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Duplication of growth hormone receptor (GHR) in fish genome: gene organization and transcriptional regulation of GHR type I and II in gilthead sea bream (*Sparus aurata*)

Alfonso Saera-Vila, Josep-Alvar Calduch-Giner, Jaume Pérez-Sánchez*

Instituto de Acuicultura de Torre de la Sal, CSIC, 12595 Ribera de Cabanes, Castellón, Spain

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Abstract

Nucleotide sequences encoding for functional growth hormone receptors (GHR) are now available in salmonids (coho and masu salmon) and other fish orders. Several authors have hypothesized a divergent evolution of salmonid GHRs, but the recent finding that a trout cDNA is related to non-salmonid (GHR type I) rather than to salmonid GHRs (GHR type II) points out a possible duplication of actively transcribed GHR genes. To address this issue, we search by RT-PCR for GHR type II in trout, gilthead sea bream, European sea bass, and turbot. Both in trout and gilthead sea bream, a cDNA sequence with all the characteristic features of masu and coho salmon GHRs (GHR type II) was found. Similarly, in European sea bass, a cDNA encoding for the intracellular domain of GHR type II was reported. No positive results were found in turbot, but searches in genome databases of fugu and zebrafish identified DNA sequences with a significant similarity to fish GHR type I and II, which are more related each other than to GHRs of tetrapods. Gene organization is, however, highly conserved through the evolution of vertebrates, and eight exons homologous to exons 2 and 4–10 of mammals were found in fish GHRs. Transcriptional regulation of GHR type I and II was also addressed by means of real-time PCR assays in gilthead sea bream. In liver and adipose tissue, GHR type I was the most abundant transcript, but the expression of GHR type I and II was practically equal in skeletal muscle. Both in liver and adipose tissue, the two GHRs were down-regulated by fasting. In skeletal muscle, the expression of GHR type I remained unaltered in fasted fish, whereas a 2- to 3-fold increase was found for GHR type II. All this provides suitable evidence for a tissue-specific regulation of fish GHRs, but further research is needed to determine whether these duplicated genes are evolved in a new or redundant fashion.

Keywords: Growth hormone receptor; Gene duplication; Genomic organization; Transcriptional regulation; Rainbow trout; Gilthead sea bream; European sea bass; Turbot

1. Introduction

Growth hormone (GH) plays a central role as a pluripotent endocrine regulator of physiological functions in fish and higher vertebrates, working through specific cell membrane receptors (GHR) that trigger a phosphorylation cascade for signaling and gene expression events (Behncken and Waters, 1999; Zhu et al., 2001). The nucleotide sequence of GHR is now available for mammals, birds, reptiles, and *Xenopus*, being included this receptor in the Class I cytokine receptor superfamily, a group of single membrane-spanning receptors which comprises among others prolactin, leptin, granulocyte colony stimulating factor, erythropoietin, and thrombopoietin receptors (Kopchick and Andry, 2000). Since the initial cloning and sequence of turbot (*Scophthalmus maximus*) (Calduch-Giner et al., 2001) and goldfish (*Carassius auratus*) GHRs (Lee et al., 2001), other fish GHRs

* Corresponding author. Fax: +964 319509.
E-mail address: jperez@iats.csic.es (J. Pérez-Sánchez).

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have been characterized in gilthead sea bream (*Sparus aurata*) (Calduch-Giner et al., 2003), black sea bream (*Acanthopagrus schlegeli*) (Tse et al., 2003), Japanese flounder (*Paralichthys olivaceus*, GenBank NCBI Accession No. AB058418), grass carp (*Ctenopharyngodon idella*, Accession No. AY283778), southern catfish (*Silurus meridionalis*, Accession No. AY336104), masu salmon (*Oncorhynchus masou*, Accession No. AB071216), and coho salmon (*Oncorhynchus kisutch*, Accession Nos. AF403539 and AF403540).

Genomic organization of turbot and gilthead sea bream GHRs has also been elucidated (Pérez-Sánchez et al., 2002), and it reveals a high conservation of exon–intron junctions through the evolution of vertebrate species despite the occurrence of an exclusive fish intron (10/10A) at the C-terminus. This intron is not alternatively spliced in black sea bream, and this fish shares a longer GHR isoform with a 31 amino acid insertion that does not alter the open reading frame. Transcripts encoding for truncated membrane-anchored forms have been reported in turbot (Calduch-Giner et al., 2001) and Japanese flounder (Accession No. AB110989). In these flatfish, the truncated GHR comprises extracellular and transmembrane domains, the first 28 amino acids of the intracellular domain and a divergence sequence of 21–26 amino acids before a stop codon is reached, which is the result of the lack of the alternative splicing of intron 9/10 (Pérez-Sánchez et al., 2002).

Amino acid alignment of full-length GHRs reveals a relative high degree of identity (35–40%) among tetrapods and non-salmonid fish with a strict conservation of the FGEFS ligand-binding domain, extracellular cysteine residues, Box 1 and Box 2 domains, and cytoplasmic tyrosine residues. Nevertheless, amino acid identity among GHRs of tetrapods and salmonids (masu and coho salmon) decreases up to 27–34% with a lack of three conserved cytoplasmic tyrosine residues (Y1, Y3, and Y6) and two extracellular cysteines (C5, C6) involved in a short disulphide link (Fuh et al., 1990). Several authors have postulated a divergent evolution of salmonid GHRs. However, recently, we have cloned and sequenced a nutritionally regulated GHR in rainbow tout (*Oncorhynchus mykiss*), which is related to GHRs of non-salmonid fish (GHR type I) rather than to GHRs of salmonids (GHR type II) (Gómez-Requeni et al., 2004a). In this scenario, we have postulated that more than one GHR gene is maintained by selection through the radiation of teleosts, and the aim of this work was to search for GHR type II in fish species with a transcribed GHR type I: rainbow trout, turbot, and two perciform fish of the Sparidae (gilthead sea bream) and Moronidae (European sea bass, *Dicentrarchus labrax*) family. The study was completed by searches for sequence similarity to fish GHR type I and II in genome databases of human, *Xenopus*, zebrafish (*Danio rerio*), and fugu (*Takifugu rubripes*). Additionally, in gilthead sea bream, genomic organization and tissue-specific regulation of GHR type II was addressed in comparison to data found for GHR type I.

2. Materials and methods

2.1. Animals

Juvenile fish of trout, gilthead sea bream, European sea bass, and turbot were fed with fish meal-based diets and grown up under natural photoperiod and temperature conditions. Sampled fish were killed by decapitation under anesthesia (3-aminobenzoic acid ethyl ester, 100 mg/L), and tissue samples were quickly frozen in liquid nitrogen and stored at −80 °C until analysis.

2.2. RNA extraction

Total RNA from liver, adipose tissue, and skeletal muscle (white muscle) were extracted by the acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). Polyadenylated RNA was isolated from total RNA with Poly-ATracts (Promega, Madison, WI). Quantity and purity of isolated RNA were determined by absorbance measures at 260 and 280 nm, and its integrity was tested by electrophoresis in formaldehyde-agarose gels.

2.3. Cloning and sequence of GHRs

After DNase I treatment, 2 μg of total RNA was reverse transcribed with 200 U Superscript II (Life Technologies, Gaithersburg, MD), using oligo$(dT)_{17}$ as anchor primer. Reactions without reverse transcriptase were included as negative controls. The resulting product was treated with RNase H (2 units) prior to PCR amplification. Degenerated primers (GHR-1, GHR-2, GHR-3, and GHR-4) were designed on the basis of available sequences of masu and coho salmon GHR type II. GHR-1 (forward, 5′-ACCARCAGCRWTWTACA ACTTGCT-3′) is located few nucleotides upstream the translation start; GHR-2 (forward, 5′-GGACYTGAG GCCAGASYTGTAC-3′) is located in Box 2; GHR-3 (reverse, 5′-TCACCACYAGNAGCTGRAACTCYT T-3′) is located 360 nucleotides upstream the stop codon; and GHR-4 (reverse, 5′-GCAATAGCATAGTC YCTACACCT-3′) is located few nucleotides downstream the stop codon. PCR amplification was made with 2 μl of liver RT reactions in a total volume of 50 μl and 2.5 U of Platinum T*aq* DNA polymerase (Life Technologies). Thirty-five cycles were carried out with denaturation at 94 °C for 45 s, annealing at 55 °C for 60 s, and extension at 72 °C for 90 s. The combination of assayed primers was GHR-1/GHR-3, GHR-2/GHR-3, and GHR-2/GHR-4. Amplified cDNAs were gel-extracted