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Molecular cloning of a putative receptor guanylyl cyclase from Y-organs of the blue crab, *Callinectes sapidus*

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Abstract

Crustacean molt-inhibiting hormone (MIH), a polypeptide produced by neurosecretory cells in eyestalk ganglia, suppresses the synthesis of ecdysteroid molting hormones by paired Y-organs. Data from several sources indicate the effects of MIH are mediated, at least in part, by a cGMP second messenger. Based on these and related findings, our working hypothesis is that the MIH receptor is a receptor guanylyl cyclase (rGC). In studies reported here, we used a PCR-based cloning strategy (RT-PCR followed by 5′- and 3′-RACE) to clone from blue crab (*Callinectes sapidus*) Y-organs a cDNA (*CsGC-YO1*) encoding a putative rGC. DNA sequence analysis revealed a 3807 base pair open reading frame encoding a 56 residue signal peptide and a 1213 residue rGC. Analysis of the deduced amino acid sequence showed that CsGC-YO1 contains the signature domains characteristic of rGCs, including an extracellular ligand-binding domain, a single transmembrane domain, a kinase-like domain, a dimerization domain, and a cyclase catalytic domain. CsGC-YO1 is most closely related to an rGC from the crayfish, *Procambarus claikii* (PcGC-M2, 58.4% identity), and rGCs from three insect species (33.1–37.5% identity). Conserved cysteine residues are similarly distributed in the extracellular domains of CsGC-YO1, PcGC-M2, and the three insect rGCs. RT-PCR revealed the CsGC-YO1 transcript is expressed in Y-organs and several other tissues. While other interpretations of the data are possible, our working hypothesis is that the cloned cDNA encodes an MIH receptor.

Keywords: Molt-inhibiting hormone; Receptor guanylyl cyclase; Ecdysteroid; *Callinectes sapidus*

1. Introduction

Crustacean molting is regulated by polyhydroxylated C-27 steroid hormones termed ecdysteroids (Lachaise et al., 1993; Skinner, 1985). Ecdysteroids are synthesized by paired Y-organs, endocrine glands located in the anterior cephalothorax. Synthesis of ecdysteroids by Y-organs is regulated, at least in part, by molt-inhibiting hormone (MIH), a polypeptide produced by neurosecretory cells in the X-organ/sinus gland complex of the eyestalks (Lachaise et al., 1993; Skinner, 1985). In vitro studies indicate MIH acts directly on Y-organs to suppress production of ecdysteroids (Mattson and Spaziani, 1985; Soumoff and O'Connor, 1982; Watson and Spaziani, 1985; Webster, 1986). Thus, a long-standing model suggests that MIH suppresses ecdysteroidogenesis during much of the molt cycle, and a molting sequence ensues when MIH secretion diminishes. Although the model remains valuable as a base from which testable hypotheses can be formulated, several lines of evidence suggest it is incomplete. For example, compounds other than MIH, including crustacean hyperglycemic hormone (CHH) (Webster, 1993; Webster and Keller, 1986) and a factor from regenerating limb buds (Yu et al., 2002), have been implicated in the regulation of Y-organs. In addition, recent evidence suggests that rates of ecdysteroid production may be influenced not only by regulatory ligands, but also by changes in the capacity of Y-organs to respond to those ligands (Nakatsuji and Sonobe, 2004).

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The intracellular signaling pathways involved in the regulation of ecdysteroidogenesis remain an area of active research (reviews: Spaziani et al., 1999, 2001). Existing data indicate that signaling pathways involving cAMP, cGMP, or both play a role in MIH-mediated regulation of ecdysteroidogenesis. In the blue crab, *Callinectes sapidus*, available data favor cGMP as the second messenger directly linked to suppression of ecdysteroid synthesis. For example, cGMP analogs, but not cAMP analogs, dose-dependently suppress ecdysteroid production by Y-organs in 6-h incubations in vitro (Han and Watson, 2001).

The enzymes that regulate synthesis of cGMP, guanylyl cyclases, fall into two major classes: the cytoplasmically localized, soluble guanylyl cyclases and the membrane associated, receptor guanylyl cyclases (rGC) (Wedel and Garbers, 2001). Soluble guanylyl cyclases are heterodimers that can be activated by nitric oxide (Wedel and Garbers, 2001). Receptor guanylyl cyclases are integral membrane proteins with five signature domains: extracellular, transmembrane, kinase-like, dimerization, and cyclase catalytic (Lucas et al., 2000). The binding of a specific ligand to the extracellular domain of the rGC activates the intracellular cyclase domain.

In preliminary experiments done in our lab, a nitric oxide donor (sodium nitroprusside) had no effect on ecdysteroid production by blue crab Y-organs in vitro (Han and Watson, unpublished). Given that result, and our previous observation that cGMP analogs dose-dependently suppress ecdysteroid production by blue crab Y-organs (Han and Watson, 2001), our working hypothesis is that the MIH receptor is an rGC. We report here the cloning of a cDNA encoding an rGC-like protein from Y-organs of *C. sapidus*. The ligand for the putative receptor has not been determined. Our future experiments will be designed to test the hypothesis that the cloned cDNA (*CsGC-YO1*) encodes an MIH receptor.

2. Methods

2.1. Experimental animals

Blue crabs, *C. sapidus*, were purchased from local markets or from Gulf Specimen Marine Laboratories, Inc. (Panacea, FL). Crabs were maintained in compartmented tanks containing artificial seawater (salinity 26–28 ppt; BIO-SEA Marinemix, Aqua Craft TM; San Carlos, CA) recirculated through a filter of crushed coral and activated charcoal. Crabs were held at 23 °C, exposed to a photoperiod of 12L:12D, and fed pieces of shrimp or fish every other day.

2.2. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Y-Organs were dissected from ice-anaesthetized intermolt crabs, frozen in liquid nitrogen, and stored at −70 °C until use. Total RNA was extracted from Y-organs using TRIzol reagent (Life Technologies, Rockville, MD). With 4 μg total RNA as template, first strand cDNA synthesis was performed using a BD SMART RACE cDNA Amplication Kit (BD Biosciences, Palo Alto, CA), with an oligo(dT) primer and instructions provided by the supplier. For PCR, degenerate primers were designed based on highly conserved amino acid sequences VYKVETIG (GC-forward: 5′-GTSTAYAAGGTRGARACNATHGG-3′) and MPRYCLFG (GC-reverse: 5′-CCRAANARRCARTANCKVGGCAT-3′) within the cyclase catalytic domain of known rGCs. Amplification was carried out in a GeneAmp 2400 PCR system with the following parameters: an initial denaturation (94 °C, 3 min), 35 cycles of denaturation (94 °C, 45 s), annealing (55 °C, 45 s), and extension (72 °C, 1 min), followed by a final extension (72 °C, 7 min). A PCR product (242 bp) of the expected size was purified, subcloned, and sequenced (see Section 2.4, below).

2.3. Rapid amplification of 3′- and 5′ cDNA ends

For rapid amplification of 3′- and 5′ cDNA ends (RACE) reactions, gene-specific primers were selected from the sequence of the 242 bp PCR product obtained by RT-PCR. 3′-RACE-Ready cDNA was synthesized using a BD SMART RACE cDNA Amplication Kit (BD Biosciences) as described above. 3′-RACE reactions were carried out using a nested PCR approach. For the first amplification, the primers were a 3′-gene-specific primer (3′GSP: 5′-GGCCCTCGAGCTGCTGGATGGGGTTCAG-3′) and the universal primer mix (UPM) provided with the kit. Amplification conditions were as follows: an initial denaturation (94 °C, 2 min), followed by 35 cycles of amplification (94 °C, 30 s; 66 °C, 30 s; 72 °C, 3 min), and a final extension (72 °C, 7 min). The primary PCR product was diluted 1:50 in tricine EDTA buffer, and used as template in a second amplification. For the second amplification, the primers were a nested gene-specific primer (3′NGSP: 5′-GGCCTACACACGGGCCCAGTCATTGCTGG T-3′) and the nested universal primer (NUP) provided with the kit. Amplification conditions were as follows: denaturation (94 °C, 2 min), followed by 15 cycles of amplification (94 °C, 30 s; 68 °C, 30 s; 72 °C, 3 min), and a final extension (72 °C, 7 min).

A 5′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Carlsbad, CA) was used for 5′ RACE. With 5 μg total Y-organ RNA as template, and a gene-specific primer selected from the previously amplified 242 bp fragment, first strand cDNA synthesis was carried out according to instructions provided with the kit. After reverse transcription, the original mRNA template was removed by treatment with RNAase Mix, and cDNA was purified using a S.N.A.P. spin column (both provided with the kit). A homopolymeric tail was added to the 3′-end of the purified cDNA using dCTP and terminal deoxynucleotidyl transferase. 5′ RACE reactions were carried out using a nested PCR approach. For the first amplification, the primers were a 5′GSP (5′-GACTGGGCCCGTGTGT AGGCCTACTCTC-3′) and the Abridged Anchor Primer provided with the kit. Amplification conditions were as follows: an initial denaturation (94 °C, 2 min), followed by 35 cycles of amplification (94 °C, 45 s; 62 °C, 30 s; 72 °C, 5 min), and a final extension (72 °C, 7 min). The primary PCR product was diluted 1:50 in tricine EDTA buffer, and used as template in a second round of amplification. For the second amplification, the primers were a 5′-nested GSP (5′-CCCCATCCAGCAGCTCGAGGGCCATAG-3′) and the Universal Anchor Primer provided with the kit. Conditions for the nested PCR were the same as those for the first PCR, with the exception that amplification was for 25 cycles.

2.4. Gel purification, cloning, sequencing, and sequence analysis

PCR products of the expected sizes were excised from gels, purified (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA), and cloned into the pGEM-T Easy Vector Systems (Promega, Madison, WI). Sequencing was performed by the UAB Core Sequence Facility. Sequence analysis was performed using the EXPASy Molecular Biology server (http://us.expasy.org), BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuisignal/sosuisignal_submit.html), Paircoil (http://paircoil.lcs.mit.edu/cgi-bin/paircoil), and Molecular Evolutionary Genetics Analysis, Version 2.1 (MEGA2.1). For sequence comparisons, rat rGCs were selected because they represent a range of vertebrate rGCs (from ligand-activated receptors to sensory receptors); invertebrate rGCs were selected based on BLAST results indicating they are mostly closely related to CsGC-YO1.