General and Comparative Endocrinology 227 (2016) 115–119

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Nucleotide sequence and expression of relaxin-like gonad-stimulating peptide gene in starfish *Asterina pectinifera*

Shogo Haraguchi [a,b], Narumi Ikeda [a], Michiko Abe [a], Kazuyoshi Tsutsui [b], Masatoshi Mita [a,*]

[a] *Department of Biology, Faculty of Education, Tokyo Gakugei University, Nukuikita-machi 4-1-1, Koganei-shi, Tokyo 184-8501, Japan*
[b] *Laboratory of Integrative Brain Sciences, Department of Biology and Center for Medical Life Science, Waseda University, Wakamatsucho 2-2, Shinjuku-ku, Tokyo 162-8480, Japan*

ARTICLE INFO

Article history:
Received 23 February 2015
Revised 9 June 2015
Accepted 29 June 2015
Available online 10 July 2015

Keywords:
Relaxin-like gonad-stimulating peptide (RGP)
Gene
Coding-DNA sequence
Transcription
Starfish

ABSTRACT

Starfish gonad-stimulating substance (GSS) is the only known invertebrate peptide hormone responsible for final gamete maturation, rendering it functionally analogous to gonadotropins in vertebrates. Because GSS belongs to the relaxin-like peptide family, we propose renaming for starfish gonadotropic hormone as relaxin-like gonad-stimulating peptide (RGP). This study examined the primary structure and expression regulation of the *RGP* gene in starfish *Asterina pectinifera*. *RGP* consisted of 3896 base pairs (bp) divided over two exons, exon 1 of 208 bp and exon 2 of 2277 bp, and one intron of 1411 bp. Promoter sequences, CAAT and TATA boxes, were present in the 5′-upstream region of the coding DNA sequence of *RGP*. The transcript was 2485 bases (b) in length. The AAUAAA polyadenylation signal was found in 3′-untranslated region over 2 kb away from the stop codon. This showed that only 14% of the *RGP* mRNA was translated into the peptide, because a size of the open-reading frame was 351 b. Furthermore, an analysis by using real-time quantitative PCR with specific primers for *RGP* showed that mRNA of RGP was expressed at high levels in the radial nerves. Expression was also observed in the cardiac stomachs, although the level was low, and trace levels were detected in the gonads, pyloric caeca and tube feet. This result suggests that the *RGP* gene is transcribed mainly in the radial nerves of *A. pectinifera*.

1. Introduction

Gonadotropins play important regulatory roles in reproduction in both vertebrates and invertebrates. The vertebrate gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are structurally and functionally conserved across various species, whereas no similar molecule has been identified in invertebrates. Among invertebrates, the first gonadotropic hormone was found in starfish. In 1959, Chaet and McConnaughy reported that an aqueous extract of starfish radial nerves could induce the shedding of gametes after injection into the coelomic cavity of ripe individuals (Chaet and McConnaughy, 1959). The active substance contained in the nerve extracts was called gonad-stimulating substance (GSS). GSS mediates oocyte maturation in starfish by acting on the ovary to produce the maturation-inducing hormone (MIH), 1-methyladenine (1-MeAde), which in turn induces the maturation of oocytes (Kanatani et al., 1969). In this sense, GSS is functionally identical to vertebrate LH, especially piscine and amphibian LHs, acting on the ovarian follicle cells to produce MIH to induce the final maturation or meiotic resumption of oocytes (Nagahama et al., 1995).

Fully 50 years since the initial finding of Chaet and McConnaughy (1959), GSS was finally purified from starfish, *Asterina pectinifera* (Mita et al., 2009b). The purified hormone was a heterodimeric peptide with a molecular weight of 4737, comprising an A-chain of 24 amino acids (aa) and a B-chain of 19 aa. Based on its cysteine motif, GSS was classified as a member of the insulin/insulin-like growth factor (IGF)/relaxin superfamily and, more precisely, to the relaxin-like peptide family. Recently, we identified another relaxin-like GSS from *Asterias amurensis* (Mita et al., 2015). Therefore, we propose that starfish gonadotropic hormone should be redesignated as relaxin-like gonad-stimulating peptide (RGP).

Previous studies have shown that the coding DNA sequence (CDS) of RGP from *A. pectinifera* consists of 351 base pairs (bp) with an open reading frame (ORF) encoding a peptide of 116 aa, including a signal peptide (29 aa), B-chain (19 aa), C-peptide (44 aa), and A-chain (24 aa) (Mita et al., 2009b). However, the complete primary structure of the *RGP* gene remains to be clarified. To elucidate the regulatory mechanism of transcription in the process of RGP biosynthesis, this study sought to determine the complete nucleotide sequences of the genomic and mRNA sequences for RGP.

---

* Corresponding author.
E-mail address: bio-mita@u-gakugei.ac.jp (M. Mita).

http://dx.doi.org/10.1016/j.ygcen.2015.06.017
0016-6480/© 2015 Elsevier Inc. All rights reserved.

Furthermore, the transcription activities of the *RGP* gene were measured in various organs of *A. pectinifera*.

2. Material and methods

2.1. Animals

A. pectinifera were collected from Yokosuka (Kanagawa, Japan), Choshi (Chiba, Japan), Ushimado (Okayama, Japan), Asamushi (Aomori, Japan), and Omura (Nagasaki, Japan). Animals were kept in circulating artificial seawater (ASW) at 15 °C and used within 2 months after collection.

2.2. Preparation of total DNA

DNA was extracted from testis of *A. pectinifera* using a DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA). The genomic DNA sequence of RGP was analyzed using a Universal GenomeWalker 2.0 Kit (Clontech, Mountain View, CA, USA) in accordance with the manufacturer's instructions.

2.3. Preparation of cDNA for RGP

Total RNA was extracted from radial nerves of *A. pectinifera* after homogenization with Sepasol (Nacalai Tesque, Kyoto, Japan) as the RNA extraction solution. The poly(A)$^+$ RNA fraction was obtained using Oligotex-dT30 (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized with a SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) in accordance with the manufacturer's instructions.

2.4. Amplification and DNA sequencing

Oligonucleotide primers for the polymerase chain reaction (PCR) were designed in accordance with sequences (DDBJ: AB496611 in *A. pectinifera*) described previously (Mita et al., 2009b). Genome DNA and RACE products encoding RGP were amplified using these primers to determine the nucleotide sequences in RGP involving the CDS and untranslated regions (UTRs).

All PCR products were electrophoresed in 1.5% agarose gels and stained with ethidium bromide. Agarose gel slices containing the PCR-product band were excised under UV illumination, and DNA was purified from the agarose plug using a QIAquick® Gel Extraction kit (Qiagen, Valencia, CA, USA), followed by an ethanol precipitation. Amplified products were cloned into a pGEM-T® easy vector in the pGEM-T® easy system (Promega, Madison, WI, USA). DNA sequence data were determined using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using a Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA).

2.5. Real-time quantitative PCR analysis

To measure the expression of *RGP* mRNA in the organs of starfish, real-time quantitative PCR (real-time qPCR) was conducted by using a StepOnePlus system (Applied Biosystems, Foster City, CA, USA) as described previously (Haraguchi et al., 2012). The PCR primers used for the amplification of starfish RGP cDNA fragments were 5′-AGCGACGTGTTGACCATGAA-3′ and 5′-GCTGTCGAT GCTTCGTTTAATG-3′. The PCR primers for β-actin were 5′-TCACAG AGCGTGGCTACTCTTTC-3′ and 5′-TGATGTCACGCACGATTTCA-3′. β-Actin was used as the internal standard. The reaction mixture contained SYBR Green Real-Time PCR Mix (Toyobo, Osaka, Japan), 400 nM each of forward and reverse primers, and 2 ng of cDNA in a final volume of 20 μl. The real-time qPCR was run with a standard cycling program, 95 °C for 3 min, 40 cycles of 95 °C, 15 s; 60 °C, 15 s; and 72 °C, 15 s. An external standard curve was generated by serial 10-fold dilution of cDNA obtained from the starfish radial nerve, which had been purified, and its concentration was measured. To confirm the specificity of the amplification, the PCR products were subjected to a melting curve analysis and gel electrophoresis. The results were normalized to the expression of β-actin using the StepOnePlus 2.0 software (Applied Biosystems, Foster City, CA, USA). Values are means ± SEM of four separate assays using different animals. All data are expressed as means ± SEM.

3. Results

Recently, we found that gonad-stimulating hormone of starfish *A. pectinifera* belonged to the relaxin-like peptide family (Mita et al., 2009b), so we propose RGP as its new name. Although RGP is a heterodimer composed of A- and B-chain with disulfide cross linkages, the CDS of RGP encodes a preprohormone sequence with a C-peptide between the A- and B-chains (Mita et al., 2009b). On the basis of the CDS, both the genomic DNA and mRNA for RGP were analyzed in this study. Total DNA was prepared from testis of *A. pectinifera*, and the mRNA was prepared from radial nerves. After reverse-transcription using a SMARTer RACE cDNA amplification Kit, the cDNA obtained was used for the analysis of *RGP* mRNA. The nucleotide sequences of gene and cDNA encoding RGP were determined by using specific primers based on the CDS of *A. pectinifera* RGP.

Previous studies have shown that the length of CDS encoding RGP is 351 bp (Mita et al., 2009b). The gene for *RGP* consisted of 3896 bp (DDBJ: LC027939) comprising 2 exons and 1 intron (Fig. 1). The lengths of exons 1 and 2 were 208 bp and 2277 bp, respectively, with an intron of 1411 bp between them. However, the mRNA encoding RGP comprised 2485 bases (b) (DDBJ: LC027938). The sequence length of the mRNA was consisted of exon 1 and exon 2 in total. Exon 1 encoded the 5′UTR (6 b), signal peptide (87 b), B-chain (57 b) and the first half of the C-peptide (58 b). The latter part of the C-peptide (74 b) and the A-chain (75 b) were encoded in exon 2.

With regard to the transcription regulation of *RGP*, the promoter sequences, CAAT (−49 to −45 bp) and TATA boxes (−31 to −27 bp), were present in the 5′-upstream region before the transcription start site in *RGP* (Fig. 2A). The AUG initiator codon in the mRNA was found at 7 b after the transcription start site. The first Met was comprised of a signal peptide for prepro-RGP.

The sequence of the intron began from GT (209–210 bp) and finished at AG (1619–1620 bp) (Fig. 2B). The intron divided the sequence encoding the C-peptide (Fig. 2B). The codon for Gly at 68 aa was derived from both exon 1 and exon 2.

Previous studies have shown that the UAA stop codon was located between 349 and 351 b in the *RGP* mRNA (Mita et al., 2009b). However, the polyadenylation signal (AAUAAA) was found at 2450–2455 b in the *RGP* mRNA (Fig. 2C). The poly(A) tail was observed after 24 b of the poly(A) signal. This showed that the length of the 3′UTR was very long and comprised 2128 b from the UAA stop codon to the poly(A) tail.

Because previous studies have shown that RGP is mainly present in radial nerves (Mita et al., 2009a), the transcriptional activity of the *RGP* gene was measured in various organs of *A. pectinifera* using real-time qPCR with specific primers for RGP. The mRNA was expressed at high level in the radial nerves (Fig. 3). Expression was also observed in the cardiac stomachs and pyloric caeca, although levels were low, and there were trace levels in the ovary, testis, and tube feet. This strongly suggests that the *RGP* gene is transcribed mainly in the radial nerves.