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Characterization of a novel growth hormone receptor-encoding cDNA in rainbow trout and regulation of its expression by nutritional state

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ABSTRACT

To clarify the divergence of the growth hormone receptor (GHR) family, we characterized a novel GHR from a teleost fish (rainbow trout). A 2357-nt cDNA was isolated and found to contain a single initiation site 71 nt from the most 5′ end, an open reading frame of 1971 nt encoding a 657-amino acid protein, and a single polyadenylation site 229 nt from the poly-A tail. Based on structural analysis, the protein was identified as a type 1 GHR (GHR1). The new GHR1 shares 42% and 43% amino acid identity, respectively, with GHR2a and GHR2b, the two type 2 GHRs isolated from trout previously. GHR1 mRNA was found in a wide array of tissues with the highest expression in the liver, red muscle, and white muscle. Fasting animals for 4 weeks reduced steady state levels of GHR1 in the liver, adipose, and red muscle. These findings help clarify the divergence and nomenclature of GHRs and provide insight into the function of duplicated GHR types.

1. Introduction

Growth hormone (GH) plays important roles in the growth, metabolism, reproduction, immune function, osmoregulation, and other physiological functions of vertebrate animals (Björnsson, 1997; Björnsson et al., 2002; Møller and Jørgensen, 2009; Nørrelund, 2005). The actions of GH are transduced through the GH receptor (GHR), a member of the class-I cytokine receptor superfamily that consist of an extracellular binding domain containing a conserved Y/FGEFS motif, a single transmembrane domain, and an intracellular domain that links to several cellular effector pathways, including JAK-STAT, ERK, and PI3K-Akt (Forsyth and Wallis, 2002; Kopchick and Andry, 2000; Waters et al., 2006).

The GHR family arose through a series of genome duplication events (1R–3R) during the course of vertebrate evolution (Ellens and Sheridan, 2013; Ellens et al., 2013; Liongue and Ward, 2007). The first two events are believed to have occurred early in chordate evolution, with the 2R event perhaps taking place before the cyclostome–gnathostome split (Van de Peer et al., 2009). The 3R event, also known as the fish-specific genome duplication (FSGD) event occurred in the actinopterygian lineage (ray-finned fishes) after the divergence from the sarcopterygian lineage (lobe-finned fishes; includes the common ancestor of the tetrapods) (Meyer and Van de Peer, 2005). The FSGD likely explains the existence of multiple GHRs that derive from distinct mRNAs in boney fish (teleosts), whereas tetrapods possess a single GHR gene (Ellens and Sheridan, 2013). Several groups of teleosts, including the salmonids underwent a subsequent independent duplication event (4R) (Meyer and Van de Peer, 2005). It is not surprising, then, that the divergence of GHRs and their nomenclature are somewhat confused (Ellens and Sheridan, 2013; Ellens et al., 2013; Fukamachi and Meyer, 2007). For example, the terms "GHR1" and "GHR2" were first used for naming GHR subtypes in tetraploid salmonids (cf. Very et al., 2005); this scheme continued for other groups of teleosts (cf. Ellens and Sheridan, 2013). The situation became even more complex by the description of a somatolactin receptor (SLR) in masu salmon that was structurally similar to GHR but appeared to preferentially bind somatolactin (SL) over GH (Fukada et al., 2005). Consequently, the relationships among the GHR1s, GHR2s, and SLRs, especially among the tetraploid salmonids, became muddled. Moreover, the functional significance of the duplicated GHRs/SLRs in teleosts was unclear.

The aim of this study was to clarify the divergence and nomenclature of GHRs. We used rainbow trout to further study the polygenic origins of GHRs and to provide insight into the functional significance of duplicated GHR subtypes.

2. Materials and methods

2.1. Experimental animals

Rainbow trout (*Oncorhynchus mykiss*) were selected because 1) as teleosts they occupy a pivotal point in vertebrate evolution (Ellens and

Abbreviations: Akt, protein kinase B; Bp, base pair; cDNA, DNA complementary to RNA; CT, threshold cycle number; ERK, extracellular signal-regulated kinase; FSGD, fish specific genome duplication; GH, growth hormone; GHR, growth hormone receptor; JAK, janus kinase; mRNA, messenger RNA; nt, nucleotide; PI3K, phosphatidylinositide 3-kinase; PRLR, prolactin receptor; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription polymerase chain reaction; SEM, standard error of the mean; SLR, somatolactin receptor; STAT, signal transducer and activator of transcription; TBE, tris–borate–ethylenediaminetetraacetic acid; UTR, untranslated region.

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Sheridan, 2013; Van de Peer et al., 2009), 2) they underwent a tetraploidization event subsequent to the FSGD (Meyer and Van de Peer, 2005), and 3) we previously isolated two GHR paralogs from them (Very et al., 2005). Juvenile fish of both sexes were obtained from Dakota Trout Ranch near Carrington, ND and transported to North Dakota State University where they were maintained in 800 L circular tanks supplied with recirculated (10% replacement volume per day) dechlorinated municipal water at 14 °C under a 12L:12D photoperiod. Fish were fed to satiety twice daily with AquaMax™ Grower (PMI Nutrition International, Brentwood, MO, USA), except 36–48 h before experimental manipulations. Animals were acclimated to laboratory conditions for at least 4 weeks prior to experiments. All procedures performed were in accordance with the *Guide for Care and Use of Laboratory Animals* (National Research Council, Washington, DC) and approved by the North Dakota State University Institutional Animal Care and Use Committee.

For the nutritional state experiments, fish were assigned randomly to one of six treatment groups (initial, fed continuously for 2 weeks, fasted for 2 weeks, fed continuously for 4 weeks, fasted for 4 weeks, and fasted for 2 weeks then refed for 2 weeks) in 100-L circular tanks (approximately 18–24 fish per tank) with a flow-through water supply at 14 °C under a 12:12L:D photoperiod. Fish were allowed to acclimate for 2 days in their experimental tank prior to the beginning of their respective nutritional regime. For those animals receiving food, feeding was suspended 24–36 h before sampling. At the time of sampling, fish were anesthetized in 0.05% (v/v) 2-phenoxyethanol, measured (body weight, length, and liver weight), bled via the severed caudal vessels, and euthanized by transection of the spinal cord. Samples of liver, mesenteric adipose tissue, white skeletal muscle, and red skeletal muscle were removed, frozen on dry ice, and stored at −80 °C for later analyses.

2.2. RNA extraction

Total RNA was extracted using TRI-Reagent® as specified by the manufacturer (Molecular Research Center, Cincinnati, OH, USA). Isolated RNA was dissolved in approximately 100 μL RNase-free deionized water. Total RNA was quantified by ultraviolet (A260) spectrophotometry and diluted to 100 ng/μL in RNase-free deionized water. RNA samples were then stored at −90 °C until further analysis.

2.3. Oligonucleotide primers and probes

Gene-specific primers used for isolation of cDNAs were custom synthesized by Sigma-Genosys (The Woodlands, TX, USA, USA). Additional primers for reverse transcription were provided in SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) and AffinityScript™ QPCR cDNA Synthesis Kit (Agilent Technologies Santa Clara, CA, USA). Gene-specific oligonucleotide primers and probes used for real-time PCR of GHR and β-actin were designed based upon our determined and known sequences (GenBank accession no. AF157514), respectively, using ABI Primer Express® Version 2 software and custom synthesized by Applied Biosystems (Life Technologies Applied Biosystems Carlsbad, CA, USA). The probes were minor-groove binding probes labeled with either FAM (GHR1) or VIC (β-actin probe). Primers and probes were used for reverse transcription and PCR without further purification.

2.4. Isolation and sequence analysis of growth hormone receptor cDNA

A two-phase approach was adopted for the isolation of selected cDNAs using and 3′-rapid amplification of cDNA ends (3′-RACE)-PCR (Phase 1) and RT-PCR (Phase 2). 3′-RACE PCR was performed using a SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA, USA) following the manufacturer's protocol. RNA from rainbow trout liver was reverse transcribed into cDNA containing the sequence tags necessary for SMART™ chemistry. The PCR was carried out using a 3′RACE gene-specific primer (Table 1) designed from partial GHR sequences in GenBank (accession nos. AF438178 and DQ452378). After an initial denaturation at 94 °C for 5 min, a 35-cycle PCR was performed with each cycle consisting of denaturation (94 °C for 30 s), annealing (65 °C for 30 s), and extension (72 °C for 1 min) phases. In the last cycle, the extension time was increased to 10 min. The PCR product was identified by electrophoresis on an agarose gel containing 1% of each OmniPur (EMD chemicals, Gibbstown, NJ, USA) and NuSieve GTG agarose (BioWhittaker Molecular Applications, Rockland, ME, USA) in 1 × Tris–borate–EDTA (TBE) buffer followed by ethidium bromide staining. The resulting PCR product was cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). Purified plasmids (75 fmol) were sequenced using the CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's protocol.

Phase 2, RNA was reverse transcribed as described earlier and PCR was carried out using primers designed from our 3′RACE product and partial GHR sequences in GenBank (accession nos. AF438178 and DQ452378) (Table 1). After an initial denaturation at 94 °C for 5 min, a 35-cycle PCR was performed with each cycle consisting of denaturation (94 °C for 1 min), annealing (65 °C for 30 s), and extension (72 °C for 2 min) phases. In the last cycle, the extension time was increased to 10 min. The resulting PCR product was visualized, cloned into the pGEM-T Easy Vector, and sequenced as described previously.

2.5. Real-time PCR assay for growth hormone receptor mRNA

2.5.1. Preparation of cDNA standards

A cDNA standard for GHR1 was synthesized by PCR. Approximately 1 μg of the full GHR cDNA product was used as template for PCR using forward and reverse gene-specific primers (Table 1) under the same conditions as described above. The resulting PCR product was visualized, cloned into the pGEM-T Easy Vector, and the sequences were verified as described previously. The amount of cDNA was quantified (A260) and diluted to 1×10^{10} copies GHR1 cDNA/μl with nuclease-free water.

2.5.2. Real-time reverse transcription PCR

From 200 ng total RNA, endogenous poly(A) + RNA was reverse transcribed in a 5 μL reaction using a SMART™ RACE cDNA Amplification Kit containing a RNase H+ reverse transcriptase and a blend of oligo (dT) and random hexamer primers according to the manufacturer's instructions. Reactions without reverse transcriptase were included as negative controls; no amplification was detected in negative controls.

mRNA levels of GHR1 were determined by real-time RT-PCR using Stratagene Brilliant II QPCR mastermix and a STRATAGENE MX3000P® Detection System (Stratagene, La Jolla, CA, USA). Real-time PCRs were carried out for samples, standards, and no-template controls in a 10 μL reaction, containing 2 μL (20 ng) cDNA, 1 μL each forward primer

Table 1
Primers and probes used for sequence analysis and for real-time quantitative PCR.

Target	Description	Sequence
GHR1	Phase I sequence analysis	
	3′RACE gene-specific primer	5′-TCCTGCCAACCCAACAAAGT-3′
GHR1	Phase II sequence analysis	
	Forward primer	5′-GAAGTCGATACCCCTCGCGCAT-3′
	Reverse primer	5′-TGGGCAGTGTAGTTTTTCTTAAGG-3′
GHR1	QPCR analysis	
	Probe	5′^{FAM}-TGCGTGTGCACTGTG-^{MGBNFQ}3′
	Forward primer	5′-TCAGACAGGAGAGGCGTACGA-3′
	Reverse primer	5′-CCAAAGTTATTGAAGGCCCTCAT-3′
β-Actin	QPCR analysis	
	Probe	5′^{VIC}-TGCTTGCTGATCCACAT-^{MGBNFQ}3′
	Forward primer	5′-GGCTTCTCTCTCCACCTTCCA-3′
	Reverse primer	5′-AGGGACCAGACTCGTCGTACTC -3′

Abbreviations: GHR, growth hormone receptor; QPCR, Real-time quantitative PCR.