Peptides 90 (2017) 100–110

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

# Molecular characterization and functional analyses of a diapause hormone receptor-like gene in parthenogenetic *Artemia*

Hui-Li Ye[a], Dong-Rui Li[a], Jin-Shu Yang[a], Dian-Fu Chen[a], Stephanie De Vos[b], Marnik Vuylsteke[b], Patrick Sorgeloos[b], Gilbert Van Stappen[b], Peter Bossier[b], Hiromichi Nagasawa[c,**], Wei-Jun Yang[a,*]

[a] College of Life Sciences, Zhejiang University, Hangzhou, Zhejiang 310058, PR China
[b] Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium
[c] Department of Applied Biological Chemistry, The University of Tokyo, Yayoi, Bunkyo, Tokyo 113-8657, Japan

ARTICLE INFO

*Article history:*
Received 24 November 2016
Received in revised form 19 January 2017
Accepted 23 January 2017
Available online 4 February 2017

*Keywords:*
Diapause
Diapause hormone (DH)
Diapause hormone receptor (DHR)
*Artemia*
RNAi
Antiserum neutralization

ABSTRACT

In arthropods, mature females under certain conditions produce and release encysted gastrula embryos that enter diapause, a state of obligate dormancy. The process is presumably regulated by diapause hormone (DH) and diapause hormone receptor (DHR) that were identified in the silkworm, *Bombyx mori* and other insects. However, the molecular structure and function of DHR in crustaceans remains unknown. Here, a DHR-like gene from parthenogenetic *Artemia* (*Ar-DHR*) was isolated and sequenced. The cDNA sequence consists of 1410 bp with a 1260-bp open reading frame encoding a protein consisting of 420 amino acid residues. The results of real-time PCR (qRT-PCR) and Western blot analysis showed that the mRNA and protein of *Ar*-DHR were mainly expressed at the diapause stage. Furthermore, we found that *Ar*-DHR was located on the cell membrane of the pre-diapause cyst but in the cytoplasm of the diapause cyst by analysis of immunofluorescence. *In vivo* knockdown of *Ar-DHR* by RNA interference (RNAi) and antiserum neutralization consistently inhibited diapause cysts formation. The results indicated that *Ar*-DHR plays an important role in the induction and maintenance of embryonic diapause in *Artemia*. Thus, our findings provide an insight into the regulation of diapause formation in *Artemia* and the function of *Ar*-DHR.



## 1. Introduction

Diapause occurs as an alternative developmental program in the life cycle of insects and crustaceans [1–4], induces cell cycle arrest accompanied by a major decline in metabolic activity, and ensures survival under unfavorable environmental conditions [5–7]. Diapause was triggered by a neuropeptide hormone in the silkworm, *Bombyx mori* and other insects, which is called diapause hormone (DH) having a conserved FXPRL-$NH_2$ motif at the C-terminus, and belonging to the pyrokinin peptide family [7,8]. DH was reported to be secreted from the suboesophageal ganglion (SG) into the hemolymph through the corpora cardiaca from where it is carried to the developing ovaries of females [7,8,10]. It then binds to a specific receptor (diapause hormone receptor, DHR) during pupal-adult development [7,9], and induces embryonic diapause in *B. mori* [7,10,11]. On the other hand, the orthology of DH was found to terminate pupal diapause in the heliothine moths, including *Helicoverpa. armigera* [12], *Helicoverpa. assulta* [13], *Heliothis virescens* [14] and *Helicoverpa zea* [15]. The pupal diapause is also under endocrine control, the mechanism of which is different from that of embryonic diapause [13–15]. The present study focuses on the embryonic diapause induced by the axis of DH and DHR.

Honma et al. first identified DHR in order to understand the molecular mechanism of the embryonic diapause triggered by DH in *B. mori* [9]. They also reported that the mRNA level was different between pupae producing diapause eggs and those producing non-diapause eggs. In ovaries of pupae producing diapause eggs, the DHR mRNA level rapidly increased after pupation, peaking after three days and declining thereafter. In the pupae producin non-diapause eggs, the mRNA level peaked in two-day-old pupae and then rapidly decreased [9].

*Abbreviations:* DH, diapause hormone; DHR, diapause hormone receptor; *Ar*-DHR, *Artemia* diapause hormone receptor-like; PBAN, pheromone biosynthesis activating neuropeptide; PBANR, pheromone biosynthesis activating neuropeptide receptor; RNAi, RNA interference.

* Corresponding author.
** Corresponding author.
*E-mail addresses:* anagahi@mail.ecc.u-tokyo.ac.jp (H. Nagasawa), w_jyang@zju.edu.cn (W.-J. Yang).

http://dx.doi.org/10.1016/j.peptides.2017.01.008
0196-9781/

DHR was reported to contain an extracellular N-terminus, an intracellular C-terminus, and seven transmembrane domains [9], which are typical features of G protein-coupled receptor (GPCR) [16]. A number of studies have shown that DH and PBAN (pheromone biosynthesis activating neuropeptide) both require the C-terminal FXPRLamide as an essential core structure for expression of biological activity [17–20]. And DHRs are also homologous with PBAN receptors (PBANRs). The PBANR hydrophobic transmembrane domains involve special sites that are responsible for PBAN stimulation and play an important role in the recognition of the most functionally critical region of the ligand (PBAN), the C-terminal five residues and amide [21,22]. Therefore, it can be inferred that DHR might have similar sites in its hydrophobic transmembrane domains. Although it is known that the DHR structures are essential to interact with DH and perform the function of inducing embryonic diapause, there has been little direct functional evidence of DHR in the formation and maintenance of embryonic diapause and the details remain to be fully elucidated. Previous report showed that knockdown of the DHR gene in *B. mori* by the transcription activator-like effector nuclease (TALEN)-based mutagenesis method let female adults lay non-diapause eggs even under diapause-inducing temperature and photoperiod conditions [23]. Moreover, in functional characterization of *B. mori* DHR using *Xenopus* oocytes, the oocytes responded to DH when injected with DHR cRNA, while they did not respond to DH lacking amide at the C-terminus, which never induces embryonic diapause in *Bombyx* pupae [9]. All these results revealed that DHR is essential for diapause induction and has been conserved to keep highly sensitive and specific interactions with DH during ligand-receptor coevolution in *B. mori*.

Parthenogenetic *Artemia* can develop into two reproductive modes, by which *Artemia* can endure and adapt to occasional extreme environments [24]. One is the ovoviviparous pathway, which gives birth to swimming nauplii, and the other is the oviparous pathway, which releases diapause cysts [25–27]. When encountering harsh environmental conditions, *Artemia* is subjected to the second pathway, in which cell division is arrested and metabolic activity is extremely reduced, enabling the organism to conserve energy [28–30]. At the molecular level, previous reports show that p26, a molecular chaperone, is adaptively up-regulated in diapause embryos and prevent irreversible protein denaturation [31–33]. Phosphorylation of cell cycle proteins (pH3S10 and pRbT356) remains at lower levels in diapause embryos, indicating that the cell cycle is arrested at this stage [27,34]. Much work has been done previously on the investigation of diapause in *Artemia*, however, the role of DH/DHR in the mechanisms of the formation and maintenance of embryonic diapause still remains unknown.

To investigate the involvement of the DH/DHR regulation system in the mechanisms of embryonic diapause, we identified a novel DHR-like gene (*Ar-DHR*) in parthenogenetic *Artemia*. It consisted of 420 amino acids with 34.0–49.2% shared identities with other known FXPRLamide peptide receptors. The mRNA and protein expression of *Ar-DHR* showed that it was highly expressed in the diapause stage, suggesting that *Ar*-DHR might be involved in embryonic diapause. Furthermore, distribution assay of *Ar*-DHR revealed that it was located on the cell membrane of pre-diapause embryos, while it accumulated in the cytoplasm of diapause cysts. *In vivo* knockdown and antiserum neutralization of *Ar*-DHR resulted in the production of nauplii instead of diapause cysts, indicating that *Ar*-DHR might function in the formation and maintenance of embryonic diapause. Thus, the identification of *Ar*-DHR should contribute to better understanding of the formation of embryonic diapause in *Artemia* and further insight into the functions of *Ar*-DHR.

## 2. Materials and methods

### 2.1. Animals

Parthenogenetic *Artemia* was collected from Gahai Lake, China. Oviparous *Artemia* were reared in 8% (w/v) artificial seawater under a 4 h light/20 h dark cycle to release diapause cysts. Ovoviviparous *Artemia* were reared in 4% (w/v) artificial seawater under a 20 h light/4 h dark cycle to produce nauplius larvae directly [25,27,31,35]. The water temperature was kept at 25 °C. Animals were fed once every two days with *Chlorella* powder (Fuqing King Dnarmsa Spirulina, China). Ovoviviparous and oviparous *Artemia* were classified by the morphology of *Artemia* shell glands [36]. Developmental stages of ovoviviparous *Artemia* were defined as early oocyte stage (oocytes residing in the ovaries), late oocyte stage (oocytes entering in the oviducts), and early embryo stage (two days after oocytes entering in the oviducts). Developmental stages of oviparous *Artemia* were defined as early oocyte stage (oocytes residing in the ovaries), late oocyte stage (oocytes entering in the oviducts), early embryo (pre-diapause) stage (two days after oocytes entering in the oviducts), diapause stage (released diapause cysts), post-diapause stage (activated diapause cysts by freezing for three months at −20 °C) [28,36,38].

### 2.2. Molecular cloning of Ar-DHR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNAs were synthesized from 1 μg of total RNA by M-MLV reverse transcriptase (TaKaRa Bio, Shiga, Japan) in a reaction volume of 10 μl.

A DHR-like open reading frame (ORF) was obtained from a genome database of *A. franciscana* Kellogg 1906 built at Ghent University Belgium (unpublished). The conserved transmembrane domains were compared with the diapause hormone receptor (DHR) gene of *Bombyx mori*, and used to design two gene-specific primers F1 and R1 (Supplemental Table 1) and first amplify partial *Ar-DHR* cDNA from parthenogenetic *Artemia* by polymerase chain reaction (PCR). To obtain the full-length *Ar-DHR* cDNA, 3′- and 5′-rapid amplification of cDNA ends (RACEs) were performed with two pairs of nested gene-specific primers (F2 and F3 for 3′-RACE; R2 and R3 for 5′-RACE) (Supplemental Table 1) using the FirstChoice RLM-RACE Kit (Ambion, Austin, TX, USA). Then primers located near the 5′- and 3′-termini of the 5′- and 3′-RACE fragments (F and R; Supplemental Table 1) were designed and used for PCR amplification to make sure that the RACE fragments belong to the same gene.

### 2.3. Quantitative real time polymerase chain reaction (qRT-PCR)

Real-time PCR reactions were performed on the Bio-Rad Mini-iOpticon Real-Time PCR System using SYBR Premix Ex Taq (TaKaRa, Japan) and *Ar-DHR* specific primers (RT-F/RT-R and Tubulin-F/Tubulin-R in Supplemental Table 1). The cycling parameters were: 40 cycles of 10 s at 95 °C (30 s only for the first cycle), 10 s at 56 °C and 10 s at 72 °C (5 min only for the last cycle). Dissociation curves were analyzed at the end of each run to determine the purity of the product and the specificity of the amplification. Relative transcript levels are presented as fold-changes calculated using the comparative CT method [38] with cDNA for tubulin as the internal reference [35,37,39,40]. All data are given as the means ± S.E.M. for independent experiments from three separate RNA pools. All statistical analyses were performed using one-way ANOVA and differences were considered significant when $P < 0.01$. Total RNAs were extracted from oviparous or ovoviviparous ovisacs of 12–20 individuals at different developmental stages (Fig. 3). For each