Stress response in the ascidian *Ciona intestinalis*: transcriptional profiling of genes for the heat shock protein 70 chaperone system under heat stress and endoplasmic reticulum stress

Tetsuya Fujikawa · Takeo Munakata ·
Shin-ichi Kondo · Nori Satoh · Shuichi Wada

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Abstract The genome of *Ciona intestinalis* contains eight genes for HSP70 superfamily proteins, 36 genes for J-proteins, a gene for a J-like protein, and three genes for BAG family proteins. To understand the stress responses of genes in the HSP70 chaperone system comprehensively, the transcriptional profiles of these 48 genes under heat stress and endoplasmic reticulum (ER) stress were studied using real-time reverse transcriptase–polymerase chain reaction (RT-PCR) analysis. Heat stress treatment increased the messenger RNA (mRNA) levels of six HSP70 superfamily genes, eight J-protein family genes, and two BAG family genes. In the cytoplasmic group of the DnaK subfamily of the HSP70 family, *Ci-HSPA1/6/7-like* was the only heat-inducible gene and *Ci-HSPA2/8* was the only constitutively active gene which showed striking simplicity in comparison with other animals that have been examined genome-wide so far. Analyses of the time course and temperature dependency of the heat stress responses showed that the induction of *Ci-HSPA1/6/7-like* expression rises to a peak after heat stress treatment at 28°C (10°C upshift from control temperature) for 1 h. ER stress treatment with Brefeldin A, a

drug that is known to act as ER stress inducer, increased the mRNA levels of four HSP70 superfamily genes and four J-protein family genes. Most stress-inducible genes are conserved between *Ciona* and vertebrates, as expected from a close evolutionary relationship between them. The present study characterized the stress responses of HSP70 chaperone system genes in *Ciona* for the first time and provides essential data for comprehensive understanding of the functions of the HSP70 chaperone system.

Keywords BAG family · *Ciona intestinalis* · ER stress · Heat stress · HSP70 superfamily · J-protein family

Introduction

Stresses that result in protein unfolding evoke various protective responses in cells including the induction of stress-responsive genes. Two types of responses to stress-induced protein unfolding are well characterized. First, heat or similar stresses that lead to the accumulation of unfolded proteins in the cytoplasm induce heat shock factor-mediated transcription of genes for chaperones and other types of proteins that deal with non-native proteins in the cytoplasm (Morimoto 1998; Voellmy 2004). Second, increases in the levels of unfolded proteins in the endoplasmic reticulum (ER) trigger a process termed the unfolded protein response (UPR). The UPR begins with the activation of ER stress sensors that signal to the nucleus to enhance the transcription of genes encoding proteins required for homeostasis of the ER (Bernales et al. 2006; Kaufman 1999; Mori 2000; Sitia and Braakman 2003; Shen et al. 2004).

The HSP70 superfamily is the best-studied group of molecular chaperones which play important roles in the

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T. Fujikawa · T. Munakata · S. Kondo · S. Wada (✉)
Department of Bioscience, Faculty of Bioscience,
Nagahama Institute of Bio-Science and Technology, Nagahama,
Shiga 526-0829, Japan
e-mail: s_wada@nagahama-i-bio.ac.jp

N. Satoh
Marine Genomics Unit,
Okinawa Institute of Science and Technology Promotion Corporation,
Uruma, Okinawa 904-2234, Japan

process of stress responses and protein metabolism (Bukau and Horwich 1998; Hartl and Hayer-Hartl 2002; Mayer and Bukau 2005). Generally, the HSP70 superfamily consists of multiple members, and each member seems to have distinct properties in terms of structure, cellular localization, function, and response to stress. In humans, 17 genes for the HSP70 superfamily are grouped into the HSP70 family, HSP110 family, and HSPA12 family (Boorstein et al. 1994; Brocchieri et al. 2008; Easton et al. 2000; Nikolaidis and Nei 2004). The HSP70 family is further divided into the DnaK subfamily and STCH family, and members of the DnaK subfamily are classified into the cytoplasmic, ER-resident, and mitochondrial type groups according to their subcellular localizations. Similarly, the HSP110 family consists of the HSP110/SSE subfamily, HSPA14 subfamily, and GRP170 subfamily, and members of the former two subfamilies localize to the cytoplasm while members of the latter subfamily localize to the ER.

The functions of the HSP70 superfamily proteins are regulated and/or modified by co-chaperones (Caplan 2003; Mayer and Bukau 2005). The J-protein and BAG families are major groups of co-chaperones of the HSP70 superfamily proteins and are responsible for the functional diversity of the HSP70 chaperone system. J-proteins are thought to affect the functions of HSP70 superfamily proteins in several ways: they stimulate the ATPase activity of HSP70 superfamily proteins, bring the substrate proteins to HSP70 superfamily proteins, and recruit HSP70 superfamily proteins to specific cellular locations and biological contexts (Craig et al. 2006; Fan et al. 2003; Hennessy et al. 2005; Walsh et al. 2004). Generally, the J-protein family consists of multiple members and the number of J-proteins is higher than that of the HSP70 superfamily proteins (e.g., 50 genes in the human genome; Qiu et al. 2006). Each member of the J-protein family interacts with one or more HSP70 superfamily proteins. BAG family proteins are also modulators of HSP70 superfamily proteins, and at least BAG1 acts as a nucleotide exchange factor for HSP70 superfamily proteins and removes ADP after ATP hydrolysis (Alberti et al. 2003; Doong et al. 2002; Takayama and Reed 2001). The BAG family is composed of less members than the J-protein family: in human, six BAG family proteins are known (Doong et al. 2002; Takayama and Reed 2001). Although the action of the members of the J-protein family and BAG family is assumed to be important in the HSP70 chaperone system, the regulation and functions of many of J-proteins and BAG family proteins remain to be elucidated.

Urochordates including ascidians are one of the three groups of chordates, and ascidians exhibit the closest invertebrate relationship with vertebrates (Delsuc et al. 2006). *Ciona intestinalis* is a cosmopolitan species of ascidians. The genome of *C. intestinalis* has been se-

quenced, and it has been shown that its genome is 159 Mb long, encodes 15,852 genes, and has a similar repertoire of genes but shows less redundancy when compared to vertebrate genomes (Dehal et al. 2002). Furthermore, a total of more than 680,000 expressed sequence tags (ESTs) have been sequenced and complementary DNA (cDNA) clones are available for most genes (Satou et al. 2002). *Cis*-regulatory regions are often compact and located near coding sequences and thus can be analyzed simply by reporter assays. The function of genes can be assessed by overexpression and/or knockdown experiments. Because of these features and advantages, it has been proposed that *C. intestinalis* can serve as a model for the genome-wide analysis of gene expression and function (Satoh et al. 2003). It is also expected that *C. intestinalis* will become a powerful new model to study the mechanism of stress responses and the functions of molecular chaperones.

We started our studies by focusing on the HSP70 chaperone system and aimed at producing a comprehensive understanding of the regulation and functions of genes for this system in *C. intestinalis*. Previously, all genes for the HSP70 superfamily, J-protein family, and BAG family have been identified in the genome of *C. intestinalis* (Wada et al. 2006). As expected, *C. intestinalis* has fewer genes than vertebrates—8, 36, and 3 genes for the HSP70 superfamily, J-protein family, and BAG family, respectively. Although a gene that encodes a J-like protein was also found, phylogenetic analysis has shown that most of the identified genes have counterpart gene(s) in vertebrates.

In the present study, we investigated the expression of all genes for the HSP70 superfamily, J-protein family (including a J-like protein), and BAG family in response to two types of stress (heat stress and ER stress) in *C. intestinalis* for the following reasons. First, the effect of heat stress and ER stress on gene expression in *C. intestinalis* has not yet been characterized. Second, the genes for the HSP70 chaperone system are expected to be good markers to investigate the response to heat stress and ER stress in this species. Third, because the characterization of stress response is useful to deduce the function of a gene as mentioned above, analysis of the responses to heat stress and ER stress in detail should provide important information to understand the functions of each member of the HSP70 chaperone system.

Materials and methods

Biological materials

C. intestinalis cultivated from April to November 2007 at the Maizuru Fisheries Research Station of Kyoto University, Kyoto, Japan was used for this study. Mature adults were transported to an inland tank of artificial seawater at

18°C and maintained in the tank for 2–10 days before the collection of gametes. Eggs and sperms were collected surgically, and eggs were fertilized with sperm from a different individual at 18°C. Embryos were allowed to develop in Millipore-filtered artificial seawater (MFSW) containing 50 µg/ml streptomycin sulfate at 18°C until they were subjected to heat stress or ER stress treatments.

At 18°C, embryos of *C. intestinalis* develop into tadpole-like larvae and hatch approximately 18 h after fertilization, and the larvae metamorphose into the sessile adult form approximately 7 h after hatching. At the beginning of metamorphosis, the larvae adhere to the bottom of the dishes, and tail regression occurs after attachment. The development of internal organs continues for 12 to 14 days after hatching (Chiba et al. 2004). Stage 3a juveniles (24 h after hatching; Chiba et al. 2004) were used in this study because juveniles of this stage develop slower than embryos before metamorphosis and are still non-feeding so that unexpected stressors that may be involved in the culturing procedure (e.g., starvation) can be excluded. Experiments with animals of other developmental stages are in progress and will be reported elsewhere.

Heat stress treatment

Sixty normal juveniles were collected under a stereomicroscope and transferred to an Eppendorf tube with 1 ml MFSW. During the operation, juveniles were kept at 18°C. The tubes were incubated at the specified temperature with block incubators. As a control, juveniles were incubated at 18°C in the same way. After incubation, MFSW was removed as much as possible and the juveniles were fixed for RNA extraction in solution D (4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyle) containing 2-mercaptoethanol (Imai et al. 2003). A set of heat shock experiments was carried out with juveniles of the same batch, and each type of heat shock experiment was repeated at least three times.

ER stress treatment

Brefeldin A and thapsigargin were dissolved in ethanol at a concentration of 10 mg/ml. Tunicamycin was dissolved in dimethylsulfoxide (DMSO) at a concentration of 20 mg/ml. The stock solutions were stored at −20°C and diluted with MFSW to the specified final concentration just before use. Sixty normal juveniles were treated with 50 µg/ml Brefeldin A, 65 µg/ml thapsigargin, or 20 µg/ml tunicamycin for 5 h and were fixed for RNA extraction. The concentrations of the drug were determined by referring previous studies that used Brefeldin A in *Xenopus* (Maroto and Hamill 2001), thapsigargin in zebrafish (Westfall et al. 2003), and tunicamycin in *Xenopus* (Miskovic and Heikkila 1999), respectively. As a control, juveniles of the same batch were treated with 6.5%

ethanol for Brefeldin A and thapsigargin or 0.1% DMSO for tunicamycin. Juveniles were kept at 18°C through the treatment. Each type of ER stress experiment was repeated at least three times.

Quantitative RT-PCR

RNA extraction, reverse transcription, and real-time reverse transcriptase–polymerase chain reaction (RT-PCR) were carried out as described previously (Imai et al. 2003) with some modifications. Total RNA was isolated from exactly 60 juveniles and treated with RNase-free DNase. Half of the total RNA was used for cDNA synthesis with oligo(dT) primer and Superscript III reverse transcriptase (Invitrogen). The other half was used for negative control experiments without the reverse transcriptase. A portion of the cDNA equivalent to one juvenile was used for one PCR reaction. All PCR reactions were performed using a LightCycler 480 instrument (Roche). LightCycler 480 SYBR Green I Master (Roche) was used for all ER stress experiments and heat stress experiments with genes for the J-protein family and BAG family, and SYBR Premix Ex Taq (Takara) was used for heat stress experiments with genes for the HSP70 superfamily. We confirmed that both reagents showed the same results. The PCR parameters were programmed according to the supplier's instructions. The CP values were calculated by the second derivative maximum method using the LightCycler 480 Basic Software. The CP value, which corresponds to the maximum of the second derivative of the amplification curve obtained by PCR, is the cycle at which the changing rate of the fluorescence level is biggest and is proportionate to the concentration of template DNA. The difference in gene expression between stressed and unstressed samples was calculated based on the difference in CP values according to the $\Delta\Delta\text{CT}$ method (Livak and Schmittgen 2001). CP values for each gene were normalized with CP values for a housekeeping gene, *Ci-CA6*, which encodes cytoplasmic actin.

The statistical analysis was performed using Student's *t* test. Stress experiments followed by determination of the amount of messenger RNAs (mRNAs) by real-time PCR were carried out three times with different batches of juvenile. The three normalized CP values were compared between those obtained from stressed and unstressed samples. We judged the significant difference between them when *P* value was <0.05. Primers used for PCR reactions were designed using Primer3 (<http://frodo.wi.mit.edu/primer3/input.htm>) and listed in Electronic supplementary materials (ESM), Table S1.

Characterization of ER-resident lectin chaperones and ER stress sensors

Identification and annotation of *Ci-CREB/ATF-d* were reported previously (Yamada et al. 2003). *Ci-CALR*, *Ci-*

CANX/CLGN, *Ci-PERK*, and *Ci-IRE1* were identified in this study for the first time. The relationships of these genes to the genes of other animals were addressed by phylogenetic analyses (ESM, Figs. S1, S2, and S3), reciprocal BLAST searches (ESM, Tables S3 and S5), and motif analyses (ESM, Tables S4 and S6) as described in a previous study (Satou et al. 2003a). All *Ciona* sequences used for the analyses are listed in ESM, Table S7. Phylogenetic analysis of the proteins was carried out as described previously (Satou et al. 2003a). BLAST searches of the *C. intestinalis* genome were conducted using the JGI genome browser (<http://genome.jgi-psf.org/ciona4/>). BLAST searches of the genome sequences of other organisms were carried out using the NCBI web site (<http://www.ncbi.nlm.gov/BLAST/>). SMART was used to search for motifs (<http://smart.embl-heidelberg.de/>).

Results

Expression profiles of genes for HSP70 superfamily proteins under heat stress

In many animals so far examined, a temperature shift of 10°C induces heat shock response (e.g., Gellner et al. 1992; Piano et al. 2002). Therefore, in the first set of experiments, juveniles raised from eggs at 18°C were subjected to heat stress at 28°C for 3 h. The juveniles were collected immediately after heat stress treatment and examined for the expression of eight genes from the HSP70 superfamily. As shown in Fig. 1, the expression levels of six genes were increased by the heat stress treatment by more than 1.5-fold with statistically significant changes (*Ci-HSPA1/6/7-like*, *Ci-HSPA2/8*, *Ci-HSPA5a*, *Ci-HSPA9B*, *Ci-HSPA4/4 L/HSPH1*, and *Ci-HYOU1*). The degree of the induction was different from gene to gene.

Ci-HSPA1/6/7-like, one of two genes that encode for cytoplasmic members of the DnaK subfamily of the HSP70 family, was the most efficiently induced among all the HSP70 superfamily genes, and the fold induction was approximately 81. In contrast, *Ci-HSPA2/8*, another gene for the members of the DnaK subfamily of the HSP70 family, was induced approximately 3.8-fold. In *C. intestinalis*, ESTs are available for most genes, and the expression level of a gene can be inferred from the EST counts found in cDNA libraries (Satou et al. 2003b). The EST counts show that *Ci-HSPA1/6/7-like* is rarely transcribed while *Ci-HSPA2/8* is actively transcribed under normal conditions (ESM, Table S2). Therefore, *Ci-HSPA1/6/7-like* is the only heat-inducible gene and *Ci-HSPA2/8* is the only constitutively active gene of the cytoplasmic group of the DnaK subfamily of the HSP70 family. This situation contrasts with other animals examined at a genome-wide level so far (see “Discussion” for detail).

Induction by heat stress was evident not only for genes for cytoplasmic members of the HSP70 superfamily but also genes for ER-resident members. *Ci-HSPA5a*, one of two ER-resident group members of the DnaK subfamily of the HSP70 family, and *Ci-HYOU1*, a member of the GRP170 subfamily of the HSP110 family, showed significant changes in mRNA levels following heat stress treatment (approximately 5.8- and 2.6-fold, respectively; Fig. 1). As will be mentioned below, they were induced by ER stress more efficiently.

Expression profiles of genes for co-chaperones for HSP70s under heat stress

Next, the heat stress responses of genes for co-chaperones for HSP70 superfamily proteins were examined. For this, juveniles cultured at 18°C were subjected to heat stress in the same conditions as described above (at 28°C for 3 h) and the expression of 37 genes of the J-protein family (including the J-like protein) and three genes of the BAG family were examined. The expression levels of eight genes from the J-protein family and two genes from the BAG family were increased by the heat stress treatment by more than 1.5-fold with significant changes (Fig. 2).

Members of the J-protein family are categorized into types A, B, and C according to their structural features (Cheetham

Fig. 1 Heat stress response of HSP70 superfamily genes in *C. intestinalis*. Fold induction of each gene by heat stress treatment at 28°C for 3 h, determined by real-time RT-PCR, is shown with a scale of log₂. Each bar represents the mean value ± SD of six independent experiments. Asterisks indicate a value that was significantly different from the control ($p < 0.05$)

and Caplan 1998; Ohtsuka and Hata 2000). Among three type A genes in *Ciona*, *Ci-DNAJA1/2/4*, a single counterpart of human *DNAJA1*, *DNAJA2*, and *DNAJA4*, was the only gene induced by the heat stress treatment with a significant change. Among 27 type C genes, only three genes (*Ci-DNAJC3*, *Ci-DNAJC7*, and *Ci-DNAJC10*) were induced by the heat treatment with significant changes. In contrast to type A and type C, four out of six members of type B were associated with significant increases in gene expression level upon heat treatment. *Ci-DNAJB1/4/5*, a single counterpart for human *DNAJB1*, *DNAJB4*, and *DNAJB5*, was the most strongly induced gene in the J-protein family.

In the BAG family, the expression of *Ci-BAG3* and *Ci-BAT3* was induced by heat stress treatment with significant changes (approximately 6.7- and 3.4-fold, respectively; Fig. 2).

Time course of expression of genes for HSP70 superfamily proteins under heat stress

To evaluate the time course of expression of genes of the HSP70 superfamily under heat stress, juveniles cultured at 1°C were subjected to heat stress at 28°C for 10, 30, 60, 120, or 180 min and examined for the expression levels of *Ci-HSPA1/6/7-like*, *Ci-HSPA2/8*, and *Ci-HSPA5a* (Fig. 3). *Ci-HSPA1/6/7-like* showed significant increases in its expression at all time points examined. Even the treatment for 10 min resulted in approximately 24-fold induction. The treatment for 60 min led to the maximum induction (approximately 760-fold). In contrast, treatment for 30 min or longer was required for more than 1.5-fold induction of *Ci-HSPA2/8* and *Ci-HSPA5a*, and their induction peaked at 120 min.

Expression profiles of genes for HSP70 superfamily proteins under heat stress at various temperatures

The effect of heat stress at different temperatures on the expression levels of genes for HSP70 superfamily proteins was examined. Juveniles cultured at 18°C were subjected to stress at 4, 18, 23, 28, 33, or 38°C for 1 h and examined for their expression levels of *Ci-HSPA1/6/7-like*, *Ci-HSPA2/8*, and *Ci-HSPA5a*. As shown in Fig. 4, the heat stress treatment at 23°C resulted in more than 1.5-fold induction of all the three genes, but the induction was not significant. In contrast, consistent with the results of the experiments described above, the 28°C treatment caused the induction of the expression of *Ci-HSPA1/6/7-like* and *Ci-HSPA2/8*. Heat stress treatments at 33°C also induced the expression of the three genes. Although *Ciona* juveniles looked alive after 1-h treatment at 38°C, heat stress treatments at 38°C led to weaker induction of *Ci-HSPA1/6/7-like* expression and down-regulation of expression of *Ci-HSPA2/8* and *Ci-HSPA5a*. Cold stress treatment at 4°C led to no significant change in the expression of *Ci-HSPA1/6/7-like*, *Ci-HSPA2/8*, or *Ci-HSPA5a*.

ER stress response can be induced by treatments with Brefeldin A in *C. intestinalis*

The ER stress responses of *C. intestinalis* were investigated. Previous studies with other eukaryotes showed that ER stress responses are induced experimentally by treatment with drugs that perturb homeostasis or protein metabolism in the ER. In order to determine whether an ER stress

Fig. 2 Heat stress response of genes of the J-protein family and BAG-family in *C. intestinalis*. Fold induction of each gene by heat stress treatment at 28°C for 3 h, as determined by real-time RT-PCR, is shown with a scale of $\log_2$. Each bar represents the mean value $\pm$ SD of three independent experiments. Asterisks indicate a value that was significantly different from the control ($p < 0.05$)

(A) *Ci-HSPA1/6/7-like* (B) *Ci-HSPA2/8* (C) *Ci-HSPA5a*

Fig. 3 Time courses of expression of three genes of the HSP70 superfamily in *C. intestinalis* treated with heat stress. Fold induction of *Ci-HSPA1/6/7-like* (a), *Ci-HSPA2/8* (b), and *Ci-HSPA5a* (c) by heat stress treatment at 28°C for 10, 30, 60, 120, and 180 min, determined by real-time RT-PCR, is plotted with a scale of log₂. Each plot represents the mean value $\pm$ SD of four independent experiments. Asterisks indicate a value that was significantly different from the control ($p < 0.05$)

response can be induced pharmacologically in *C. intestinalis*, the effect of Brefeldin A, which is known to act as ER stress inducer (Hunziker et al. 1992; Ma and Hendershot 2002; Hussain and Ramaiah 2007), was examined on expression of markers for ER stress response. Brefeldin A blocks protein transport from the ER to the Golgi apparatus by inhibiting a GTP exchange factor required for retrograde trafficking and results in an overload of transport-arrested proteins in the ER. Juveniles were treated with Brefeldin A for 5 h and examined for the expression of genes for ER-resident lectin chaperones (*Ci-CALR* and *Ci-CANX/CLGN*: *Ci-CALR* is an orthologue for human *CALR*; *Ci-CANX/CLGN* is a single counterpart for human *CANX* and *CLGN*; see ESM, Fig. S1 and Tables S3, S4, and S7 for annotation of these genes) and ER stress sensors [*Ci-CREB/ATF-d*, *Ci-PERK*, and *Ci-IRE1*: *Ci-CREB/ATF-d* is a single counterpart for human *ATF6* and *CREBL1* (Yamada et al. 2003); *Ci-PERK* and *Ci-IRE1* are orthologues for human *PERK* and *IRE1*, respectively; see ESM Figs. S2 and S3 and Tables S5, S6, and S7]. As shown in Fig. 5, the expression levels of *Ci-PERK*, *Ci-IRE1*, and *Ci-CALR* showed significant increases as a result of Brefeldin A treatment. This result suggested that *C. intestinalis* possesses a conserved mechanism for ER stress responses and Brefeldin A can induce the response in this animal as expected.

Expression profiles of genes for HSP70 superfamily proteins and their co-chaperones under ER stress

Then, the effect of Brefeldin A on the expression levels of genes for the HSP70 chaperone system was examined. Juveniles were treated with Brefeldin A for 5 h and examined for their expression levels of eight genes of the HSP70 superfamily, 37 genes of the J-protein family (including the J-like protein), and three genes of the BAG family.

In the HSP70 superfamily, the expression levels of four genes were increased by Brefeldin A treatment by more than 1.5-fold with significant changes (*Ci-HSPA1/6/7-like*, *Ci-HSPA5a*, *Ci-HSPA5b*, and *Ci-HYOU1*; Fig. 6). *Ci-HSPA5a* and *Ci-HSPA5b* belong to the ER-resident group of the DnaK subfamily of the HSP70 family. *Ci-HYOU1* is a member of the GRP170 subfamily of the HSP110 family. Therefore, all of the ER-resident members of the HSP70 superfamily were shown to be ER stress-inducible.

Among the J-protein family genes, the expression levels of four genes were increased by more than 1.5-fold with

Fig. 4 Expression of three genes of the HSP70 superfamily in *C. intestinalis* treated with heat stress at various temperatures. Fold induction of *Ci-HSPA1/6/7-like* (a), *Ci-HSPA2/8* (b), and *Ci-HSPA5a* (c) by heat stress treatment at 4, 18, 23, 28, 33, and 38°C for 1 h, as determined by real-time RT-PCR, is plotted with a scale of $\log_2$. Each plot represents the mean value $\pm$ SD of three independent experiments. Asterisks indicate a value that was significantly different from the control ($p < 0.05$)

significant changes following Brefeldin A treatment (*Ci-DNAJC1*, *Ci-DNAJC3*, *Ci-DNAJC10*, and *Ci-DNAJD1/TIM14*; Fig. 7). In contrast to HSP70 superfamily genes and J-protein family genes, no BAG family genes were induced by Brefeldin A treatment (Fig. 7).

It has been shown that ER stress responses can also be induced by tunicamycin (Elbein 1991) or thapsigargin (Thastrup et al. 1990), which have different mechanisms of action to Brefeldin A. Tunicamycin is an inhibitor of protein glycosylation, which is essential for protein folding in the ER. Thapsigargin is an inhibitor of a calcium pump of the ER and causes the depletion of ER calcium stores, which plays an important role in the protein folding in the ER. To test whether these drugs cause similar effects on gene expression to Brefeldin A, the expression of *Ci-HSPA5a* in juveniles treated with tunicamycin or thapsigargin for 5 h was examined. As shown in Fig. 6, tunicamycin increased the *Ci-HSPA5a* expression level approximately 3.3-fold with a significant change. Thapsigargin also increased *Ci-HSPA5a* expression approximately 2.7-fold, although the change was not statistically significant.

Discussion

The present study characterized the stress responses of HSP70 chaperone system genes in *C. intestinalis* for the first time and provides essential data for the systematic understanding of the function of the HSP70 chaperone system in this animal, a novel model for stress biology.

Expression profiles of HSP70 chaperone system genes are conserved between *Ciona* and vertebrates

The results of the present study are summarized in Fig. 8. The following genes were judged to be heat-stress-inducible: six genes of the HSP70 superfamily (*Ci-HSPA1/6/7-like*, *Ci-HSPA2/8*, *Ci-HSPA4/4L/HSPH1*, *Ci-HSPA5a*, *Ci-HSPA9B*, and *Ci-HYOU1*), eight genes of the J-protein family (*Ci-DNAJA1/2/4*, *Ci-DNAJB1/4/5*, *Ci-DNAJB2/3/6/7/8*, *Ci-DNAJB9*, *Ci-DNAJB11*, *Ci-DNAJC3*, *Ci-DNAJC7*, and *Ci-DNAJC10*), and two genes of the BAG family (*Ci-BAG3* and *Ci-BAT3*). In vertebrates, *HSPA1A*, *HSPA1B*, *HSPA1L*, *HSPA6*, *HSPA8*, *HSPH1*, and *HYOU1* of the HSP70 superfamily, *DNAJA1*, *DNAJA4*, *DNAJB1*,

(A) *Ci-HSPA1/6/7-like*

(B) *Ci-HSPA2/8*

(C) *Ci-HSPA5a*

Fig. 5 Response of genes of ER stress sensors (*Ci-CREB/ATF-d*, *Ci-PERK*, and *Ci-IRE1*) and ER-resident lectin chaperones (*Ci-CALR* and *Ci-CANX/CLGN*) to ER stress treatment in *C. intestinalis*. Fold induction of each gene following Brefeldin A treatment for 5 h, as determined by real-time RT-PCR, is shown with a scale of log₂. Each bar represents the mean value $\pm$ SD of four independent experiments. Asterisks indicate a value that was significantly different from the control ($p < 0.05$)

DNAJB4, *DNAJB5*, *DNAJB6*, *DNAJB9*, *DNAJC3*, and *DNAJC7* of the J-protein family, and *BAG3* of the BAG family have been shown to be heat-inducible (Abdul et al. 2002; Murray et al. 2004; Pagliuca et al. 2003; Szustakowski et al. 2007; Trinklein et al. 2004). Therefore, the battery of genes of the HSP70 chaperone system induced upon heat stress seems to be well conserved between *Ciona* and vertebrates (ESM, Table S8).

This finding also holds true for the ER stress response. The present study identified four HSP70 superfamily genes (*Ci-HSPA1/6/7-like*, *Ci-HSPA5a*, *Ci-HSPA5b*, and *Ci-HYOU1*) and four J-protein family genes (*Ci-DNAJC1*, *Ci-DNAJC3*, *Ci-DNAJC10*, and *Ci-DNAJD1/TIM14*) as ER stress-inducible in *Ciona*. In vertebrates, HSPA5, HYOU1, DNAJB9, DNAJB11, DNAJC1, DNAJC3, DNAJC10, and SEC63 proteins are localized to the ER, and the gene expression of HSPA5, HYOU1, DNAJB9, DNAJC3, DNAJC10, and SEC63 are induced by ER stress treatment (Cunnea et al. 2003; Lee et al. 2003; Lecca et al. 2005; Nakanishi et al. 2004; Shen et al. 2002; Yan et al. 2002). Therefore, *Ciona* and vertebrates share at least four evolutionarily conserved ER stress response gene groups of the HSP70 chaperone system: *Ci-HSPA5a/Ci-HSPA5b/HSPA5*, *Ci-HYOU1/HYOU1*, *Ci-DNAJC3/DNAJC3*, and *Ci-DNAJC10/DNAJC10* (ESM, Table S8).

A number of *Ciona* genes were judged to be induced by neither heat stress nor ER stress. It is possible that some of these genes are induced by these stresses under experimental conditions other than those applied in the present study. However, most of these genes have vertebrate counterpart(s) that also show no inducible expression under these stress conditions. Such genes may be induced by other types of stresses or may not be induced by any stresses intrinsically and act in non-stress response processes as shown for some vertebrate genes (e.g., *DNAJC6* and *GAK* in clathrin uncoating; Eisenberg and Greene 2007).

The cytoplasmic group of the DnaK subfamily of the HSP70 family in *C. intestinalis* shows remarkable simplicity

The present study shows that *C. intestinalis* has only one heat-inducible gene (*Ci-HSPA1/6/7-like*) and only one constitutively active gene (*Ci-HSPA2/8*) in the cytoplasmic group of the DnaK subfamily of the HSP70 family. In contrast, all animals that have been examined genome-wide so far have multiple heat-inducible genes of this group. Therefore, although the gene expression profiles of the HSP70 chaperone system seem to be conserved between *C. intestinalis* and vertebrates as discussed above, the *Ciona*

Fig. 6 ER stress response of HSP70 superfamily genes in *C. intestinalis*. Fold induction of each gene following Brefeldin A treatment, *Ci-HSPA5a* following tunicamycin treatment (TU), and *Ci-HSPA5a* following thapsigargin treatment (TG) for 5 h, as determined by real-time RT-PCR, is shown with a scale of log₂. Each bar represents the mean value $\pm$ SD of four independent experiments. Asterisks indicate a value that was significantly different from control ($p < 0.05$)

Fig. 7 ER stress response of genes for the J-protein family and BAG-family in *C. intestinalis*. Fold induction of each gene following Brefeldin A treatment for 5 h, as determined by real-time RT-PCR, is shown with a scale of $\log_2$. Each bar represents the mean value $\pm$ SD of three independent experiments. Asterisks indicate a value that was significantly different from control ($p < 0.05$)

system shows remarkable simplicity as compared to the systems of other animals in this respect.

In humans, for example, seven genes belong to the cytoplasmic group of the DnaK subfamily of the HSP70 family (*HSPA1A*, *HSPA1B*, *HSPA1L*, *HSPA2*, *HSPA6*, *HSPA7*, and *HSPA8*; Brocchieri et al. 2008). Phylogenetic analysis showed close relationships among *HSPA1A*, *HSPA1B*, and *HSPA1L*, between *HSPA2* and *HSPA8*, and between *HSPA6*

and *HSPA7* (Brocchieri et al. 2008). According to the expression profiles, these genes are divided into heat-inducible genes (*HSPA1A*, *HSPA1B*, *HSPA6*, and *HSPA7*) and constitutively active genes (*HSPA1L*, *HSPA2*, and *HSPA8*; Brocchieri et al. 2008).

The previous phylogenetic analysis suggested that *Ci-HSPA2/8* is a single counterpart for human *HSPA2* and *HSPA8* (Wada et al. 2006). Therefore, it is likely that

Fig. 8 Summary of genes induced in *C. intestinalis* juveniles by heat stress (28 C, 3 h), ER stress (50 μ g/ml Brefeldin A, 5 h), by both stimuli, or neither. Genes that showed a more than 1.5-fold increase in their expression with a significant difference were considered as stress-inducible

constitutively active expression is an ancestral feature inherited by *Ci-HSPA2/8*, *HSPA2*, and *HSPA8*. On the other hand, the relationship of *Ci-HSPA1/6/7-like* and human genes was unclear in the previous analysis because *Ci-HSPA1/6/7-like* locates outside of the other members of the cytoplasmic group of the DnaK subfamily of the HSP70 family in the phylogenetic tree (Wada et al. 2006). The finding in the present study that *Ci-HSPA1/6/7-like* is heat-inducible, together with the results of the phylogenetic analysis of human genes, suggests that *Ci-HSPA1/6/7-like*, *HSPA1A*, *HSPA1B*, *HSPA1L*, *HSPA6*, and *HSPA7* share a heat-inducible ancestor.

In *Drosophila melanogaster*, the cytoplasmic group of the DnaK subfamily of the HSP70 family contains seven heat-inducible genes (*hsp70Aa*, *hsp70Ab*, *hsp70Ba*, *hsp70Bb*, *hsp70Bbb*, *hsp70Bc*, and *hsp68*) and three constitutively active genes (*hsc70-1*, *hsc70-2*, and *hsc70-4*; Bettencourt and Feder 2001; Girardot et al. 2004; Kristensen et al. 2005; Maside et al. 2002; Sørensen et al. 2005). In *Caenorhabditis elegans*, the cytoplasmic group of the DnaK subfamily of the HSP70 family consists of four genes (*hsp70-1*, *hsp70-7*, *hsp70-8*, and *hsp70-9*), and *hsp70-7*, *hsp70-8*, and *hsp70-9* are heat-inducible (Heschl and Baillie 1990; Kim et al. 2001; Nikolaidis and Nei, 2004). Because the phylogenetic analyses suggested that *Drosophila hsc70-1* and *Drosophila hsc70-4* are related to *HSPA2*, *HSPA8*, and *Ci-HSPA2/8* (Wada et al. 2006) and *Caenorhabditis hsp70-1* is related to *Drosophila hsc70-4* (Nikolaidis and Nei 2004), these genes may have been derived from a constitutively active ancestor. As for other *Drosophila* and *Caenorhabditis* genes, it is difficult to deduce the phylogenetic relationships to human and *Ciona* genes because of high intraspecific sequence similarity of *Drosophila* and *Caenorhabditis* genes (Nikolaidis and Nei 2004; Wada et al. 2006).

C. intestinalis responds to heat stress in a temperature-dependent manner

In the present study, heat stress treatment at 28°C but not 23°C resulted in enhanced expression of *Ci-HSPA1/6/7-like*, *Ci-HSPA2/8*, and *Ci-HSPA5a*. Bellas et al. (2003) examined the effect of temperature on embryonic development of *C. intestinalis* and reported that development was arrested at temperature below 16°C and above 24°C while it progressed normally at temperatures from 18°C to 23°C. Therefore, the results of the two experiments are consistent and suggest that the physiological conditions of *C. intestinalis* are normal at a temperature from 18°C to 23°C and treatment at higher temperatures interferes with the homeostasis of this animal. Kroihner et al. (1992) reported that heat treatment at 25°C and 28°C stimulates the metamorphosis of *C. intestinalis* and suggested a correlation between the induction of metamorphosis and the synthesis of heat shock proteins. If this idea holds true, the data indicate that heat treatment even at 25°C

induces the expression of heat shock protein genes. Experiments under more detailed conditions are required to determine the minimal temperature that leads to heat shock response in *C. intestinalis*. The involvement of heat shock proteins in metamorphosis is another interesting issue for a future study.

The induction of expression of *Ci-HSPA1/6/7-like*, *Ci-HSPA2/8*, and *Ci-HSPA5a* was also observed upon heat stress treatment at 33°C. In contrast, heat stress treatment at 38°C resulted in reduced expression of the three HSP70 family genes as compared to that obtained by treatment at 28°C and 33°C. Therefore, it is possible that heat stress treatment at 38°C is so severe that cellular machinery required for the induction of expression of the three HSP70 family genes is damaged. A study of the cytotoxic effects of heat stress treatment is required to test this possibility.

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