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Alternative splicing of a single precursor mRNA generates two subtypes of Gonadotropin-Releasing Hormone receptor orthologues and their variants in the bivalve mollusc *Crassostrea gigas*

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Abstract

Despite their economic importance, only very little information is available regarding (neuro)endocrine regulation of reproduction in bivalve molluscs. To gain insights into the molecular control of gonadic development of these animals, G protein-coupled receptors (GPCR) specifically expressed in the gonad of the pacific oyster *Crassostrea gigas* were investigated. One such receptor, *Cg*-GnRH-R, an oyster GPCR orthologue of vertebrate GnRH receptors clearly involved in the control of oyster gametogenesis was first identified [Rodet, F., Lelong, C., Dubos, M.P., Costil, K. and Favrel, P., 2005. Molecular cloning of a molluscan Gonadotropin-Releasing Hormone receptor orthologue specifically expressed in the gonad. Biochim Biophys Acta 1730 187–95.]. We report here the characterization of multiple transcripts encoding GnRH-R orthologues (*Cg*-GnRH-RII-L/*Cg*-GnRH-RII-S) including a truncated receptor (*Cg*-GnRH-R-TF) and demonstrate they are generated by the alternative splicing of a single mRNA precursor. The differential structure of these receptors suggests that *Cg*-GnRH-R on one hand and *Cg*-GnRH-RII-L/*Cg*-GnRH-RII-S on the other hand constitute two receptor subtypes with regard to ligand specificity. Pattern of expression of these transcripts suggests that *Cg*-GnRH-R cognate ligand is specifically involved in the control of gametogenesis while *Cg*-GnRH-RII-L and *Cg*-GnRH-RII-S ones likely do not control reproductive functions specifically. Hypothesis on the involvement of this family of receptors in signalling both GnRH and APGWamide in molluscs is discussed.

Keywords: Mollusc; Reproduction; Gonad; G Protein-coupled receptor

1. Introduction

Reproduction in bivalve molluscs follows a seasonal cycle and is controlled by both environmental parameters and (neuro) endocrine factors. As (neuro)peptides and (neuro)peptide receptors occupy the highest hierarchic position in the regulation of most animal physiological processes including reproduction, the identification of key regulatory (neuro) peptides or (neuro)peptide receptors steering the reproductive cycle in bivalve molluscs promises the provision of innovative molecular tools for improving hatchery production of these economically important animals. Most (neuro)peptide receptors

Abbreviations: AKH, adipokinetic hormone; *Cg*-GnRH-R; *Crassostrea gigas* GnRH-like receptor; EC, extracellular loop; GnRH, Gonadotropin-Releasing Hormone; GPCR, G protein-coupled receptor; IC, intracellular loop; ORF, open reading frame; TM, transmembrane domain.

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belong to the G protein-coupled receptor (GPCR) family, one of the largest categories of proteins encoded by animal genomes. To gain insights into the molecular control of gonadic regulation of bivalve molluscs, GPCRs expressed in the gonad of the pacific oyster *Crassostrea gigas* were investigated. One such receptor, *Cg*-GnRH-R, an oyster GPCR orthologue of vertebrate GnRH receptors clearly involved in the control of oyster gametogenesis was recently characterized (Rodet et al., 2005). Chordates harbour two or three GnRH-Rs (Illing et al., 1999; Millar et al., 2001; Jodo et al., 2003; Kusakabe et al., 2003). In the protostomian lineage, *Drosophila* displays two orthologous receptors (Hauser et al., 1998; Cazzamali et al., 2002) whereas the molluscan *Octopus vulgaris* exhibits only a single GnRH-like receptor (Kanda et al., 2006). Thus, the question arises as whether *C. gigas* possesses one or several orthologous receptor (s). This paper reports the characterization of various oyster GnRH-R orthologues generated by alternative splicing of a single precursor mRNA.

2. Material and methods

2.1. Animals

Adult oysters *C. gigas* were purchased from a local oyster farm (Normandie, France).

2.2. RT-PCR and 5′- and 3′-RACE-PCR

Total RNA from various tissues of the pacific oyster *C. gigas* was isolated using Tri-Reagent (Sigma, USA). Poly(A^+)RNA was isolated using polythymidine coupled to magnetic beads according to the manufacturer's instructions (Dynal). Reverse transcription was carried out using oligo$(dT)_{17}$ as primer, 1 μg mRNA and 200 U Moloney murine leukemia virus reverse transcriptase (Promega). Two degenerated primers corresponding to transmembrane regions 3 and 6 of various GPCRs (Probst et al., 1992; Tensen et al., 1998) were designed: TM-3, 5′-CGYSATYRSSITKGACMGSTA-3′; TM-6r, 5′-CGGSMIC-CARCAGAISRYRAA-3′. Y=C or T, K=G or T, R=A or G, S=C or G, M=A or C, I: inosines. cDNA amplification was performed for 48 cycles at 95 °C/40 s, 50 °C/2 min, and 72 °C/2 min. A 462 bp PCR fragment recovered from enriched germ cell cDNAs was purified, subcloned into pGEM-T easy vector using a TA cloning kit (Promega) and sequenced using an ABI Prism Big Dye terminator cycle sequencing kit (PE Biosystems). Full-length cDNA was generated by 5′- and 3′-RACE using Marathon cDNA amplification kit (Clontech). Doubled stranded cDNA from oyster enriched germ cells was ligated to adaptors and 25 ng of this template was used to PCR amplify 5′- and 3′-RACE fragments using adaptor specific primers and gene specific primers deduced from the initial 462 bp fragment sequence. This allowed the characterization of the *Cg*-GnRH-R cDNA on one hand (Rodet et al., 2005) and the identification of part of the *Cg*-GnRH-RII-L 5′ end on the other hand. From this PCR fragment, using the specific primers GnRH-RII-5′ (5′ CAG AAT CAG CAT GAT CCG GCC 3′) and GnRH-RII-3′ (5′ CCC CTT AGT CTG AAC GAT GCG 3′), *Cg*-GnRH-RII-L cDNA was cloned by another round of 5′- and 3′-RACE. To check the actual contiguity of 3′ and 5′ RACE fragments and to generate a single fragment, RT-PCR was then performed with the specific primers GnRH-RII-sense (5′ ATG AAT TCC GAC GCG GAT GC 3′) and GnRH-RII-antisense (5′ TCA GTG CTT GTC CAT CGT CAC TG 3′), for 5 cycles at 95 °C/1 min, 65 °C/1 min and 72 °C/4 min °; 5 cycles at 95 °C/1 min, 60 °C/1 min and 72 °C/4 min °; 30 cycles at 95 °C/1 min, 58 °C/1 min and 72 °C/4 min. This resulted in the generation of three fragments of 1558 bp, 1422 bp and 1358 bp encoding *Cg*-GnRH-RII-S, *Cg*-GnRH-RII-L and *Cg*-GnRH-R-TF respectively (see S2).

2.3. Screening of genomic library

A genomic library of *C. gigas* was constructed in λ-$DASH_{II}$ (Stratagene) according to the manufacturer's instructions. A total of 1.8×10^6 independent clones were recovered (Herpin et al., 2002). After amplification, a total of 10^5 recombinant λ-DASH phages were plated, adsorbed to Nylon membranes, screened at high stringency with three random primed ^{32}P labeled fragment cDNAs (specific activity 10^9 dpm/ μg). Probe I encompasses part of the specific sequence of *Cg*-GnRH-R up to the common region; probe II corresponds to the specific 5′ extremity of *Cg*-GnRH-RII-L, *Cg*-GnRH-RII-S and *Cg*-GnRH-R-TF and probe III is a fragment which extends from the common region to the 3′ end of the various transcripts (Fig. 2A). Positive clones were purified as described by Sambrook et al. (1989), subjected to restriction analysis and Southern blot hybridization using the original probe to confirm that the λ-DASH clones contained *Cg-gnrh-r* specific genomic sequences. The genomic organization of the *Cg-gnrh-r* gene was subsequently determined by sequencing subcloned positive genomic fragments. Exon/intron boundaries were determined by comparing the genomic sequence to the sequence of the various characterized transcripts.

2.4. Real-time quantitative PCR

Quantitative RT-PCR analysis was performed using the iCycler apparatus (Bio-Rad). Total RNA was isolated from adult tissues using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions. After treatment during 20 min at 37 °C with 1U of DNase I (Sigma) to prevent genomic DNA contamination, 1 μg of total RNA was reversed transcribed using 1 μg of random hexanucleotidic primers (Promega), 0.5 mM dNTPs and 200U M-MLV Reverse Transcriptase (Promega) at 37 °C for 1 h in the appropriate buffer. The reaction was stopped by incubation at 70 °C for 10 min. iQ™ SYBR Green supermix PCR kit (Biorad) was used for real-time monitoring of amplification (5 ng of template cDNA, 40 cycles: 95 °C/15 s, 60 °C/15 s) with the following primers: Q-*Cg*-GnRH-R-IIs (5′-ACGACACCACGGAAATGC-3′) and Q-*Cg*-GnRH-RIIa (5′-GCCACGATGAACATGCAC-3′) as sense and antisense primers respectively. Accurate amplification of the target amplicon was checked by performing a melting curve. Using QsACTIN (5′-GCCCTGGACTTCGAACAA-3′) and