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Molecular cloning and characterization of growth hormone receptor and its homologue in the Japanese eel (*Anguilla japonica*)

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Abstract

Two cDNAs encoding growth hormone receptor (GHR)-like genes, eGHR1 and eGHR2, were isolated from Japanese eel (*Anguilla japonica*) liver tissue. The putative eel GHR proteins showed conserved structural features of vertebrate GHRs, including six cysteine residues and a YGEFS motif in the extracellular domain, a single transmembrane region, and proline-rich box 1 and box 2 domains. Northern blot analysis showed a single eGHR1 transcript in liver, while two sizes of eGHR2 transcripts, thought to be produced by alternative splicing, were present. RT–PCR revealed that eGHR1 and eGHR2 transcripts were widely distributed throughout the whole body of the Japanese eel. Moreover, the results of binding assays showed the specific binding of growth hormone to recombinant eGHR1. Since these putative eGHR proteins show all characteristics of the GHR family, we conclude that eGHR1 and eGHR2 cDNA encode two different GHRs in Japanese eel. We confirmed the ligand specificity of eGHR1 by binding assay, and further research is needed to allow characterization of the binding capability of eGHR2.

Keywords: Growth hormone receptor; Growth hormone; Japanese eel; Cloning; Binding assay; Prolactin; Somatolactin; Dig; Cytokine receptor family

1. Introduction

Growth hormone (GH) has many important physiological roles in the control of growth, metabolism, and cellular differentiation in vertebrates. The actions of GH are initiated by its binding to a specific receptor, GH receptor (GHR), localized on the cell membrane of target tissues, which induces a phosphorylation cascade for signaling and gene expression (Herrington and Carter-Su, 2001; Zhu et al., 2001; Kühn et al., 2002). GHR cDNA was first isolated from rabbit and human liver tissue (Leung et al., 1987). Subsequently, GHR cDNAs have been cloned from several tetrapod vertebrate classes (Argetsinger and Carter-Su, 1996; Ohkubo et al., 1998; Zhang et al., 2000; Huang and Brown, 2000). Recently, the GHR cDNAs were also isolated from several fishes (Lee et al., 2001; Calduch-Giner et al., 2001; Tsu et al., 2003; Calduch-Giner et al., 2003; Kajimura et al., 2004; Nakao et al., 2004; Fukada et al., 2004). GH actions are mediated through GHR, which has been little studied in teleosts, leaving molecular mechanisms of action open to speculation.

The structure of GHR has been elucidated through cDNA cloning in a number of vertebrates, in which the characterization of GHR has been also extensively investigated. GHR is a single membrane-spanning receptor and is a member of cytokine class I receptor superfamily including receptors for prolactin (PRL), erythropoietin, hematopoietin, granulocyte–macrophage colony stimulating factor, interferons, and several interleukins (Zhu et al., 2001). The members have an extracellular N-terminal ligand binding domain and a single transmembrane domain. Certain common characteristics were found within the superfamily. The extracellular domain (ECD) contains several pairs of conserved cysteine residues and a WSXWS motif, which is replaced by YGEFS in GHR proteins.

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There are two conserved regions called box 1 and box 2 domains in the intracellular domain. These characteristic structures of GHR were conserved in fish GHRs.

No molecular information was available as regards GHR in Japanese eel (*Anguilla japonica*), although liver tissue had specific GH binding sites (Hirano, 1991; Mori et al., 1992). Cloning of its cDNA is indispensable for better characterization of the Japanese eel GHR. It was generally accepted that only one gene encoding GHR existed in vertebrates; however, we identified two different GHR-like genes, eGHR1 and eGHR2, from Japanese eel. A recent study has also demonstrated the presence of two different GHR genes in fish (Saera-Vila et al., 2005). In the present study, we characterized the two cDNAs, possibly derived from different genes each encoding a GHR. Moreover, the specific binding of GH to recombinant eGHR1 was examined by binding assay.

2. Materials and methods

2.1. Cloning of eGHR1 cDNA

Total RNA was extracted from liver tissue of female eels (*Anguilla japonica*, 22.5–25.7 cm in total length, TL) by the acid guanidium isothiocyanate–phenol–chloroform extraction method using ISOGEN (Nippongene, Tokyo, Japan). Polyadenylated RNA was subsequently isolated from total RNA with Oligotex-dT-30 (Takara, Shiga, Japan). The cDNA was synthesized from oligo(dT) primed mRNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for 1 h at 42 °C.

To amplify eGHR1 cDNA fragments by RT–PCR, degenerate primers were designed from consensus sequences of GHRs. The forward primer was 5′–TGGAA(A/G)GA(A/G)TG(T/C)CC(A/T/C/G)GA(T/C)TA–3′ designed from WKECPDY sequence. The reverse primer was 5′–TAGGTC(A/T/G)G(T/G)G(C/G)TCA(T/C/G)GTA(C/G)CC–3′ designed from GYLTPDL sequence in masu salmon GHR (Fukada et al., 2004). Subsequently, both 5′- and 3′-RACE were performed using a Marathon cDNA amplification kit (Clontech Laboratories, Palo Alto, CA, USA). To confirm the nucleotide sequence of eGHR1 cDNA, cDNA fragments were sequenced that included the open reading frame (ORF) of eGHR1. The fragments were obtained by RT–PCR using PLATINUM® *Taq* DNA Polymerase High Fidelity (Invitrogen) with proofreading ability.

2.2. Cloning of eGHR2 cDNA

To amplify eGHR2 cDNA fragments by RT–PCR using cDNA prepared from female eel liver tissue, the primer, 5′–GAGGACATAGTGCACCCTGA–3′, designed from EDIVHPD sequence in eGHR1 and a degenerate primer, 5′–CGTCCAGA(A/G)TC(A/G)TC(A/G)TC(T/C)TT–3′, designed from KDDDSGR sequence corresponding to well-conserved regions of consensus sequences of other GHRs species, were used. The amplified fragment was cloned, sequenced and used to screen a λZAP II cDNA library constructed from randomly primed eel liver mRNA. Because both the 5′- and 3′-terminus of the positive clone obtained from the library screening were truncated, 5′- and 3′-RACE were performed. To confirm the nucleotide sequence, cDNA fragments including the ORF of eGHR2 were amplified by RT–PCR using PLATINUM® *Taq* DNA Polymerase High Fidelity.

2.3. cDNA sequence analysis

All cDNA fragments were subcloned into a pCR®2.1 plasmid vector using a TA cloning kit (Invitrogen), and sequenced by the dideoxy chain termination method using the BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Sequence determination was performed on ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

2.4. Structural and phylogenetic tree analyses

Predictions of the signal peptide and transmembrane region were performed using prediction server of the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk) based on the deduced amino acid sequences of the obtained cDNAs. The homology search of the amino acid sequences was carried out using the FASTA in the DNA Data Bank of Japan website (http://www.ddbj.nig.ac.jp/search/fasta-j.html). The amino acid sequences were aligned using Clustal W in the DNA Data Bank of Japan website (http://www.ddbj.nig.ac.jp/search/clustalw-j.html) (Thompson et al., 1994) and subjected to the Clustal W analysis to construct a phylogenetic tree using a neighbor-joining method (Saitou and Nei, 1987). The Clustal W analysis was performed using default settings and relative branch support was evaluated by bootstrap analysis. NJ plot software was used to prepare a graphical view of the phylogenetic tree (http://pbil.univ-lyon1.fr/software/njplot.html) (Perrière and Gouy, 1996). We sorted available fish GHRs to two groups, type I and type II, according to a previous report (Saera-Vila et al., 2005) and used in this analysis.

2.5. Northern blot analysis

Total RNA was extracted using ISOGEN from liver samples of female eels (65–68 cm in TL). After denaturing at 70 °C for 10 min, 5 μg poly $(A)^+$-RNA was electrophoresed on a 1% agarose gel containing 16% formaldehyde, and then transferred onto a nylon membrane (Hybond-N^+, Amersham Bioscience, Piscataway, NJ, USA). The membrane was baked at 70 °C for 1 h. The cDNA fragments encoding the ECD of eGHR1 and eGHR2 were labeled with [α-^{32}P] dCTP using the Random Primer Plus Extension Kit (NEN, Boston, MA, USA) and served as probes. The membrane was pre-hybridized in hybridization buffer (6× SSC containing 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA and 0.5% SDS) at 65 °C for 3 h. After overnight hybridization at 65 °C with the ^{32}P-labeled probe in hybridization buffer containing calf thymus DNA (100 μg/mL), the membranes were washed with 1× SSC containing 0.1% SDS, before their analysis using a BAS 2000 Bio-Image Analyzer (Fujifilm, Tokyo, Japan).