Effect of chimeric relaxin-like gonad-stimulating peptides on oocyte maturation and ovulation in the starfish *Asterias rubens* and *Aphelasterias japonica*

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Abstract

A relaxin-like gonad-stimulating peptide (RGP), comprising two peptide chains (A- and B-chains) linked by two inter-chain bonds and one intrachain disulfide bond, acts as a gonadotropin in starfish. RGP orthologs have been identified in several starfish species, including *Patiria pectinifera* (PpeRGP), *Asterias rubens* (AruRGP) and *Aphelasterias japonica* (AjaRGP). To analyze species-specificity, this study examined the effects on oocyte maturation and ovulation in ovaries of *A. rubens* and *A. japonica* of nine RGP derivatives comprising different combinations of A- and B-chains from the three species. All nine RGP derivatives induced spawning in *A. rubens* and *A. japonica* ovaries. However, AruRGP, AjaRGP and their chimeric derivatives were more potent than peptides containing the A- or B-chain of PpeRGP. Three-dimensional models of the structures of the RGP derivatives revealed that residues in the B-chains, such as Asp^{B6}, Met^{B10} and Phe^{B13} in PpeRGP and Glu^{B7}, Met^{B11}, and Tyr^{B14} in AruRGP and AjaRGP, respectively, are likely to be involved in receptor binding. Conversely, it is likely that Arg^{A18} in the A-chain of AruRGP and AjaRGP impairs binding of these peptides to the PpeRGP receptor in *P. pectinifera*. In conclusion, this study provides new insights into the structural basis of RGP bioactivity and RGP receptor activation in starfish.

Key words: Relaxin-like gonad-stimulating peptide; Starfish; Gonadotropin; Species-specificity; Chimeric derivatives; Receptor

1. Introduction

In 1959, Chaet and McConnaughy (1959) first reported that a water extract of radial nerve cords from the Forbes starfish (sea star) *Asterias forbesi* induces shedding of gametes when injected into the coelomic cavity of ripe animals. The gonadotropin present in starfish radial nerve cord extracts is not, however, a pituitary-type glycoprotein hormone but is a relaxin-like neuropeptide that was originally named gonad-stimulating substance (GSS). Purification and structural identification of GSS revealed that, like relaxins, it comprises two different peptides – A- and B-chains with two inter-chain disulfide bonds and one intra-chain disulfide bond (Mita et al., 2009; Mita 2013). Thus, GSS was renamed as relaxin-like gonad-stimulating peptide (RGP) (Mita, 2016, 2019).

Although RGP is the primary mediator of oocyte maturation and ovulation in starfish, the effect of RGP is indirect. It acts on the ovary to produce the second mediator 1-methyladenine (1-MeAde), which is the maturation-inducing hormone (MIH) of starfish (Kanatani et al., 1969; Kanatani, 1979, 1985). Thus, RGP stimulates ovarian follicle cells around oocytes to produce 1-MeAde (Mita et al., 2009). This action is mediated through the activation of its receptor, G-proteins and generation of cyclic-AMP by adenylyl cyclase (Mita and Nagahama, 1991; Mita et al., 2009; Mita et al., 2011a, 2011b, 2012, 2013, 2014).

Previous studies have determined the chemical structures of RGP in several species of starfish (Ikeda et al., 2015; Lin et al., 2017; Mita et al., 2015a, 2015b; Mita and Katayama, 2016; Smith et al., 2017), which include PpeRGP in *Patiria* (*Asterina*) *pectinifera* (Mita et al., 2009), AamRGP in *Asterias amurensis* (Mita et al., 2015a), and AjaRGP in *Aphelasterias japonica* (Mita and Katayama, 2016). PpeRGP shares a high level of sequence similarity with RGP in other starfish species of the order Valvatida in the class Asteroidea (Mita, 2016) but less sequence conservation between PpeRGP and RGP in *A. amurensis* and *A. japonica*, both of which belong to the order Forcipulatida. It is noteworthy, therefore, that neither AamRGP

nor AjaRGP induce spawning of ovarian fragments from *P. pectinifera*, whereas AamRGP is active on *A. japonica* ovary (Mita et al., 2015a; Mita and Katayama, 2016). In contrast, PpeRGP induces spawning of ovaries from *A. amurensis* and *A. japonica* as well as *P. pectinifera* (Mita et al., 2015a; Mita and Katayama, 2016). Thus, the action of PpeRGP is less species-specific than AamRGP and AjaRGP.

Because RGP is a heterodimeric peptide, the A- and/or B-chain of RGP could be involved in receptor binding. Previous studies have shown that a 'relaxin-specific receptor-binding cassette' (Arg XXX Arg XX Ile/Val) in the B-chain of vertebrate relaxins is important for receptor binding (Büllesbach and Schwabe, 1988, 2000, 2005). Despite its similarity with relaxins, however, the RGP sequence does not possess the vertebrate 'relaxin-specific receptor-binding cassette' in the B-chain (Mita, 2016). Therefore, it is likely that other residues in the B-chain of RGP are involved in receptor binding.

Little is known about mechanisms of RGP species-specificity. To obtain new insights into the structural basis of the interaction of RGP with its receptor, our objective here was to examine the effects of PpeRGP, AamRGP, AjaRGP, and their chimeric derivatives on oocyte maturation and ovulation in ovaries of *A. amurensis* and *A. japonica*. However, previous studies have reported that assaying spawning activity in *A. amurensis* ovaries is difficult because ovaries immediately spawn 'spontaneously' when isolated in seawater (Cloud and Schuetz, 1973; Shirai, 1974). Recently, we reported that the chemical structure of RGP is identical in *A. amurensis* (AamRGP) and *Asterias rubens* (AruRGP). Furthermore, we also found that spontaneous spawning does not occur immediately when ovaries from *A. rubens* are isolated in seawater and spawning is induced by AruRGP (Lin et al., 2017). Therefore, for this study we used ovaries from *A. rubens* and *A. japonica* for spawning assays to investigate the species-specificity and structure-activity relationships of RGP.

2. Materials and methods

2.1. Animals

Starfish, *P. pectinifera* and *A. japonica* were collected from Asamushi (Aomori, Japan). *A. rubens* were obtained from a fisherman based at Whitstable (Kent, UK). Animals were kept in circulating artificial seawater aquarium at 15°C (*P. pectinifera* and *A. japonica*) or 12°C (*A. rubens*) and were used within 2 months after collection.

2.2. Peptide synthesis

RGP and chimeric derivatives were synthesized as described previously (Katayama and Mita, 2016; Mita et al., 2019). In brief, A- and B-chains were prepared by the ordinary 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis. Three disulfide bonds were regioselectively formed by dimethyl sulfoxide (DMSO) oxidation, *S*-pyridylsulfenyl-directed thiolysis and iodine oxidation reactions. MALDI-TOF mass spectra were recorded using an Autoflex spectrometer (Brucker). Amino acid composition was determined using a LaChrom amino acid analyzer (Hitachi, Tokyo, Japan) after hydrolysis with 6 M HCl solution at 150^{o}C for 2 h in a vacuum-sealed tube.

2.3. Induction of oocyte maturation and ovulation

Bioactivity of synthetic chimeric RGPs was assayed using ovarian fragments from *A. rubens* and *A. japonica* as described previously (Shirai, 1986). Modified van't Hoff's artificial seawater (ASW) adjusted to pH 8.2 with 0.02 M borate buffer was prepared (Kanatani and Shirai, 1970) and the ovaries of mature female starfish were excised and cut using scissors into small fragments containing only a few lobes. The ovarian fragments were then incubated in ASW containing peptides at a range of concentrations (0.4 – 50 nM) for 30 – 60 min. The

samples were examined to determine whether or not spawning had occurred and were scored (Shirai, 1986) as follows: (+++) spawning occurred and most oocytes had matured; (++) about 50% of oocytes had matured, (+) a few oocytes had matured and (−): no spawning occurred. The scores were converted to numerical values (+++ = 100; ++ = 67; + = 33; − = 0) so that the effective dose for inducing gamete spawning in 50% of ovarian fragments could be determined graphically. Means ± SEM were determined from four separate assays using ovaries from different animals.

2.4. Three dimensional structures

Three-dimensional (3D) structures of RGP derivatives were produced using SWISS-MODEL with default settings (https://swissmodel.expasy.org/; Guex et al., 2009; Benkert et al., 2011; Bertoni et al., 2017; Bienert at al., 2017; Waterhouse et al., 2018). The structural template consisting of sequences combined with the signal peptide to B-chain and the C-peptide to A-chain among three kinds of pre-proRGPs was automatically set by the software to the solution structure of human insulin or IGF (PDB code, 2GF1), as described previously (Mita et al., 2019). The modeled structures were visualized with Pymol (http://www.pymol.org) and then the signal and C-peptides were eliminated. The predicted 3D structure models showed three disulfide cross-linkages; two interchain bonds between the A- and B-chains, and an intrachain bond within the A-chain. It is considered that the 3D models are close to the native structures.

3. Results

All nine of the RGP derivatives tested induced spawning of ovarian fragments from *A. rubens* and *A. japonica*. Furthermore, ovarian fragments from *A. rubens* and *A. japonica* when placed into ASW alone did not undergo spawning within 60 min of incubation.

However, spawning occurred 'spontaneously' after incubation for 2 hours or more. Because spawning induced by RGP occurred within a period of up to 60 min, the effective dose of each RGP for induction of oocyte spawning in 50% of ovarian fragments (EC_{50}) could be determined for *A. rubens* and *A. japonica* ovaries. The EC_{50} values for peptides comprising A- and B-chains from AruRGP and/or AjaRGP were less than 1.5 nM (0.31 – 1.4 nM), whereas the EC_{50} values observed for peptides comprising A- and/or B-chains from PpeRGP ranged from 2.4 nM to as high as 16.0 nM (Table 1). These findings are consistent with the greater sequence similarity shared by AruRGP and AjaRGP in comparison with PpeRGP (Fig. 1) and in accordance with phylogenetic relationships – i.e. *A. rubens* and *A. japonica* both belong to the order Forcipulatida whereas *P. pectinifera* belongs to the order Valvatida.

Determination of the relative potency of the RGP derivatives provided a basis for examination of structure-activity relationships with reference to the predicted 3D structures of PpeRGP, AruRGP and AjaRGP generated using SWISS-MODEL. The 3D structures of PpeRGP (Fig. 2A), AruRGP (Fig. 2B), and AjaRGP (Fig. 2C) were in accordance with previously reported data (Mita et al., 2019), showing that the overall structure of the B-chain is very similar in all three molecules. Furthermore, this consistency in structure can also been identified in the ninety degree rotated 3D structures of PpeRGP (Fig. 2D), AruRGP (Fig. 2E) and AjaRGP (Fig. 2F).

Vertebrate relaxins bind to their receptors via 'the receptor-binding cassette' of the B-chain (Büllesbach and Schwabe, 1988, 2000, 2005). Therefore, we examined the RGP sequences to identify residues in the middle region of B-chains corresponding to 'the receptor-binding cassette', which include Asp^{B6}, Met^{B10} and Phe^{B13} for PpeRGP and Glu^{B7}, Met^{B11} and Tyr^{B14} for AruRGP and AjaRGP, respectively (Fig. 1). Examination of the 3D structure models reveals that the side–chains of these residues are located on the external surface of the helical region of the B-chain and are separated by single helical turns in

PpeRGP (Figs. 2A and D), AruRGP (Figs. 2B and E), and AjaRGP (Figs. 2C and F). Therefore, it seems likely that these residues in the B-chain of RGP may have an important role in binding of RGP to its receptor(s).

The 3D models revealed variability in the structure of A-chains of PpeRGP, AruRGP and AjaRGP. Thus, Pro^{A17} in PpeRGP (Figs. 2A and D) and Arg^{A18} in AruRGP (Figs. 2B and E) and AjaRGP (Figs. 2C and F) are located near the B-chain, respectively. In contrast, the side-chains of Ser^{A18} and Val^{A22} in PpeRGP (Fig. 2D) and Asp (Glu)^{A19} and Gln^{A23} in AruRGP and AjaRGP (Figs. 2E and F) are orientated away from the B-chains.

4. Discussion

It has been shown that the starfish gonadotropin RGP, formerly known as GSS, generally acts non-species specifically, but with some exceptions (Noumura and Kanatani, 1962; Chaet, 1966a, b). However, it was not possible to investigate the structural basis of RGP bioactivity and species-specificity until recently, when the chemical structures of RGP from different starfish species were determined. Each RGP molecule is a heterodimer composed of two different peptides, A- and B-chains with two inter-chain bonds and one intra-chain disulfide bond (Mita et al., 2009; Mita, 2016, 2019). The sequences of RGP in *A. rubens* (AruRGP) and *A. japonica* (AjaRGP) are quite similar to each other, with three residues out of a total of twenty-five being different in the A-chains - Pro^{A1}, Leu^{A13} and Gln^{A20} in AruRGP and Thr^{A1}, Thr^{A13} and Glu^{A20} in AjaRGP (Fig. 1). Furthermore, only one of the twenty residues of the B-chains are different in AruRGP and AjaRGP - Glu^{B2} in AruRGP and Pro^{B2} in AjaRGP. In contrast, the sequence of PpeRGP is quite different to those of AruRGP and AjaRGP (Fig. 1). There are seven residues (Ser^{A1}, Ser^{A4}, Ala^{A7}, His^{A13}, Pro^{A17}, Ser^{A18} and Val^{A22}) that differ in the A-chain and three residues (Ala^{B17}, Val^{B18} and Ser^{B19}) that differ in the B-chain.

To investigate the species-specificity of RGP and the structural basis of the interaction of

195 RGP with its receptor, we have chemically synthesized nine RGP derivatives comprising A-
196 (RGP-A) and/or B- (RGP-B) chains from *P. pectinifera*, *A. rubens* and *A. japonica*. We tested
197 these nine RGP derivatives on ovarian tissue from *P. pectinifera* and found that only peptides
198 containing the PpeRGP A-chain induced oocyte maturation and ovulation, with RGP
199 derivatives that contain the A-chain of AruRGP or AjaRGP failing to induce spawning of *P.*
200 *pectinifera* ovarian tissue (Mita et al., 2019; Table 1). In contrast, here we found that all nine
201 of the RGP derivatives induced spawning of ovarian fragments from *A. rubens* and *A.*
202 *japonica* (Table 1). Because RGP acts on follicle cells around oocytes in ovarian tissue (Mita
203 et al., 1987, Mita et al., 2009), the RGP receptor(s) expressed in follicle cells of *P. pectinifera*
204 exhibits high species-specificity in binding RGP, whereas the RGP receptor(s) expressed in
205 follicle cells of *A. rubens* and *A. japonica* exhibit low species-specificity. However,
206 comparison of the EC_{50} values of the RGP derivatives revealed that, consistent with
207 phylogenetic relationships, molecules containing RGP A- and/or B-chains from the two
208 species belonging the order Forcipulatida, *A. rubens* and *A. japonica*, were more potent as
209 inducers of spawning of ovarian fragments from these two species than RGP molecules
210 containing A- and/or B-chains from *P. pectinifera* (order Valvatida). More specifically, the
211 EC_{50} values of PpeRGP and chimeric derivatives containing the PpeRGP B-chain substitution
212 (AruRGP-A /PpeRGP-B and AjaRGP-A /PpeRGP-B) were particularly high when tested on
213 ovarian fragments from *A. japonica*. These findings strongly suggest that the B chain of RGP
214 is important for receptor binding and bioactivity. Furthermore, these findings provided a basis
215 for comparison of the structures of the nine derivatives to determine more specifically which
216 residues may be important for receptor binding and bioactivity.
217 Previous studies have shown that the 'relaxin-specific receptor-binding cassette' (Arg
218 XXX Arg XX Ile/Val) in the B-chain of vertebrate relaxins is important for receptor binding
219 (Büllesbach and Schwabe, 1988, 2000, 2005). Although the B-chain of RGP sequences do not

possess the vertebrate relaxin-specific receptor-binding cassette, the corresponding amino acid residues in B-chains are Asp^{B6}, Met^{B10} and Phe^{B13} for PpeRGP and Glu^{B7}, Met^{B11}, and Tyr^{B14} for AruRGP and AjaRGP, respectively. 3D models of the structures of RGPs reveal that these residues are separated by single helical turns in the B-chains of RGPs. The predicted 3D structure models of the B-chain appear to be quite similar among RGPs including the chimeric derivatives (Mita et al., 2019). Furthermore, structural differences between Asp^{B6} in the PpeRGP B-chain and Glu^{B7} in the AruRGP and AjaRGP B-chains and between Phe^{B13} in the PpeRGP B-chain and Tyr^{B14} in the AruRGP and AjaRGP B-chains appear to adversely affect the affinity of RGP receptors in *A. rubens* and *A. japonica* for RGP derivatives containing the B-chain of PpeRGP. Therefore, this strongly suggests that Asp^{B6}, Met^{B10} and Phe^{B13} in PpeRGP and Glu^{B7}, Met^{B11} and Tyr^{B14} in AruRGP and AjaRGP are specifically involved in binding to RGP receptor proteins.

Previous studies have shown that chimeric RGP derivatives containing the A-chains of AamRGP (= AruRGP) and AjaRGP do not induce spawning in *P. pectinifera* ovaries (Mita et a., 2019). Based on the 3D structural models, the Pro^{A17} of PpeRGP and Arg^{A18} of AruRGP and AjaRGP were located near the B-chain, respectively. Because the side chain of arginine is positively charged and is larger than the side chain of proline, it seems likely that this impairs binding of RGP derivatives containing Arg^{A18} to *P. pectinifera* RGP receptor. Conversely, because Ser^{A18} and Val^{A22} of PpeRGP or Asp (Glu)^{A19} and Gln^{A23} of AruRGP and AjaRGP are predicted to be located away from the B-chains, these residues may not affect receptor binding.

Further insights into the structural basis for RGP species-specificity and bioactivity will be obtained if the RGP receptor(s) can be identified in starfish. Therefore, this now represents a high priority for future research on RGP signaling.

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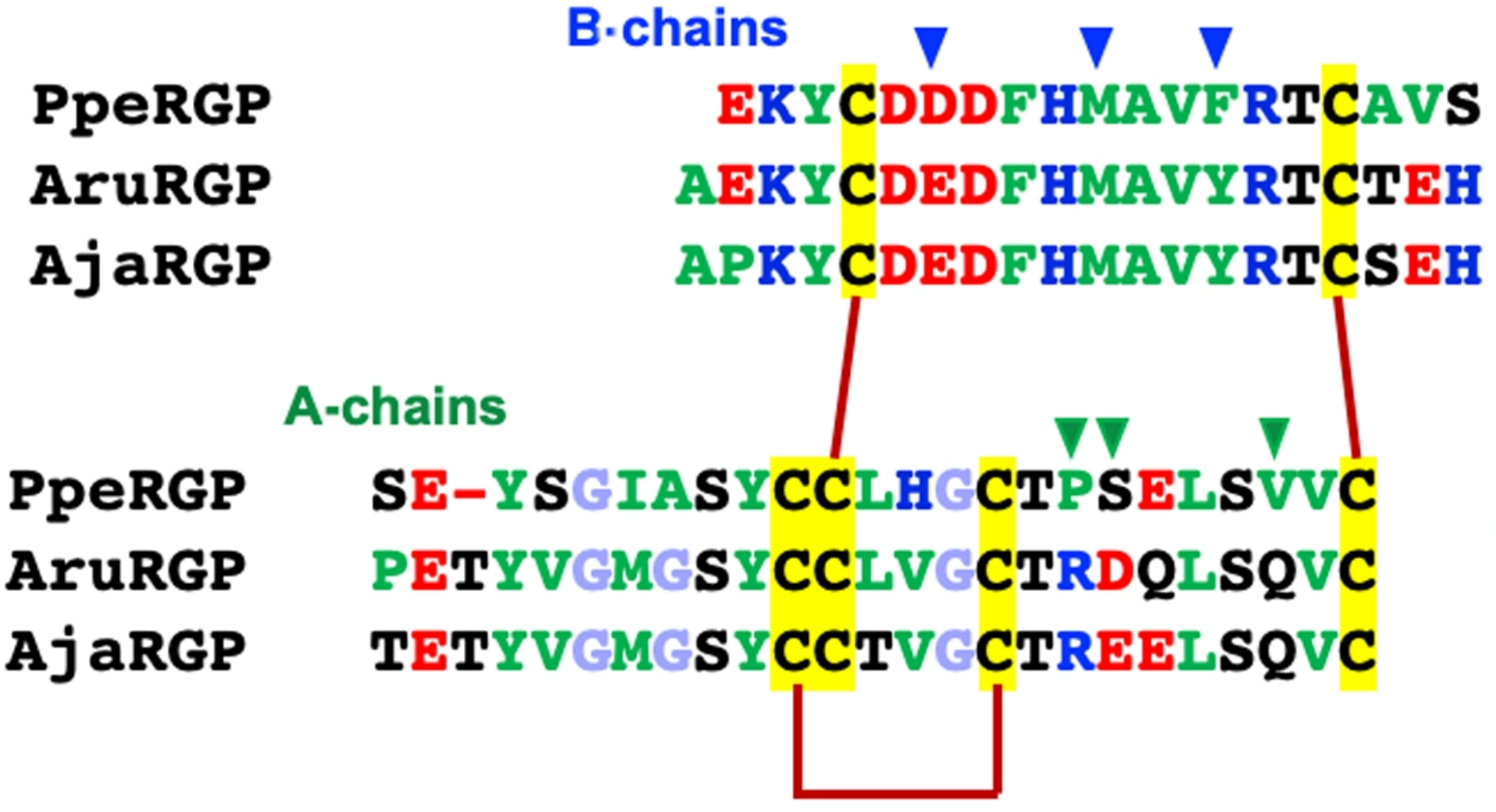
Figure legends

Fig. 1. Amino acid alignments of A- and B-chains in starfish relaxin-like gonad-stimulating peptides (RGPs) of *Patiria pectinifera* (PpeRGP), *Asterias rubens* (AruRGP), and *Aphelasterias japonica* (AjaRGP). To illustrate the conserved features, the amino acid types are color coded according to their properties, with basic residues in blue (Arg, Lys and His), acidic residues in red (Glu and Asp), hydrophobic residues in green (Ala, Val, Ile, Phe, Trp, Tyr, Pro and Met), hydrophilic in black (Ser, Thr, Asn and Gln) and glycine in light blue. The cysteine residues are highlighted in yellow and disulfide bonds are shown with solid dark lines. Key amino acids in the A- and B-chains that are discussed in this paper are shown with green and blue arrow heads, respectively.

Fig. 2. Three-dimensional (3D) models of the structures of relaxin-like gonad-stimulating peptides (RGPs) of *Patiria pectinifera* (A, D), *Asterias rubens* (B, E), and *Aphelasterias japonica* (C, F). Images D, E, F are the same 3D structures as A, B, C, respectively, but are rotated 90 degrees horizontally. The side chains of selected amino acids in the A-chains (green) and B-chains (blue) are shown and labelled. Underlined residues are discussed in this paper because of their potential involvement in receptor binding. Each 3D structure model was built using SWISS-MODEL, as described in the methods.


B-chains
PpeRGP EKYCDDDFHMAVFRTCAVS
AruRGP AEKYCDEDFHMAVYRTCTEH
AjaRGP APKYCDEDFHMAVYRTCSEH
A-chains
PpeRGP SE-YSGIASYCCLHGCTPSELSVVC
AruRGP PETYVGMGSYCCLVGCTRDQLSQVC
AjaRGP TETYVGMGSYCCTVGCTREELSQVC

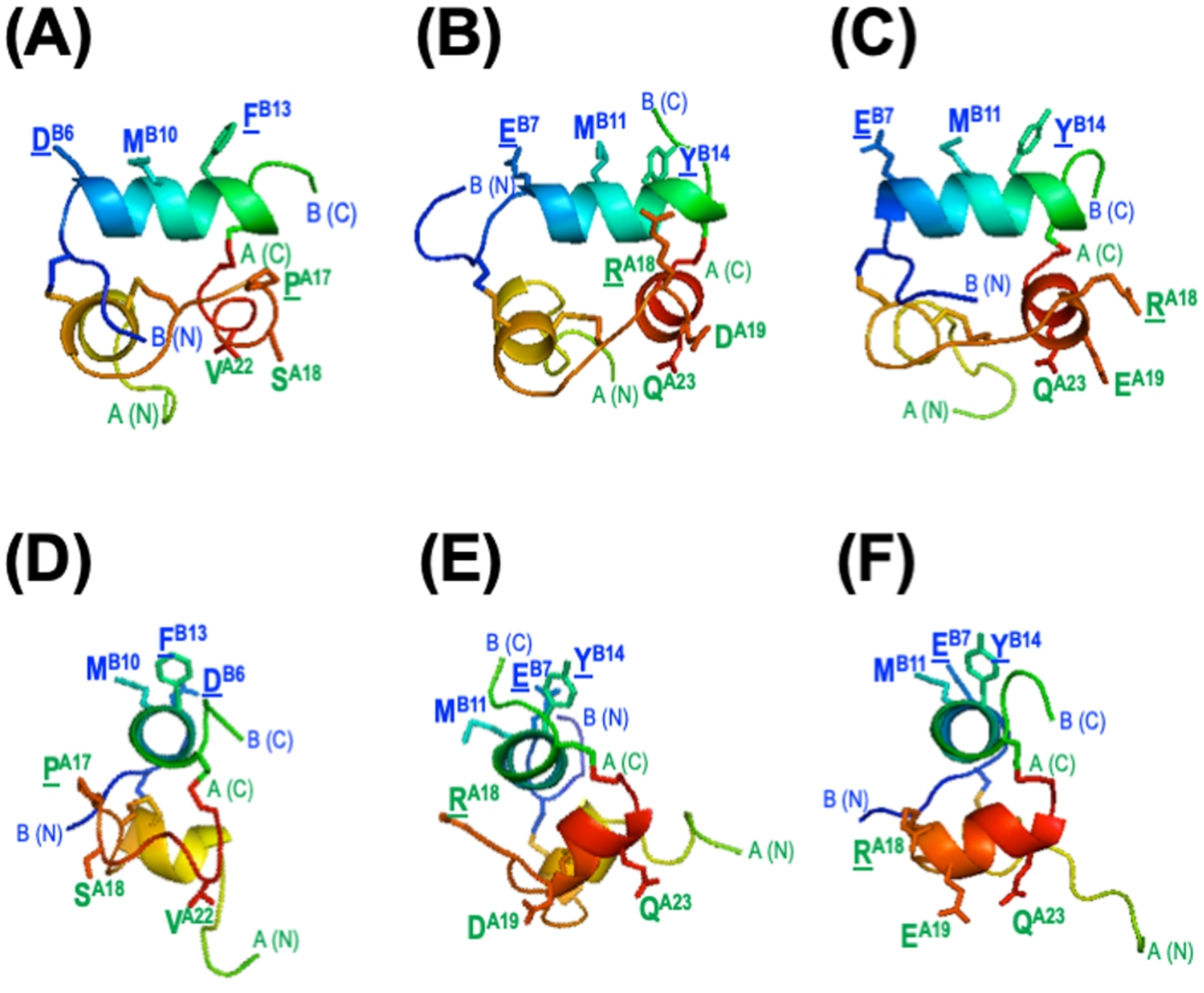

(A)
DB6
MB10
FB13
B (C)
A (C)
PA17
B (N)
VA22
SA18
A (N)
(B)
EB7
MB11
B (C)
YB14
B (N)
RA18
A (C)
DA19
A (N)
QA23
(C)
EB7
MB11
YB14
B (C)
A (C)
B (N)
RA18
QA23
EA19
A (N)
(D)
FB13
MB10
DB6
B (C)
PA17
A (C)
B (N)
SA18
VA22
A (N)
(E)
B (C)
EB7
YB14
MB11
B (N)
A (C)
RA18
A (N)
DA19
QA23
(F)
EB7
YB14
MB11
B (C)
A (C)
B (N)
RA18
EA19
QA23
A (N)

Table 1. Effects of RGPs and their chimeric derivatives on the induction of oocyte maturation and ovulation in ovarian fragments of *Patiria pectinifera* (A), *Asterias rubens* (B), and *Aphelasterias japonica* (C)

(A) *Patiria pectinifera* ovary

	Ppe-B	Aru-B	Aja-B
Ppe-A	1.1 ± 0.2	13.9 ± 2.6	9.8 ± 1.7
Aru-A	No effect	No effect	No effect
Aja-A	No effect	No effect	No effect

(B) *Asterias rubens* ovary

	Aru-B	Aja-B	Ppe-B
Aru-A	0.75 ± 0.10	1.0 ± 0.1	4.2 ± 0.6
Aja-A	1.4 ± 0.4	1.3 ± 0.1	3.0 ± 0.4
Ppe-A	2.5 ± 0.4	2.4 ± 0.2	2.9 ± 0.2

(C) *Aphelasterias japonica* ovary

	Aja-B	Aru-B	Ppe-B
Aja-A	0.32 ± 0.01	0.33 ± 0.01	16.0 ± 0.6
Aru-A	0.36 ± 0.01	0.31 ± 0.01	8.1 ± 0.5
Ppe-A	4.6 ± 0.2	6.6 ± 0.6	11.8 ± 0.7

Ovarian fragments were incubated with various concentrations of chimeric RGP derivatives for 1 h. The effective dose for induction of oocyte spawning in 50% of ovarian fragments (EC_{50}) was determined from four experiments. Values are means ± SEM of four separate assays using different animals. Peptides used were examined up to 50 nM. EC_{50} values less than 2 nM or greater than 2 nM are shown in red or blue, respectively.