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Short Communication

Molecular cloning, expression pattern, and immunocytochemical localization of a gonadotropin-releasing hormone-like molecule in the gastropod mollusk, *Aplysia californica*

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Abstract

Successful reproduction in vertebrates depends upon the actions of gonadotropin-releasing hormone (GnRH). Despite the wide presence of GnRH in Phylum Chordata, GnRH has not been isolated in protostomes other than the common octopus. To provide information on the evolution of this critical hormone, we isolated the full-length cDNA of a GnRH-like molecule from the central nervous system of a gastropod mollusk, the sea hare *Aplysia californica*. The open reading frame of this cDNA encodes a protein of 147 amino acids. The molecular architecture of the deduced protein is highly homologous to that reported for the prepro-octopus GnRH (oct-GnRH) and consists of a putative signal peptide, a GnRH dodecapeptide, a downstream processing site, and a GnRH-associated peptide (GAP). The deduced amino acid sequence of the *Aplysia* GnRH (ap-GnRH) is QNYHFSNGWYAG and differs from oct-GnRH by only two amino acids. The transcript for ap-GnRH is widely expressed in the central nervous system (CNS), the ovotestis, and the atrial gland, an exocrine gland. Immunocytochemistry (ICC) using an antiserum against oct-GnRH detected immunoreactive neurons in all CNS ganglia examined, and the staining was abolished by the preadsorption of the antiserum with synthetic ap-GnRH. In sum, ap-GnRH sequence is the first gastropod GnRH-like molecule to be elucidated. Further, it represents one of the only two GnRH-like molecules found outside Phylum Chordata. These data refute the possibility that oct-GnRH arose singly in cephalopods by convergent evolution and provide valuable support for an ancient origin of GnRH during metazoan evolution.

Keywords: *Aplysia californica*; Sea hare; GnRH; cDNA cloning; mRNA expression; Gastropod; Nervous system

1. Introduction

In vertebrates, gonadotropin-releasing hormone (GnRH) is a decapeptide hormone critical for reproduction. Its presence was once thought to be restricted to members of Phylum Chordata, but increasing evidence suggests otherwise. A large number of studies reported the presence of GnRH-immunoreactivity (ir) and bioactivity in the gastropods (Goldberg et al., 1993; Young et al., 1999; Zhang et al., 2000; Tsai et al., 2003), bivalves (Pazos and Mathieu, 1999; Nakamura et al., 2007), polyplacophorans (Gorbman et al., 2003), cephalopods (Di Cosmo and Di Cristo, 1998; Di Cristo et al., 2002), cnidarians (Anctil, 2000; Twan et al., 2006), and platyhelminthes (Anctil and Tekaya, 2005). Importantly, a dodecapeptide containing the structural core of chordate GnRH was isolated from the central nervous system (CNS) of the common octopus (Iwakoshi et al., 2002). Although the octopus GnRH (oct-GnRH) deviates from the common chordate GnRH motif of having ten amino acids, it contains several features common to all forms of chordate GnRH. These include an N-terminal pyroglutamyl residue and a C-terminal amidated glycine residue, the general conservation of the N-terminal amino acids (when Asn^2 and Tyr^3 are removed), and the conservation of the C-terminal Pro^{11} and Gly^{12} residues

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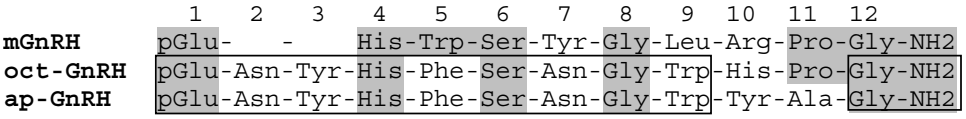

Fig. 1. Amino acid sequences of oct-GnRH aligned with ap-GnRH and mammalian GnRH (mGnRH) for comparison. Amino acids identical among the three peptides are shaded. Amino acids identical between oct-GnRH and ap-GnRH are boxed.

(see Fig. 1). Further, the oct-GnRH prohormone contains the highly conserved dibasic cleavage site (Lys^{14}-Arg^{15}) downstream of Gly^{12} (Iwakoshi et al., 2002), another universal feature of chordate GnRH prohormones.

Until now, oct-GnRH was the only non-chordate GnRH sequence elucidated. To ensure oct-GnRH did not arise in a single group as the result of convergent evolution, GnRH sequences in additional groups of protostomes must be examined. Characterization of GnRH in other protostomes will also allow us to decipher functional and structural features that are critical enough to warrant strong conservation. As a first step towards these goals, we elucidated the sequence of a GnRH-like molecule in a marine gastropod mollusk, *Aplysia californica*. Several features make *A. californica* an ideal model for this study. First, GnRH-ir is present in the CNS and hemolymph of *A. californica* (Zhang et al., 2000; Tsai et al., 2003). Second, *A. californica* possess a well-identified CNS and reproductive axis (Kandel, 1979) that should greatly facilitate our understanding of neural and reproductive roles of GnRH. Lastly, the recent publication of *A. californica* CNS transcriptome (Moroz et al., 2006) is a tremendous resource that aids in the search for vertebrate neuropeptide homologues in mollusks. With the help of the CNS transcriptome database, we cloned the first gastropod GnRH-like molecule (ap-GnRH) in *A. californica*. Further, we examined the pattern of ap-GnRH transcript expression and peptide localization to provide an initial characterization of the distribution of this neuropeptide.

2. Materials and methods

2.1. Animals and tissue collection

Sexually mature wild-caught *A. californica* (100–200 g in mass) were purchased from Alacrity Marine Biological Services (Redondo Beach, CA). Animals were kept in a 400-gal tank with circulating artificial seawater (Instant Ocean) that was continuously filtered through biological and chemical filters. Animals were maintained between 15 and 18 °C and fed Romaine lettuce daily. All animals were anesthetized by an injection of 1/3 body volume of isotonic magnesium chloride before sacrifice. For RNA preparation, CNS ganglia and peripheral tissues were collected, snap-frozen on dry ice, and stored at −70 °C until RNA isolation. For immunocytochemistry (ICC), the CNS ganglia were dissected, pinned out on a Sylgard-lined dish, and immersion fixed overnight in Bouin's fixative. Ganglia were stored in 70% ethanol until ICC.

2.2. RNA isolation

RNA from pooled CNS, individual ganglia, and peripheral tissues were isolated using the lithium chloride method previously described (Querat et al., 1991). RNA samples were quantified based on absorbance at 260 nm. All samples were pretreated with RNase-free DNase (Promega, Madison, WI) to eliminate genomic DNA contamination prior to reverse transcription (RT).

2.3. Oligonucleotide primers

In silico search of *Aplysia* CNS transcriptome was conducted with TBLASTN program using the PAM30 matrix. Searches using oct-GnRH dodecapeptide as the query sequence revealed two *A. californica* cDNA fragments (Accession Nos. EB190103 and EB187791) containing deduced amino acid sequences highly similar to oct-GnRH. We denoted these sequences as ap-GnRH1 (562 bp) and ap-GnRH2 (653 bp). Both sequences contained an open reading frame but lacked a portion of the 5′-untranlated region (UTR) and the majority of 3′-UTR. The two sequences differ from one another by only five nucleotides. The gene-specific oligonucleotide primers used for 5′ and 3′ rapid amplification of cDNA ends (RACE) and RT-polymerase chain reaction (PCR) were designed based on the common regions of these two sequences and listed in Table 1. Searches using vertebrate GnRH decapeptide motif failed to produce any positive hits. Primers for *A. californica* actin (ap-actin) were designed based on the corresponding cDNA sequence (Accession No. U01352).

2.4. Cloning of full-length cDNA for prepro-ap-GnRH

The full-length cDNA sequence for the prepro-ap-GnRH was obtained by 5′ and 3′ RACE. Total RNA isolated from the CNS (5 μg) was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) in a 20 μl reaction that contained 0.5 μM BRL-A1, 0.5 μM Smart III adapter primer, 0.5 mM dNTP, 1× first-strand reaction buffer, 10 mM dithiothreitol, and 200 U reverse transcriptase. The reaction was incubated at 42 °C for 60 min, and the reverse transcriptase was heat-inactivated at 70 °C for 15 min.

An initial PCR was performed to amplify the open reading frame of EB190103 and EB187791 using primers ApGS1 and ApGA3. PCR was performed in a 25-μl reaction mixture containing 1 μl of the first-strand

Table 1
Primers used to amplify ap-GnRH cDNA

Primer	Sequence
BRL-A1	5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTT TTTTTTTT-3′
Smart III	5′-AAGCAGTGGTATCAACGCAGAGTGGCCATTA TGGCCGGG-3′
BRL-A2	5′-GGCCACGCGTCGACTAGTAC-3′
Smart P1	5′-AAGCAGTGGTATCAACGCAGAGT-3
Smart P2	5′- ATCAACGCAGAGTGGCCATTATG-3′
ApGS1	5′-GTAGTAGTTAGACGCCAGGAA-3′
ApGS2	5′-AGCAGTAACAGTGGCTTGGACG-3′
ApGS3	5′-GAGGCAGCGAGAATACAGAGG-3′
ApGS4	5′-CCGCCACCACCACTCTTTTC-3′
ApGA1	5′-CGAAACCACGCCCACTCAAGC-3′
ApGA2	5′-GCTGCTGTCGGCGTCTGTGA-3′ApGA3
ApGA3	5′-CCAAGTTGTCTGCGAGGCTGT-3′
ApGA4	5′-CTCACCAACGCCGAAACCAC-3′
ActinS1	5′-GGTATTGTGTTGGACTCTGG-3′
ActinA1	5′-TGATGGAGTTGAAGGTGGTC-3′