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The RNA-editing deaminase ADAR is involved in stress resistance of *Artemia* diapause embryos

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**ABSTRACT**

The most widespread type of RNA editing, conversion of adenosine to inosine (A→I), is catalyzed by two members of the adenosine deaminase acting on RNA (ADAR) family, ADAR1 and ADAR2. These enzymes edit transcripts for neurotransmitter receptors and ion channels during adaptation to changes in the physical environment. In the primitive crustacean *Artemia*, when maternal adults are exposed to unfavorable conditions, they release diapause embryos to withstand harsh environments. The aim of the current study was therefore to elucidate the role of ADAR of *Artemia* diapause embryos in resistance to stress. Here, we identified *Artemia* ADAR (Ar-ADAR), which harbors a putative nuclear localization sequence (NLS) and two double-stranded RNA-binding motifs (dsRBMs) in the amino-terminal region and an adenosine deaminase (AD) domain in the carboxyl-terminal region. Western blot and immunofluorescence analysis revealed that Ar-ADAR is expressed abundantly in post-diapause embryos. Artemia (n = 200, three replicates) was tested under basal and stress conditions. We found that Ar-ADAR was significantly induced in response to the stresses of salinity and heat-shock. Furthermore, in vivo knockdown of Ar-ADAR (n = 100, three replicates) by RNA interference induced formation of pseudo-diapause embryos, which lack resistance to the stresses and exhibit high levels of apoptosis. These results indicate that Ar-ADAR contributes to resistance to stress in *Artemia* diapause embryos.

**KEYWORDS**

ADAR; *Artemia*; diapause; RNA editing; RNA interference; stress resistance

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Artemia, a primitive crustacean that resides in inland salt lakes, alternates between ovoviviparous or oviparous reproduction in response to environmental variation (Clegg & Trotman, 2002; Slegers, 1991). In favorable environments, i.e. in the presence of sufficient oxygen and food, as well as appropriate salinity and pH, Artemia produce nauplius larvae directly; otherwise, they release diapause embryos in which metabolism and cell proliferation are completely suspended (quiescence). Diapause embryos can withstand unfavorable conditions such as low oxygen, UV radiation, and variations in pH, temperature, and salinity. Diapause can be terminated by certain environmental conditions such as desiccation, dehydration, low temperature, or chemical treatment, leading to active post-diapause embryos (Drinkwater & Clegg, 1991). This property makes Artemia a good model for the study of biology in extreme environments (MacRae, 2016; Willis & Clegg, 2001). Although progress has been made in understanding Artemia embryonic diapause in stress resistance, many features of this process remain to be elucidated.

The aim of the current study was to test the hypothesis that an ADAR has a role in stress resistance of Artemia diapause embryos. Hence, we used degenerate PCR and rapid amplification of cDNA ends (RACE) to obtain the complete cDNA sequence of an ADAR from Artemia (Ar-ADAR). Phylogenetic analysis revealed that Ar-ADAR is in the crustacean clade and closely related to ADAR from Daphnia pulex. Western blotting confirmed that Ar-ADAR was present in Artemia diapause embryos, and that it was abundant in post-diapause embryos but absent in other stages. Moreover, Ar-ADAR expression was markedly augmented in response to extreme stresses (high salinity and heat-shock). RNA interference (RNAi)-mediated knockdown of Ar-ADAR resulted in release of pseudo-diapause embryos that lacked the ability to withstand harsh environments. These results indicate that Ar-ADAR is regulated by various stresses and is involved in stress resistance of Artemia diapause embryos.

Methods

Culture and collection of samples of Artemia parthenogenetica

Diapause embryos of Artemia parthenogenetica were harvested from Gaahi Lake (Qinghai Province, China). These diapause embryos were activated into post-diapause embryos by soaking in saturated artificial seawater (Blue Starfish, Zhejiang, China) for 48 h and freezing at –20 °C for three months. Next, the post-diapause embryos were hydrated at 4 °C for 5 h, and then incubated in 2.5% artificial seawater at 28 °C with continuous light for 24 h until they hatched out. Swimming nauplii (first larval stage of crustaceans) were reared in 8% (w/v) artificial seawater under light: dark (LD) cycles of 4 h L (12:00 h–16:00 h): 20 h D for oviparous Artemia or in 4% (w/v) artificial seawater under 16 h L (07:00 h–23:00 h): 8 h D for ovoviviparous Artemia. The water temperature was kept at 28 °C. The water was supplemented with Chlorella powder (Fuqing King Dnarmsa Spirulina Co. Ltd., Zhejiang, China) every two days.

The developmental stages were defined as follows: Early oocyte biogenesis stage (Early), oocytes reside in ovaries; Middle oocyte biogenesis stage (Middle), oocytes enter oviducts; and Late oocyte biogenesis stage (Late), the eggs enter the uterus. A sufficient amount of both oviparous and ovoviviparous Artemia was placed in an ice bath for 1–2 min until they were lightly anesthetized. Then the ovaries, ovisac or uterus were dissected. Diapause embryos (Dia) and post-diapause embryos (Post-dia) were washed with phosphate buffered saline, pH 7.4 (PBS) three times. Nauplii (Nau) were filtered through a strainer to remove the seawater, and then washed with PBS three times. All of these samples were snap-frozen in liquid nitrogen, and stored at –80 °C for protein extraction.

Cloning of full-length Ar-ADAR cDNA

Based on an alignment of AD domains of ADARs in different organisms, two sets of degenerate primers were designed (dAr-ADAR F1, dAr-ADAR F2, dAr-ADAR R1 and dAr-ADAR R2; sequences are provided in Table 1). To prepare a cDNA template for PCR amplification, total RNA was extracted from Artemia using Trizol reagent (Invitrogen, Carlsbad, CA). RNA concentration was quantitated on a Genova UV/visible spectrophotometer at 260 nm. First-strand cDNA was synthesized from 1 µg of total RNA using the SuperScript/First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA), and then used as a template for PCR. In the first round of PCR, Ar-ADAR dF1 and Ar-ADAR dR1 primers were used with the following program: 94 °C for 4 min; 35 cycles of 94 °C for 30 s, 45 °C for

| Primer   | Length (bp) | Position | Direction | Sequence (5’–3’)
|----------|-------------|----------|-----------|------------------|
| Ar-ADAR dF1 | 18          | 906–923  | F         | TNAATGAYT5SYCAYGGCNG
| Ar-ADAR dF2 | 18          | 1079–1096| F         | ACNCDCCDTGTGGDGAT
| Ar-ADAR dR1 | 20          | 1372–1389| R         | GCCMBSABYRTTCARC
| Ar-ADAR dR2 | 18          | 1274–1291| R         | YTRTCMSWRCACCAT
| RT– Ar-ADAR F | 20          | 1109–1128| F         | GTGTACAAAGACATGGGAAA
| RT– Ar-ADAR R | 20         | 1254–1273| R         | CTGTAGAAAGTCTTTACCA
| Tubulin F  | 20          | 446–465  | F         | GCAGTGTCCTACAAGGGTTTC
| Tubulin R  | 22          | 74–795   | R         | ATCAAAAGGCAGCCTGGGGTG
| 3’AR-ADAR F1 | 26          | 1080–1105| F         | CAGGCCTCCTTGATGGATGCAAGATT
| 3’AR-ADAR F2 | 28          | 1121–1148| F         | CGGGGAAGCTCTTGCTGTGGATTGCA
| 5’AR-ADAR R1 | 28          | 1213–1240| R         | CGGCAGTTCAAGGCCAGATTTCC
| 5’AR-ADAR R2 | 29          | 1151–1179| R         | CGGCAGTTCAAGGCCAGATTTCC
| shAr-ADAR F | 25          | 32–48    | F         | GTCTGAGAAGTCAACACACACAGA
| shAr-ADAR R | 25          | 519–541  | R         | CGGCATCTGGTTCCTGGGGGCCTAG

F and R indicate forward and reverse directions, respectively. Underlined regions represent recognition sequences of restriction endonucleases, which were added to the primers to facilitate cloning and do not hybridize with Artemia sequences.
30 s, and 72 °C for 30 s; and a final extension at 72 °C for 10 min. Following the first round of PCR, nested PCR was performed using the first PCR product as the template and the degenerate primers Ar-ADAR df2 and Ar-ADAR dr2, with the following program: 94 °C for 4 min; 35 cycles at 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 10 min. A cDNA fragment of approximately 180 bp was amplified and subcloned into vector pUCm-T (Takara Bio, Shiga, Japan) for sequencing analysis. RACE was performed using the primers 5Ar-ADAR F1, 5Ar-ADAR F2, 3Ar-ADAR R1 and 3Ar-ADAR R2 (Table 1) and adapter primers from the FirstChoice RLM-RACE Kit (Ambion, Austin, TX) separately, with the following program: 94 °C for 4 min; 35 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min; and a final extension at 72 °C for 10 min. Fragments of 1307 bp and 884 bp were obtained by 5′RACE and 3′RACE, respectively. The full-length Ar-ADAR cDNA sequence was obtained from the overlapping RACE fragments. The sequence of this cDNA has been submitted to the GenBank database with accession number KX011870.

**Bioinformatics**

The sequenced cDNA was edited and analyzed using Lasergene 7 (DNAStar Inc., Madison, WI), and the amino acid sequence of the peptide was predicted using the websites PredictProtein (http://www.predictprotein.org/) and Scratch Protein Predictor (http://www.ics.uui.edu/~baldig/scratch/). Blast searches (both blastx and blastn) were performed on the NCBI website (http://www.ncbi.nlm.nih.gov/blast). A conserved domain tool (http://www.ncbi.nlm.nih.Gov/Structure/cdd/wrpsb.cgi), the online analysis software ExPASy (http://prosite.expasy.org/), and Phyre2 (www.sbg.bio.ic.ac.uk/phyre2) were used to predict structure and functional domains. SignalP4.0 (http://www.cbs.dtu.Dk/services/SignalP/) was used to predict signal peptide and transmembrane regions. A phylogenetic tree was constructed with ClustalX 2.0 and the MEGA 6 software using the neighbor-joining (NJ) method. The bootstrap method was used to evaluate the accuracy of the tree (bootstrap value = 1000). The nucleotide sequence of Ar-ADAR was submitted to GenBank/DDBJ/EMBL with accession number KX011870.

**Double-stranded RNA (dsRNA) of Ar-ADAR construction and production**

For preparation of dsRNA, the plasmid PET-T7, which contains two inverted T7 promoter sites flanking the multiple cloning sites, was used as the expression vector (Dai et al., 2008). To obtain the reconstructed plasmid expressing Ar-ADAR-dsRNA, a 510 bp fragment in the coding region of the Ar-ADAR gene was amplified with specific primers (shAr-ADAR F and shAr-ADAR R; Table 1), and subcloned into PET-T7 at the XbaI and EcoRI sites. The recombinant plasmid was transformed into *Escherichia* (E.) coli DH5α and sequenced to confirm the inserted nucleotide sequence, and then transformed into *E. coli* HT115 to express dsRNA. A plasmid expressing GFP-dsRNA (Dai et al., 2011), constructed as described previously, was used as a negative control.

The production and purification of dsRNA used modified versions of protocols described previously (Yodmuang et al., 2006). The recombinant plasmid was transformed into *E. coli* HT115. The recombinant clone was inoculated into 100 ml of LB (lysogeny broth) (Sangon Biotech, Shanghai, China) medium and cultured to achieve an OD600 of 0.4 at 37 °C. To induce the expression of dsRNA, IPTG (L-isopropyl β-D-1-Thiogalactopyranoside) (Sangon Biotech, Shanghai, China) was added to a final concentration of 0.4 mM for 4 h. The bacterial cell was precipitated by centrifugation and washed once with PBS buffer, then re-suspended in 2 ml buffer containing 1 M NH4Ac and 10 mM EDTA. The same volume of phenol (pH 4.5): chloroform (1:1) was added into the mixture. The sample was incubated at 65 °C for 20 min (denatured) and then cooled down slowly (renatured). To eliminate single-stranded RNA in the loop region of the stem loop structure and endogenous RNA from the bacterial host strain, RNase A buffer (500 mM NaCl, 25 mM Tris–HCl, pH 7.5) and 2.5 mg/ml RNase A were added and then incubated for 30 min at 37 °C. Double-stranded RNA was extracted from bacterial lysate by phenol (pH 4.5): chloroform (1:1). Each dsRNA obtained from this preparation appeared as a single band in agarose gel electrophoresis and corresponded to its expected size. dsRNA concentration was estimated at OD260 and adjusted to a final concentration of 10 μg/ml prior to storage at −80 °C until use.

**Injection**

Diapause-destined *Artemia* adults were injected with 1 μg of Ar-ADAR-dsRNA or GFP-dsRNA using an Ultra-MicroPump II equipped with a Micro4TM MicroSyringe pump controller (World Precision Instruments Inc., Sarasota, FL). After injection, *Artemia* were cultured in 8% artificial seawater with 4 h light exposure per day. One week later, RNA and proteins were extracted from the offspring as described previously (Dai et al., 2011), and real-time quantitative PCR and western blotting analyses were performed to assess RNAi efficiency. Three independent groups were analyzed.

**Real-time quantitative PCR**

Total RNAs were extracted from offspring of non-treated, GFP-dsRNA, and Ar-ADAR-dsRNA-induced *Artemia* using TRIzol reagent (Invitrogen, Carlsbad, CA). The extracted RNA was quantified by measuring the absorbance at 260 nm with a Genova UV/visible spectrophotometer. First-strand cDNAs were prepared from the total RNA specimens as described above. After reverse transcription, real-time PCR reactions were performed on the Bio-Rad MiniOpticonTM Real-Time PCR System using SYBR Premix Ex TaqTM (TaKaRa Bio, Shiga, Japan) and gene-specific primers to amplify Ar-ADAR and tubulin as an internal control (RT-Ar-ADAR F: GTGATACAGATCAGGGAAG and RT-Ar-ADAR R: CGTTAGAAGTCTTTCACCAC for Ar-ADAR; Tubulin F: GCA GTGGTCTACAAGGTTTC and Tubulin R: ATCAAAACGAA
The relative amounts of mRNAs were analysed using the comparative CT method (Schmittgen & Livak, 2008). All data were expressed as the mean ± SEM of three independent repetitions.

All statistical analyses were performed by one-way analysis of variance, and p < .01 was considered significant.

**Western blot analysis**

The anti-Ar-ADAR antibody was raised in rabbit (HuaAn Biotechnology Co. Ltd., Zhejiang, China) against peptides containing the partial amino acid sequences KRQAEDTVDLRSSLSC and CYGNPKFNATAEGPP, which represented the best epitopes with more hydrophilic amino acids. Proteins were extracted from each sample using the Trizol reagent and quantitated using the Bradford method. Fifty microgram samples of each protein were subjected to SDS-PAGE (10% or 12.5% gel), and the resolved proteins were electrotransferred to a nitrocellulose membrane at 8 V for 1 h in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 10% methanol). After blocking, the membrane was incubated overnight at 4 °C with antibody against Ar-ADAR (1:1000), Tubulin (1:5000), artemin (1:10,000), or p26 (1:10,000). After two washes in TBST (20 mM Tris, 137 mM NaCl, pH 7.6) for 10 min, the membrane was incubated with horseradish peroxidase (POD)-goat anti-rabbit IgG (1:25,000; Roche, Mannheim, Germany) or POD-goat antimouse IgG (1:25,000; Roche, Mannheim, Germany) for 40 min. After washing, detection was performed using the BM Chemiluminescence Western Blotting Kit (Roche, Mannheim, Germany). Bands on blots were quantified by measuring the intensities using ImageJ. These intensities were normalized against those of the loading control, Tubulin. The ratio of Ar-ADAR intensities using ImageJ. These intensities were normalized against Ar-ADAR (1:1000), Tubulin (1:5000), artemin (1:10,000), or p26 (1:10,000). After two washes in TBST (20 mM Tris, 137 mM NaCl, pH 7.6) for 10 min, the membrane was incubated with horseradish peroxidase (POD)-goat anti-rabbit IgG (1:25,000; Roche, Mannheim, Germany) or POD-goat antimouse IgG (1:25,000; Roche, Mannheim, Germany) for 40 min. After washing, detection was performed using the BM Chemiluminescence Western Blotting Kit (Roche, Mannheim, Germany). Bands on blots were quantified by measuring the intensities using ImageJ. These intensities were normalized against those of the loading control, Tubulin. The ratio of Ar-ADAR was quantified by the normalized intensities and described in histograms.

Statistical significance was determined using Student’s t-test.

**Immunofluorescence**

Late oocytes and diapause or post-diapause embryos were decapsulated with 3% sodium hypochlorite, fixed with 4% paraformaldehyde, paraffin-embedded, and cut into 6 μm sections using a microtome (Leica EM UC6). The slides were deparaffinized in xylene and rehydrated. Antigen retrieval was performed by boiling in 10 mM sodium citrate buffer (pH 6.0) for 10 min, followed by cooling at room temperature for 30 min. Moreover, the single cells were extruded and collected from post-diapause embryos, and adhered to the slides directly. Then the cells and sections were washed with PBS, blocked with 5% BSA (Sigma-Aldrich, St. Louis, MO) in PBS for 1 h, incubated at 4 °C overnight with anti-Ar-ADAR antibody (1:100), and then incubated with anti-rabbit Alexa 647-conjugated secondary antibody (1:200) (Invitrogen, Carlsbad, CA). The single cells from post-diapause embryos were also counterstained with DAPI (Sangon Biotech, Shanghai, China). Signals were detected by confocal microscopy. All confocal images were collected using a Zeiss LSM 510 and Zeiss 5 Live confocal microscope equipped with a 20 × or 63 ×, 1.4 NA objective lens. Excitation at 650 nm and observation at 665 nm was used to detect Alexa Fluor 647 staining (Invitrogen, Carlsbad, CA).

**Stress treatments**

Nauplii were subjected to the following stress treatments. For heat-shock, nauplii were exposed in 2.5% artificial seawater at 42 °C in a water bath for 10, 20 or 30 min (n = 200, three replicates). For salinity stress, nauplii were immersed in 2.5%, 10%, 15% and 20% NaCl with continuous rotation for 24 h (n = 200, three replicates). Nauplii collected after exposure to heat-shock (30 min) and 20% NaCl were incubated in 2.5% artificial seawater for 7 h under laboratory conditions. About 90% nauplii resumed the ability to swim freely and the live larvae were obtained as the stress-released specimens.

In RNAi-treated and control diapause embryos, hatching rates were monitored to determine resistance to salinity and heat-shock. To test the response to extreme salinity, RNAi-treated embryos, non-treated, and control diapause embryos and post-diapause embryos (activation) (n = 100, three replicates) were hatched in 2.5% and 20% NaCl for 24 h at 28 °C with continuous light for 24 h. To test the response to heat-shock, RNAi-treated, non-treated, and control post-diapause embryos (activation) (n = 100, three replicates) were incubated in a water bath at 42 °C for 30 min, and then incubated in 2.5% artificial seawater to monitor hatching rate.

All data are given as means ± SEM from three independent replicates. All statistical analyses were performed using a one-tailed, paired t-test, and p < .01 was taken to indicate a significant difference.

**TUNEL assay**

After injection of dsRNAs, non-treated, control and test Artemia embryos were fixed with 4% paraformaldehyde, paraffin-embedded, and cut into 6 μm sections using a microtome (Leica EM UC6). The terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay was performed using the DeadEnd Colorimetric TUNEL System (Promega, Madison, WI).

**Statistical analyses**

Tests are described in the Methods sections above.

**Results**

**Characterization of Ar-ADAR cDNA**

Based on an alignment of ADAR from Daphnia, Drosophila, zebrafish, Xenopus, mouse, and human, we designed two pairs of ADAR degenerate primers encoding a conserved peptide in the deaminase domain (FHLYIS/NTAPCGD and TMSCSDKIL/ARWN). Degenerate PCR on Artemia cDNA yielded a 180 bp fragment encoding a 60 amino acid peptide with greatest identity to the ADAR family. A complete Ar-ADAR cDNA was obtained by 5′ and 3′ RACE, and then amplified using gene-specific primers to confirm they originated from a single transcript. The full-length Ar-ADAR sequence was
Figure 1. Nucleotide sequences of Ar-ADAR cDNA and its deduced amino acid sequence, and a phylogenetic tree of the amino acid sequences of various ADAR. (a) Nucleotide and amino acid residue numbers are indicated on the left. Start (ATG) and stop codons (TAA) are in bold. Letters with light gray background represent the putative nuclear localization signal (NLS). Two dsRNA-binding motifs (dsRBMs) are shown with dark gray background. The adenosine deaminase (AD) domain is on black background. Termination codons are denoted by asterisks. Putative polyadenylation signals (AATAAA) are underlined. Nucleotide sequencedata have been submitted to the GenBank database with the accession numbers KX011870. The conserved amino acids of the catalytic core in the AD domain containing Zn$^{2+}$ binding coordinated by H296, C353 and C417, and IP6 binding sites R302, R303, K420, K532, Y560, K564, K574, W589 and K592 are marked by white boxes. (b) The ADARs used in this analysis and their accession numbers in GenBank/EMBL/DDBJ or SWISS-PROT databases are as follows: Artemia parthenogenetica ADAR (this work), Homo sapiens ADAR1 (XP_011507362), Homo sapiens ADAR2 (XP_011527727), Homo sapiens ADAR3 (NP_061172), Mus musculus ADAR1 (XP_006501815), Mus musculus ADAR2 (XP_006513125), Danio rerio ADAR1 (NP_571671), Danio rerio ADAR2 (XP_005161744), Harpegnathos saltator ADAR (EFN83585), Apis mellifera ADAR (XP_006563390), Drosophila melanogaster ADAR (NP_569940), Marsupenaeus japonicus ADAR (XP_570092), Daphnia pulex ADAR (NP_570092), Danio rerio ADAR1 (NP_571671), Danio rerio ADAR2 (XP_005161744), Harpegnathos saltator ADAR (EFN83585), Apis mellifera ADAR (XP_006563390), Drosophila melanogaster ADAR (NP_569940), Marsupenaeus japonicus ADAR (AHK23065), Daphnia pulex ADAR (EFX81797) and Caenorhabditis elegans ADAR (AAC25097). The tree was constructed using the neighbor-joining method. Bootstrap percentage values for 1000 replicate analyses are shown at branching points. The bar at the bottom shows the branch length corresponding to the mean number of differences (0.1) per residue along each branch.
1964 bp in length, including the flanking 31 bp 5'UTR, the 82 bp 3'UTR, and the 1851 bp open reading frame (ORF) encoding a putative 616-amino acid peptide. A canonical polyadenylation signal (AATAAA) was present downstream of the stop codon (TGA) (Figure 1(a)). The Ar-ADAR cDNA and the encoded peptides were subjected to BLAST searches, revealing that Ar-ADAR has high sequence identity with ADAR enzymes. The deduced Ar-ADAR harbors a putative N-terminal region, and an AD domain in the C-terminal region. The catalytic core in the AD domain containing Zn^2+ binding coordinated by H296, C353, and C417, and IP6 (the phosphorylated derivative of myo-inositol) binding sites R302, R303, K420, K532, Y560, K564, K574, W589 and K592 are conserved (Figure 1(a)). These results indicate that ADARs have been conserved, both structurally and functionally, over the course of evolution.

To examine the evolutionary relationships of Ar-ADAR with other species and various ADAR family members (ADAR1, ADAR2 and ADAR3), we constructed a phylogenetic tree based on the amino acid sequences from ten species, using the method of Hanks and Hunter (Hanks & Hunter, 1995). The phylogenetic tree was constructed by the NJ method, and statistical significance of groups within trees was evaluated using the bootstrap method with 1,000 replications. Analysis of the tree (Figure 1(b)) revealed that the ADAR3 family is quite close to the ADAR2 family, and Ar-ADAR is closer to the ADAR2/ADAR3 family than the ADAR1 family. ADARs were grouped into three main clusters: the vertebrate cluster, comprising Homo sapiens, Mus musculus, Danio rerio and Danio rerio; the insect cluster, comprising Harpegnathos saltator, Apis mellifera and Drosophila melanogaster; and the crustacean cluster, comprising Marsupenaeus japonicus, Artemia parthenogenetica and Daphnia pulex. The Artemia Ar-ADAR is in the crustacean clade, closely related to ADAR from Daphnia pulex.

**Ar-ADAR is abundantly expressed in post-diapause embryos of Artemia**

*Artemia* has two reproductive modes: oviparous and ovoviviparous (Figure 2(a)). Under favorable conditions, *Artemia* produces nauplii directly by ovoviviparous reproduction, whereas, under adverse environments, *Artemia* releases diapause embryos by oviparous reproduction. The dormancy of diapause embryos can be terminated by certain environmental stimuli (e.g. dehydration, freezing), resulting in activation into post-diapause embryos; subsequently, when external conditions become favorable, the embryos receive appropriate signals and proceed to hatch as nauplii. To detect the function of Ar-ADAR, we performed western blots to analyze the expression level of Ar-ADAR in the two reproductive pathways. As Figure 2(b) shows, Ar-ADAR was expressed specifically in diapause embryos (eight-fold higher than in nauplii) and abundantly in post-diapause embryos (50-fold higher than in nauplii). By contrast, the protein was expressed at a low level during oocytogenesis and in hatched nauplii. These results were also confirmed by immunofluorescence analysis (Figure 2(c)). Moreover, Ar-ADAR was distributed both in the cytoplasm and nucleus of cells of post-diapause embryo (Figure 2(d)). The results indicate that Ar-ADAR might be important for formation and/or maintaining of post-diapause in *Artemia*.

**RNAi treatment of Ar-ADAR induces formation of pseudo-diapause embryos**

To investigate the function of Ar-ADAR in *Artemia* further, we performed *in vivo* knockdown of Ar-ADAR by dsRNA interference. After injection of diapause-destined *Artemia* adults with 1 µg of Ar-ADAR-dsRNA, the Ar-ADAR mRNA level in the offspring was decreased to less than 5% of that in non-treated *Artemia* and control *Artemia* injected with a green fluorescent protein (GFP)-specific dsRNA (GFP-dsRNA) (Figure 3(a)), and Ar-ADAR protein expression also decreased significantly (Figure 3(b)). P26 and artemin (protein and RNA chaperones) were expressed abundantly in non-treated and control diapause embryos, but their levels decreased markedly in knockdown pseudo-diapause embryos, in which the embryos were not in the diapause state and continued to develop (Figure 3(c)). Offspring produced by females injected with Ar-ADAR-dsRNA, GFP-dsRNA or non-treated were superficially similar, but, following decapsulation, the non-treated and dsGFP-treated diapause embryos were uniform and dense, whereas the dsAr-ADAR-treated pseudo-diapause embryos were uneven and more transparent (Figure 3(d)).

**Pseudo-diapause embryos induced by Ar-ADAR RNAi lose the ability to withstand harsh environments**

Ar-ADAR is expressed specifically in *Artemia* diapause and abundantly in post-diapause embryos, which can withstand severe environments. To investigate the relationship between Ar-ADAR and stress resistance, we exposed embryos to high salinity or heat-shock. Western blotting revealed that Ar-ADAR was gradually upregulated in response to 2.5%, 10%, 15%, or 20% salinity, or to heat-shock at 42 °C for 10, 20, or 30 min (Figure 4(a)). Specifically, Ar-ADAR levels were 6–7-fold higher under extreme salinity (20%) or heat-shock (42 °C, 30 min) than in basal conditions. These results indicated that Ar-ADAR is induced by stress and is involved in the stress resistance of *Artemia* diapause embryos.

Next, we tested the effect of stress on hatching rate in Ar-ADAR RNAi offspring. For this purpose, we subjected pseudo-diapause (Ar-ADAR RNAi) embryos, control and non-treated post-diapause (activated) embryos to the stresses. Following high salinity and heat-shock stress treatments, the hatching rate was dramatically lower in pseudo-diapause embryos than in non-treated and control post-diapause embryos (Figure 4(b)).

**Knockdown of Ar-ADAR induces apoptosis**

Under favorable conditions, the hatching rate of non-treated and control post-diapause embryos (activation) was no less than 60%. Although the pseudo-diapause embryos induced by Ar-ADAR RNAi could be hatched directly, their hatching
Figure 2. Expression of Ar-ADAR at each developmental stage of oviparous and ovoviviparous Artemia. (a) Two reproductive pathways of Artemia. Under favorable conditions, swimming nauplius larvae are formed directly in an ovoviviparous pathway; to withstand adverse environments, Artemia undergoes an oviparous developmental pathway to release diapause embryos. Diapause embryos must be activated by some factors into post-diapause cysts, and then can be hatched out into nauplii. Early, early embryogenesis; Middle: middle embryogenesis; Late: late embryogenesis; Dia: diapause embryos; Post-dia: post-diapause embryos; Nau: Nauplius. Scale bars, 1 mm. (b) Western blot analysis of Ar-ADAR expression at each developmental stage. Tubulin was used as a loading control. The relative band intensities were quantitated using ImageJ software, and the ratio of Ar-ADAR to Tubulin is presented. Values are means ± SEM (n = 3). Statistical significance was determined by a one-tailed t-test, **p < .01. (c) Immunofluorescence analysis of Ar-ADAR. Light microscopy (LM) images were acquired to show overall morphology. Ar-ADAR, Ar-ADAR detected by Alexa Fluor 647-conjugated secondary antibody. DAPI, nuclei counterstained with DAPI. Merge, DAPI and Alexa Fluor 647 were merged. The cell is marked by a white dashed circle. Scale bars, 50 μm.
rate was no more than 40% (Figure 5(a)). Based on the speculation that embryos depleted of Ar-ADAR might contribute to the initiation of apoptosis, we subjected Artemia to Ar-ADAR interference and evaluated the extent of apoptosis. Ar-ADAR RNAi embryos exhibited more severe apoptosis than non-treated and control groups (Figure 5(b)). Thus, knockdown of Ar-ADAR induces apoptosis and compromised the hatch rate.

Discussion

In the current study, we found that Ar-ADAR was expressed specifically in Artemia in-diapause embryo, and abundantly in post-diapause embryos. Moreover, Ar-ADAR expression was markedly augmented in response to extreme stresses (high salinity and heat-shock). RNA interference (RNAi)-mediated knockdown of Ar-ADAR in vivo resulted in release of pseudodiapause embryos, which lacked the ability to withstand harsh environments. Our study supports a central role for AR-ADAR in stress resistance of Artemia diapause embryos.

Although the central role of A→I RNA editing in the central nervous system in mammals and Drosophila is well established, the physiological regulation and functional roles of ADAR-mediated RNA editing in tissues other than the brain and other organisms remain unclear. In this study, we report

Figure 3. Effect of knockdown of Ar-ADAR on diapause embryo formation in Artemia. (a) Real-time quantitative PCR analysis of Ar-ADAR mRNA expression in embryos produced by females non-treated (Intact), treated with green fluorescent protein (GFP)-specific (Control) or Ar-ADAR-specific RNAi (RNAi). mRNA levels were normalized to those of tubulin mRNA. Data are represented as means ± SEM of n = 3 replicates. **p < .01 by one-tailed t-test. (b) Western blot analysis of Ar-ADAR protein expression in the control and Ar-ADAR knockdown embryos. Tubulin was used as a loading control. The relative band intensities were quantified using ImageJ software, and the ratio of Ar-ADAR to Tubulin is presented. Values are means ± SEM (n = 3). Statistical significance was determined by one-tailed t-test, *p < .01. (c) Western blot analyses of p26 and artemin after RNAi. Tubulin was used as a loading control. (d) Phenotypes of embryos after RNAi. Scale bars, 50 μm.
our novel findings that Ar-ADAR is prominently expressed in post-diapause embryos in Artemia, and that its expression is regulated by stresses, specifically high salinity and heat-shock. Importantly, pseudo-diapause embryos, which were released from females treated with in vivo Ar-ADAR RNAi, lost the ability to withstand harsh environments. Our results indicate that, in addition to its functions in the central nervous system, ADAR plays an important role in stress resistance. However, the substrates edited by Ar-ADAR in Artemia in diapause and stress resistance remain to be elucidated in detail.

As crucial enzymes that catalyze the conversion of A to I, ADARs have been conserved both structurally and functionally throughout evolution. Three ADAR gene family members, ADAR1, ADAR2, and ADAR3, have been identified in vertebrates (Nishikura, 2016), whereas only a single ADAR2-like gene, dADAR, is known to be present in Drosophila melanogaster (Palladino et al., 2000). Two ADAR genes, CeADR1 and CeADR2, exist in Caenorhabditis elegans (Tonkin et al., 2002); two splicing isoforms of ADAR2 have been identified in squid (Palavicini et al., 2009); and ADAR1 and ADAR2, but not ADAR3, were identified by screens in sea urchin and sea anemone (Jin et al., 2009). In this study, we cloned and sequenced ADAR from Artemia. Ar-ADAR is closer to human ADAR2/ADAR3 than ADAR1 based on the NJ tree analysis. One NLS is predicted to exist in the N-terminal region of Ar-ADAR; thus the enzyme could localize in the nucleus, which was confirmed by immunofluorescence analysis, consistent with a role in A→I editing of pre-RNA. Ar-ADAR also has two copies of dsRBMs in the N-terminal region and one conserved AD domain in the C-terminal region. The sequence
PCGDARIF is identical to the corresponding sequence in human ADAR2, but differs from human ADAR1, which contains PCGDGALF at that position. In addition, Ar-ADAR does not contain Z-DNA-binding domains, which are characteristic of ADAR1 proteins, or an R domain as in ADAR3. These data indicate that Ar-ADAR is an orthologue of human ADAR2. Moreover, secondary structure analysis indicated that both dsRBMs (amino acids 37–98 and 135–206, respectively) of Ar-ADAR adopt the highly conserved α–β–β–β–α configuration.

The catalytic core in the AD domain of Ar-ADAR contains Zn$^{2+}$ binding and IP6 binding sites are conserved. This indicates ADARs were conserved both structurally and functionally over the course of evolution.

In human, squid, and Drosophila, conversion of A to I by RNA editing occurs primarily in the transcripts of the central nervous system, thereby diversifying the functions of ion channels and many other proteins. Although RNA editing by ADAR2 plays essential roles in the central nervous system (Bhalla et al., 2004; Burns et al., 1997; Rueter et al., 1995; Wang et al., 2000b), limited information is available regarding the functions and regulation of ADAR2 in other organism and tissues. Expression of ADAR2 recovered by some specific drugs has been reported (Tomaselli et al., 2013). Moreover, the expression and activity of ADAR2 are dramatically elevated in response to nutritional and metabolic cues, and depletion of this protein can influence the cellular exocytotic output by glucose in pancreatic islets and beta-cells (Gan et al., 2006). In this study, the expression of Ar-ADAR was induced by an increase in salinity or heat-shock. Specifically, the expression level of Ar-ADAR increased 6–7-fold under extreme stresses, e.g. high salinity (20% NaCl) or long heat-shock (42°C, 30 min). Thus, ADAR2 could be considered as a marker of a stress response.

Inactivation of ADAR gene family members has significant physiological consequences, as seen from the phenotypic alterations of ADAR mutants in various species. Mice with a homozygous ADAR2 null mutation experience repeated episodes of epileptic seizures and die several weeks after birth due to under-editing of the GluR-B Q/R site (Higuchi et al., 2000). Mutant Drosophila with a homozygous deletion in the dADAR gene exhibit brain-related changes, such as temperature-sensitive paralysis, uncoordinated locomotion, and age-dependent neurodegeneration, presumably resulting from deficient editing of important dADAR target genes such as Na$^+$- (para), Ca$^{2+}$- (Dmca1A), and glutamate-gated Cl$^-$ channels (DrosGluCl-α) (Palladino et al., 2000). C. elegans strains that contain homozygous deletions of both CeADR1 and CeADR2 exhibit defective chemotaxis (Tonkin et al., 2002). In this study, pseudo-diapause embryos released from females subjected to Ar-ADAR knockdown were more uneven and transparent and lost the ability to resist the stresses of high salinity and heat-shock. Specifically, pseudo-diapause embryos in which embryogenesis proceeded contains more apoptotic cells. Obviously, ADAR is critical for cell survival, especially under conditions of extreme stress and more research is needed to identify the specific dsRNA(s) targeted by Ar-ADAR in Artemia.

The stress resistance of Artemia diapause embryos is a consequence of both the outer shell and internal molecular events. The shell is composed of lipoprotein, chitin, hematin, metals and organic extracellular matrix peptides (SGEG1, SGEG2 and chitin-binding proteins A, B and C) that protect diapause embryos from environmental stresses (Clegg, 2005; Dai et al., 2011; Liu et al., 2009; Ma et al., 2013; Tanguay et al., 2004). Within the cell, p26, artemin, trehalose and glycerol protect against the damaging effects of heat, dehydration and cold (King et al., 2014; Viner & Clegg, 2001; Willis & Clegg, 2001). P26, a diapause-specific protein found in Artemia cysts, functions as a molecular chaperone, preventing heat- and reduction-induced protein aggregation in vitro.

![Figure 5. Knockdown of Ar-ADAR induces apoptosis. (a) Hatching rate of non-treated (Intact), control post-diapause embryos (activation) and pseudo-diapause embryos induced by Ar-ADAR RNAi. Triplicate experiments were performed for each stress. In every group, 100 cysts were allowed to hatch; the hatching rate was calculated from the resultant number of live nauplii. Statistical significance was determined by a one-tailed t-test, *p < .05. (b) Apoptosis was evaluated using the TUNEL assay. Images show representative TUNEL-stained sections from embryos of Artemia injected with dsGFP or dsAr-ADAR. Scale bars, 25 μm.](image-url)
Artemia alternates between ooviparous and oviparous reproduction in response to environmental conditions. In diverse environments, Artemia releases diapause embryos that remain in a quiescent state characterized by halted metabolism and cell cycle arrest. Consequently, Artemia diapause embryos are tolerant to harsh stressors such as extreme salinity, high pH, large temperature differences, intermittent dry conditions, anoxia, and UV radiation; these stresses can terminate dormancy, resulting in activation into post-diapause embryos. In this study, we report our novel findings that expression of Ar-ADAR increases in Artemia diapause embryos, and that the protein accumulates in activated post-diapause embryos. In addition, Ar-ADAR was induced by stresses. Together, these observations indicate that Ar-ADAR plays an important role in tolerance of harsh environments by Artemia diapause embryos. Further studies of the function and substrates of ADAR enzymes in Artemia will improve our understanding of the fundamental physiology of A→I RNA editing, as well as the basic biology of Artemia diapause embryos.

Conclusions

Our results demonstrate that Ar-ADAR is involved in the stress resistance of Artemia diapause embryos. Our findings provide insights into the function and regulation of ADAR, as well as the mechanisms underlying the stress resistance of Artemia diapause embryos.

Note

1. The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank/EBI Data Bank with accession number(s) KX011870.

Disclosure statement

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