improved in R6/2-HSF1Tg mice, we analyzed their brains. Levels of Hsps did not increase in the brains of HSF1Tg and R6/2-HSF1Tg mice, as no active HSF1 was expressed (Fig. 6C). Weight loss and atrophy of the brain in R6/2-HSF1Tg mice was not ameliorated as compared with R6/2 mice (Fig. 6D and E). Furthermore, the numbers of inclusion-positive nuclei in the cortex and striatum of 45–87-day-old R6/2-HSF1Tg mice were similar to those of R6/2 mice (Fig. 6E). These results clearly indicated that expression of an active HSF1 even in nonneural tissues improves the life span of Huntington disease mice.

**DISCUSSION**

Hsps, which act as molecular chaperones, are composed of diverse families of proteins that control folding of cellular proteins (12). Members of each family have specific roles in assisting folding of proteins and usually cooperate with others that belong to different families. Therefore, we expected that combined overexpression of a set of Hsps in cells
might more strongly suppress aggregate formation and subsequent cell death. In fact, the overexpression of both Hsp70 and Hsp40 synergistically inhibited inclusion formation in some culture cells (15–17) and in Drosophila (22). However, we showed here that the inhibitory effects of overexpression of combined Hsps on Q81-GFP inclusion formation in HeLa and PC12 cells were at similar levels to those of overexpression of a single Hsp (Fig. 3). The inhibitory effects of aggregate formation by Hsps may depend on the nature of abnormal proteins and cell types in which Hsps are differentially expressed. Importantly, the overexpression of an active human HSF1 inhibited the inclusion formation much more strongly than that of any Hsp, and overexpression of chicken HSF1 had some inhibitory effects without elevating Hsp levels. Our results indicated that the inhibitory effects of HSF1 on inclusion formation are not only mediated through the up-regulation of major Hsps but also through regulation of other Hsps or unidentified gene products.

HSF1 acts as a cell survival factor against various stresses such as high temperatures. Interestingly, chicken HSF1 has little ability to induce Hsps in heat shock conditions but has a marked cell survival function (49). Therefore, this function of HSF1 is mediated through regulation of unknown target genes other than major heat shock genes. This study further expanded the idea that the unidentified target genes of HSF1 may play roles in the inhibition of aggregate formation, as well as cell survival. A comprehensive chromatin immunoprecipitation analysis revealed that HSF1 binds to a lot of genes in vivo in human cells under normal growth conditions and that many of the genes are never induced after heat shock (52). Furthermore, HSF1, as well as HSF4, were shown to directly regulate growth factor genes such as FGF and IL-6 genes (41, 53). It is necessary to clarify which target gene is responsible for the inhibition of aggregate formation.

Polyglutamine-mediated neurodegeneration and eye phenotype are suppressed by overexpression of Hsp70 and Hsp40 in Drosophila (6, 21, 22), and overexpression of small Hsp or Hsp104 suppresses polyglu-
tamine aggregation in body wall muscle and extends life span in Caenorhabditis elegans (20, 54). In mice, overexpression of Hsp70 improved the motor phenotypes of SCA1 and spinobulbar muscular atrophy model mice (7, 23). In contrast, overexpression of Hsp70 and Hsp40 did not suppress polyglutamine toxicity in rod photoreceptor in SCA7 model mice (34). Furthermore, overexpression of Hsp70 in R6/2 Huntington model mice had no effect on life span or neuronal phenotypes and delayed aggregate formation only a little (32, 33). Thus, no one has been able to markedly suppress phenotypes, or even polyglutamine aggregates, by overexpressing Hsps in R6/2 Huntington model mice. In this study, we used these R6/2 model mice and showed that overexpression of an active HSF1 in nonneural tissues suppressed at least polyglutamine aggregates in skeletal muscle and markedly extended life span. Our observations suggested significant beneficial effects of the over-

FIGURE 4. An active HSF1 inhibits nonspecific injury of the skeletal tissue. A, expression levels of HSF1 in transgenic mice expressing hHSF1ΔRD (HSF1Tg). Tissue extracts of HSF1Tg mice were prepared and subjected to Western blot analysis using an anti-HSF1 antibody. An asterisk indicates endogenous HSF1, and an arrow indicates the product of transgene, hHSF1ΔRD. Adrenal gl., adrenal gland. B, weights of quadriceps of 52- and 66-day-old wild-type (WT), R6/2, and R6/2-HSF1Tg mice. Means and standard deviations of weights of six samples are shown. C, CSAs of type I and type II fibers of quadriceps in 66-day-old mice were estimated. Means and standard deviations of CSAs from each three mice are shown. Representative sections of R6/2/HSF1 mice look almost normal. Scale bar, 1 μm (100 nm in inset).

FIGURE 5. An active HSF1 inhibits inclusion formation in the skeletal tissue. A, the percentages of inclusion-positive nuclei in the quadriceps. Slices of the quadriceps isolated from 45–87-day-old R6/2 and R6/2-HSF1Tg mice were stained with anti-Huntingtin antibody (Ht) and with 4’,6-diamidino-2-phenylindole (DAPI). The numbers of nuclei with or without Huntingtin-positive inclusion were counted. Means and standard deviations of the percentages of nuclei containing the inclusion from six samples (upper) and representative pictures (lower) are shown. Arrows indicate inclusions. B, expression levels of HSF1 and Hsps in the skeletal muscle and heart. Extracts of the quadriceps isolated from 66-day-old mice were subjected to Western blot analysis using each specific antibody. WT, wild type.
expression of an active HSF1 on tissues expressing polyglutamine expansion.

It is unclear how the expression of polyglutamine expansion in nonneural tissues affects disease progression in human Huntington disease or in R6/2 Huntington disease model mice. Huntington protein is expressed in many tissues (55), and weight loss in several organs such as the heart and liver is observed in patients (56). In R6/2 mice, inclusion bodies are observed in a variety of tissues including the skeletal muscle, heart, liver, and pancreas (50). The severe muscle atrophy in R6/2 mice may mimic the wasting in human patients, and this mouse develops diabetes, a feature mimicking the elevated diabetes rate in human patients (57). We showed here that the overexpression of an active HSF1 in nonneural tissues not only partially restores weight loss of the skeletal muscle but also markedly expands life span. Our observations indicated that polyglutamine expansion has toxic effects on the skeletal muscle and suggested that suppression of the toxic effects on the skeletal muscle might have a substantial impact on the disease progression of Huntington disease.

One therapeutic strategy for polyglutamine disease would be to elevate molecular chaperones that assist normal protein folding and prevent abnormal folding and aggregation formation (58). It was shown that treatment with arimoclomol, a coinducer of heat shock proteins by activating HSF1, delays disease progression in superoxide dismutase mutant mice (59). Our results suggested that a reagent that activates HSF1 might have beneficial effects at least on life span in Huntington disease and other neurodegenerative diseases, although it does not cross the blood-brain barrier.

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