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Molecular cloning of prepro-thyrotropin-releasing hormone cDNAs from the common carp *Cyprinus carpio* and goldfish *Carassius auratus*

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Abstract

To expand our knowledge on the evolution of prepro-thyrotropin-releasing hormone (ppTRH) from fish to tetrapods, sequences of ppTRH cDNAs from two cyprinid teleosts, the common carp *Cyprinus carpio* and goldfish *Carassius auratus*, were determined. Degenerate primers were designed based on the conserved regions between the zebrafish ppTRH sequence identified from the zebrafish EST database and the sockeye salmon ppTRH sequence, and PCR amplification was performed. Full-length ppTRHs were confirmed from ppTRH cDNAs obtained by 5′- and 3′-rapid amplification of cDNA ends. The common carp ppTRH cDNA encodes 187 amino acids including 6 copies of the TRH progenitor sequence (Lys/Arg-Arg-Gln-His-Pro-Gly-Lys/Arg-Arg), whereas the goldfish ppTRH cDNA encodes 231 amino acids including 8 copies of the TRH progenitor sequence. The molecular phylogenetic analysis of the ppTRH sequences reflected the predicted pattern of species classification. The common carp, goldfish, and zebrafish ppTRHs have some unique characteristics. The common carp and zebrafish ppTRHs are smaller than that of the goldfish mainly due to the absence of 29 and 17 consecutive amino acids, respectively. The deleted region includes one or two TRH progenitor sequences flanked by some glutamate residues, similar to the glutamate-rich regions of human ppTRH. Hydropathy profiles showed that the presence of a TRH progenitor sequence in the C-terminal hydrophilic region is a characteristic of teleosts and human ppTRHs. These observations may provide clues to a better understanding of the molecular evolution of ppTRH.

Keywords: Prepro-thyrotropin-releasing hormone; Common carp; Goldfish; Molecular evolution

1. Introduction

Thyrotropin-releasing hormone (TRH) was first isolated from porcine and ovine hypothalamus (Burgus et al., 1970; Schally et al., 1969). It is well known that TRH stimulates release of thyroid-stimulating hormone (TSH), growth hormone (GH), and prolactin (PRL) from the pituitary in mammals. TRH may also function as a neurotransmitter or neuromodulator in the central nervous system and in the gastrointestinal tract (Jackson, 1982). In teleosts, on the other hand, TRH stimulates release of pituitary hormones other than TSH, such as PRL (Barry and Grau, 1986; Kagabu et al., 1998), GH (Kagabu et al., 1998; Trudeau et al., 1992), and α-melanocyte-stimulating hormone (Lamers et al., 1994). In non-mammalian vertebrates, corticotropin-releasing hormone (CRH) is known to stimulate TSH secretion (Denver, 1999).

TRH is synthesized as a large precursor protein, prepro-TRH (ppTRH) that contains multiple copies of the TRH progenitor sequence (-Lys/Arg-Arg-Gln-His-Pro-Gly-Lys/Arg-Arg-), and proteolytic cleavage at these loci can yield multiple copies of TRH. So far,

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sequences of ppTRH cDNA have been determined in human (Yamada et al., 1990), mouse (Satoh et al., 1992), rat (Lechan et al., 1986), *Xenopus* (Bulant et al., 1992; Kuchler et al., 1990; Richter et al., 1984), and sockeye salmon (Ohide et al., 1996). The repetitive number of the TRH progenitor sequence varies among species: human (6 copies), rat (5 copies), *Xenopus* (7 copies), and sockeye salmon (8 copies). Although similarities in ppTRH primary structure are very low, their hydropathy profiles show a high degree of similarity. These results suggest that the three-dimensional structures of ppTRHs are highly conserved, although their primary structures have diverged during the course of vertebrate evolution (Ohide et al., 1996).

To gain a better understanding of the molecular evolution of ppTRH, it is important to characterize ppTRH from other vertebrate species. In the present study, considering the enormous radiation of the fishes, we isolated and characterized ppTRH cDNAs from two teleosts, the common carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*). These fish belong to the same family *Cyprinidae*, but the numbers of TRH progenitor sequences are different because of several deleted regions. Hydropathy profiles of ppTRHs indicated that the presence of a TRH progenitor sequence in the C-terminal hydrophilic region is unique to fish and human. The molecular phylogenetic analysis of the ppTRH sequences reflected the predicted pattern of species classification.

2. Materials and methods

2.1. Preparation of total RNA

The common carp and goldfish were obtained from local suppliers. Fish were anesthetized in 2-phenoxyethanol (0.6 ml/L water) and decapitated. The brains were quickly dissected out. Total RNA was immediately extracted using RNA extraction solution (Isogen, Nippongene, Toyama, Japan) and used for cDNA synthesis using Ready-To-Go T-primed first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Tokyo, Japan) according to manufacturer's instructions.

2.2. Amplification of partial cDNAs

Four degenerate oligonucletide primers (ZF TRH-F1: 5′-CTGCTC/GCGCTCCATC/TCTC-3′; ZF TRH-F2: 5′-CAGCCA/GGAGTGGA/CTGGA/TG-3′; ZF TRH-R1: 5′-TGC/GCGCTTCTCCAA/GCTC-3′; and TRH-Rev: 5′-CG/TC/TTTICCIGGA/GTGC/TTGIC-3′) were designed and used for PCR amplification of partial cDNAs.

The common carp partial cDNA was amplified in a reaction mixture (total volume of 20 μl) consisting of 1× PCR buffer, dNTPs (200 μM), Thermoprime Plus DNA Polymerase (0.5 U; ABgene, Epsom, UK), ZF TRH-F1 (0.5 μM), TRH-Rev (0.5 μM), and cDNA template (0.5 μl). The PCR conditions were: denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min and then 72 °C for 10 min. A 10-fold dilution of the reaction mixture used in the first PCR served as a template for a second PCR with the same primers and conditions.

The goldfish partial cDNA was amplified in a reaction mixture (total volume of 50 μl) consisting of *Pfu*-buffer, dNTPs (200 μM), *Pfu* DNA Polymerase (1.25 U; Stratagene, La Jolla, CA), ZF TRH-F2 (0.5 μM; 5′-ends phosphorylated), ZF TRH-R1 (0.5 μM; 5′-ends phosphorylated), and cDNA template (0.5 μl). The PCR conditions were: denaturation at 94 °C for 2 min, followed by 40 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min and then 72 °C for 2 min.

2.3. Rapid amplification of cDNA ends (RACE)

To obtain full-length ppTRHs from the brain of common carp and goldfish, 3′- and 5′-RACE were performed. Poly(A)$^+$RNA was extracted from total RNA using Oligotex-dT30 (Japan Synthetic Rubber/Nippon Roche, Tokyo, Japan), and adaptor-ligated cDNAs were synthesized using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions.

Gene specific primers were designed for RACE based on the partial cDNA sequences: for the common carp: carp TRH-GSP1 (5′-GTCTCTGGAGCAGGTGAT GCTGG-3′) for 3′-RACE; carp TRH-GSP2 (5′-TT TGTGGAGCAGCATCAGGTATCG-3′) for 5′-RACE; for the goldfish: GF TRH-GSP1 (5′-CACCG ACTACGAAGACGAGGCTG-3′) for 3′-RACE; GF TRH-GSP2 (5′-CTCCAGCATCATGTGCTCCAGA G-3′) for 5′-RACE. The reaction mixture (total volume of 20 μl) consisted of 1× PCR buffer, dNTPs (200 μM), Thermoprime Plus DNA Polymerase, gene specific primer (0.5 μM), AP1 (5′-CCATCCTAATACGACT CACTATAGGGC-3′; 0.5 μM), and cDNA template (0.5 μl). The PCR conditions were: denaturation at 94 °C for 3 min, followed by 5 cycles of 94 °C for 30 s and 72 °C for 2 min, 5 cycles of 94 °C for 30 s and 70 °C for 2 min, and 30 cycles of 94 °C for 30 s and 68 °C for 2 min and then 72 °C for 10 min.

2.4. Confirmation of the obtained sequences

The nucleotide sequences obtained by 5′- and 3′-RACE over the coding region were reconfirmed by PCR amplification using the gene specific primers, carp TRH-5′GSP (5′-CACTGCAGGACAGAGCTGTG-3′) and carp TRH-3′GSP (5′-CAGCTCAGACAATTCAGTG AACTC-3′) for the common carp, and GF TRH-5′GSP (5′-CTACAGGTGAATCACTACTGCAGG-3′) and