ArHsp40, a type 1 J-domain protein, is developmentally regulated and stress inducible in post-diapause *Artemia franciscana*

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Abstract Upon diapause termination and exposure to favorable environmental conditions, cysts of the crustacean *Artemia franciscana* reinitiate development, a process dependent on the resumption of metabolic activity and the maintenance of protein homeostasis. The objective of the work described herein was to characterize molecular chaperones during post-diapause growth of *A. franciscana*. An Hsp40 complementary DNA (cDNA) termed *ArHsp40* was cloned and shown to encode a protein with an amino-terminal J-domain containing a conserved histidine, proline, and aspartic acid (HPD) motif. Following the J-domain was a Gly/Phe (G/F) rich domain, a zinc-binding domain which contained a modified CXXCXGXG motif, and the carboxyl-terminal substrate binding region, all characteristics of type I Hsp40. Multiple alignment and protein modeling showed that ArHsp40 is comparable to Hsp40s from other eukaryotes and likely to be functionally similar. qRT-PCR revealed that during post-diapause development, *ArHsp40* messenger RNA (mRNA) varied slightly until the E2/E3 stage and decreased significantly upon hatching. The immunoprobings of Western blots demonstrated that ArHsp40 was also relatively constant until E2/E3 and then declined dramatically. The drop in ArHsp40 when metabolism and protein synthesis were increasing was unexpected and demonstrated developmental regulation. The reduction in ArHsp40 at such an active life history stage

indicates, as one possibility, that *A. franciscana* possesses additional Hsp40s, one or more of which replaces ArHsp40 as development progresses. Increased synthesis upon heat shock established that in addition to being developmentally regulated, ArHsp40 is stress inducible and, because it is found in mature cysts, ArHsp40 has the potential to contribute to stress tolerance during diapause.

Keywords Molecular chaperone · Hsp40 · J-domain · Post-diapause development · Stress · Crustacean · *Artemia franciscana*

Introduction

The J-domain proteins, termed Hsp40 in eukaryotes and DnaJ in prokaryotes, are important co-chaperones of Hsp70 and its bacterial counterpart DnaK (Vos et al. 2008; Li et al. 2009; Kampinga and Craig 2010; Baaklini et al. 2012; Sarbeng et al. 2015). The J-domain proteins form a heterogeneous family with members minimally possessing a conserved J-domain. The J-domain is arranged primarily as four helices, and it interacts with Hsp70/DnaK, possibly only when both the chaperone and co-chaperone associate with ATP (Mayer and Bukau 2005; Vos et al. 2008; Li et al. 2009; Kampinga and Craig 2010; Ahmad et al. 2011). A highly conserved histidine, proline, and aspartic acid (HPD) motif occurs in the J-domain between helix II and III, and it is crucial for binding to Hsp70, activation of Hsp70 ATPase, and protein folding. There are three main categories of J-domain proteins including type I with a G/F-rich region thought to mediate chaperone function by recognizing subsets of client conformers, facilitate binding to Hsp70, and provide molecular flexibility to aid transfer of substrate from Hsp40 to Hsp70/DnaK (Ahmad et al. 2011; Stein et al. 2014), a zinc-binding domain which has two

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functionally dissimilar zinc-binding sites and is potentially involved in substrate interaction (Linke et al. 2003; Tiwari et al. 2013), and a carboxyl-terminal area that in cooperation with other domains combines with substrate (Lu and Cyr 1998a, b; Cuéllar et al. 2013; Tiwari et al. 2013). Type II J-domain proteins resemble type I but lack the zinc-binding domain. Type III J-domain proteins possess a J-domain that may appear anywhere in the sequence, but neither a G/F nor zinc-binding region. Types I and II Hsp40s are general co-chaperones and they combine with many substrates, but each type III protein has a limited client repertoire (Vos et al. 2008; Li et al. 2009; Kampinga and Craig 2010).

J-domain proteins, some of which are stress inducible, possess chaperone activity in their own right, interacting with substrate proteins and protecting them from irreversible denaturation before transport to Hsp70 and folding (Lu and Cyr 1998a, b; Li et al. 2009; Kampinga and Craig 2010; Ahmad et al. 2011; Baaklini et al. 2012; Borges et al. 2012; Cuéllar et al. 2013; Tiwari et al. 2013; Ludewig et al. 2015). J-domain proteins activate the ATPase of Hsp70 thereby favoring strong interaction of Hsp70 with substrates which promotes the folding of nascent and partially denatured proteins (Mayer and Bukau 2005; Vos et al. 2008). The J-domain proteins function with nucleotide exchange factors (NEFs) such as Bag-1 and Hsp110 in eukaryotes and GrpE in bacteria to foster efficient Hsp70-dependent protein folding (Mayer and Bukau 2005; Kampinga and Craig 2010; Baaklini et al. 2012). With Hsp110 and Hsp70, J-domain proteins Hdj1 in mammalian cells and either Sis1 or Ydj1 in yeast aid in the disaggregation and refolding of proteins in cell-free systems and in prion propagation, processes thought to involve stimulation of ATPase in Hsp70 and Hsp110 by Hsp40 (Shorter 2011; Torrente and Shorter 2013; Summers et al. 2013; Reidy et al. 2014). Members of the J-domain protein family influence diverse physiological processes such as growth under normal and stress conditions in plants (Petti et al. 2014; Park and Kim 2014; Ohta and Takaiwa 2014), apoptosis in mammalian cells (Sinha and D'Silva 2014; Lee et al. 2015), tumor cell proliferation (Huang et al. 2014), and protection against bacterial infection (Song et al. 2014), through binding of select substrates and interaction with Hsp70.

The extremophile crustacean, *Artemia franciscana*, undergoes oviparous development resulting in the production of cysts which are gastrula-stage embryos enclosed in a chitinous shell (Liang and MacRae 1999; MacRae 2003; Dai et al. 2011; Ma et al. 2013; MacRae 2016). Upon release from females, cysts enter a state of metabolic dormancy termed diapause, characterized in *Artemia* by extreme resistance to environmental and physiological stress (Clegg 1997, 2011; MacRae 2003, 2005, 2010, 2016). Diapause terminates upon exposure to external cues such as desiccation and cold, and the cysts either enter quiescence if conditions are unfavorable for growth or undergo post-diapause development into swimming

larvae (nauplii) (MacRae 2003; Robbins et al. 2010). The survival of *A. franciscana* cysts during diapause and quiescence depends on molecular chaperones including the small heat shock protein (sHsp) p26 (Sun et al. 2004; Villeneuve et al. 2006; King and MacRae 2012), the ferritin homolog artemin (Chen et al. 2007; Hu et al. 2011; King et al. 2014) and Late Embryogenesis Abundant (LEA) proteins (Warner et al. 2010; Toxopeus et al. 2014). These proteins disappear subsequent to diapause termination and have, at most, a minor part in post-diapause development of *A. franciscana*.

Hsp70 has a role in the growth of *A. franciscana* (Clegg et al. 2000; Willsie and Clegg 2002) and protection against stress, including heat and pathogenic bacteria (Sung et al. 2008, 2009a, b; Baruah et al. 2011), but information on its major co-chaperone, Hsp40, is lacking as it is for most crustaceans. With this in mind, a search was initiated for J-domain proteins in *A. franciscana* resulting in the cloning and sequencing of a complementary DNA (cDNA), termed *ArHsp40*, encoding a type I Hsp40. The synthesis of *ArHsp40* is developmentally regulated during post-diapause development, undergoing a dramatic decrease upon hatching of nauplii. Additionally, *ArHsp40* is heat inducible during post-diapause development, indicating a role in stress tolerance, and because it occurs in cysts newly released by females, it may also contribute to stress tolerance during diapause.

Materials and methods

Culture of *A. franciscana*

Nonhydrated, post-diapause *A. franciscana* cysts from the Great Salt Lake (INVE Aquaculture Inc., Ogden, UT, USA) were placed in filtered and autoclaved seawater (23 ‰) from Qingdao Harbor, hereafter termed seawater, and incubated 20 h at 25 °C. Emerged *A. franciscana* nauplii (E2) (Fig. 1a) (Go et al. 1990; Liang and MacRae 1999; Jiang et al. 2011), representing a life history stage actively engaged in protein synthesis and thus likely to contain Hsp40, were individually harvested from the culture with a Pasteur pipette under a dissecting microscope and homogenized to obtain RNA as described below. *A. franciscana* cysts were also hydrated for at least 3 h on ice in distilled H₂O, collected by filtration, washed with cold distilled H₂O, and incubated with vigorous shaking in seawater at 27 °C. Samples were collected for RNA and protein extraction at time 0 which was the start of incubation at 27 °C following hydration (cysts), after 5 h (cysts) and 10 h (cysts and emerged E1) and as emerged, membrane-enclosed E2/E3 nauplii, all of which were homogenized (Fig. 1a). After hatching of E2/E3 nauplii, first instar larvae (Fig. 1b) were collected by phototaxis and either homogenized immediately or incubated in seawater for 16 h at 27 °C to obtain second instar larvae (Fig. 1c) (Langdon et al. 1991). Second instar

Fig. 1 Post-diapause developmental stages of *A. franciscana*. Post-diapause cysts of *A. franciscana* were incubated in seawater, collected and homogenized for mRNA and protein preparation. **a** Cysts and emerging nauplii, **b** instar 1 nauplii, and **c** early instar 2 nauplii. Early and late stages of instar 2 nauplii are morphologically similar to one another. E1 emerged 1 nauplius, E2 emerged 2 nauplius, E3 emerged 3 nauplius, C cyst, ES eye spot

larvae were collected after incubation at 27 °C for either 16 h (early instar 2) or 26 h (late instar 2). Light micrographs of cysts and other life history stages of developing *A. franciscana* were obtained with an Olympus SZ61 stereo-microscope and an Infinity 1–1 camera (Lumenera, Ottawa, ON, Canada).

Cloning of *A. franciscana* Hsp40 cDNA

RNA obtained from homogenates of *A. franciscana* E2 nauplii with the RiboPure™ kit (Ambion, Austin, TX, USA) according to manufacturer's instructions was quantified by spectrophotometry. Single-strand cDNA was synthesized from 2 µg of RNA with the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, Burlington, ON, Canada).

To obtain a partial *Hsp40* cDNA from *A. franciscana*, the NCBI Expressed Sequence Tag (EST) database was searched for Hsp40 sequences using *Daphnia pulex* Hsp40 (Accession number EFX77852.1) as reference. Based on sequence comparisons, primers for the amplification of *A. franciscana* *Hsp40* cDNA were synthesized (Shanghai Sangon Biological Engineering Technology and Service Co., Ltd., Shanghai, China) (Table 1). PCR mixtures contained 2.5 µl 10× Taq Buffer, 2 µl 25 mM MgCl₂, 0.5 µl dNTP mixture at 10 mM each, 1 µl of each primer at 10 µM, 0.5 µl cDNA, 17.25 µl H₂O, and 0.25 µl Taq polymerase at 5 U/µl (Shanghai Sangon Biological Engineering and Service Co., Ltd). PCR was at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 8 min. PCR products, resolved in 1 % agarose gels and purified with the Quickclean III PCR Extraction Kit (GenScript USA Inc., Piscataway, NJ, USA), were ligated into the TA vector pGEM (Fermentas, Ottawa, ON, Canada) and used to transform competent TOP10 *Escherichia coli* (Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.). Recombinant pGEM vectors containing cDNA inserts of the appropriate size were isolated with the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, Oakville, ON, Canada), and the inserts were sequenced (Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.) revealing a partial *Hsp40* cDNA.

Full-length *A. franciscana* *Hsp40* cDNA was obtained by 5'- and 3'-RACE using the FirstChoice® RLM-RACE Kit

Table 1 Primers used in the cloning of Hsp40 cDNA from *A. franciscana*

Primers	Sequences (5'-3')	Position	T _m (°C)
Partial ArHsp40 Forward	TTTGGTGGTGGTATGGGT	355	56.9
Partial ArHsp40 Reverse	CGTCAATAACCTCGCCTT	652	56.3
5' RACE Outer Primer	GCTGATGGCGATGAATGAACACTG		
5' RACE Inner Primer	CGCGGATCCGAACACTGCGTTTGCTGGCTTTG ATG		
5' RACE Gene specific Outer Primer Reverse	CCAGAACAATCGTGACAAAC	632	56.6
5' RACE Gene specific Inner Primer Reverse	CACATTTCTGAGTAGCACCTT	538	56.9
3' RACE Outer Primer	GCGAGCACAGAATTAATACGACT		
3' RACE Inner Primer	CGCGGATCCGAATTAATACGACTCACTATAGG		
3' RACE Gene specific Outer Primer Forward	AGCGTCACCCTTGGAGAAT	421	59.7
3' RACE Gene specific Inner Primer Forward	CGAAATGTGAAGGTCGTGG	491	60.1
Full-length ArHsp40 Forward	GCGGATCC ATGCCAAAGGAAACAGGGTT	76	60.7
Full-length ArHsp40 Reverse	CGCCAT GGCT ACTGGGTTGGACATTGAACTC	1290	59.9

Primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd., Shanghai, China. Position denotes the first base of the primer in the full-length cDNA; T_m (°C) indicates the annealing temperature. Bolded sequences in the full-length forward and reverse primers are respectively restriction sites for BamHI and NcoI

(Ambion Applied Biosystems, Austin, TX, USA) and primers based on the sequence of the partial *A. franciscana* Hsp40 cDNA clone (Table 1). The 5'- and 3'-RACE products were resolved in 1 % agarose, purified as described above, cloned, and sequenced. A full-length clone of *Artemia* Hsp40 was obtained by PCR using forward and reverse primers, respectively containing BamHI and NcoI restriction sites (Table 1) and single-strand cDNA from emerged E2 nauplii as template. Reaction mixtures contained 5-μl 10× Taq buffer with 25 mM MgCl₂, 5 μl dNTP mixture with each nucleotide at 10 mM, 2 μl of each primer, 1-μl cDNA template, 34.5 μl nuclease-free H₂O, and 0.5 μl i-pfu DNA polymerase at 2.5 U/μl (iNtRON Biotechnology, Inc., Korea). PCR was at 94 °C for 2 min, 35 cycles of 94 °C for 20 s, 61 °C for 10 s, and 72 °C for 30 s, followed by 3 min at 72 °C. Analysis and purification of PCR products were as described above. The cDNA fragments and the His-tagged expression vector pRSET B (Invitrogen) were digested with BamHI and NcoI, and products were purified as described above. The vector and cDNA were mixed with 2 μl 10× T4 DNA ligase buffer, 0.2 μl of T4 DNA ligase (TaKaRa, Shiga Japan), and 17.8 μl nuclease-free H₂O. Following transformation of competent BL21(DE3) pLysS *E. coli* (Shanghai Sangon Biological Engineering and Service Co., Ltd) inserts cloned in pRSET B were sequenced revealing a full-length Hsp40 cDNA clone from *A. franciscana* termed ArHsp40.

Sequence analysis of ArHsp40

Nucleotide and deduced amino acid sequences, the latter obtained with ExpASY <http://www.expasy.org/spdbv>, were submitted to the National Center of Biotechnology International (NCBI) database for BLASTN and BLASTP searches <http://www.ncbi.nlm.nih.gov/>. Multiple sequence alignments were made with ClustalW version 2.0 <http://www.ebi.ac.uk/Tools/msa/clustalw2>. Protein structural predictions were made with Phyre2 <http://www.sbg.bio.ic.ac.uk/phyre2>.

ArHsp40 mRNA quantitation in post-diapause *A. franciscana*

TRIzol® (Invitrogen, Burlington, ON, Canada) was employed to recover RNA from seven different post-diapause life history stages of *A. franciscana* (Fig. 1). All RNA samples were incubated with DNaseI and then heated at 65 °C for 5 min. RNA was quantified by spectrophotometry and single-strand cDNA was synthesized from 2 μg of RNA with the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, Burlington, ON, Canada). For qRT-PCR, the forward and reverse primers, respectively 5'-GTGCATCAGTTGAGCGTCAV-3' and 5'-TGCTGAACCATTCAGGAG C-3', were used to amplify an

ArHsp40 cDNA fragment of 194 bp. An α -tubulin DNA fragment of 276 bp was amplified as internal standard using the forward and reverse primers, respectively 5'-CGAC CATAAAAGCGCAGTCA-3' and 5'-CTAC CCAGCACCACAGGTCTCT-3'. Primers were used at 1 mM. qPCR was conducted with a QuantiTect® SYBR® Green PCR Kit (Qiagen, Mississauga, ON, Canada) in a Rotor-Gene RG-3000 system (Corbett Research, Sydney, NSW, Australia) using 0.5 μ l cDNA as template. Copy numbers of *ArHsp40* and α -tubulin cDNAs were determined by the use of Rotor-Gene 6 Software (Corbett Research).

ArHsp40 quantitation in post-diapause *A. franciscana*

A. franciscana at the life history stages shown in Fig. 1 were homogenized on ice with a micropestle (Kimble Chase, Vineland, NJ, USA) in Pipes buffer (100 mM Pipes, 1 mM MgCl₂, 1 mM EGTA, pH 7.4) containing proteolytic enzyme inhibitors (Halt Protease Cocktail, #87,786, Pierce Biotechnology, Rockford, IL, USA) and centrifuged at 12,000g for 10 min at 4 °C. After determination of protein concentration by the Bradford assay, 40 μ g of protein from each supernatant was loaded in separate lanes of 12.5 % SDS polyacrylamide gels. Following electrophoresis, gels were either stained with Coomassie or transferred to nitrocellulose and incubated for 20 min at room temperature with polyclonal antibodies raised to either mammalian Hsp40 (ADI-SPA-400) (Enzo Life Sciences, Farmingdale, NY, USA), or to the ArHsp40 peptide, 331-VKFPDVINPALIPQLE-346, (Anti40-type 1) (Abiocode, Agoura Hills, CA, USA) and to tyrosinated α -tubulin (Anti-Y) (Xiang and MacRae 1995). Anti40-type 1 reacted specifically with a protein of the appropriate size on Western blots produced by IPTG-induced bacteria transformed with *ArHsp40* cDNA in the plasmid pRSET-B, but not with any protein in extracts from bacteria transformed with empty vector. ArHsp40 ADI-SPA-400 and Anti-Y were diluted 1:1000 in TBS (10 mM Tris, 140 mM NaCl, pH 7.4) and Anti40-1 was diluted 1:5000 in PBS (10 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Membranes were washed after exposure to primary antibody and incubated with HRP-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich, Oakville, ON, Canada) diluted 1:10,000 in either TBS (ADI-SPA-400 and Anti-Y) or PBS (Anti40-type1) (King and MacRae 2012). Subsequent to membrane washing, antibody-reactive proteins were visualized with ECL plus Western blotting detection reagents (GE Healthcare, Baie d'Urfe, Quebec, Canada) and a DNR Bio-Imaging Systems MF-Chemi-BIS 3.2 gel documentation system. Immunoreactive proteins were quantitated with Image Studio Software (Li-Core Biosciences, Lincoln, NE, USA), and band intensities for ArHsp40 and tyrosinated α -tubulin were compared at each developmental stage examined. PINK

Plus Prestained Protein Ladder (FroggaBio, Toronto, ON, Canada) was used as the molecular mass marker.

Heat induction of ArHsp40

First instar *A. franciscana* nauplii grown at 27 °C were collected 4 h after hatching when they contained little ArHsp40, washed with seawater on 5- μ m nylon mesh woven filters (Spectrum Labs Inc., Rancho Dominguez, CA, USA), and then incubated with aeration in 20 ml seawater in Corex tubes at 39 °C for 1 h in a programmable water bath (VWR International LLC, Mississauga, ON, Canada). Heat shock at 39 °C was chosen on the basis of previous experiments (Liang and MacRae 1999). Animals heat shocked at 39 °C for 1 h were either homogenized immediately in Pipes buffer or allowed to recover at 27 °C for 2, 4, 6, and 8 h and then homogenized. After protein determination by the Bradford method, 40 μ g of each cell-free homogenate was resolved in SDS polyacrylamide gels, blotted to nitrocellulose and probed with antibodies to either ArHsp40 or tyrosinated α -tubulin as described above. ArHsp40 and tyrosinated α -tubulin were quantitated with Image Studio Software (Li-Core Biosciences).

Statistical analysis

One-way ANOVA was used to determine if ArHsp40/tubulin messenger RNA (mRNA) and protein means differed significantly across developmental stages and during heat shock treatments.

Results

Cloning of *ArHsp40* cDNA

A cDNA consisting of 1546 nucleotides was obtained from emerged (E2) nauplii of *A. franciscana* by 5'- and 3'-RACE (Fig. 2). The cDNA (Accession Number KP300765) contained a 5'-untranslated region (UTR) of 75 nucleotides and, following the stop codon, a 3'-UTR of 256 nucleotides with a poly(A) tail. An open reading frame (ORF) of 1212 bps encoded a polypeptide of 404 amino acid residues with a predicted molecular mass of 45.0 kDa and a theoretical pI of 6.73. The lack of a signal sequence indicated that the protein encoded by the cDNA remained in the cytosol. The protein contained an amino terminal J-domain, composed of amino acid residues 5Thr-Asp65 with the highly conserved HPD motif in position 34–36 (Fig. 2). Protein modeling using the online server Phyre2 (<http://www.sbg.bio.ic.ac.uk/phyre2>) and Swiss-PDB viewer 4.1.0 (<http://www.expasy.ch/>) indicated that the J-domain consisted of 4 α -helices with a loop region between helices II and III where the HPD motif resided (Fig. 3). The J-domain was followed by a Gly/Phe (G/F)-rich

The immunoprobings of Western blots containing cell-free protein extracts disclosed ArHsp40 and tyrosinated α -tubulin in all life history stages of *A. franciscana* examined (Fig. 6). ArHsp40 and tyrosinated α -tubulin blotted from SDS polyacrylamide gels to nitrocellulose respectively had molecular masses of approximately 45 and 55 kDa. The amount of ArHsp40 in protein extracts was relatively constant in pre-emerged and emerged nauplii and then decreased dramatically upon hatching to the point that it was barely visible on Western blots containing protein extracts from instar 2 nauplii (Fig. 6a, b). No change was observed for tyrosinated α -tubulin during the stages of post-diapause development examined (Fig. 6c). The immunoprobings of Western blots was repeated

qRT-PCR, using α -tubulin mRNA as the internal standard, revealed that during post-diapause development, *ArHsp40* mRNA varied slightly until the E2/E3 stage and then declined significantly upon hatching (Fig. 5). Similar results were observed in initial semiquantitative experiments by the use of RT-PCR (not shown).

Fig. 4 Sequence alignment of Hsp40s. The amino acid sequence of ArHsp40 from *A. franciscana* was aligned with Hsp40s from the species listed in Table 2. Identical amino acid residues are indicated by

asterisk and similar amino acid residues are indicated by *colon*. The domains of ArHsp40 are indicated by *labeled black lines* above the sequence

Table 2 ArHsp40 shares identity with Hsp40 from other organisms

Species	Class	Identity (%)	Accession number
<i>Caenorhabditis elegans</i>	Chromadorea	49	NP_493570.1
<i>Aedes aegypti</i>	Insecta	64	ABF18277.1
<i>Culex quinquefasciatus</i>	Insecta	65	XP_001844792.1
<i>Daphnia pulex</i>	Branchiopoda	67	EFX77852.1
<i>Acyrtosiphon pisum</i>	Insecta	64	NP_001119620.1
<i>Megachile rotundata</i>	Insecta	67	XP_003699212.1
<i>Spodoptera frugiperda</i>	Insecta	65	AHA86298.1
<i>Dendroctonus ponderosae</i>	Insecta	66	ERL94599.1
<i>Tribolium castaneum</i>	Insecta	66	XP_971446.1
<i>Helobdella robusta</i>	Clitellata	61	XP_009028927.1
<i>Biomphalaria glabrata</i>	Gastropoda	60	XP_013094153.1
<i>Homo sapiens</i>	Mammalia	63	NP_001530.1
<i>Saccoglossus kowalevskii</i>	Enteropneusta	63	XP_002734268.1
<i>Branchiostoma floridae</i>	Leptocardii	63	P_002591298.1

The % identity between ArHsp40 and individual Hsp40s from different organisms was determined by BLASTP at <http://www.ncbi.nlm.nih.gov>

several times using different protein preparations, and the results were highly repeatable.

ArHsp40 synthesis was heat inducible

Immunoprobings of Western blots revealed that ArHsp40 increased significantly in instar 1 nauplii upon 1 h of heat shock, the shortest time tested, and stayed at this level for the first 2 h of recovery at 27 °C, increasing approximately 10-fold

relative to control nauplii (Fig. 7). ArHsp40 levels decreased significantly upon further recovery, declining to control values after 8 h.

Discussion

A type I Hsp40 cDNA termed *ArHsp40* was cloned from emerged (E2) nauplii of *A. franciscana*, one of the few crustaceans where this molecular chaperone has been identified (Lee et al. 2012; Zhang et al. 2013). Acquisition of *ArHsp40* cDNA required cloning of a partial *Hsp40* sequence by RT-PCR using primers designed on the basis of results obtained by searching data bases with *Hsp40* cDNA from *D. pulex*, followed by 5'/3'-RACE. In common with other type 1 Hsp40s, ArHsp40 possesses an amino-terminal J-domain featuring the highly conserved HPD motif, a G/F-rich region, a ZBD with four CXXCXGXG motifs, one of which ended in K rather than G, and a carboxyl-terminal region thought to bind substrate. The cysteine residues in the CXXCXGXG motifs of the HSP40 ZBD are absolutely conserved, but an equivalent substitution of lysine for glycine at positions corresponding to 204 in ArHsp40 occurs in other species (Martinez-Yamout et al. 2000). The substitution is tolerated structurally even though it introduces a positive charge into a region of electro-negativity, suggesting functional consequences, but the significance of this change is unknown. Sequence alignment and protein modeling showed that ArHsp40 was similar to Hsp40s from other species suggesting that it functions in the same way. Hsp40s serve as molecular chaperones, and they transport bound substrates to Hsp70 for folding and/or degradation, processes involving the stimulation of Hsp70 ATPase by Hsp40 (Ahmad et al. 2011; Carmel et al. 2011; Baaklini

Fig. 5 *ArHsp40* mRNA decreased during post-diapause development of *A. franciscana*. qRT-PCR, using α -tubulin as internal standard, was employed to amplify a 194-bp fragment of *ArHsp40* mRNA in samples obtained from seven post-diapause life history stages of *A. franciscana*. Lane 1, 0 h cysts; lane 2, 5 h cysts; lane 3, 10 h cysts/E1 nauplii; lane 4, E2/E3 nauplii; lane 5, instar 1 nauplii; lane 6, early instar 2 nauplii; lane 7, late instar 2 nauplii. The copy numbers of *ArHsp40* and α -tubulin cDNAs, determined by Rotor-Gene 6 Software (Corbett Research), were compared to one another. The experiment was done in duplicate with RNA from animals prepared at different times. Error bars represent standard deviation. The asterisks indicate that the mean copy number of *ArHsp40* mRNA transcripts is statistically different from the *ArHsp40* mRNA copy number in cysts at development time 0, $p < 0.05$. Different numbers of asterisks indicate that copy numbers are statistically different from one another

Fig. 6 ArHsp40 decreased during post-diapause development of *A. franciscana*. Cell-free protein homogenates prepared from seven post-diapause life history stages of *A. franciscana* were resolved in 12.5 % SDS polyacrylamide gels and either stained with Coomassie (a) or blotted to nitrocellulose and stained with antibody to either ArHsp40 (b) or tyrosinated α -tubulin (c). The bands formed on the blots by antibody-reactive proteins were quantitated with Image Studio Software and the ratio of ArHsp40/tyrosinated α -tubulin was calculated (d). Lane 1, 0 h cysts; lane 2, 5 h cysts; lane 3, 10 h cysts/E1 nauplii; lane 4, E2/E3 nauplii; lane 5, instar 1 nauplii; lane 6, early instar 2 nauplii; lane 7, late instar 2 nauplii. Error bars represent standard deviation. The asterisks indicate that the amount of ArHsp40 is statistically different from the amount of ArHsp40 in cysts at development time 0, $p < 0.05$. Different numbers of asterisks indicate that the amounts of ArHsp40 are statistically different from one another

et al. 2012; Borges et al. 2012; Torrente and Shorter 2013; Summers et al. 2013; Tiwari et al. 2013; Cuéllar et al. 2013; Reidy et al. 2014).

qRT-PCR and immunoprobings of Western blots respectively revealed that *ArHsp40* mRNA and protein exist in all life history stages of *A. franciscana* examined and that they declined in amount upon hatching, such that they became difficult to detect. Moreover, ArHsp40 synthesis

Fig. 7 ArHsp40 synthesis was induced by heat shock in post-diapause *A. franciscana*. Instar 1 nauplii, collected as described in “Materials and methods,” were heat shocked at 39 °C 1 h, sampled and then allowed to recover at 27 °C with sampling at 2-h intervals. Protein extracts were prepared from animals incubated only at 27 °C (control), after heat shock for 1 h and upon recovery at 2-h intervals, resolved in 12.5 % SDS polyacrylamide gels and either stained with Coomassie (a) or blotted to nitrocellulose and stained with antibody to either ArHsp40 (b) or tyrosinated α -tubulin (c). The bands formed on the blots by antibody-reactive proteins were quantitated with Image Studio Software, and the ratio of ArHsp40 to tyrosinated α -tubulin was calculated (d). Lane C, no heat shock; HS, 1 h at 39 °C; 2R, 4R, 6R, and 8R, recovery at 27 °C, respectively, for 2, 4, 6, and 8 h. Error bars represent standard deviation. The asterisks indicate that the amount of ArHsp40 is statistically different from the amount of ArHsp40 in nonstressed nauplii, $p < 0.05$. Different numbers of asterisks indicate that the amounts of ArHsp40 are statistically different from one another

is induced by thermal stress. These results indicated, in addition to a role for ArHsp40 in stress tolerance, that ArHsp40 influences post-diapause growth and development and that this effect decreases or even terminates upon hatching, this the first demonstration of Hsp40 developmental regulation in a crustacean. By comparison,

differential regulation of *Hsp40* gene expression takes place within tissues and diapause-induced eggs of the insect *Bombyx mori* (Sasibhushan et al. 2013; Li et al. 2016). The occurrence of ArHsp40 in metabolically active life history stages of *A. franciscana* indicates an involvement in protein homeostasis during post-diapause growth, which, because the protein is found in undeveloped cysts, may also be true for diapause-destined embryos.

Hsp110, Hsp70, sHSPs, and Hsp40, the latter possessing increased functional effectiveness when in mixed classes of J-domain proteins, comprise a protein disaggregase in metazoans whereby denatured proteins retrieved from aggregates are refolded (Shorter 2011; Torrente and Shorter 2013; Summers et al. 2013; Reidy et al. 2014; Nillegoda and Bukau 2015; Nillegoda et al. 2015). Proteins within diapause and quiescent cysts of *A. franciscana*, as well as in larvae and adults, may denature and aggregate when encountering stresses such as desiccation and heat. A disaggregase machinery in *A. franciscana* involving the stress-inducible ArHsp40 would enhance the refolding of aggregated proteins thus favoring growth and development. ArHsp40 may also assist Hsp70 in the liberation and refolding of partially denatured proteins thought to be protected during diapause in *Artemia* by association with sHsps and artemin (Liang and MacRae 1999; Hu et al. 2011; King and MacRae 2012; King et al. 2014).

Hsp70, Hsp40, and other HSPs are elevated in cold-adapted larvae of the gall fly, *Eurosta solidaginis*, suggesting that these molecular chaperones contribute to protein preservation during stress (Zhang et al. 2011). The mRNA for Hsp40, among several other molecular chaperones, is upregulated during recovery from cold stress in *Drosophila melanogaster*, implying that Hsp40 works in concert with other chaperones to repair protein chilling injury (Colinet et al. 2010), just as it could during recovery from heat shock and from stresses associated with diapause and quiescence. mRNAs encoding Hsp70 and Hsp40 build up concurrently in adults of the flesh fly *Sarcophaga crassipalpis* exposed to hypoxia and then decline more or less coordinately throughout recovery under normoxic conditions (Michaud et al. 2011), implying linked functions, initially to prevent irreversible denaturation of proteins and then to ensure efficient protein refolding. Based on transcript quantization, Hsp40 contributes to the thermo-adaptation of *D. melanogaster* along a microclimate gradient demonstrating a role in stress tolerance (Carmel et al. 2011). The coordinated synthesis of Hsp70 and ArHsp40 in *A. franciscana*, should it occur during early post-diapause development, would suggest that these proteins cooperate to maintain protein homeostasis, as proposed for diapausing *B. mori* (Sasibhushan et al. 2013).

Conclusions

cDNA encoding a J-domain protein with a molecular mass of approximately 45 kDa and termed *ArHsp40* was cloned from post-diapause *A. franciscana* nauplii. Based on sequence and structural analyses, ArHsp40 is a type 1 Hsp40. *ArHsp40* mRNA and protein appeared in post-diapause *A. franciscana* life history stages, and both were maintained at relatively constant levels until the E2/E3 life history stages, after which they declined precipitously upon hatching. ArHsp40 has the potential to influence protein homeostasis in metabolically active post-diapause life history stages of *A. franciscana*, but the results suggest that it is replaced by another Hsp40 at hatching. Additionally, because ArHsp40 synthesis is induced by heat shock, suggesting a role in stress resistance, and it occurs in mature cysts, ArHsp40 has the potential to influence protein homeostasis during embryogenesis and in diapausing/quiescent cysts of *A. franciscana*.

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