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Isolation of cDNA encoding the precursor to lamprey gonadotropin- releasing hormone-I from the brain of the sea lamprey, *Petromyzon marinus*

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SUMMARY

Lampreys are only one of the two earliest evolved extant vertebrates belonging to the class Agnatha. Two gonadotropin-releasing hormones (GnRH), lamprey GnRH-I and -III, have been isolated and shown to act as neurohormones controlling reproduction in the sea lamprey, *Petromyzon marinus*. In the present study, a full length cDNA encoding the lamprey GnRH-I was isolated and characterized. The cDNA consisted of 641 bp which included an open reading frame of 261 bp encoding the 87 amino acid sequence of prepro-lamprey GnRH-I. The lamprey GnRH-I precursor had the same tripartite structure as the other known GnRH precursors consisting of a 24 residue signal peptide followed by the lamprey GnRH-I decapeptide and a Gly-Lys-Arg processing and cleavage site connecting a GnRH-associated peptide (GAP). These data provide new information and insights into our understanding of the molecular evolution of GnRH.

XIII International
Congress of
Comparative
Endocrinology

Yokohama, Japan
16-21 November 1997

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a regulatory neurohormone central in the control of reproduction in vertebrates. Currently, nine primary structures of GnRH have been determined in various representatives of vertebrates and two in invertebrates. Included in this family are the structures of GnRHs of three fish species of ancient origin, an agnathan, the sea lamprey, *Petromyzon marinus*, (lamprey GnRH-I and -III) (Sherwood et al., 1986; Sower et al., 1993); an elasmobranch, the spiny dogfish shark, *Squalus acanthias*, (dogfish GnRH and chicken GnRH-II) (Lovejoy et al., 1992); and a holocephalan, the ratfish, *Hydrolagus colliei*, (chicken GnRH-II) (Lovejoy et al., 1991). These GnRHs share a basic core sequence of pGlu¹-His²-Trp³-Ser⁴-X⁵-X⁶-X⁷-X⁸-Pro⁹-Gly¹⁰-NH₂. The hormone precursors to these GnRHs have also been identified and also have maintained the same tripartite structure which consists of a signal peptide, GnRH and processing site, and the GnRH associated peptide.

The GnRHs of the sea lamprey have been extensively studied to understand the early evolution of vertebrate GnRHs. Lamprey GnRHs have a distinct amino acid sequence among vertebrate GnRHs by having a substitution of the glycine at position 6 (Glu⁶ in lamprey GnRH-I or Asp⁶ in lamprey GnRH-III), which is believed to be crucial for receptor binding.

In this study, cloning and characterization of the cDNA encoding lamprey GnRH-I was attempted to determine its phylogenetic relationship with the genes encoding GnRHs of tetrapods and gnathostomata. It has been proposed that there have been two periods of gene duplication during the course of vertebrate evolution, one of which occurred close to the origin of the vertebrates and the other which occurred later in the early evolved teleosts. Therefore, the data from these studies add new information and insights into our understanding gene duplication during the early development of vertebrates and of the molecular evolution and functional diversity of these hormones.

MATERIALS AND METHODS

Brain tissue was dissected from sexually maturing adult sea lampreys which were captured during their upstream migration at the Cocheco River salmon ladder in Dover, NH. The brain tissue was immediately frozen on liquid nitrogen and stored at -80°C.

Total RNA was extracted from the frozen tissue using a polytron homogenizer and ISOGEN (Nippon Gene) according to instructions of the manufacturer. Poly (A+) RNA was isolated using Stratagene's Poly (A+) RNA Quick isolation kit.

The first strand of cDNA was synthesized by MMLV reverse transcriptase (Promega) using poly (A+) RNA as a template and oligo-(dT)₁₅ as a primer. Lamprey cDNA was used as a template to prepare lamprey GnRH-I cDNA by rapid amplification of cDNA ends (RACE) as described by Frohman et al. (1988). A degenerate GnRH primer (5' CAGCAYTRITCIYAYSATGGAARCCIGGIGG 3'; Operon Technologies) was designed according to the amino acid sequence of lamprey GnRH-I and -III. This GnRH primer and oligo-(dT)₁₅ were used for 3' RACE to amplify downstream of the GnRH coding sequence. PCR fragments were subcloned into the pGEM7 (Promega) or pT7Blue (Novagen) vector. The DNA sequence was analyzed by an ABI sequencer (model# 373A) using dye labeled terminators. Possible candidates of GnRH cDNA were selected by examining the DNA sequence coding for the dibasic amino acid processing site following the GnRH primer sequence.

Antisense primer (5' CTGATCAGCTTGGTGTGGGC 3') was made to anneal approximately 75 nucleotides downstream of the GnRH primer site of the putative GnRH clones obtained by 3' RACE. A poly C tail was introduced at the 3' end of the cDNA using terminal transferase and dCTP (Kimmel and Berger, 1987). Antisense primer and oligo-(dG)₁₅ primer was used for 5'-RACE and resulting PCR fragments were subcloned. Southern hybridization of PCR clones was performed to identify GnRH clones using the GnRH primer as a probe. The

complete cDNA sequence was obtained by the overlapping sequence of several PCR clones.

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RESULTS AND CONCLUSIONS

The complementary DNA encoding the neuropeptide GnRH-I was isolated from sexually maturing sea lamprey brains based on the principle of RACE (rapid amplification of cDNA ends) described by Frohman (1988). An approximately 600 bp fragment (PCR1) was observed on gel electrophoresis as a dominant product of 3'-RACE. The DNA sequence coding for the dibasic protease cleaving site, Lysyl-Arginine, was found immediately following the GnRH primer sequence. According to the sequence of PCR1, antisense primer was designed to amplify the 5'-end of the cDNA coding region. This resulted in an approximately 250 bp fragment (PCR 2) which hybridized to radiolabeled GnRH primer. The DNA sequence coding for lamprey GnRH-I was found in PCR2. Gene-specific primers were designed for use in subsequent RT-PCR amplifications which resulted in five different PCR clones to confirm the complete sequence of lamprey GnRH-I cDNA.

The sequence of the entire cDNA for lamprey GnRH-I consists of 641 nucleotides (nt) and includes 34 nt of the upstream untranslated region, a 261 nt long prepropeptide, and a 346 nt long downstream UTR through the putative polyadenylation signal sequence to the poly-A tail. The translated region of the cDNA encodes a peptide of 87 amino acids. The deduced prepropeptide sequence contains the typical tripartite structure of known GnRH prohormones, a 24 amino acid signal sequence, the GnRH decapeptide, a glycine-lysine-arginine processing and cleavage site, and a putative GnRH associated peptide consisting of 50 amino acid residues.

Lamprey GnRH-I and the following processing site were between 60 and 70% identical to those of tetrapod and teleost GnRH precursors. The processing site, Gly-Lys-Arg, was preserved throughout 500 million years of GnRH evolution. The signal peptide of lamprey GnRH-I had no significant homology (an average of 20% identity) with those of tetrapod and teleost GnRHs, however the common feature of signal peptides in GnRH precursors, that it is rich in hydrophobic amino acids and/or rich in strong alpha-helix formers, e.g., leucine,

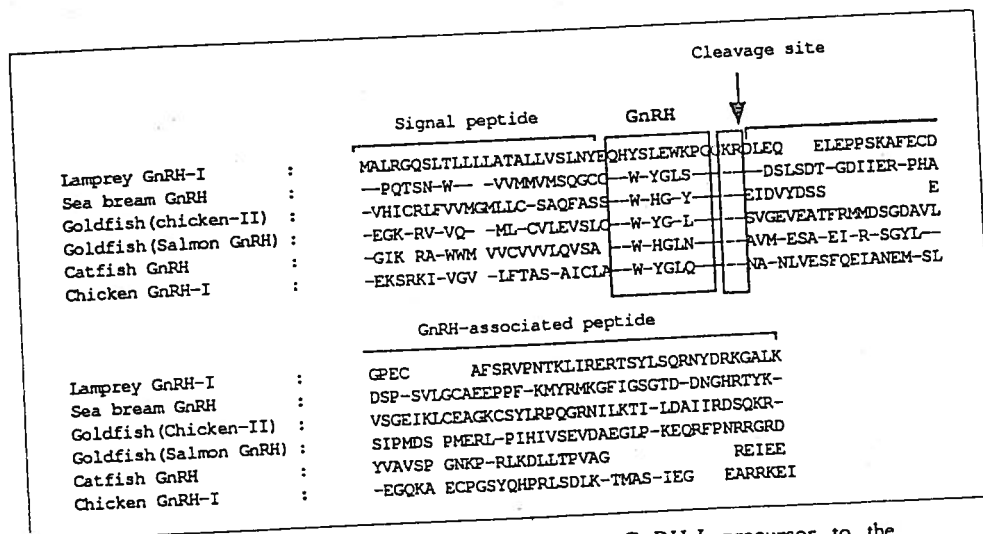


Figure 1. Sequence comparison of the lamprey GnRH-I precursor to the precursors to sea bream GnRH (Gothilf et al., 1995), goldfish chicken GnRH-I and salmon GnRH (Lin and Peter, 1996), catfish GnRH (Bogerd et al., 1994), chicken GnRH-I (Dunn et al., 1993), and mammalian GnRH (Seeburg and Adelman, 1984).

methionine, and alanine, can be recognized in the signal peptide of lamprey GnRH-I. The least conserved region (an average of 16% of homology) in the lamprey GnRH-I precursor throughout evolution was GAP, however, the GAP of lamprey GnRH-I precursor was rich in hydrophilic amino acids which is a common feature of GAPs in other GnRH precursors. The contrast between hydrophobic signal and hydrophilic GAP probably serve to maintain the right orientation and confirmation of precursors during processing in the endoplasmic reticulum.

In summary, characterization of brain hormone genes from an extant representative species of the oldest lineage of vertebrates is particularly important for understanding the molecular evolution and functional diversity of these hormones. In addition, these data demonstrate that there are common vertebrate mechanisms in the control of reproduction by GnRH.

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