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A relaxin-like gonad-stimulating peptide from the starfish *Aphelasterias japonica*

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ABSTRACT

Relaxin-like gonad-stimulating peptide (RGP) in starfish is the first identified invertebrate gonadotropin responsible for final gamete maturation. In this study, a new ortholog RGP was identified from *Aphelasterias japonica*. The DNA sequence encoding *A. japonica* RGP (AjaRGP) consists of 342 base pairs with an open reading frame encoding a peptide of 113 amino acids (aa), including a signal peptide (26 aa), B-chain (20 aa), C-peptide (42 aa), and A-chain (25 aa). AjaRGP is a heterodimeric peptide with disulfide cross-linkages. Comparing with *Asterias amurensis* RGP (AamRGP) and *Patiria* (=*Asterina*) *pectinifera* RGP (PpeRGP), the amino acid identity levels of AjaRGP with respect to AamRGP and PpeRGP are 84% and 58% for the A-chain and 90% and 68% for the B-chain, respectively. This suggests that AjaRGP is closer to AmaRGP rather than PpeRGP. Although chemical synthetic AjaRGP can induce gamete spawning and oocyte maturation in ovarian fragments of *A. japonica*, the ovary of *P. pectinifera* fails to respond to AjaRGP. This suggests that AjaRGP acts species-specifically.

1. Introduction

Chaet and McConnaughy (1959) first reported that an aqueous extract of starfish radial nerves could induce the shedding of gametes when injected into the coelomic cavity of ripe animals. The active substance contained in the nerve extract was considered to be a peptide hormone named gonad-stimulating substance (GSS) (Kanatani and Shirai, 1967, 1969). Although GSS is an initial trigger for oocyte maturation in starfish, the effect of GSS is indirect. Resumption of meiosis in immature oocytes and release from the ovary are induced by a second mediator, maturation-inducing hormone (MIH), identified as 1-methyladenine (1-MeAde) in starfish (Kanatani et al., 1969; Kanatani, 1985). Thus, GSS plays an important role in 1-MeAde production in ovarian follicle cells (Hirai and Kanatani, 1971; Hirai et al., 1973) by way of activation of its receptor, G-protein, and adenylyl cyclase (Mita et al., 1987, 1989; Mita and Nagahama, 1991). In this sense, GSS is functionally identical to vertebrate luteinizing hormone (LH), especially piscine and amphibian LHs, acting on ovarian follicle cells to produce MIH to induce the final maturation or meiotic resumption of the oocyte (Nagahama et al., 1995).

Fifty years since the initial finding of Chaet and McConnaughy (1959), GSS was finally purified from the radial nerves of starfish *Patiria* (=*Asterina*) *pectinifera* (Mita et al., 2009). The purified hormone is a heterodimer composed of two different peptides, A- and B-chains, with disulfide cross-linkages. Based on its cysteine motif, starfish GSS is classified as a member of the insulin/insulin-like growth factor (IGF)/relaxin superfamily and, more precisely, it belongs to a relaxin-like peptide family (Mita et al., 2009). Thus, GSS in starfish *P. pectinifera* is designated as relaxin-like gonad-stimulating peptide (RGP) (Haraguchi et al., 2016).

Recently, RGP molecules were identified in several species of starfish. The chemical structures of RGP in *Certonardoa semiregularis* (Ikeda et al., 2015), *Patiria miniata* (Haraguchi et al., 2016), and *Acanthaster planci* (Mita et al., 2015b) are almost the same as that of *P. pectinifera*. Because *P. pectinifera*, *P miniata*, *C. semiregularis*, and *A. planci* belong to the Order Valvatida in the Class Asteroidea, this suggests that the chemical structure of *P. pectinifera* RGP is conserved among starfish of the Order Valvatida beyond species.

In contrast, the chemical structure of RGP identified from *Asterias amurensis* of the Order Forcipulatida is quite different from that of RGP in *P. pectinifera* (Mita et al., 2015a). This suggests that the RGP molecule of starfish in the Order Forcipulatida is different from that in the Order Valvatida. However, little is known about the chemical structure of RGP in other species of starfish in the Order Forcipulatida. The starfish *Aphelasterias japonica* of the Order

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Forcipulatida is an endemic Japanese species and inhabits the inter-tidal zone in northern Japanese waters. To elucidate whether the RGP molecule is conserved in starfish of the Order Forcipulatida, we tried to identify *A. japonica* RGP.

2. Materials and methods

2.1. Animals

Starfish *A. japonica* were collected from Asamushi (Aomori Prefecture, Japan). *P. pectinifera* were also collected from Asamushi, Yokosuka (Kanagawa Prefecture, Japan) and Ushimado (Okayama Prefecture, Japan).

2.2. cDNA cloning

Total RNA was extracted from radial nerves of *A. japonica* using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) as an RNA extraction solution. A Poly(A)$^+$ RNA was prepared from total RNA using Oligotex-dT30 (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized using a SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) in accordance with the manufacturer's instructions. Oligonucleotide primers for the cDNA cloning of RGP were designed from sequences of RGP in *P. pectinifera* (DDBJ: AB496611) and *A. amurensis* (LC040882) as follows: 5′-RACE forward primer, 5′-GTTGGGCTATGCTTAAATTT-3′ and 3′-RACE reverse primer, 5′-GCTACTATCGACAACTGAGA-3′. 5′- and 3′-RACE products encoding RGP were amplified using these primers to determine the open-reading frame (ORF) of RGP in the cDNA from *A. japonica*. Polymerase chain reaction (PCR) using ExTaq (Takara Bio Inc., Shiga, Japan) was performed as follows: denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 45 s, and a final extension at 72 °C for 10 min.

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 with the use of 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).

The signal peptide of the preproRGP was determined using SignalP 4.1 Server (Petersen et al., 2011).

2.3. RGP synthesis

Peptides of RGP of *P. pectinifera* (PpeRGP) and *A. japonica* (AjaRGP) were synthesized essentially according to the method for synthesizing insulin-like peptide from the prawn *Marsupenaeus japonicus* as described previously (Katayama et al., 2014). AjaRGP-BΔAla^1 truncated alanine at the N-terminus (Ala^1) of the B-chain in AjaRGP was also synthesized. RGP of *A. amurensis* (AamRGP) was purchased from Peptide Institute Inc. (Osaka, Japan).

2.4. Effect of synthetic RGP

The seawater used was modified van't Hoff's artificial seawater (ASW) adjusted to pH 8.2 with 0.02 M borate buffer (Kanatani and Shirai, 1970). RGP activity was assayed biologically using ovarian fragments of *A. japonica* or *P. pectinifera* as described previously (Shirai, 1986). The ovary of a mature female in *A. japonica* or *P. pectinifera* was excised and cut into small fragments, each of

Fig. 2. The heterodimeric structures of relaxin-like gonad-stimulating peptide (RGP) in *Aphelasterias japonica* (AjaRGP), *Asterias amurensis* (AamRGP), and *Patiria* (=*Asterina*) *pectinifera* (PpeRGP). AjaRGP-BΔAla^1 is truncated at Ala^1 of the B-chain in AjaRGP. The cysteine bridges are shown as red, solid lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

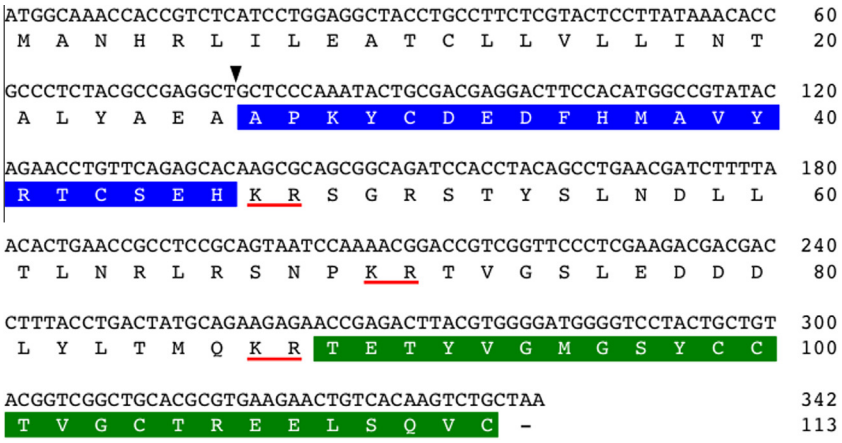

Fig. 1. Coding DNA and predicted amino acid sequences of relaxin-like gonad-stimulating peptide (RGP) in starfish *Aphelasterias japonica*. Sequences of the A and B chains are shown in green and blue boxes, respectively. Characters shown with red underlines indicate basic dipeptides that are the sites of proteolytic cleavage. An inverted triangle shows the deduced cleavage site of the signal peptide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)