Heat Shock Protein Accumulation Is Upregulated in a Long-Lived Mutant of Caenorhabditis elegans

Glenda A. Walker,1 Tiffany M. White,3 Gawain McColl,1 Nicole L. Jenkins,1 Sandra Babich,2 E. Peter M. Candido,2 Thomas E. Johnson,3 and Gordon J. Lithgow1

1School of Biological Sciences, University of Manchester, United Kingdom.
2Department of Biochemistry, University of British Columbia, Vancouver, Canada.
3Institute of Behavioral Genetics, University of Colorado, Boulder.

We present evidence for elevated levels of heat shock protein 16 (HSP16) in an intrinsically thermotolerant, long-lived strain of Caenorhabditis elegans during and after heat stress. Mutation of the age-1 gene, encoding a phosphatidylinositol 3-kinase catalytic subunit, results in both extended life span (Age) and increased intrinsic thermotolerance (Itt) in adult hermaphrodites. We subjected age-synchronous cohorts of worms to lethal and nonlethal thermal stress and observed the accumulation of a small (16–18 kd) heat-shock-specific polypeptide detected by an antibody raised against C. elegans HSP16. Strains carrying the mutation hx546 consistently accumulated HSP16 to higher levels than a wild-type strain. Significantly, overaccumulation of HSP16 in the age-1(hx546) strain following heat was observed throughout the adult life span. A chimeric transgene containing the Escherichia coli β-galactosidase gene fused to a C. elegans HSP16-41 transcriptional promoter was introduced into wild-type and age-1(hx546) backgrounds. Heat-inducible expression of the transgene was elevated in the age-1(hx546) strain compared with the wild-type strain under a wide variety of heat shock and recovery conditions. These observations are consistent with a model in which Age mutations exhibit thermotolerance and extended life span as a result of elevated levels of molecular chaperones.

LIFE span of Caenorhabditis elegans is determined, in part, by a pathway resembling the insulin signaling pathway in mammals. Mutations in some genes encoding components of the pathway cause increased mean and maximum life span (Age phenotype), slow the rate of aging, and also enhance resistance to stress (1–3).

Almost all Age mutations are pleiotropic, affecting early life history traits as well as longevity (1,3). Many of the proposed mechanisms of life-span extension by Age mutations come from studies of these associated phenotypes. During development, the insulinlike signaling pathway is one of several pathways that regulate the formation of a specialized larval diapause stage, called the dauer larvae (4). Dauer larvae develop from L1 worms during nutritional deprivation and in response to a pheromone signal. The dauer larva is nonfeeding, nonreproducing, stress resistant, and capable of surviving four to eight times longer than the adult (5).

Loss-of-function mutations of either daf-2, which encodes a protein similar to vertebrate insulin-receptor (6), or age-1, which encodes a potential catalytic subunit of phosphatidylinositol-3-kinase (PI3K) (7), extend adult hermaphrodite life span by up to 100% (8,9). Loss-of-function mutations of daf-16 (which encodes a forkhead transcription factorlike protein) (10,11) suppress both the Age phenotype and Daf-c phenotypes of age-1 and daf-2 mutations (12–14). These mutations are also suppressed by mutations in daf-18, which encodes a protein similar to the human PTEN, which exhibits its phosphatidylinositol 3,4,5-trisphosphate (PIP3) 3-phosphatase activity (15,16). Additionally, genes encoding homologues of mammalian Akt/PKB and PDK-1 proteins act in this signaling pathway between age-1 and daf-16 (17). Taken together, an insulinlike signaling pathway is necessary for normal development and normal life span of C. elegans. The cell and tissue specificity of the action of this pathway has been investigated by using a series of daf-2 genetic mosaics (18). It appears that daf-2 may function in many cell types, but its activity in neurons and support cells is sufficient to determine development and life span.

The mechanism of life-span extension in the Age mutants remains unknown, but we have proposed that it results in part from the overaccumulation of molecular chaperone molecules (19–21). This was prompted by the observation that mutations leading to extended life span also confer intrinsic thermotolerance (Itt) (19,20,22). Organisms respond to thermal stress by accumulating heat shock proteins (HSPs), many of which exhibit molecular chaperone activity (23). The HSPs are encoded by distinct gene families, and expression of individual genes can confer resistance to lethal heat stress (24); for example, hsp104 confers tolerance to a number of environmental stresses in Saccharomyces cerevisiae (25), and inducible hsp70 confers thermotolerance in Drosophila embryos (26). The small HSP family, characterized by an α-crystallin domain, also contributes to thermotolerance. The hsp27 accumulates and is phosphory-
lated in response to a number of stimuli, including heat shock (27). The protein is an actin polymerization modulator and appears to be a key modulator of stress-activated apoptosis as a consequence of its role in F-actin accumulation following stress (28). The relationship between hsp27 and thermotolerance has been investigated in a number of heterologous expression studies. Expression of human hsp27 in rodent cells confers thermotolerance (27) and expression of Drosophila hsp27 in COS cells leads to tolerance of thermal and oxidative stress (29).

We have investigated the expression of a small hsp gene in Caenorhabditis elegans encoding the hsp27 homologue, HSP16. As many as eight genes, at three loci, encode the members of the HSP16 family (30). HSP16-2 is known to form large oligomeric complexes and to function as a molecular chaperone (31). We have measured the accumulation of HSP16s under a variety of heat shock and recovery conditions, in young through to late-life adults and in both wild-type strains and a long-lived age-1 mutant strain. This study demonstrates that the mutation, age-1(hx546), confers an upregulation of HSP16 genes in response to thermal stress and consequently that an insulinlike signaling pathway regulates at least one family of hsp genes in C. elegans.

METHODS

Strains and Culture of Nematodes

The wild-type Bristol (N2) strain and TJ1052 [age-1(hx546)]II were obtained from the Caenorhabditis Genetics Center. All strains were routinely grown at 20°C on small nematode growth media (NGM) agar plates (32,33). Synchronous populations of hermaphrodites were established by picking eggs from mixed populations onto new plates and allowing them to develop at 20°C.

Life Span and Thermotolerance Assays

Life span and thermotolerance assays were undertaken as previously described (19,20). Briefly, to assess thermotolerance, two populations of twenty-five 3- or 4-day-old hermaphrodites were shifted from 20°C to 35°C and scored for touch-provoked movement and pharyngeal pumping. Animals not pumping or responding to touch were scored as dead. Survival was scored on two populations of age-synchronized hermaphrodites at 20°C on NGM agar plates. Statistical analysis was performed by standard methods (19).

Antibody Generation

Polyclonal antibodies to HSP16-2 were prepared in rabbits as previously described (34).

Preparation of Worm Extracts and Western Blotting

Four-day-old adult hermaphrodites were transferred from 20°C and placed at either 30°C, 33°C, or 35°C for various lengths of time up to 24 hours. Sixty adult animals were counted and picked at each time point into S-basal (33) in a siliconized eppendorf tube, washed once in S-basal, and frozen in liquid N₂. No larvae were present in these samples.

For quantitative Western analysis, two identical gels were prepared. One gel was incubated in 20 ml of Sypro-red protein stain (Molecular Probes; Eugene, OR) for 50 minutes. The stained gel was visualized by using an ultraviolet light source and was photographed with a Sypro-red photographic filter (Cat. #S-6656). Following transfer of the second gel, the membrane and the gel were stained with Ponceau S and Coomassie Blue, respectively, to verify successful transfer. The membrane was incubated with primary rabbit anti-C. elegans HSP16 antibody diluted 1:10,000 in blocking buffer and then with secondary, goat anti-rabbit IgG antibody/ horseradish peroxidase conjugate (Sigma; Poole, Dorset, England), diluted 1:5000 in blocking buffer for 1.5 hours on a shaker. Detection was undertaken with chemiluminescent reagents (SuperSignal, Pierce; Rockford, IL) and standard autoradiography. For comparison of worms across life span, signals were normalized to that for heat shocked, young wild-type worms.

Transgene Copy Number Determination

Chromosomal DNA was prepared from Tj830, Tj831, and N2 (wild-type) strains and probed with the plasmid pBlue-script, which contains the bacterial lacZ. Identical restriction fragments were observed for both transgenic strains. Copy number was assessed by probing with a control catalase gene. Autoradiographs were digitally scanned and the signal was quantified by using the Sigma Gel software package. The signal was normalized for length and specific activity of probe.

Generation of Aged Worms

Three-day-old hermaphrodites were transferred from NGM plates and then aged in liquid media. Hermaphrodites and their progeny were separated by culturing populations in 40 μm nylon sieves. At each age tested, three replicates of 60 adult worms were treated with a 2-hour exposure at 35°C with a 24-hour recovery at 20°C, and then 40 survivors were collected as above and frozen in liquid N₂. No larvae were present in these samples. Quantitative Western analysis was performed as described above.

Histochemical β-Galactosidase Staining

Three- to four-day gravid hermaphrodites were raised at 20°C were shifted to 30°C, 33°C, or 35°C. At indicated times, plates were removed and both adults and larvae were washed off in S-basal immediately or allowed to recover at 20°C for 30 minutes to 10 hours. After gentle centrifugation, the S-basal was removed and the worms were frozen with liquid N₂. Samples were histochemically stained to detect β-galactosidase. For histochemical staining, worms were thawed on ice, methanol permeabilized, and washed in 10 mM of Tris buffer. Staining solution was added [21 mM of NaH₂PO₄, 104 mM of Na₂HPO₄, 0.01 mM of MgCl₂, 5 mM of K₃Fe(CN)₆, 5 mM of K₄Fe(CN)₆, 40 μg ml of sodium dodecyl sulfate (SDS), 240 μg ml of XGal, and 1.2% N,N-dimethylformamide], and worms were incubated in a 37°C water bath overnight before examination. The worms were scored blind by two researchers for both the intensity and location of staining.

RESULTS

Accumulation of HSP16 in Young Worms During Heat Shock

We examined the accumulation of HSP16 during heat shock at 30°C, 33°C, and 35°C in age-1(hx546) and wild-
type worms. Hermaphrodite worms were grown at 20°C for 4 days and then were subjected to heat shock. Every 2 hours during the heat shock, subpopulations of worms were analyzed for HSP16 levels. Upon heat shock at 30°C, 33°C, or 35°C, rapid accumulation of a heat shock protein species of approximately 16–18 kd was detected in both wild-type and age-1(hx546) worms. Quantitation of signals on the Western blots indicates that HSP16 accumulates preferentially in age-1(hx546) worms at all heat shock temperatures tested. Figure 1 illustrates the results of four replicate experiments in which, after 4 hours at 35°C, the age-1(hx546) worms accumulated approximately five times more detectable HSP16 than wild-type strains. By the eighth hour of the heat shock, the levels of HSP16 continued to rise in both strains, and wild-type worms were beginning to die. As previously reported, there was no detectable HSP16 at 20°C in young wild-type worms. (35,36). Likewise, HSP16 was not detected in age-1(hx546) worms at 20°C (data not shown).

**Abundance of HSP16 in Young Worms During Recovery From Heat Shock**

A significant age-1-associated over-accumulation of HSP16 was observed during recovery from heat shock. Synchronous, 4-day-old populations of hermaphrodites were heat shocked at 35°C for 2 hours and then returned to 20°C for periods up to 72 hours. Subsamples of 60 worms were analyzed on quantitative Western blots. In four independent experiments (Figure 2), HSP16 continued to accumulate in wild-type worms during the first 24 hours following the termination of the 2-hour heat shock and then declined over the next 48 hours. However, worms carrying the age-1 mutation maintained elevated HSP16 for a further 24 hours. Thus, during recovery from heat shock, age-1(hx546) worms accumulate up to eightfold higher levels of HSP16.

**Accumulation of HSP16 in Aged Worms During Recovery From Heat Shock**

The abundance of HSP16 in response to a heat shock stress at various ages across life span is shown in Figure 3. The long-lived age-1(hx546) mutants exhibited elevated induced levels of HSP16 at all ages examined (Mann-Whitney test, p < .05), with the exception of day 12. No constitutive expression of HSP16 was detected until day 16, where low levels were observed in both genotypes (data not shown).
shown). This constitutive expression did not significantly differ between genotypes and amounted to approximately 4% relative to induced expression in 4-day-old wild-type worms.

Construction of Strains Carrying HSP16::LacZ Reporter Construct

In order to further investigate the age-1-associated overaccumulation of HSP16 we constructed wild-type and age-1(hx546) strains containing a chromosomally located reporter transgene (zIs1). We obtained strain BW1601 from W. Wood (University of Colorado) that contained an extrachromosomal array (ctEx5) in which the divergent transcriptional promoter from the HSP16A locus is ligated to the bacterial lacZ coding region (pPD49.83) to form a translational fusion (Figure 4A). This construct had been co-injected with the dominant rol-6 marker (38) and Rol worms picked resulting in BW1601, which was then subjected to /H9253 irradiation to induce chromosomal integration. Strains exhibiting high-frequency roller phenotype were then backcrossed five times to either N2 (wild-type) or TJ1052 [age-1(hx546)]. The resulting strains [TJ830 (zIs1) and TJ831 (zIs1; age-1(hx546))] were then assessed for Age and Itt (Figure 4). TJ831 is Age compared to TJ830 (Figure 4B; p < .001). TJ831 also exhibited the Itt phenotype when compared with N2 (wild-type) and TJ830 (Figure 4C; p < .0001). The structure and copy number of the transgenic array zIs1 was examined in both strains by Southern blotting. Identical restriction fragments and very similar copy number (100 copies) were observed for both strains (data not shown).

We tested whether the presence of multiple copies of the hsp16 transcriptional promoter in zIs1 affected the age-1-associated HSP16 overaccumulation previously observed. The accumulation of endogenous HSP16 during heat shock in TJ830 and TJ831 was measured (Figure 4D). Synchronous cultures of 4-day-old hermaphrodites were heat shocked at 35°C. Survival was monitored during the heat shock in two populations of 25 animals. TJ831 was significantly more thermotolerant than TJ830 (p < .0001). At each time point, subsamples of 60 animals were rapidly frozen for Western analysis. HSP16 accumulates in both strains at 35°C, but after 8 hours at 35°C, there is at least a fivefold...
difference in abundance of HSP16 between the two strains. Hence the presence of extra copies of the HSP16 transcriptional promoter does not suppress the age-1-associated overaccumulation of HSP16 (Figure 4D).

**Reporter Gene Expression in an Age-1(hx546) Strain**

We carried out a series of heat shock and recovery protocols and examined β-galactosidase activity. In the absence of heat shock, no β-galactosidase activity (β-gal) was detected in either TJ830 or TJ831 (data not shown). Heat shocks at 30°C, 33°C, and 35°C all induce β-galactosidase activity in both strains as revealed by histochemical staining. Distinct tissue-specific expression patterns were observed for different heat shock conditions, and considerable variation in the extent of staining was observed between different experiments. We recorded the number of animals in a sample exhibiting staining in either the pharynx, developing embryos, or throughout the entire body of the worm. A series of heat shock and recovery conditions were identified in which TJ830 and TJ831 exhibited distinct responses. For example, after a 30-minute heat shock at 35°C, followed by a 1-hour recovery, age-1(hx546) worms exhibited β-gal activity throughout but no activity was detectable in wild-type worms. A series of 30-minute heat shocks at 35°C followed by variable recovery periods were undertaken to test the reproducibility of this finding (Table 1 and Figure 5). In six out of seven independent experiments, age-1(hx546)-specific elevation of β-galactosidase activity was observed in that the proportion of worms exhibiting β-gal activity was greater, as was the intensity of β-gal staining. It should be noted that there was considerable variation between experiments in the degree of response from both strains. Figure 5 illustrates a recovery time course following a 30-minute heat shock at 35°C. Worms were stained 1, 2, and 10 hours after the heat shock; only very faint staining was observed in all wild-type worms, whereas 48 of 50 age-1 mutant worms exhibited extensive β-gal activity. Similar time courses were performed after heat shocks at 30°C and 33°C with similar results (data not shown).

**Table 1. β-galactosidase Activity Following Mild Heat Shock and Recovery**

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<th>Experiment</th>
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<th>Pharynx†</th>
<th>Whole Body†</th>
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*Worms were recovered at 20°C following a 30-min heat shock at 35°C.
†Number of worms staining/number of worms scored.

**Figure 5. Histochemical staining for β-galactosidase activity in transgenic lines.** Cohorts of TJ831[zIs1; age-1(hx546)] and TJ830[zIs1] transgenic worms were grown at 20°C until 4 days of age and then heat shocked at 35°C for 30 minutes. The worms were then returned to 20°C to recover and subpopulations were frozen on liquid N2. A, wild type, 1-hour recovery; B, wild type, 2-hour recovery; C, wild type, 10-hour recovery; D, age-1(hx546), 1-hour recovery; E, age-1(hx546), 2-hour recovery; F, age-1(hx546), 10-hour recovery.

**DISCUSSION**

Because many Age mutations also confer intrinsic thermotolerance, we have proposed that Age and lrt are mechanistically related (19–21). We have investigated the accumulation of a family of small heat shock proteins (HSP16) homologous with mammalian HSP27. Previous studies have shown that HSP16 genes are not expressed in the absence of thermal stress (35,39,40). Here we have shown that when hsp16 gene expression is induced during heat shock, the resulting level of HSP16 is dependent upon AGE1, a protein that acts in an insulin-like signaling pathway in C. elegans.

We examined strains of nematode worms mutant in age-1 during thermal stress and recovery from thermal stress. Under both conditions, HSP16 accumulates in the age-1-mutant worms to levels up to eight times higher than in wild-type worms. We also examined worms at seven different ages across the life span and found that adult age-1 mutants, when challenged by heat shock, generally accumulated greater levels of HSP16 compared with wild-type strains of the same chronological age. Mutation of age-1 also influenced the levels of a reporter activity (β-galactosidase) under the transcriptional control of an HSP16 promoter. Histochemical
staining for \( \beta \)-galactosidase activity of worms exposed to different heat shock and recovery regimes revealed a number of mild heat shock conditions in which \( age-1(hx546) \) conferred enhanced induction of the transgene. There was considerable variation in the response of both strains between experiments, but the \( age-1 \) background consistently led to higher levels of \( \beta \)-galactosidase activity in individual worms and also to a greater proportion of worms showing activity. This was true in adults, larvae, and developing embryos visible within the hermaphrodite worms.

These reporter gene experiments suggest that altered HSP16 levels in Age worms arise from an alteration of gene expression, although we have not ruled out the possibility that the turnover rate of HSP16 protein is also altered in the mutant strain. As the \( lacZ \) reporter transgene contains the 5\'-untranslated region of \( hsp-16 \), the gene expression alteration may be at the level of translation or the rate of accumulation of the \( hsp16 \) mRNA. The regulation of HSP genes has not been extensively studied in the nematode, although the \( hsp-16 \) promoter does contain sequences that are similar to the binding site for the heat shock factor (HSF) transcriptional regulator (40). Mutation of the \( age-1 \) does not seem to cause a constitutive derepression of \( hsp16 \) gene expression, but when \( hsp16 \) is induced, wild-type AGE1 appears to repress expression. Therefore, mutation of \( age-1 \) may increase the level of \( hsp16 \) gene transcription, perhaps by increasing the activity of HSF or by reducing the activity of a negative regulator such as heat shock factor binding protein (HSF-BP1) (41).

The results presented here are consistent with the observation that \( age-1 \) mutants are Ilt (20) and that an inhibitor of PI3K activity, LY294002, causes thermotolerance (42). However, it is unlikely that expression of the \( hsp16 \) gene family alone accounts for all the stress phenotypes associated with these mutants. Mutation of \( age-1 \), for example, also results in resistance to oxidative stress (43,44), ultraviolet radiation stress (45) and high concentrations of heavy metals (46). Consistent with these phenotypes, \( age-1 \) mutant worms have elevated levels of the antioxidant enzymes superoxide dismutase and catalase (43,44) and also overexpress at least one metallothionein gene (46).

Taken together, these results suggest that the insulinlike signaling pathway coordinately regulates the expression of a range of stress proteins. Furthermore, this is consistent with a role for this pathway in dauer formation, as dauers are also resistant to a range of environmental stresses (47).

The results presented here support an emerging picture of the role of stress proteins during normal aging (2,22). Acclimation of both \( C. \) elegans and Drosophila to heat shock also results in extended life span (20,48). Drosophila carrying extra copies of the inducible \( hsp70 \) gene exhibit even greater heat-acclimation-associated increase in survival (49). In addition, a series of publications illustrate that overexpression of antioxidant enzyme genes extends the life span of Drosophila (50–52).

We propose that the relationship between thermal stress and life span is based upon the rate at which conformationally altered protein accumulates both during thermal stress and during aging under nonstressed conditions (53–56). As molecular chaperones promote the refolding of protein from a nonnative to a native state and prevent aggregate formation, the presence of chaperones during both thermal stress and aging may be advantageous. Two additional observations are consistent with this view. The first is that HSP expression is induced during normal aging in Drosophila. Inducible forms of \( hsp22 \) and \( hsp70 \) are expressed in the absence of any environmental stress and, it is likely that this is due to the accumulation of oxidatively damaged proteins (57). We have also observed the accumulation of small amounts of HSP16 at late ages in the worm (data not shown). The second observation is that caloric restriction, in which rodent life span is significantly increased by a controlled reduction in caloric intake, leads to a more robust heat shock response and overexpression of \( hsp70 \), \( hsp27 \), and \( hsp-90 \) (58). Taken together, these findings suggest that molecular chaperones are candidates for longevity-associated proteins.

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Tiffany White is currently with the Department of Microbiology, University of Colorado, Denver. Glenda Walker is currently with the Department of Veterinary Parasitology, University of Glasgow, Scotland. Gawain McColl and Nicole Jenkins are currently with the Buck Institute for Age Research, Novato, California.

Address correspondence to Gordon Lithgow, The Buck Institute for Age Research, Novato, CA 94949. E-mail: glithgow@buckinstitute.org

References


