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Cloning of invertebrate gonadotropin-releasing hormone receptor (*GnRHR*)-like gene in Yesso scallop, *Patinopecten yessoensis*

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ABSTRACT

Although the invGnRH peptide was characterized from the nerve ganglia in bivalves, its signaling mechanism is nevertheless unclear. The objective of this paper was to identify the invGnRHR cDNA from Yesso scallop as a first step for understanding of invGnRH in bivalve neuroendocrine system. We performed PCR cloning mediated with transcriptome survey and expression analysis with various tissues of the scallop. Our results showed that we identified an invGnRHR-like cDNAs from not only Yesso scallop Patinopecten yessoensis but also Pacific oyster *Crassostrea gigas*. In addition, we subsequently identified an adipokinetic hormone receptor (AKHR)-like and AKH-like cDNA pair from the scallop. Comparison of the tissue distributions of both receptor mRNAs suggested functional divergence of two homologous neuropeptides. In brief, py-GnRHR-like mRNA showed broad distribution in various tissues including the nerve ganglia, while py-AKHR-like mRNA was expressed in the nerve ganglia and restricted to some limited peripheral tissues. The findings suggested that both py-GnRH and py-AKH signals were utilized via their own receptors. qPCR assays revealed their receptor mRNA expression in the gonads during a maturation, showing that py-GnRHR-like mRNA in the pre-mature gonads was higher than other mature stages.

1. Introduction

Gonadotropin-releasing hormone receptors (GnRHRs) act for the reception of GnRH signaling to release LH and FSH, being as a firm neuroendocrine system in vertebrates (Zohar et al., 2010). However, once we look to invertebrates, there is very limited knowledge about ancestral GnRHR and GnRH: For example, timing of the appearance of ancestral genes of vertebrate GnRHR and GnRH pair in invertebrates is still unknown. Functional orthologues of the vertebrate GnRHR and GnRH in invertebrates are not fully elucidated. Recent studies with next-generation sequencing have shed a ray of light on the gene evolution of neuropeptide receptors and their ligands in Cnidarian and Protostomian (Roch et al., 2011; Grimmelikhuijzen and Hauser, 2012; Hauser and Grimmelikhuijzen, 2014; Roch et al., 2014). These studies proposed the presence of GnRHRs and GnRHs in wide variety of invertebrates and suggested a scenario of co-evolution of an ancestral GnRHR and GnRH pair (Grimmelikhuijzen and Hauser, 2012; Hauser and Grimmelikhuijzen, 2014). Specifically, Roch et al. (2014) reported that the GnRHR superfamily in invertebrates consists of four GPCR members (i.e., invertebrate GnRH receptor (invGnRHR), corazonin receptor (CrzR), adipokinetic hormone receptor (AKHR) and AKH/Crz related peptide receptor (ACPR)). By gene duplications in invertebrates, they proposed the GnRHR superfamily has evolved as follows: (i) Amphioxus and ancestral species of Protostomia and Deuterostomia possess both vertebrate GnRHR (GnRHR) and invGnRHR but no AKHR. (ii) Mollusks and annelids possess both invGnRHR and AKHR. Hauser and Grimmelikhuijzen (2014) dubbed invGnRH as CrzR for mollusks and annelids. However, they also confessed that the Crzs of both mollusks and annelids lack any of the structural properties of arthropod Crzs, suggesting that corazonins (Crzs) are neuropeptides specific to insects and crustaceans (Roch et al., 2011). Hence, we hereby support the term "invGnRH" for mollusks and annelids. (iii) Arthropods possess CrzR and AKHR. In addition some insects (e.g., mosquito, Bombyx, Nasonia and Rhodnius) have above two (i.e., CrzR and AKHR) and

Abbreviations: GnRHR, gonadotropin-releasing hormone receptor; LH, luteinizing hormone; FSH, follicle stimulation hormone; GPCR, G protein-coupled receptor; invGnRHR, invertebrate GnRHR; CrzR, corazonin receptor; AKHR, adipokinetic hormone receptor; ACPR, AKH/Crz related peptide receptor; CNS, central nervous system; RACE, rapid amplification of cDNA ends; pI, isoelectric point; Mw, molecular weight; OXTR, oxytocin receptor; VPR, vasopressin receptor; TM, transmembrane; VG, visceral ganglion; CPG, cerebral and pedal ganglia; ISH, in situ hybridization; TR-FIA, time-resolved fluoresce immunoassay.

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ACPR that was evolved from AKHR (Grimmelikhuijzen and Hauser, 2012).

To the best of our knowledge in mollusks, *invGnRHR* cDNAs were identified in octopus *Octopus vulgaris* (*oct-GnRHR*, (Kanda et al., 2006)), sea hare *Aplysia californica* (*ap-GnRHR*, (Sun et al., 2012)) and limpet *Lottia gigantea* (*lim-GnRHR*, (Roch et al., 2011)). Unfortunately, very limited knowledge of their biological activity is currently available from above mentioned mollusks. For a cephalopod, contractile effect was induced by oct-GnRH in the heart (Iwakoshi et al., 2000), oviduct (Iwakoshi-Ukena et al., 2004), and radula retractor muscle (Kanda et al., 2006). In addition, stimulatory effects of oct-GnRH for sex steroid production (i.e., the immunoassayable progesterone (P), testosterone (T) and 17β-estradiol (E_2)) were found in gonads (Kanda et al., 2006). Indeed, *oct-GnRHR* mRNA is expressed in the central nervous system (CNS), peripheral nervous systems and several peripheral tissues (e.g., stomach, heart, branchia, oviduct, ovary, egg and testis) (Iwakoshi-Ukena et al., 2004; Kanda et al., 2006). These findings suggested that oct-GnRHR receives the signal not only for muscle contraction in peripheral tissues but also for several brain controls for autonomic function, feeding, memory and movement (Kanda et al., 2006; Minakata et al., 2009). Meanwhile, for a gastropod, *ap-GnRHR* mRNA is expressed in CNS (including all central ganglia), posterior foot, ovotestis and heart (Sun et al., 2012). The in vivo ap-GnRH injection into Aplysia did not affair any reproductive parameter, but it might modulate the muscular contraction for controlling parapodia, foot and head movement (Tsai et al., 2010; Sun and Tsai, 2011). Thus, the limited knowledge from above two mollusks suspected that the protostomian invGnRHR acts as a transmitter in both CNS and peripheral tissues for various physiological actions (Sun et al., 2012).

For bivalves, we have investigated the neuroendocrinological regulation in bivalve reproduction (Osada and Treen, 2013). The previous study cloned *invGnRH* genes from two marine bivalves that are commercially important in Asia (Treen et al., 2012): Pacific oyster *Crassostrea gigas* and Yesso scallop *Patinopecten yessoensis*. For the scallop, we identified mature GnRH peptide (py-GnRH) from the nerve ganglia by mass spectrometry (Nagasawa et al., 2015b), suggesting the presence of invGnRH signaling in marine bivalves. In addition, sequential reports suggested bioactivities of py-GnRH to reproductive parameters (Treen et al., 2012; Nagasawa et al., 2015a). Moreover, the presence of receptive mechanism for py-GnRH in the gonad was proposed by both organ culture (Nakamura et al., 2007; Treen et al., 2012) and in vivo administration experiments (Nagasawa et al., 2015a). However, no *invGnRHR* cDNA has been identified from bivalve. In order to understand the invGnRH signaling in bivalves, we therefore aimed to clone the receptor gene for the first step. We hereby carried out in silico mining and identified the full-length *invGnRHR*-like cDNA from Yesso scallop. Likewise, we also cloned *AKHR*-like and *AKH*-like cDNAs from the scallop and performed phylogenic analysis to overview genetic relationship between *invGnRHRs* and *AKHRs*. Their mRNA distributions were RT-PCR analyzed in the nerve ganglia and peripheral tissues.

2. Materials & methods

2.1. Sample collection

Two-year-old farmed Yesso scallops (*P. yessoensis*) were purchased several times from the local commercial supplier (Mutsu Bay, Aomori and Ogatu Bay, Miyagi, Japan) in 2015 and 2016. Their maturational status of gonads were well described in the previous study (Osada et al., 2007). The shipped scallops were kept in 120-liter aquarium for a few days at 10 °C with filtration system supplying sufficient dissolved oxygen for recovery from the transport stress. Various tissues (i.e., lip, foot, cerebral and pedal ganglia (CPG), visceral ganglion (VG), testis, ovary, digestive gland, mantle, gill and hemocytes) were sampled and stored in RNA*later* stabilization solution (Thermo Scientific, Waltham, MA) at −30 °C for subsequent RNA extraction. To perform the detailed expression analysis, the nerve ganglia (i.e., CPGs and VGs) were sampled from the two-year-old scallops ($n = 3$) at non-reproductive stage (their sex were indistinguishable) in September 2014. In addition, both testes ($n = 3$) and ovaries ($n = 3$) were sampled from two-year-old scallops at beginning of maturation stage in November 2015. Because scallop testis at mature stage possesses very high content of sperms resulting in failure of RNA extraction, testis at mature stage was unusable for the RT-PCR analysis. As for hemocytes collection, hemolymph was withdrawn by a needle from heart and pooled from several individuals (females: $n = 6$, males $= 3$). Approximately 15 ml of the pooled hemolymph for each sex was centrifuged at 4 °C for 15 min at 1500 rpm. Then, the supernatant was aspirated, and the pellet of hemocytes was stored in RNA*later* stabilization solution as above mentioned.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from various tissues using RNeasy mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol and quantified by spectrophotometry with a NanoDrop ND-1000 instrument (Thermo Scientific). RNA integrity was assessed by electrophoresis on a 1% (*w*/*v*) agarose gel. Total RNA (1 μg) was transcribed to cDNA using the high capacity cDNA reverse transcription kits (Life Technologies, Tokyo, Japan).

2.3. Transcriptome survey and RACE cloning

Local blast system was built with Yesso scallop transcriptome sequences (SRX047537, (Hou et al., 2011) with our unpublished data) by using BLAST+ applications (NCBI; https://blast.ncbi.nlm.nih.gov). Transcriptome survey by tblastn was carried out with known invGnRHR amino acid sequences of mollusks (e.g., Aplysia, octopus and limpet) and then the candidate contig was identified for further RACE cloning. Total RNA extracted from the scallop ovary was used for cDNA synthesis with SMARTer RACE cDNA amplification kit (TaKaRa-Bio, Shiga, Japan) according to the manufacturer's instructions. The 5′- or 3′-RACE was carried out with gene specific primers based on the contig sequence identified form the above local blasting (Table 1). The PCR fragments amplified by Takara Ex Taq HS (TaKaRa-Bio) were cloned into pGEM-T easy vectors (Promega, Madison, WI) and sequenced (Macrogen, Seoul, South Korea).

2.4. Bioinformatic analyses

Deduced amino acid sequences of *py-GnRHR*-like cDNA were generated from the cloned full-length coding sequences by EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) and used subsequent bioinformatic analysis. Sequence identity and similarity were obtained from blastp suite-2sequences (http://blast.ncbi.nlm.nih.gov). Transmembrane helix and secondary structure were predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and MEMSAT (http://www.sacs.ucsf.edu/cgi-bin/memsat.py), respectively. Amino acid sequences were aligned with the corresponding orthologs in molluskan species using Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenic analysis of GnRHR, OXTR, and VPR superfamilies from vertebrates and invertebrates was performed. Then, the detailed phylogenic analysis of invGnRHRs and AKHRs in mollusk, annelid and arthropod was performed. For identification of *invGnRHR*-like cDNA in the oyster, we performed protein blast (NCBI) with a putative py-GnRHR amino acid sequences as a query. For phylogenic and sequence similarity analyses, all amino acid sequences were trimmed within the region from TM1 to TM7 as similar uses for GPCR superfamilies in the previous report (Roch et al., 2011). Trimmed sequences were aligned by Clustal W2 and used for Bayesian inference (MrBayes v3.1.2, mrbayes.csit.fsu.edu) using a mixed model of amino acid substitution (1,000,000 generations, sampling every 10th generation and burning the first 10,000 trees).