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Molecular cloning, gene structure, molecular evolution and expression analyses of thyrotropin-releasing hormone receptors from medaka (*Oryzias latipes*)

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ABSTRACT

Molecular cloning of thyrotropin-releasing hormone receptors (TRHR) was performed in a model teleost fish, medaka (*Oryzias latipes*). Four subtypes of TRHR were cloned and named them as TRHR1a, TRHR1b, TRHR2 and TRHR3 based on their similarity to known TRHR subtypes in vertebrates. TRHR1a, TRHR1b, TRHR2, and TRHR3 of medaka encode 416, 398, 451, and 386 amino acid residues, respectively. Comparison of cDNA sequences of medaka TRHR subtypes with respective genomic DNA sequences revealed gene structures: TRHR1a, TRHR1b and TRHR3 genes consist of two exons while the TRH2 gene consists of five exons. Molecular phylogenetic analyses depicted the molecular evolution of TRHR in vertebrates: From the ancestral molecule, TRHR2 diverged first and then TRHR1 and TRHR3. Reverse transcription-polymerase chain reaction analyses revealed the sites of TRHR expression: Expression of TRHR1, TRHR1b and TRHR2 subtypes has been confirmed in the brain, pineal organ, retina and pituitary gland. In addition, TRHR1b is expressed in spleen, digestive tract and skin, and TRHR2 in testis, ovary and gill. TRHR3 is widely expressed in various tissues. These results indicate that in medaka, TRH might exert multiple functions mediated by different TRHR subtypes expressed in each tissue.

1. Introduction

Thyrotropin-releasing hormone (TRH; pGlu-His-Pro-NH_2) is the first hypophysiotrophic peptide isolated from the hypothalamus in mammals [28,5]. In mammals, TRH is known to stimulate secretion of thyroid-stimulating hormone (TSH), growth hormone (GH) and prolactin (PRL). However, stimulation by TRH of GH release is seen under abnormal conditions such as pituitary tumor [9]. TRH might act as a neuromodulator or neurotransmitter both in the central and peripheral nervous systems as well [13].

In teleosts, TRH neurons have been reported to localize in the olfactory bulbs, ventral telencephalon, preoptic nucleus, hypothalamus, rostrodorsal tegmentum, and medulla oblongata in the common carp, sockeye salmon, brown trout and zebrafish [11,1,6,7]. TRH-immunoreactivity is also distributed throughout the brain. TRH stimulates synthesis and release of GH, PRL, α-MSH and POMC-derived peptides from the pituitary gland in teleosts [3,32,14].

Effects of TRH are mediated through its specific receptor, TRH receptor (TRHR). TRHR locates at the surface of target cells. TRHR is a member of the G-protein coupled receptor family with seven α-helix transmembrane domains. TRHR has a relatively short N-terminal and a long C-terminal [10]. The C-terminal of TRHR diverged among species during molecular evolution. Three TRHR subtypes have been identified so far: TRHR1, TRHR2 and TRHR3. These subtypes show different ligand-binding properties and affinities to TRH varies among subtypes [34,24,19]. However, functions of each TRHR subtype remain to be determined. In addition, TRHR1 isoforms have been reported in human and rat: The shorter isoform is produced due to frame shift caused by alternative splicing [35].

In teleosts, cDNA and gene structure of TRHR have been reported only for TRHR1 and TRHR2 in white sucker [12]. Thus, the presence of TRHR3 in teleosts remains to be demonstrated. To elucidate the functions of TRH mediated through different TRHR subtypes in fish, we therefore cloned cDNA encoding TRHR subtypes and analyzed their gene structures and expression sites in medaka, a model teleost fish in which developmental, genetic, physiological, pharmacological and toxicological studies have been performed [22,15].

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2. Materials and methods

2.1. Preparation of cDNA

Experimental animals used in this study were handled in compliance with the Guidelines for the Care and Use of Laboratory Animals at Utsunomiya University. The adult medaka (both sex, 0.2–0.3 g in body weight) was obtained from a local supplier. Fish were maintained under light–dark (LD) 14:10 (light on 05:00–19:00) conditions at 25 °C. The fish were anaesthetized in icy water around mid-light (11:00–13:00) and decapitated. The brains were immediately dissected out from 10 fish and immediately used for RNA extraction.

Total RNA was extracted using ISOGEN (Nippon gene, Tokyo, Japan) and first-strand cDNA was synthesized from the total RNA (5 μg) using a Ready-To-Go T-Primed First-Strand Kit (GE Healthcare, Tokyo, Japan) according to the manufacturer's instructions. For rapid amplification of cDNA ends (RACE), poly(A)$^+$ RNA was extracted from total RNA using a Oligotex-dT30 (Japan Synthetic Rubber/Nippon Roche, Tokyo, Japan), and adaptor-ligated cDNA were synthesized using the Marathon cDNA Amplification Kit (Clonetech, Palo Alto, CA) according to the manufacturer's instructions.

2.2. Design of gene specific primers (GSP) for medaka TRHR

TBLASTN searches were performed at Medaka Genome Project by NIG DNA Sequencing Center (http://dolphin.lab.nig.ac.jp/medaka/; golw_scaffold Hd-rR (200406)) using deduced amino acid sequences of TRHR in white sucker (TRHR1, AF288367; TRHR2, AF288368) and *Xenopus laevis* (TRHR1, AJ420782; TRHR2, AJ420781; TRHR3, AJ420780) on August 14, 2004. Sequences with high homology to TRHR (scaffold934, scaffold4066, scaffold4739, scaffold 4989, scaffold 7898) were searched out. TBLASTN searches were performed at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using putative coding sequence (CDS) of these scaffolds. Then, scaffold4066 and scaffold7898 were predicted to encode exon 1 and exon 2 of putative TRHR1 of medaka, respectively. Scaffold4989 was predicted to encode both exons 1 and 2 of TRHR1 as well. The former (scaffold4066 and scaffold7898) and the latter (scaffold4989) were designated as medaka TRHR1a and TRH1b, respectively. Scaffold934 and scaffold4739 were predicted to encode both exons 1 and 2 of TRHR2 and TRHR3; thus designated as medaka TRHR2 and TRHR3, respectively. In order to amplify the putative CDS of medaka TRHR, gene specific primers (mdTRHR1a-F1, mdTRHR1a-F2, mdTRHR1a-R1 and mdTRHR1a-R2 for TRHR1a, mdTRHR1b-F1, mdTRHR1b-F2, mdTRHR1b-R1 and mdTRHR1b-R2 for TRHR1b, mdTRHR2-GSP1, mdTRHR2-NGSP1, mdTRHR2-GSP2 and mdTRHR2-NGSP2 for TRHR2, mdTRHR3-F1, mdTRHR3-F2, mdTRHR3-R1 and mdTRHR3-R2 for TRHR3; Table 1) were designed and used for polymerase chain reaction (PCR) and RACE.

2.3. PCR

For CDS of TRHR1a, TRHR1b, and TRHR3, PCR was performed in a total volume of 20 μl containing: 1 × *Pfu* buffer; dNTP, 200 μM; cDNA from the medaka brain, 0.5 μl; phosphorylated sense and antisense primers, 5 μM; *Pfu* DNA polymerase (Stratagene, La Jolla, CA), 1.25 U. Amplification was performed in 96-well GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The PCR conditions were: denaturation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, and then 72 °C for 10 min.

For RACE of TRHR2, the reaction mixture (total volume of 20 μl) containing: 1 × *LA* PCR Buffer II; dNTPs, 200 μM; *TaKaRa LA Taq*, 0.5 U; gene specific primer, 0.5 μM; AP1 or AP2 (Clontech), 0.5 μM; cDNA template, 0.5 μl. The PCR conditions were: denaturation at 94 °C for 2 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, 70 °C for 30 s and 72 °C for 2 min, 30 cycles of 94 °C for 30 s and 68 °C 30 s and 72 °C for 2 min.

Table 1
Primers used for cDNA cloning and tissue distribution studies of medaka TRHR subtypes.

Primer	Oligonucleotide sequence
mdTRHR1a-F1	5′-TCTGAAAGGTGCACAGAGTTC-3′
mdTRHR1a-F2	5′-GGGATCACCTACTTCCAGTACC-3′
mdTRHR1a-R1	5′-GGACCACGTTGTCGTAGACT-3′
mdTRHR1a-R2	5′-CAGAATCCCTGACATTCAGATA-3′
mdTRHR1b-F1	5′-CCACTTCAGTTTCACGGATTAC-3′
mdTRHR1b-F2	5′-TACCTGTCAGACATCCAGGAG-3′
mdTRHR1b-R1	5′-GGAGTTCTTGCAACTCGTCT-3′
mdTRHR1b-R2	5′-CAGGCACTCAGCAAACAGTA-3′
mdTRHR2-GSP	1 5′-GTGCACGGTGTCCAGAGCAAAGCG-3′
mdTRHR2-NGSP	1 5′-TACTGCATGCTGTGGCTCTTTCTCGTG-3′
mdTRHR2-GSP2	5′-GCGCTGTTGGCGTAAATGCATGTCCG-3′
mdTRHR2-NGSP	2 5′-ACCAAGCATTCAGATAGGGAGTGGACAC-3′
mdTRHR3-F1	5′-TTGTACTCATTGGGCTTGAAC-3′
mdTRHR3-F2	5′-CAGGGTCACTACAACAGTACCAG-3′
mdTRHR3-R1	5′-GTCGATGAAGGAGTTGATGACT-3′
mdTRHR3-R2	5′-CATTGTCCTGACAATTCTTGC-3′
AP1	5′-CCATCCTAATACGACTCACTATAGGGC-3′
AP2	5′-ACTCACTATAGGGCTCGAGCGGC-3′
mdTRHR2-RTF1	5′-GCAGTACAGGTGTATGAATTATTC-3′
mdTRHR2-RTR1	5′-CCATCCCTTAACTCTGAAGAC-3′
medaka β-actin-Fw	5′-CAGACACGTATTTGCCTCTG-3′
medaka β-actin-Fw	5′-CAAGTCGGAACACATGTGCA-3′

2.4. Sequencing

PCR products were separated by electrophoresis using 2% agarose gel containing ethidium bromide. DNA bands of interest were excised under ultraviolet light exposure and purified by a QIAEXII Gel Extraction Kit (QIAGEN). Extracted DNA fragments were ligated into pBluescript II SK (−) (Stratagene). Then, JM109 competent cells were transformed according to the manufacture's direction. Plasmid DNA was purified from the transformed cells (white colonies) after overnight culture in LB broth by LaboPass Plasmid Mini Purification Kit (COSMO Genetech, Seoul, Korea). Nucleotide sequences were analyzed using ABI PRISM 3100 Genetic Analyzer using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). At least 5 independent clones were sequenced and consensus was taken to avoid PCR errors.

2.5. Sequence analysis

Conjugation of cDNA sequences was performed by GENETYX (Genetyx Corporation, Tokyo, Japan). Deduced amino acid sequences of medaka TRHR subtypes were aligned by Clustal W [31]. Transmembrane regions of TRHR were predicted from deduced amino acid sequences using the transmembrane prediction using hidden markov models (TMHMM; http://www.cbs.btu.dk/services/TMHMM/; [29]. Gene structures of medaka TRHR subtypes were predicted by online mapping at UT Genome Browser Medaka (Medaka 1.0; http://medaka.utgenome.org/).

2.6. Molecular phylogenetic analysis

Deduced amino acid sequences of TRHR were obtained from the DNA Data Bank of Japan (DDBJ)/ the European Molecular Biology Laboratory (EMBL)/ the National Center for Biotechnology Information (NCBI) and used for molecular phylogenetic analysis: human