Expression of CeHSP17 Protein in Response to Heat Shock and Heavy Metal Ions

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Abstract: Small heat shock proteins (sHSP) are ubiquitously found in all organisms, and with other heat shock proteins (HSP) such as HSP60, HSP70, HSP90, HSP100 made up the molecular chaperone family. They are involved in a wide range of biological processes which include among others cell resistance to biological and environmental stress conditions. In this study, we show by western blotting that CeHSP17, an sHSP of Caenorhabditis elegans, is significantly induced by high temperatures. Furthermore, in response to metal stress, the CeHSP17 protein expression was significantly induced by cadmium and zinc at high concentration of clearly cytotoxic range in wild-type C. elegans. Altogether, our results show the involvement of CeHSP17 protein in both environmental and biological stresses in C. elegans and establish for the first time the expression pattern of the CeHSP17 protein in response to thermal and metal stress conditions in C. elegans. The responses of CeHSP17 protein expression may serve as potential sensitive biomarker for metal-induced toxicity monitoring and environmental risk assessment.

Key words: biomarker, cadmium, CeHSP17, C. elegans, small heat shock proteins, zinc.

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Organisms respond to environmental challenges which include among others thermal and metal stress by accumulating HSP. Based on current understanding, the biological function of HSP is to carry on the generalized “quality control” to other proteins in the cell and play an important role in the process of “life, aging, disease, and death” of the protein (Hartl and Hayer-Hartl, 2002). In vitro, HSP have a kind of chaperone-like activity, by preventing the irreversible aggregation of denatured (or partially denatured) substrate proteins (also called client proteins) and help in the correct folding of the substrate proteins. In vivo, HSP play a central role in protein homeostasis: they safeguard the structure conformation and folding of nascent proteins, assist in the assembly and disassembly of protein complexes, in protein degradation, etc. (Bukau and Horwich, 1998; Hartl and Hayer-Hartl, 2002; Kim et al., 2007). HSP are classified by their molecular weight into HSP60, HSP70, HSP90, HSP100, and sHSP (Parssell and Lindquist, 1993; Candido, 2002).

The sHSP as a family of molecular chaperones are characterized by low molecular mass of 12 to 23 kDa (MacRae, 2000), possession of a conserved α-crystallin domain, and formation of large oligomers (Cobb and Pettrash, 2000; Haslbeck et al., 2005; Sun and MacRae, 2005). Among the molecular chaperone families, sHSP are the only known ATP-independent chaperones (Jakob et al., 1993) and act as “holdase” for partially folded intermediates under stress conditions, which can be released and refolded at optimal conditions with the help of ATP-dependent chaperones (Wang and Spector, 2000; Cashikar et al., 2005). Small heat shock proteins are ubiquitously present in entire living organisms from bacteria to human cells (Caspers et al., 1995; Bult et al., 1996; Narberhaus, 2002; Laksanalamai and Robb, 2004).

The nematode C. elegans, is a model organism for molecular and ecotoxicological studies based on certain characteristics such as sensitivity to different kinds of stress, ease of culture, transparent body, small body size, short life span, complete and well-characterized genome, etc. (Brenner, 1974). It has 16 sHSP (Ding and Candido, 2000; Haslbeck et al., 2005), one of which is CeHSP17. However, relatively little known information is available about CeHSP17 molecular function and properties in C. elegans (Candido, 2002). Recently, we have reported our findings on the ability of CeHSP17 protein to enable growth of Escherichia coli cells at a lethal temperature of 50°C (Ezemaduka et al., 2014) and mechanistically show that CeHSP17 protein could exhibit chaperone-like activity in preventing the stress-induced aggregation of model substrate proteins through the formation of super-molecular assemblies (Zhang et al., 2015). Despite these findings, we are still challenged with what CeHSP17 protein does as a molecular chaperone in C. elegans. Therefore, the aim of the present study is to characterize the molecular functional roles of the CeHSP17 protein in C. elegans adaptation to both thermal and metallic stress conditions. In lieu of the above, we subjected age-synchronized worms to both lethal and nonlethal thermal stress and varied heavy metal toxicity. We measured the accumulation of CeHSP17 under a variety of heat shock and recovery state, in young through aged adult wild-type worms, and at different concentrations of cadmium and zinc ions. Our study provides evidence that elevated temperatures significantly induce the expression of CeHSP17. Also, that cadmium and zinc metals induce CeHSP17 expression especially at high concentration of clearly cytotoxic range.

Materials and Methods

Nematode strain, maintenance, and propagation: Wild-type C. elegans N2 strains (var. Bristol) used in this study.
were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN). Worms were kept at 20°C on nematode growth medium (NGM) agar plates and fed with OP50 strain *E. coli* according to the standard protocol (Brenner, 1974). Age-synchronized adult worms obtained from eggs isolated from gravid adult hermaphrodites by sodium hypochlorite treatment (Fabian and Johnson, 1994) were used in all experiments.

Heat stress assay: For heat shock experiments, three replicates of 3-12-d-old synchronous adult hermaphrodites grown on NGM plates were transferred from 20°C to 35°C-42°C for various lengths of time up to 8 hr. At 1-2 hr intervals, the worms were removed and recovered at 20°C for 2 hr to 72 hr. At each temperature and age, 60 survivors were harvested and boiled in SDS-loading buffer to be analyzed by 10% Tricine SDS-PAGE and western immunoblotting. Heat stress assays were performed in triplicate and repeated at least twice.

Cadmium and zinc treatment for CeHSP17 protein expression: Young adults (3-d-old) from age-synchronous populations were transferred from NGM plates into 24-well microtiter plates containing 0.5 ml of K-medium (53 mM NaCl, 32 mM KCl) with Cd (0.1 and 8 mM) or Zn (0.1 and 6 mM) or K-medium without the metal salt (zero concentration) used as control. Zinc was selected because this metal ion is a trace metal essential for all life forms. Cadmium, although a nonessential metal with no known biological function in metazoa, represents a common industrial pollutant. Worms were incubated at 20°C or 30°C for 8 hr, recovered by centrifugation, and washed several times with M9 buffer. Approximately 50 (50 ± 1) live worms were picked and boiled in SDS-loading buffer to be analyzed by 10% Tricine SDS-PAGE and western immunoblotting. The toxicity tests were performed at least twice in triplicate.

Immunoblotting analysis and antibodies: Immunoblotting analysis of *C. elegans* worm extracts was performed as previously described (Ezemaduka et al., 2014). Briefly, 50, 60, and 80 worm lysates containing about 1.1, 1.4, and 2.0 mg/ml total protein concentration, respectively, were resolved on 10% Tricine SDS-PAGE and transferred to polyvinylidene difluoride membranes. Western blotting was performed using primary rabbit anti-CeHSP17 polyclonal antibody (Ezemaduka et al., 2014). The secondary antibody, goat anti-rabbit IgG–horseradish peroxidase conjugate was obtained from Transgene. Protein bands were visualized with nitroblue tetrazolium (Amresco, Solon, OH) and 5-bromo-4-chloro-3-indolylphosphate (Promega, Madison, WI). Actin monoclonal antibody purchased from Santa Cruz Biotech (product no. sc47778; Dallas, TX) was used as the loading control in all western blot analysis.

Statistical analysis: The levels of CeHSP17 protein in each sample were normalized against the level of actin in the same samples and shown as the mean values ±SE. Student *t*-test was applied to study the relationship between the normalized levels of the protein in treated groups and those of the nonexposed controls. Differences were considered significant when *P* < 0.05. All statistical analyses were conducted using SPSS 12 (SPSS, Chicago, IL). At least, three replicates were performed for statistical purposes.

**RESULTS**

Effects of temperature on CeHSP17 protein level in *C. elegans*: To determine whether CeHSP17 protein is inducible by thermal stress, we examined the expression of CeHSP17 at different temperature treatments in wild-type worms. Synchronized 3-d-old worms grown at 20°C were subjected to 25, 30, 35, and 40°C temperature treatments, and CeHSP17 protein levels were analyzed by western blotting (Fig. 1). On shifting temperature treatments other than the *C. elegans* physiological growth temperature of 20°C (herein referred to as control temperature), a marked increase in the level of CeHSP17 protein was detected in wild type-worms at 25, 30, and 35°C (Fig. 1A, lane 2, 4, and 6) and no increase in the level of CeHSP17 was detected in *C. elegans* heat shocked at 40°C lethal temperature of (Fig. 1, lane 8). It is worth noting that at 20°C, the expression of CeHSP17 is at a very low level which presumably is a characteristic of heat inducibility rather than constitutive expression. Significant increase of about threefold in CeHSP17 protein expression was seen at 30°C (Fig. 1A, lane 4) when compared with 25, 35, and 40°C temperature treatments (Fig. 1A, lanes 2, 6, and 8). However, subsequent exposure of worms to a higher temperature of 42°C which would be rather deleterious resulted in upregulation of CeHSP17 accumulation in worms (Fig. 1, lanes 3, 5, and 7). Furthermore, the expression level of CeHSP17 in wild-type worms showed a significant increase from 4 to 6 hr, with a peak value of threefold more than that of the control after heat shock at 30°C, then it gradually decreased and returned to the control level at 8 hr (Fig. 1B).

CeHSP17 abundance in young worms during recovery from heat shock: Synchronous 3-d-old worms were heat shocked at 30°C for 2 hr and allowed to recover at 20°C for 12 hr up to a period of 72 hr. Approximately 80 worms were picked at each indicated time interval and analyzed by western blotting. As shown in Fig. 2, the accumulation of CeHSP17 was upregulated and maintained in wild-type worms even after the 72-hr heat shock treatment. Subsequently, we investigated whether the CeHSP17 protein is upregulated in response to thermal stress at various ages across the *C. elegans* life span. Data in Fig. 3 showed an induced level of CeHSP17 at all tested ages of wild-type worms recovering from 30°C heat shock treatment. CeHSP17 accumulates in 8- and 12-d-old worms to levels up to two- and threefold, respectively, more than in unstressed 4-d-old worms. Notably, the CeHSP17 protein shows little increase in basal expression as the worms get older, although the increase was not statistically significant.

Effect of metal stress on the expression of CeHSP17 in *C. elegans*: Given the fact that sHSP synthesis can be highly
induced by many environmental stimuli such as extreme temperature, oxidative stress, heavy metals, and toxins (Courgeon et al., 1988; Kitagawa et al., 2000; Li et al., 2010; Ezemaduka et al., 2014), it is of no doubt that CeHSP17 may also be induced by metal stress. We therefore investigated the expression of CeHSP17 in worms treated with heavy metal ions. After 20 hr of exposures to different concentrations of Cd and Zn metals at 20°C, western blot analysis confirmed that the CeHSP7 protein is strongly induced by Cd²⁺ and Zn²⁺ at their highest metal concentration of 8 and 6 mM, respectively (Fig. 4), with the highest point of about three- and twofold higher than that of the control group, respectively. However, the CeHSP17 protein level showed moderate increase after exposure to 0.1 mM of Cd (Fig. 5A) compared with 0.1 mM of Zn (Fig. 5B).

Effect of heat shock and metal toxicity on the expression of CeHSP17 protein in C. elegans: To further assess if the CeHSP17 protein is upregulated by combined thermal and metal stress in C. elegans, the protein expression was analyzed in synchronous young adult populations subjected to different concentrations of cadmium and zinc at 30°C. As shown in Fig. 5, worms exposed to Cd and Zn at 30°C exhibited increase in the expression of CeHSP17 protein. The CeHSP17 protein level for 0.1 and 8 mM Cd-treated worms at 30°C showed moderate increase than that found in untreated control (Fig. 5A). Zinc exposures also resulted in an increase in CeHSP17 protein expression at all assayed concentrations, with significant increase about twofold more than that found in control worms after exposure to 0.1 mM Zn at 30°C for 8 hr (Fig. 5B).

DISCUSSION

Small heat shock proteins as a molecular chaperone are basically known by the rapid induction of their expression in response to stresses such as heat, metal, reactive oxygen species, etc. Therefore, they are defined as a molecular chaperone...
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(Leung et al., 2008), one of the objectives of this study was to evaluate the early response of the CeHSP17 protein under different environmental stressors, including temperature and heavy metals. In the present work, we characterized the biological function of CeHSP17 protein from *C. elegans* under a variety of experimental conditions and showed for the first time that this protein is highly inducible by temperature shifts and heavy metal ions.

Our initial experiments showed that when worms were treated at temperature above 20°C (*C. elegans* physiological growth temperature) for 1 hr, the expression of CeHSP17 increased, and the CeHSP17 protein expression appeared to be significantly up-regulated by a heat shock at 30°C (Fig. 1), as expected because high temperature is the classic inducer of sHSP, suggesting that CeHSP17 may play an important role in preventing the natural host from thermal stress damage or may assist to repair the damages caused by such thermal insult. Similarly, *C. elegans* HSP16 (Dixon et al., 1990) and other sHSP from mammalian cells (Landry et al., 1989), plants (Soto et al., 1999; Basha et al., 2004), and prokaryotes (Torok et al., 2001) have been demonstrated to be induced by heat, and correlated with protection against thermal stress.

It is worthy to note that the CeHSP17 protein accumulation showed increase following subsequent treatment at 42°C lethal temperature (Fig. 1). This suggests that CeHSP17 is not only highly induced in response to sublethal heat shock treatments, but the increased

by their ability to bind to denatured proteins arising from such stress, and prevent their irreversible aggregation. As *C. elegans* is considered an excellent animal model for biomedical and environmental toxicology

![Fig. 3. Basal and heat shock expression of CeHSP17 protein across Caenorhabditis elegans life span. Worms at different age across the life span were either cultured at 20°C or exposed at 30°C and recovered after exposure at 20°C for 2 hr. The worm samples were prepared for western blot analysis with antibodies against CeHSP17. The CeHSP17 basal expression level in 4-d-old untreated control worms was set to one. Significant differences (P < 0.05) from same age are denoted by "*" and significant differences (P < 0.05) between same temperature are denoted by "#."](image)

![Fig. 4. Expression levels of the CeHSP17 protein in worms treated with 0, 0.1, and 8 mM of CdCl₂ (A), and 0, 0.1, and 6 mM of ZnCl₂ (B). The expression level of untreated control worms (0 mM) was set to one. Significant differences *P ≤ 0.05 compared with control. Data are presented as mean "±SEM."](image)
expression also preconditions the worms against subsequent high temperature challenge that would rather be lethal to the organism development. Several studies have reported HSP to correlate with acquired stress resistance termed hormesis, which is the ability of a moderate stress to protect the animal from a subsequent and lethal stress (Lindquist and Craig, 1988; Jaattela and Wissing, 1992; Mailhos et al., 1993; Hercus et al., 2003). The detectable decrease in the expression of CeHSP17 at a high temperature of 40°C, and even at subsequent 42°C degree challenge is not clear, but may be explained by the lack of protein translation at such high temperatures which hinder the steps involved in protein synthesis (Sciandra and Subjeck, 1984). In addition, the expression changes of CeHSP17 at different ages across the *C. elegans* life span were different even at same temperature stress (Fig. 3), implicating that the impact of temperature stress on different age might be different.

Regarding CeHSP17 induction by metals, our data also show that high concentrations of cadmium and zinc, which is clearly cytotoxic in wild-type *C. elegans* (Dietrich et al., 2016), seemed the most potent inducer of the CeHSP17, which appeared to be highly upregulated by up to three and twofold, respectively (Fig. 4). This presumably would reflect the cellular requirement of CeHSP17 protein to confer the natural host with protection against exogonously imposed environmental stress. It also suggests that the CeHSP17 protein might be a useful biomarker for assessing cadmium and zinc, and should be assayed for other heavy metals.

Studies reported by others have indicated the induction of sHSP by Cd²⁺ and Zn²⁺ in human lens epithelial cells, plants, insects, fish, etc., (Hawse et al., 2003; Yi et al., 2006; Sonoda et al., 2007; Yang et al., 2012). CeHSP17 activation even at lower metal concentration at elevated temperature, as demonstrated for Zn²⁺ (Fig. 5B), implicates that the heat shock response is not only a marker for irreversible cytotoxicity (as usually seen at higher concentrations) but also for low-level toxicity. It is worthy to note the detectable similarity in the expression of CeHSP17 in *C. elegans* exposed to cadmium and zinc. This may be explained based on the fact that cadmium and zinc are closely related metals, both placed in the same group 12 on the periodic table, and based on their size and electron configuration similarities, could bind to identical macromolecular structure via nitrogen, oxygen, and sulfur (Brzoska and Moniuszk-Jakoniuk, 2001).

In conclusion, our data from this study show that CeHSP17 in *C. elegans* conceivably senses the cellular stress caused by heat, as well as environmental pollutants such as cadmium and zinc. The responses of CeHSP17 protein expression may be an early biomarker for toxicity monitoring and environmental risk assessment. However, future studies would determine if other heavy metals induce CeHSP17 protein expression, the specific cell components or processes targeted by CeHSP17 protein during thermal and metal stress, respectively, as are their interacting partners and client proteins necessary for explaining the mechanism involved in such molecular function.
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