Comparative Biochemistry and Physiology, Part B 207 (2017) 15–21

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

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

Molecular cloning and characterization of an estrogen receptor gene in the marine polychaete *Perinereis aibuhitensis*

Linlan Lv [a], Xuexing Dong [a,b,*], Fu Lv [a], Weihong Zhao [a], Yebin Yu [a], Wenping Yang [a]

[a] Key Laboratory of Aquaculture and Ecology of Coastal Pool of Jiangsu Province, Department of Ocean Technology, Yancheng Institute of Technology, Yancheng, Jiangsu 224003, China
[b] College of Fisheries and Life Science, Shanghai Ocean University, Shanghai 201306, China

ARTICLE INFO

Article history:
Received 22 September 2016
Received in revised form 30 November 2016
Accepted 3 February 2017
Available online 7 February 2017

Keywords:
cDNA cloning
Perinereis aibuhitensis
Estrogen receptor
Tissue distribution
17β-Estradiol

ABSTRACT

Estrogen receptors (ERs) are the primary mediators of estrogen signaling, and play crucial roles in the reproduction and development of vertebrates. The full-length cDNA of *Perinereis aibuhitensis* estrogen receptor (*paER*) was cloned and characterized for the first time. The positions of the cysteine residues and the residues around them, which constitute two zinc finger motifs and a P-box, are conserved in both vertebrates and invertebrates. A phylogenetic analysis revealed that *paER* is an orthologue of ER in the polychaete *Platynereis dumerilii*. A tissue distribution analysis of *paER* mRNA showed that it is expressed in various tissues, including the body wall, head, esophageal gland, esophagus, stomach, and most strongly in the intestines. Its expression was also measured in *P. aibuhitensis* after exposure to 17β-estradiol (E2) for 48 h. The *paER* mRNA levels in the body wall were measured after 6, 12, 24, and 48 h in E2-exposed and control animals. However, no significant differences in *paER* expression were observed between them at any time point. This report describes the first molecular characterization of full-length *paER* and its tissue-specific expression in *P. aibuhitensis*.

1. Introduction

Estrogen plays significant roles in reproduction, development, growth, and sexual differentiation, exerting its actions through the ligand-induced transcriptional activation of the estrogen receptor (ER) in vertebrates (Wallen, 2005; Heldring et al., 2007; Nelson and Habibi, 2013). ERs belong to the nuclear receptor superfamily of diverse proteins. ER orthologues have been cloned and characterized in mollusks (Kajiwara et al., 2006; Keay et al., 2006; Matsumoto et al., 2007; Bannister et al., 2007; Castro et al., 2007; Stange et al., 2012; Ni et al., 2013; Nagasawa et al., 2015; Tran et al., 2016a), amphioxus (Bridgham et al., 2008; Paris et al., 2008; Katsu et al., 2010), and annelids (Keay and Thornton, 2009). However, compared with vertebrates, much less is known about the functions of invertebrate ERs. Luciferase gene reporter assays have demonstrated that the ERs of mollusks (Thornton et al., 2003; Keay et al., 2006; Matsumoto et al., 2007; Bannister et al., 2007) and amphioxus (Bridgham et al., 2008; Paris et al., 2008; Katsu et al., 2010) are not activated by steroidal estrogens and seem unable to bind estrogen. However, the ERs of *Platynereis dumerilii* and *Capitella capitata* display the classic properties of vertebrate ERs, including the recognition of EREs and a specific sensitivity to low concentrations of estrogens. They can also be activated or antagonized by synthetic estrogens or anti-estrogens, respectively, including environmental ER disruptors (Keay and Thornton, 2009).

17β-Estradiol (E2) is the most potent form of endogenous estrogen and constitutes a major part of the estrogenic activity in sewage effluents and surface waters (Ra et al., 2011). Studies of vertebrates have shown that E2 can induce the expression of the vitellogenin (*vtg*) gene *via* a genomic mechanism driven by specific ERs (Teo et al., 1998). Most reports of the induction of VTG by E2 in aquatic invertebrates have focused on mollusks. Several studies have shown that the injection of E2 causes an increase in VTG (Osada et al., 2003; Matozzo and Marin, 2008) and that E2 induces *ER* expression in *Saccostrea glomerata* (Tran et al., 2016b). However, in other studies, E2 failed to induce the production of yolk protein in mollusks after their exposure to E2 *in vivo* (Riffeser and Hock, 2002; Won et al., 2005). Exposure to E2 failed to induce *ER* expression in *Mytilus edulis* at the ripe stage in their gonadal maturation cycle (Puinean et al., 2006). In contrast, a significant increase in *ER2* mRNA expression was observed in the same species (*M. edulis*) exposed to estrogens in the early stage of gametogenesis (Ciocan et al., 2010). Ciocan et al. (2011) reported significant upregulation of ER in the early stages of testis development compared with that in mature mussels. These data suggest that estrogens are important in the reproductive process in bivalves (Ciocan et al., 2011). However, the role of estrogen and its mechanism of action in invertebrates are far from clear.

Polychaetes are usually the most abundant taxon in marine benthic communities and play a major role in the functioning of these

* Corresponding author at: Department of Ocean Technology, Yancheng Institute of Technology, Yancheng, Jiangsu 224003, China.
E-mail address: ycitdong@126.com (X. Dong).

http://dx.doi.org/10.1016/j.cbpb.2017.02.001
1096-4959/

communities (Hutchings, 1998). Estradiol has been detected in the coelomic fluid of male and female *Nereis virens* and E2 also stimulated vitellin (Vn) protein accumulation in *in vitro* cultures of eleocytes (Garcia-Alonso and Rebscher, 2005; Garcia-Alonso et al., 2006). Estrogen-activated ERs have been identified in *Platynereis dumerilii* and *Capitella capitata*, in the first reported instance of ERs in invertebrates. To better understand the molecular basis of the polychaete reproductive endocrine system and the evolution of vertebrate steroid hormone receptors, we cloned the full-length cDNA encoding ER from *Perinereis aibuhitensis*. We also investigated the tissue distribution of *paER* mRNA and the changes in its expression during exposure to E2.

2. Materials and methods

2.1. Animal collection and acclimation

Perinereis aibuhitensis specimens were collected from the tidal sediments of the Sheyang estuary in Yancheng, Jiangsu Province, China. The animals were transferred from the field to an ice cooler. They were acclimated under laboratory conditions (17 ± 0.5 °C, salinity 25 ± 1, pH 8.0 ± 0.1). After a week, various tissues, including the body wall, head, esophageal gland, esophagus, stomach, and intestines were isolated, immediately preserved in RNAstore Reagent (Tiangen Biotech, Beijing, China), and stored at −80 °C until analysis.

2.2. E2 exposure

Specimen collection and acclimation are described in Section 2.1. Healthy worms ($n = 210$; 1.86 ± 0.12 g wet weight) were transferred to glass tanks ($20 \times 20 \times 40$ cm^3) containing 2 L of artificial seawater supplemented with different concentrations of E2 for 48 h. A stock E2 solution of 10 mg/L was prepared by dissolving E2 in acetone, and then diluting it to the desired concentrations with artificial seawater. The concentrations of E2 used in the experiment were 0.1, 1, 10, 100, and 1000 ng/L. A negative control group and a solvent control (0.01% v/v acetone) group were included with three replicate tanks for each experimental group. Each group contained 30 individuals. The worms were not fed throughout the experiment, and the artificial seawater containing E2 was changed each day during exposure. The body wall tissues were sampled after 6, 12, 24, and 48 h and stored at −80 °C.

2.3. RNA preparation

Total RNA was extracted from the tissues (isolated as described above) using RNAiso Plus (TaKaRa, Dalian, China), following the manufacturer's instructions. The quality of the RNA was assessed with 1% agarose gel electrophoresis. The RNA concentration was calculated from the absorbance at 260 nm. The RNA samples were then dissolved in RNase-free water and stored at −80 °C until further analysis.

2.4. Molecular cloning of cDNA encoding ER

2.4.1. First-strand cDNA synthesis

cDNA was synthesized with a PrimeScript™ RT reagent Kit (TaKaRa), according to the manufacturer's instructions. The cDNA was assayed with 2% agarose gel electrophoresis and then stored at −20 °C. The first-strand cDNA from the body wall was used to clone the full-length cDNA of *ER* and the first-strand cDNAs of the other tissues were used for the gene expression analysis.

2.4.2. Primers, amplification, and sequencing

We designed one primer pair, *paER*-F and *paER*-R, based on the highly conserved regions of the published *ER* cDNA sequences of other invertebrates, using the CODEHOP PCR primer design method (http://blocks.fhcrc.org/codehop.html). The partial target gene was amplified with PCR. The PCR products were gel purified, cloned into the pMD19-T vector (TaKaRa), and sequenced by Sangon Biological Engineering (Shanghai) Co., Ltd. (Shanghai, China).

The full-length *paER* cDNA sequence was obtained using rapid amplification of cDNA ends (RACE) with a 5′/3′ rapid-amplification of cDNA ends (RACE) kit (TaKaRa), according to the manufacturer's protocol. Gene-specific primers (GSPs) were designed based on the sequence of the cDNA fragment. Briefly, for the 5′-RACE reactions, we used the NUP primer (supplied with the kit) and a reverse primer (5′-GSP1). 3′-GSP1 and the UPM primer were used for the 3′-RACE. The 5′/3′ RACE products were purified, cloned, and sequenced by Sangon Biological Engineering (Shanghai) Co., Ltd. All the primers used in this study are shown in Table 1.

2.4.3. Bioinformatic and phylogenetic analyses

The sequence of the cloned cDNA was confirmed with the BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The open reading frame (ORF) was predicted with ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf). A multiple protein sequence alignment of ERs (*Platynereis dumerilii*, ACC94156.1; *C. capitata*, ACD11039.1; *Crassostrea gigas*, EKC32720.1; *M. galloprovincialis* BAF34908.2; *Homo sapiens*, AAA52399.1) was constructed with the ClustalW multiple alignment program (http://www.ebi.ac.uk/clustalw). The amino acid sequence, protein molecular weight, and isoelectric point (PI) of paER were predicted with ExPASy (http://us.expasy.org/tools/protparam.html). A phylogenetic analysis was performed with the neighbor-joining algorithm in the Mega 6.0 program (Tamura et al., 2011). A bootstrap analysis was conducted with 1000 replicates.

2.5. Gene expression analysis

2.5.1. Quantitative PCR

The expression of the *paER* mRNA in the body wall of *P. aibuhitensis* exposed to E2 was quantified with a SYBR Green quantitative real-time reverse transcription PCR method. The level of *paER* mRNA in the head, esophageal gland, esophagus, stomach, and intestinal tissues of *P. aibuhitensis* was also measured. *paER* expression was analyzed with the primers paER-Q-F and paER-Q-R (Table 1). The actin gene was used as the internal reference gene, amplified with the primer pair actin-Q-F and actin-Q-R. Quantitative PCR (qPCR) was performed in triplicate with the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and the SYBR Premix Ex Taq II (Tli RnaseH Plus) Kit (TaKaRa). PCR efficiency (E) was determined for each primer pair by constructing standard curves for serially diluted samples to ensure that E ranged from 95% to 105% ($R^2 > 0.99$). The PCRs were performed in a total volume of 20 μL containing 10 μL 2× SYBR Premix Ex Taq (TaKaRa), 1 μL of cDNA template, 0.4 μL of each primer (10 μmol/L), and 8.2 μL of double-distilled water. The qPCR conditions were 95 °C for 3 min, and 39 cycles of 95 °C for 5 s and 58 °C for 30 s. A dissociation curve analysis of the amplification products was performed at the end

Table 1
Sequences of the PCR primers used in this study.

Primer code	Sequence (5′ → 3′)	Application of primer
paER-F	ACCGGCGGAAGTCCTGYCARGCNTG	Degenerate primer
paER-R	GCACAGCAGCTCCATCCARCA	For 5′-RACE
paER-GSP1(R1)	GCCTCGATATCTGCCATATTTCTCCCAC	
paER-GSP1(R5)	TGGCAGCTCTTGCGCCGATGT	
paER-nGSP1(R2)	CCGACGCACAACAGTTCCATCCAG	
paER-nGSP1(R6)	GTCCTGGTCCACAGGAGAATGTGAGGT	
paER-GSP2	GCCATCAATAGTGACCCAGGACCCC	For 3′-RACE
paER-nGSP2	TGGAGCAAAACGATCTGCCCACGAA	
actin-Q-F	ATCTACGAGGGCTACGCT	For quantitative PCR
actin-Q-R	CGTGGTGGTGAAGGAGTAG	
paER-Q-F	AACGAGAGTTCGGAGAGCAA	
paER-Q-R	ATGGTGAAGGGATGAGGATG	

N, A/T/C/G; Y, C/T; R, A/G; D, G/A/T.