The synthesis of diapause-specific molecular chaperones in embryos of *Artemia franciscana* is determined by the quantity and location of heat shock factor 1 (Hsf1)

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Received: 15 October 2018 / Revised: 21 December 2018 / Accepted: 11 January 2019 / Published online: 30 January 2019
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Abstract

The crustacean, *Artemia franciscana*, displays a complex life history in which embryos either arrest development and undertake diapause as cysts or they develop into swimming nauplii. Diapause entry is preceded during embryogenesis by the synthesis of specific molecular chaperones, namely the small heat shock proteins p26, ArHsp21, and ArHsp22, and the ferritin homolog, artemin. Maximal synthesis of diapause-specific molecular chaperones is dependent on the transcription factor, heat shock factor 1 (Hsf1), found in similar amounts in cysts and nauplii newly released from females. This investigation was performed to determine why, if cysts and nauplii contain comparable amounts of Hsf1, only cyst-destined embryos synthesize diapause-specific molecular chaperones. Quantification by qPCR and immunoprobings of Western blots, respectively, demonstrated that *hsf1* mRNA and Hsf1 peaked by day 2 post-fertilization in embryos that were developing into cysts and then declined. *hsf1* mRNA and Hsf1 were present in nauplii-destined embryos on day 2 post-fertilization, but in much smaller amounts than in cyst-destined embryos, and they increased in quantity until release of nauplii from females. Immunofluorescent staining revealed that the amount of Hsf1 in nuclei was greatest on day 4 post-fertilization in cyst-destined embryos but could not be detected in nuclei of nauplius-destined embryos at this time. The differences in quantity and location of Hsf1 explain why embryos fated to become cysts and eventually enter diapause synthesize p26, ArHsp21, ArHsp22, and artemin, whereas nauplius-destined embryos do not produce these molecular chaperones.

Keywords Heat shock factor 1 · Transcription factor · Molecular chaperone · Diapause · *Artemia franciscana*

Introduction

Diapause, a physiological process regulated by complex patterns of gene expression, is characterized by reduced metabolism, sequestration of nutrient reserves, interruption of development, declining water content, and increased tolerance to environmental and physiological stress (Denlinger 2002; Košťál 2006; MacRae 2010). Diapause can be divided into stages, including initiation, maintenance, and termination (Košťál 2006; Košťál et al. 2017), and it is triggered by environmental signals such as photoperiod, temperature change, food depletion, and crowding, although animals may enter diapause as an obligate part of their life history without the

need for an environmental trigger (Košťál 2006; MacRae 2010; Reznik and Voinovich 2016). Animals remain dormant until diapause termination is triggered by an environmental signal (Robbins et al. 2010), generally occurring when favorable growth conditions are likely to prevail. Diapause may be precociously terminated in many organisms by artificial stimuli such as mechanical shaking, electrical or temperature shock, injury, infection, or treatment with solvents and other chemicals (Robbins et al. 2010). After diapause termination, either development resumes or quiescence ensues wherein organisms remain dormant while unfavorable environmental conditions persist, but resume development when circumstances improve (MacRae 2005).

The crustacean, *Artemia franciscana*, exhibits a complex life history as embryos develop within females into either swimming larvae (nauplii) that molt and become adults, or gastrulae enclosed in a rigid shell (cysts) that enter diapause (MacRae 2003; Liang and MacRae 1999). Diapausing *A. franciscana* exhibit extremely low metabolic activity

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(Clegg et al. 1996; Clegg 1997) and greatly enhanced stress tolerance, the latter dependent on the cyst wall (MacRae 2016; Dai et al. 2011; Ma et al. 2013), trehalose (Clegg 1965), late embryonic abundant (LEA) proteins (Warner et al. 2010; Toxopeus et al. 2014; Moore et al. 2016), and the diapause-specific ATP-independent molecular chaperones, p26 a small heat shock protein (sHsp) (Jackson and Clegg 1996; Liang and MacRae 1999; Clegg 2011; King and MacRae 2012; King et al. 2013) and artemin, a ferritin homolog (Chen et al. 2003, 2007; Clegg 2011; King et al. 2014). Maximum synthesis of diapause-specific molecular chaperones in *A. franciscana* is dependent on the transcription factor, heat shock factor 1 (Hsf1) (Tan and MacRae 2018).

A. franciscana *hsf1* cDNA has been cloned and sequenced (Tan and MacRae 2018) revealing an amino terminal winged helix-turn-helix DNA binding domain (DBD) that, based on the activity of other Hsfs, binds to upstream regulatory elements termed heat shock elements (HSEs) and promotes gene expression (Neudegger et al. 2016). The primary targets of Hsfs in many organisms are the genes that encode heat shock proteins or molecular chaperones (Brunquell et al. 2016; Mahat et al. 2016; Takii et al. 2017; Li et al. 2017) but many other genes are also activated by Hsfs (Barna et al. 2012, 2018; Li et al. 2016, 2017; Nair et al. 2017; Gomez-Pastor et al. 2018).

Hsf1 is present in similar amounts in cysts and nauplii newly released from females, but only embryos destined to become cysts synthesize diapause-specific molecular chaperones. To investigate why this is the case, Hsf1 was examined, revealing that embryos of *A. franciscana* destined to become cysts and enter diapause synthesized more Hsf1 during development than did embryos destined to become nauplii. Moreover, Hsf1 localized to nuclei in cyst-destined embryos beginning at about 2 days post-fertilization but could not be detected in the nuclei of nauplius-destined embryos. The results demonstrate that synthesis of diapause-specific molecular chaperones and entry into diapause are determined, at least in part, by the quantity and location of Hsf1 in developing embryos of *A. franciscana*.

Methods

Culture of *A. franciscana*

A. franciscana cysts from the Great Salt Lake (INVE Aquaculture Inc., Ogden, UT, USA) were hydrated overnight at 4 °C in distilled water, collected by filtration, washed with cold distilled water followed by filtered and autoclaved sea water from Halifax Harbour, hereafter called sea water, and incubated in sea water with vigorous shaking at room temperature. Nauplii were harvested and grown in sea water at room temperature with gentle aeration. After sufficient growth to

allow visual differentiation, males and females captured individually were incubated separately in two containers with gentle aeration in sea water at room temperature. Male and female *A. franciscana* were mated in 5-cm diameter Petri dishes containing sea water at room temperature with nauplii and cysts collected and used in experiments as they were released from females. Animals were fed daily with *Isochrysis* sp. (clone synonym *T-Iso*) obtained from the Provasoli-Guillard National Centre for Culture of Marine Phytoplankton (West Boothbay Harbor, ME, USA). The research described in this paper was performed in accordance with the ethical guidelines provided by the Canadian Council on Animal Care (CCAC). The University Committee on Laboratory Animals (UCLA) of Dalhousie University approved the research and assigned Protocol Number 117-36.

Quantification of *hsf1* mRNA and Hsf1 in oocytes, embryos, cysts, and nauplii of *A. franciscana*

Oocytes were obtained from *A. franciscana* females prior to fertilization and developing embryos were collected 2 and 4 days after fertilization. Cysts and nauplii were harvested immediately after release from females. For the collection of oocytes and embryos, females were immobilized on a cold slide under an SZ61 stereomicroscope (Olympus Canada, Inc., Markham, ON, Canada). Egg sacs containing oocytes and embryos were then excised and frozen in liquid nitrogen. RNA and protein were prepared as described (Tan and MacRae 2018). To quantify *Hsf1* mRNA, qRT-PCR was performed with recovered RNA using forward 5'-GTCC TCCTTG CTTTCGCTATTT-3' and reverse 5'-TGTC GGCTTCCTGGTCTGATTC-3' primers with α -tubulin as internal standard (Tan and MacRae 2018). Protein samples were resolved in SDS polyacrylamide gels, transferred to nitrocellulose membranes (Bio-Rad), and probed with antibody to Hsf1 (Tan and MacRae 2018) and tyrosinated α -tubulin (Xiang and MacRae 1995) followed by HRP-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich, Oakville, ON, Canada). Immunoreactive protein bands were quantified with Image Studio Software (Li-Core Biosciences, Lincoln, NE, USA).

Localization of Hsf1 in oocytes, embryos, cysts, and nauplii of *A. franciscana*

Immunofluorescent staining, essentially as described (Liang et al. 1997; Liang and MacRae 1999), was employed to detect Hsf1 in *A. franciscana*. Oocytes and developing embryos at 2 and 4 days post-fertilization destined to become cysts and nauplii and freshly released cysts and nauplii were collected, gently crushed on poly-L-lysine coated slides (Sigma-Aldrich), and fixed immediately with methanol for 5–10 min at –20 °C. Slides were washed in ice-cold PBS (137 mM

NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) 3 times for 10 min and incubated in 2 changes of PBSAT [0.5% (w/v) BSA, 0.75% (w/v) Triton X-100 in PBS] for 2–3 h. The samples were incubated at room temperature for 1 h with antibody to Hsf1 diluted 1:200 in PBSAT and then overnight at 4 °C. After three washes of 10 min each in PBSAT, samples were incubated at room temperature for 2 h in FITC-conjugated goat anti-rabbit IgG antibody (Abcam Inc., Toronto, ON, Canada) diluted 1:200 in PBSAT. Slides were washed in PBS 3 times for 10 min followed by a 5-min wash in distilled water prior to incubation in 0.5% (w/v) 4',6-diamidino-2-phenylindole (DAPI) (Fisher Scientific, Ottawa, ON, Canada) for 2 min. Slides were rinsed in distilled water, mounted in a drop of Vectashield (Vector Laboratories, Burlingame, CA, USA), cover slipped and sealed with nail polish. The slides were examined with a Zeiss laser scanning confocal microscope LSM 710 (Olympus Canada Inc., Toronto, ON, Canada). As a control for specificity, antibody to Hsf1 mixed with peptide antigen (Abbiotec, San Diego, CA, USA; Tan and MacRae 2018) and PBSAT without Hsf1 antibody were used to probe slides that contained samples from cyst-destined embryos at 2 days post-fertilization.

Statistics

To assess differences, one-way ANOVA Dunnett *t* tests were performed for mRNA and protein at various stages of embryo development. Analysis was carried out using Microsoft Excel 2013. All data were plotted as mean $\pm$ SD unless otherwise stated.

Results

Quantification of *hsf1* mRNA in oocytes, embryos, cysts, and nauplii of *A. franciscana*

q-PCR using α -tubulin mRNA as internal standard showed that *hsf1* mRNA increased significantly in diapause-destined embryos 2 days after fertilization and then decreased as development progressed (Fig. 1). In comparison, much lower amounts of *hsf1* mRNA appeared by day 2 post-fertilization in nauplius-destined embryos and it increased until nauplii exited females, although not to the level of *hsf1* mRNA in newly released cysts (Fig. 1).

Quantification of Hsf1 in oocytes, embryos, cysts, and nauplii of *A. franciscana*

Immunoprobings of Western blots using tyrosinated α -tubulin as internal standard showed that Hsf1 increased significantly by day 2 post-fertilization in cyst-destined embryos and then decreased until cyst release from females (Fig. 2a, c). By

comparison, Hsf1 appeared at day 2 post-fertilization in nauplius-destined embryos but in much lower quantities than in cyst-destined embryos (Fig. 2b, c). Hsf1 increased until nauplii were released from females, reaching a slightly higher but significantly different level than Hsf1 in newly released cysts (Fig. 2b, c).

Localization of Hsf1 in oocytes, embryos, cysts, and nauplii of *A. franciscana*

Antibody raised to Hsf1 reacted with samples obtained by crushing embryos 2 days post-fertilization on glass slides, but immunofluorescence disappeared when the primary antibody was omitted from the staining protocol or was greatly diminished when mixed with the Hsf1 peptide antigen against which the antibody was raised, indicating that the antibody recognized Hsf1 (not shown). Examination by confocal microscopy did not detect Hsf1 in either cyst or nauplius destined oocytes (Figs. 3 and 4). Hsf1 was however observed surrounding nuclei from cyst-destined embryos at both stages of development examined and from cysts. A small amount of Hsf1 was detected in nuclei at 2 days post-fertilization, reaching a peak by 4 days, and then declining in nuclei from cysts (Fig. 3). A small amount of Hsf1 exterior to nuclei was initially observed at day 2 post-fertilization in nauplius-destined embryos and persisted in freshly released nauplii where it was first detected in nuclei (Fig. 4). That Hsf1 was located throughout nuclei after entry into the organelle was confirmed by optical sectioning (Fig. 5).

Discussion

Diapausing and quiescent cysts of *A. franciscana* possess four diapause-specific molecular chaperones termed p26, ArHsp21, ArHsp22, and artemin, at least two of which are important contributors to the remarkable resistance of cysts to physiological and environmental stress (King and MacRae 2012; King et al. 2013, 2014). These molecular chaperones are synthesized during embryogenesis and their maximal production depends on Hsf1, a transcription factor found in similar amounts in cysts and nauplii freshly released from females (Tan and MacRae 2018). Why then, if cysts and nauplii contain similar amounts of Hsf1, do only cyst-destined embryos synthesize p26, ArHsp21, ArHsp22, and artemin? In other words, how is it that Hsf1 contributes to development in such a way that embryos become cysts that enter diapause rather than nauplii that develop directly into adults?

qPCR and immunoprobings of Western blots, respectively, demonstrated that *hsf1* mRNA and Hsf1 were essentially absent from oocytes whether they were fated to become cysts or nauplii. By 2 days post-fertilization, *hsf1* mRNA and Hsf1 had

Fig. 1 *hsf1* mRNA quantification during development of *A. franciscana*. RNA was prepared from cyst-destined (gray) and nauplius-destined (black) oocytes, embryos on days 2 and 4 after fertilization and freshly released cysts (gray) and nauplii (black). *hsf1* mRNA was quantified by q-PCR using α -tubulin as internal standard. Inset, enlarged view of *hsf1* mRNA in nauplius-destined oocytes and embryos and nauplii. The

experiments were done in triplicate and error bars show standard deviation. The values represented by bars labeled with different letters were significantly different ($p < 0.05$) from one another. Asterisks indicate that the amounts of mRNA were significantly different ($p < 0.05$) between samples from cyst-destined and nauplius-destined embryos on the day specified

increased dramatically in cyst-destined embryos but were barely detectable in nauplius-destined embryos. As development progressed beyond 2 days, *hsf1* mRNA and Hsf1

decreased in cyst-destined embryos but increased in nauplius-destined embryos, yielding similar amounts of Hsf1 in cysts and nauplii freshly released from females. Although

Fig. 2 Hsf1 quantification during development of *A. franciscana*. Protein extracts from cyst-destined and nauplius-destined oocytes and embryos on days 2 and 4 post-fertilization and freshly released cysts and nauplii were resolved in SDS polyacrylamide gels, blotted to nitrocellulose and reacted with antibodies to Hsf1 and tyrosinated α -tubulin. **a**, **b** Representative examples of Western blots probed with antibody to Hsf1 and tyrosinated α -tubulin. **c** Immunoreactive bands were quantified with

Image Studio Software and the ratio of Hsf1 to tyrosinated α -tubulin was determined. Experiments were done in duplicate and error bars show standard deviation. Bars with different letters are significantly different ($p < 0.05$) from one another. Asterisks indicate that the amounts of protein were significantly different ($p < 0.05$) between samples from cyst-destined and nauplius-destined embryos on the day specified

Fig. 3 Hsfl localization in cyst-destined oocytes and developing embryos and in cysts of *A. franciscana*. Cyst-destined oocytes (0) and embryos 2 and 4 days after fertilization (2, 4) and cysts freshly released from females were gently crushed on poly(L) lysine-coated slides and reacted with antibody that recognized Hsfl followed by FITC-conjugated goat anti-rabbit IgG antibody. Samples were then stained with DAPI, mounted in mounting media, cover slipped, and examined by confocal microscopy. DAPI, DAPI staining; FITC, antibody staining; scale bar, 10 μ m

there was a large discrepancy in the amounts of Hsfl in embryos destined to become cysts versus nauplii, Hsfl was observed in nauplius-destined embryos allowing, in principle, the synthesis of diapause-specific molecular chaperones. Because Hsfl must enter the nucleus to trigger transcription (Wang and Lindquist 1998), immunofluorescent staining of samples from *A. franciscana* oocytes, embryos, cysts, and nauplii was performed to determine if nuclei contained Hsfl. The intensity of immunofluorescence staining in samples as determined visually correlated with the amounts of Hsfl obtained by the quantitative probing of Western blots and showed that Hsfl entered nuclei in cyst-destined embryos but not nauplius-destined embryos 2 days after fertilization. Because Hsfl did not enter nuclei in appreciable quantities, perhaps in part because it was present in low amounts, diapause-specific molecular chaperones were not synthesized in nauplius-destined embryos.

If Hsfl entering the nucleus determines the synthesis of diapause-specific molecular chaperones, then it should appear before or concurrently with their mRNAs and it must be active in order to promote transcription. The synthesis of *p26* mRNA

Fig. 4 Hsfl localization in nauplius-destined oocytes and developing embryos and in nauplii of *A. franciscana*. Nauplius-destined oocytes (0) and embryos 2 and 4 days after fertilization (2, 4) and nauplii freshly released from females were gently crushed on poly(L) lysine-coated slides and reacted with antibody that recognized Hsfl followed by FITC-conjugated goat anti-rabbit IgG antibody. Samples were then stained with DAPI, mounted in mounting media, cover slipped, and examined by confocal microscopy. DAPI, DAPI staining; FITC, antibody staining; scale bar, 10 μ m

in diapause-destined embryos begins 2 days post-fertilization and is followed on day 3 by *p26* production (Liang and MacRae 1999). *ArHsp21* mRNA appears in diapause-destined embryos on day 2 post-fertilization (Qiu and MacRae 2008a) whereas *ArHsp22* mRNA emerges in diapause-destined embryos on day 3 post-fertilization, with both increasing until cyst release (Qiu and MacRae 2008b). Thus, the up-regulation of Hsfl and its localization to nuclei in cyst-destined embryos coincides with the initial production of mRNAs encoding diapause-specific molecular chaperones, showing that it is appropriately positioned in the cell to initiate transcription of their genes. Hsfl decreases as diapause-destined embryos develop indicating that a large amount of Hsfl is not needed once molecular chaperones essential for diapause reach a threshold. Hsfl in cysts after release from females may be required for the residual synthesis of proteins required for diapause as metabolism declines and the re-initiation of development upon diapause termination.

Hsfl has been linked to diapause, molecular chaperones, and stress tolerance in organisms other than *A. franciscana*, usually indirectly via demonstrating a relationship between

Fig. 5 Optical sections of immunofluorescently stained nuclei from developing *A. franciscana*. Cyst-destined embryos of *A. franciscana* 4 days post-fertilization were gently crushed on poly(L) lysine-coated slides and reacted with antibody that recognized Hsf1 followed by FITC-conjugated goat anti-rabbit IgG antibody. Samples were then stained with DAPI, mounted in mounting media, cover slipped, and examined by confocal microscopy. Panels 1–8, continuous series of 0.79 μm optical sections. DAPI, DAPI staining; FITC, antibody staining. Scale bar, 10 μm

the quantities of Hsf1 and chaperones or their mRNAs. For example, active (phosphorylated) and inactive forms of Hsf1 increase in the cold hardy larvae of the goldenrod gall moth, *Epiblema scudderiana* as winter approaches and the insect enters diapause, this increase coinciding with augmentation of several molecular chaperones (Zhang et al. 2018). In the larvae of another cold hardy goldenrod gall moth, *Eurosta solidaginis*, activated Hsf1 increases in coordination with several molecular chaperones as temperatures cool and diapause ensues, but unlike in *E. scudderiana*, Hsf1 declines in mid-winter and remains low until spring (Zhang et al. 2011). Upon diapause termination by incubation at 5 $^{\circ}\text{C}$, the mRNA for Hsf1 increases in the silkworm, *Bombyx mori*, and this is thought to up-regulate the transcription of the genes encoding Hsp70a and Samui (Kihara et al. 2011). Interestingly, the African chironomid, *Polypedilum vanderplanki*, exhibits concurrent increases in mRNAs encoding Hsf1 and a variety of molecular chaperones during anhydrobiosis or desiccation (Gusev et al. 2011). *A. franciscana* cysts often experience desiccation during diapause.

Hsf1 may affect entrance into and maintenance of diapause via mechanisms that involve proteins other than the HSPs. In the L1 larval stage of the nematode, *Caenorhabditis elegans*, HSF-1 is activated by the dauer pheromone produced in response to crowding stress leading to developmental diapause or dauer which is broken upon return to favorable growth conditions without the need for a discrete environmental signal (Barna et al. 2012, 2018). HSF1 regulation of dauer is thought to occur via its effect on at least three signal transduction pathways including insulin/IGF-1, TGF β , and cyclic guanosine monophosphate/guanylate cyclase (cGMP/GC) (Barna et al. 2018).

Conclusions

Hsf1, shown previously to regulate the synthesis of p26, ArHsp21, ArHsp22, and artemin, is present in similar amounts in newly released cysts and nauplii although nauplius-destined embryos do not synthesize diapause-specific molecular chaperones. We showed herein that Hsf1 was more abundant in cyst-destined embryos than in nauplius-destined embryos and that Hsf1 located to nuclei in the former but not in the latter. Additionally, Hsf1 decreased in cyst-destined embryos after day 2 post-fertilization whereas it increased in nauplius-destined embryos. These data support the conclusion that Hsf1 has different roles in the two types of embryos, especially in the synthesis of molecular chaperones, but also in other aspects of embryo growth and development. The work described in this study constitutes the first demonstration that regulation of the synthesis of diapause-specific molecular chaperones in *A. franciscana* resides in the differential synthesis and localization of Hsf1 in developing

embryos and that via this mechanism, Hsf1 has an important role in determining whether embryos develop into cysts or nauplii.

Funding information The work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant (Number RGPIN/04882-2016) to THM and by a scholarship from the Chinese Scholarship Council to JT.

Compliance with ethical standards

The research described in this paper was performed in accordance with the ethical guidelines provided by the Canadian Council on Animal Care (CCAC). The University Committee on Laboratory Animals (UCLA) of Dalhousie University approved the research and assigned Protocol Number 117-36.

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