The co-existence of two growth hormone receptors in teleost fish and their differential signal transduction, tissue distribution and hormonal regulation of expression in seabream

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Abstract

Two genomic contigs of putative growth hormone receptors (GHRs) were identified in fugu and zebrafish genomes by *in silico* analysis, suggesting the presence of two GHR subtypes in a single teleost species. We have tested this hypothesis by cloning the full-length cDNA sequence of a second GHR subtype from the black seabream in which the first GHR subtype had been previously reported by us. In addition, we had also cloned the sequences of both GHR subtypes from two other fish species, namely the Southern catfish and the Nile tilapia. Phylogenetic analysis of known GHR sequences from various vertebrates revealed that fish GHRs cluster into two distinct clades, viz. GHR1 and GHR2. One clade (GHR1), containing 6 to 7 extracellular cysteine residues, is structurally more akin to the non-teleost GHRs. The other clade (GHR2), containing only 4 to 5 extracellular cysteine residues, is unique to teleosts and is structurally more divergent from the non-teleost GHRs. In addition, we had examined the biological activities of both GHR subtypes from seabream using a number of reporter transcription assays in cultured eukaryotic cells and demonstrated that both of them were able to activate the Spi 2-1 and β -casein promoters upon receptor stimulation in a ligand specific manner. In contrast, only GHR1 but not GHR2 in seabream could trigger the c-fos promoter activity, indicating that the two GHR subtypes possess some differences in their signal transduction mechanisms. Also, the expression of GHR2 is significantly higher than GHR1 in many tissues of the seabream including the gonad, kidney, muscle, pituitary and spleen. *In vivo* hormone treatment data indicated that cortisol upregulated hepatic GHR1 expression in seabream but not GHR2, whereas testosterone decreased hepatic GHR2 expression but not GHR1. On the other hand, hepatic expression of both GHR1 and GHR2 in seabream was decreased by estradiol treatment.

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Introduction

Growth hormone (GH) interacts with GH receptor (GHR) on target tissues to evoke the post-receptor signaling events. Apart from promoting somatic growth (Kopchick & Andry 2000), GH is also involved in other biological functions such as reproduction (Trudeau 1997), immunity (Yada *et al.* 1999), and osmoregulation in fish (Sakamoto *et al.* 1997). GHR belongs to the hematopoietin cytokine receptor superfamily (Moutoussamy *et al.* 1998) that includes GHR, prolactin receptor (PRLR), and a number of other cytokine receptors. Certain common characteristics are found among members of this superfamily. For example, all members of this superfamily possess an extracellular N-terminal ligand-binding domain and a single trans-membrane domain. The extracellular domain of each

receptor contains several pairs of conserved cysteine residues and two fibronectin III domains. A WSXWS motif is present in almost all members of this superfamily but in the case of mammalian GHR this is substituted by an YXXFS motif. In addition, there are two conserved regions within the intracellular domain termed Box 1 and Box 2 that are important for signal transduction of the receptor. The proline-rich Box 1 region is the site of Janus kinase 2 (JAK2) binding and is essential for the signaling functions of GHR (VanderKuur *et al.* 1995). Box 2 contains about 15 amino acid (aa) residues and is believed to be involved in the internalization of the receptor (Govers *et al.* 1999).

The GHR cDNA sequences of more than 10 fish species have been reported. In general, fish GHRs share some common features as their mammalian orthologs including the conserved positions of several pairs of

extracellular cysteine residues as well as the presence of the Box 1 and Box 2 regions. However, when the aa sequences of various fish GHRs were aligned, it was intriguing to note that they were clustered into two clades. One clade, represented by the reported GHRs of most teleosts, contains 6 to 7 extracellular cysteine residues. The other clade, represented by the reported salmonid GHRs, contains 5 extracellular cysteine residues. This initial observation (Tse *et al.* 2003) has led to the suggestion that these two types of fish GHRs represent two lineages of GHR in teleost evolution with some fish species expressing one type of GHR and the rest expressing the other. This assumption is now challenged by the finding that two genomic contigs of putative GHRs could be identified in both the fugu (*Fugu rubripes*) and zebrafish (*Danio rerio*) genomes, suggesting the presence of two subtypes of GHR in a single teleost species. The present study reports the identification and functional expression of a novel GHR subtype (sbGHR2) in black seabream (*Acanthopagrus schlegelii*) which is distinct from the classical GHR (sbGHR1) previously reported by us (Tse *et al.* 2003). During the preparation of this manuscript, Saera-Vila *et al.* (2005) have reported the sequences of two GHRs in gilthead seabream (*Sparus aurata*). However, no functional study on this additional fish GHR subtype has been performed and it is not known whether this second GHR subtype in fish bears any biological significance. In the present study we have expressed this newly identified sbGHR2 in cultured eukaryotic cells and demonstrated that it is functionally active. We have also compared its signal transduction activities viz-à-viz sbGHR1. In addition, the tissue distribution of the two GHR subtypes in seabream and their responsiveness in gene expression to different hormones have also been investigated. Furthermore, we have isolated the full-length sequences of both GHR subtypes from two other fish species, viz. the Southern catfish (*Silurus meridionalis*) and Nile tilapia (*Oreochromis niloticus*), providing further evidence for the co-existence of two GHR subtypes in a single fish species.

Materials and methods

Identification of genomic contigs in fugu and zebrafish genomes corresponding to GHR

Genomic contigs corresponding to GHR in fugu and zebrafish were identified by comparing the known fish GHR sequences with the fugu and zebrafish genome sequences available on the National Center for Biotechnology Information (NCBI) database (Poulter & Butler 1998), using the Basic Local Alignment Search Tool (BLAST) program from which the putative fugu and zebrafish GHR1 and GHR2 cDNA sequences could be deduced.

Isolation of full-length sbGHR2 cDNA sequence

A cDNA fragment of the sbGHR2 was obtained by a nested polymerase chain reaction (PCR) approach as described previously (Tse *et al.* 2003) using a seabream liver cDNA library as the template. In the first round, the PCR was carried out with an initial denaturation at 94 °C for 2 min, followed by 36 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. This was followed by a final step at 72 °C for 10 min. In the nested PCR, the reaction conditions were identical to the first round of PCR except that the annealing temperature was increased to 58 °C. The degenerate primers sbGHR2-F1 and sbGHR2-R3 were used in the first round of PCR whereas sbGHR2-F2 and sbGHR2-R2 were used in the nested reaction. Sequence information of all the primers used in the present study is listed in Table 1. The amplicons were resolved on a 1% agarose gel and the target DNA fragment was purified by the QIAquick Gel Extraction Kit (Qiagen). The fragment was then cloned into pBlueScript II SK+ vector (Stratagene, La Jolla, CA, USA) and the sequence determined using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystem, Forster City, CA, USA) on an ABI Prism 3100 auto-sequencer (Applied Biosystem).

Gene specific primers for sbGHR2 were designed from the determined nucleotide sequence of the cDNA fragment obtained. The full-length cDNA sequence of sbGHR2 was then obtained by a semi-nested PCR approach using the seabream liver cDNA library as template. Two rounds of PCR were performed to obtain the 5' end sequence. In the first round, a 36-cycle PCR (94 °C 30s, 58 °C 30s, 72 °C 45s) was performed using T7 and sbGHR2-R5 as primers. In the second round of PCR, T7 and sbGHR2-R4 were used as primers in a 30-cycle reaction of identical conditions as the first round. The 3' end sequence was similarly obtained. The first round of PCR was a 25-cycle reaction (94 °C 30s, 55 °C 30s, 72 °C 2.5 min) using sbGHR2-F4 and pSport1.1 as primers. In the second round PCR, sbGHR2-F5 and pSport1.1 were used as primers in a 36-cycle reaction of identical conditions as the first round.

Isolation of full-length GHR1 and GHR2 cDNA sequences from Southern catfish and Nile tilapia

The full-length GHR1 and GHR2 sequences of two other teleost species, viz. Southern catfish and Nile tilapia, were obtained from the liver of the fish by RT-PCR followed by rapid amplification of cDNA ends (RACE).

Southern catfish GHR1: Southern catfish liver first strand cDNA was synthesized using the SMART RACE First Strand cDNA Synthesis Kit (BD Biosciences, Palo Alto, CA, USA). A semi-nested RT-PCR approach was employed to obtain a cDNA fragment of the Southern

Table 1 Nucleotide sequences of the primers used

Primers	Sequences
sbGHR2-F1	GAG TGG VGT GAR TGT CCT MRC TA
sbGHR2-F2	GAG TGC TTC TTC AAT GAA A
sbGHR2-F4	ACA CTG AAT GAA AGT CTG AGC GGC
sbGHR2-F5	TGT GAT AAG CAC CCA TCG GTC TCT
sbGHR2-R2	AGA TCA GGG TCA CAG CAG CTG GC
sbGHR2-R3	CAG AAG GAG GCT GTT CTG CC
sbGHR2-R4	AAA CTC GAC ACT TTC GCC GGG ATG
sbGHR2-R5	CAG TCC GTG TCC AGG TCT GTG AGC
sbGHR1-F4	GTT TCC TCC CTG GAT TGG CTG T
sbGHR1-R3	GAA ATC CGG CGC ATA GGT CGA
sbActin-F	ACC CAG ATC ATG TTC GAG ACC
sbActin-R	ATG AGG TAG TCT GTG AGG TCG
scGHR1-1	CCN AAR ATH AAR GGN ATH GAY CC
scGHR1-2	GAY CCN TGG GTN AGR TTY AT
scGHR1-3	TCN SWN ACY TGN GCR TAR AAR TC
scGHR1-F1	CCC AAC TTG TAT CCT GAC GAT
scGHR1-F2	CCC TCT GGT CAG CAG GAG CAA CT
scGHR1-R1	CCA GGG TCG AGC ACC ATC AGC TGG ATA
scGHR1-R2	GGT GTG GAG TTG CTC CTG CTG ACC AGA
scGHR1-5'UTR	GGA GTA GAT CGG AGC CCT GGA
scGHR1-3'UTR	CCT CAT GAC ATG CAT GTA CAC AT
scGHR2-F1	AAR AHA TYG TGK WWC CAG ACC C
scGHR2-R1	AGA TCA GGG TCA CAG CAG CTG GC
scGHR2-3'-F1	GAC AAT GGC AAG GAC ACA CAG G
scGHR2-3'-F2	CAT GCT GAT CCT GAT TGT GCT GTC GC
scGHR2-5'-R1	TCR ATG AAY TCC ACC CAG GG
scGHR2-5'-R2	AAT CCC CAA AGT TGT AGC CCC TC
CDSIII/3'PCR	ATT CTA GAG GCC GAG GCG GCC GAC ATG-d(T) ₃₀ N ₋₁ N(N=A,G,C, or T;N ₋₁ =A,G, or C)
SMART IV	AAG CAG TGG TAT CAA CGC AGA GTG GCC ATT ACG GCC GGG
ntGHR1-F1	CCA TCA GAT GAG CAA CTT CTG AAA AGT
ntGHR1-R1	ACT TCC TGG TGA ATC AGC CTT A
ntGHR2-R1	TCR ATG AAY TCC ACC CAG GG
ntGHR2-F2	GCT CGG TGG GAA AGA GTT TGG AGA
ntGHR2-F3	AGA GTG GGA AGC TTA GGG AGT CGA C
ntGHR2-R2	CTT CAT ACT GCA GCT TCA TCC ACC C
ntGHR2-R3	CAG CAG CGT CCA GTT CAG GCT GAC A
CHO-Actin-F	ATT GTC ACC AAC TGG GAC GAT A
CHO-Actin-R	CTT TGA TGT CAC GCA CAA TTT C
pSport1.1	ACG CGT ACG TAA GCT TGG ATC C
T7	TAA TAC GAC TCA CTA TAG GG

catfish GHR1, using scGHR1-1 and scGHR1-3 as primers in the first round of PCR, and scGHR1-2 and scGHR1-3 as primers in the second round of PCR. Then 3' RACE and 5' RACE were performed to obtain the 3' and 5' cDNA ends respectively. Four gene specific primers were used for the RACE reactions: viz. scGHR1-F1, scGHR1-F2, scGHR1-R1, and scGHR1-R2. After sequencing the RACE products, two gene specific primers, viz. scGHR1-5'UTR and scGHR1-3'UTR, were further designed from the 5'-untranslated region (UTR) and 3'-UTR respectively, and were used to amplify the full-length sequence from first-strand cDNA prepared from liver. The sequence was then determined again to confirm the validity of the entire nucleotide sequence. This final validation step, which

has been applied to all the full-length cDNA sequences reported in the present study, is essential to ensure that the composite sequences obtained from the various PCRs and RACE reactions indeed correspond to the real full-length sequences.

Southern catfish GHR2: A cDNA fragment was obtained from the liver first strand cDNA using scGHR2-F1 and scGHR2-R1 as primers. Based on the determined sequence of this cDNA fragment, gene specific primers were designed to obtain the 3' and 5' ends by RACE. For 3' RACE, the first round of PCR was performed using scGHR2-3'-F1 and CDSIII/3'PCR as primers, followed by a nested PCR using scGHR2-3'-F2 and CDSIII/3'PCR as primers. For 5' RACE, the first round of PCR was performed using

SMART IV and scGHR2-5'-R1 as primers, followed by a nested PCR using SMART IV and scGHR2-5'-R2 as primers.

Nile tilapia GHR1: According to the sequence of Mozambique tilapia GHR and other known GHRs, two primers, viz. ntGHR1-F1 and ntGHR1-R1, located in the 5'-UTR and 3'-UTR respectively, were designed to amplify the entire coding region of the Nile tilapia GHR1 from the liver first strand cDNA.

Nile tilapia GHR2: The primer scGHR1-R1 was used to obtain a fragment of the Nile tilapia GHR2 using a single primer approach (Parks *et al.* 1991, Hermann *et al.* 2000). For 3' RACE, the first round of PCR was performed using ntGHR2-F2 and CDSIII/3'PCR as primers, followed by a nested PCR using ntGHR2-F3 and CDSIII/3'PCR as primers. For 5' RACE, the first round of PCR was performed using ntGHR2-R2 and SMART IV as primers, followed by a nested PCR using ntGHR2-R3 and SMART IV as primers.

For all the PCRs described in this section, the reaction was carried out with an initial denaturation at 94 °C for 3 min, followed by 33–35 cycles of 94 °C for 30 s, 52–57 °C for 30 s and 72 °C for 40 s to 1.5 min. This was followed by a final step at 72 °C for a further period of 10 min.

Phylogenetic analysis of GHR sequences from various vertebrates

Nucleotide and aa sequence editing, comparison and alignment were performed using Editseq and Megalign from the DNASTar package (DNASTAR, Madison, WI, USA). The multiple alignment software Clustal X (Thompson *et al.* 1997) was employed to construct the phylogenetic tree.

Expression of sbGHR1 and sbGHR2 in seabream tissues

RT-PCR was performed to assess the relative expression of sbGHR1 and sbGHR2 in various seabream tissues. First strand cDNA was synthesized from 3 µg total RNA prepared from seabream tissues using Oligo-dT₁₈ as primer and MMLV-H⁻ reverse transcriptase as enzyme (GeneSys, Borehamwood, Herts, England). Two pairs of gene specific primers, one pair specific for sbGHR1 (sbGHR1-F4 and sbGHR1-R3) and the other pair specific for sbGHR2 (sbGHR2-F5 and sbGHR2-R5), were used to amplify the two GHR transcripts from seabream tissues. The sbGHR1 transcript gave an amplicon size of 938 bp whereas the one for sbGHR2 was 443 bp. Negative control was also carried out on samples derived from mock reverse transcription reactions (i.e. no reverse transcriptase added) to rule out any possible contamination of RNA samples by genomic DNA. A pair of β-actin primers (sbActin-F and

sbActin-R) was used in parallel for normalization of the receptor expression level. After an initial denaturation at 94 °C for 2 min, a 29-cycle PCR was carried out at 94 °C for 30 s, 58 °C for 30 s and 72 °C for either 1 min (for sbGHR1) or 30 s (for sbGHR2). This was followed by a final step at 72 °C for 10 min. For the β-actin amplification, a 25-cycle PCR was used with an extension time of 30 s, other conditions being identical. The cycle number adopted for each gene was initially determined by a validation test in each case in which the PCR was performed as described but terminated at different cycle numbers. A profile of the amount of PCR product generated at different cycle numbers was thus constructed and the cycle number finally chosen for the gene expression study was within the linear region of the amplification. This validation is important to ensure that the amount of PCR product reflects the amount of template in the original sample. The PCR products were then resolved on a 1.5% agarose gel and stained with ethidium bromide. The band intensities, obtained by densitometric scanning, were analyzed using the Multi-Analyst software (Bio-Rad). Difference in intensity due to size difference of the two sbGHR amplicons has also been taken into account in the calculation of the relative expression values (Harms *et al.* 2000). The target band intensity was divided by the β-actin band intensity in each tissue for normalization.

Functional expression of sbGHR1 and sbGHR2 in cultured eukaryotic cells

The full-length receptor cDNAs cloned from seabream, viz. sbGHR1 and sbGHR2, were functionally expressed in cultured Chinese hamster ovary (CHO-K1) cells (American Type Culture Collection, Manassas, USA) and their biological activities were studied. CHO cells were transiently transfected with 50 ng of a pcDNA3.1 vector (Invitrogen) carrying the entire coding region of sbGHR2 between the *EcoR* V and *Not* I sites. The sbGHR1 expression plasmid used has been reported previously (Tse *et al.* 2003). Briefly, 500 ng of a luciferase reporter plasmid containing either the rat serine protease inhibitor (Spi 2.1) promoter in pGL2 vector (kindly provided by Dr Nils Billestrup at the Hagedorn Research Institute, Gentofte, Denmark), the rat β-casein promoter in pLucDSS or the human c-fos promoter in pFL711 (both kindly provided by Dr K L Yu at the Department of Zoology, University of Hong Kong) were co-transfected together with either one of the two GHR expression constructs. After 6 h of transfection, either recombinant seabream GH (sbGH) (Groppe, Adelaide, Australia), or purified GH (sGH), prolactin (sPRL) or somatolactin (sSL) from salmon (kindly provided by Dr. Penny Swanson at the Northwest Fisheries Science Center, Seattle, WA, USA) was added. The cells were incubated with the respective hormones for a further

20 h. Afterwards, the cells were then washed and lysed for determination of luciferase activities. To detect the expression levels of the two receptors in the transfected CHO cells, total RNA of the control group in each case was extracted and used in the determination of receptor expression by RT-PCR using the same reaction conditions as described in the previous section for tissue distribution studies except that the number of PCR cycles was 28. A pair of gene specific primers for hamster β -actin, viz. CHO-Actin-F and CHO-Actin-R, was employed in parallel RT-PCR to normalize the gene expression results.

Hormonal regulation of sbGHR1 and sbGHR2 gene expression in seabream liver *in vivo*

Black seabream (body length: 22.5 ± 0.3 cm, body weight 234.5 ± 11 g) of both sexes were purchased from a local marine fish farm. Prior to the experiments fish were acclimatized for at least one month in the Marine Science Laboratory of the Chinese University of Hong Kong. Throughout the experiments, fish were kept in fully aerated seawater and were exposed to natural photoperiod at ambient temperature ($24\text{--}29^\circ\text{C}$) in May 2004. Fish were fed with commercially available fish pellet diet until satiety during the experimental period. Fish were injected with either cortisol ($5\mu\text{g/g}$) (Sigma), 17β -estradiol ($2\mu\text{g/g}$) (Sigma), testosterone ($2\mu\text{g/g}$) (Sigma), sbGH ($1\mu\text{g/g}$) (Groppe, Adelaide, Australia) or ipamorelin (10 ng/g) (NEOMPS SA, Strasbourg, France) intraperitoneally once daily for 4 days. Cortisol, estradiol and testosterone were dissolved in olive oil. Ipamorelin and sbGH were dissolved in 0.8% (w/v) NaCl solution. The injections were performed at 10 a.m. every day and the tissues were collected from the fish at 3 p.m. on the day of the sacrifice. Olive oil or 0.8% (w/v) NaCl solution was used as the vehicle in the respective control groups. The fish were then killed by decapitation and tissues were stored in a -70°C freezer after being snap frozen. The expression levels of sbGHR1 and sbGHR2 were assessed by RT-PCR using gene specific primers as detailed in the previous section on tissue distribution studies.

Data analysis

All data were expressed as mean values $\pm$ S.E.M. ($n=6$). Data were considered statistically significant at $P<0.05$ using either one-way analysis of variance (ANOVA) or unpaired *t*-test.

Results

Identification of GHR2 in teleosts

When various fish GHR cDNA sequences were compared with the fugu genome on the NCBI database

using the BLAST program, two genomic contigs with high scores and low expect values were identified (NCBI entry numbers: CAAB01001386, CAAB01001701), suggesting that the fugu genome probably contains two GHR genes. By adopting the same strategy, two genomic contigs with high scores and low expect values were also identified on the zebrafish genome (NCBI entry numbers: CR352327, BX530077). Interestingly, the deduced sequences of the two putative GHRs in fugu and zebrafish correspond to the two types of GHRs reported in various fish species in terms of their structural similarity. These observations are highly suggestive that two types of GHRs could be found concomitantly in a single fish species and the two clades of GHR sequences reported in various fish species so far only represent part of the whole picture. Based on the sequence information available on the two types of fish GHR, degenerate primers (Table 1) were designed for the amplification of both types of GHR from three teleost species.

A novel cDNA transcript showing sequence homology to our previously reported seabream GHR (sbGHR) (Tse *et al.* 2003) was isolated from the black seabream liver cDNA library. We name our previously reported sequence as sbGHR1 and the newly identified sequence herein as sbGHR2. This new sequence contains 3875 bp encompassing a 5'-UTR of 102 bp, a coding region of 1776 bp and a 3'-UTR of 1997 bp including the poly(A) tail. The coding region encodes a protein of 591 aa with a putative single transmembrane domain (Fig. 1). Several characteristic landmarks of GHR including the FGEFS motif, the Box 1 and Box 2 regions, and some of the conserved extracellular cysteine residues and intracellular tyrosine residues could be located. However, sbGHR1 and sbGHR2 possess features that are distinctly different from each other (Fig. 2). First, the number of extracellular cysteine residues is different. While sbGHR1 possesses 7 extracellular cysteine residues, sbGHR2 has only 5. Second, sbGHR1 contains 9 intracellular tyrosine residues but sbGHR2 contains only 6. Moreover, the overall sequence identity between sbGHR1 and sbGHR2 is rather low, being 58.1% at the nucleotide level and 33.7% at the aa level (Table 2).

The co-existence of two GHR subtypes in a single fish species was further demonstrated in two other teleost species. Both GHR1 and GHR2 cDNAs could be identified in Southern catfish and Nile tilapia. The Southern catfish GHR1 (scGHR1) cDNA contains 1958 bp encoding for a protein of 602 aa whereas the Southern catfish GHR2 (scGHR2) cDNA contains 2300 bp encoding for a protein of 553 aa. On the other hand, the Nile tilapia GHR1 (ntGHR1) cDNA contains 1908 bp encoding for a protein of 635 aa whereas the Nile tilapia GHR2 (ntGHR2) cDNA contains 1725 bp encoding for a protein of 574 aa. Similar to the

Journal of Molecular Endocrinology (2006) **36**, 23–40

sbGHR1	MSVSSSSSSSSSSSTSNLLLLLVSSLDWLSTRGSALAMDHMTSSAPVGPHTFCVSREQETFR	65
sbGHR2	MYLLQLDPTMAASLT--LLFFLYIFT-----SSALESTSEQVHPQRDPQLTGCVSANMETFR	56
sbGHR1	WSPGGFHNLSPPGALRVFYLLKKDSP--TSEWKECPYSHLKR-ECFFDVNHTSVVVPYQMQLRG	126
sbGHR2	WNVGTLQNLKSGELRLFYINKLSPLDSPKEWTECPHYSTRPNECFNENHTSIWTFYRVQLRS	121
sbGHR1	QNNVTYLDDEDYCFIVENIVRPDPVSLNWTLLNISPSGLSYDVMVTWEPPPSADVGAGWMRIEYE	191
sbGHR2	RDESTVYDENK-FTVEAIVQDPDPDLTWTTLNESLSGAYYDVLSSWKPPQSAVDATGWMTLQYE	185
sbGHR1	IQYTERNTSNWEALEMPHTQQTIIYGLQIGKEYEVHIRMQAFVKFGEFSDSVFIQVTEIPSQD	256
sbGHR2	VQYRSVSSDQWKVMDPVISTHRSLYGLKHNVNHEVRVRMRMLAGKEFGEFSDSIFIH---IPAKV	247
sbGHR1	SNVPFKLALIFGVLGILILILLIGISQQPRLMMILLPPVPAPKIKGIDPELLKKGKLDLNFILS	321
sbGHR2	SSFPVVALLFGALCLVAILMLVIISQQEKLFIILLPPVPGPKIRGIDPELLKEGKRLRELTSL-	307
sbGHR1	GGGMGGLSTMAPDFVQDEFPWVEFIEVDAEDADAAEKEENQGSQTQRLDLPQPVSHHMNIGCAN-	385
sbGHR2	---GGPPNLRPELYNNDPWVEFIDLIDEEQG---DKLTDLDTCLMH-----RSLSSNCTPI	362
sbGHR1	AVSFPDDDSGRASCYDPDLHDQDTLMLMATLLPGQPEAGEDSFDDV-ESAPVIERSERPLMQTQT	449
sbGHR2	SIGFRDDDSGRASCCDPLPSDPEASPFHPLIPNQTLSKEVSCQTASEPSSPVQSPASGEPPFAA	425
sbGHR1	GGPQTWNTDFVAQVSNVMPSGGVVLSPGQQLRLQESTSATEDEAQKKGKGSSEDEVKAQKELQF	514
sbGHR2	PGREA-----MYTQVSEVRSSGKVLSPPEEQTEVEQTTGKDTEKDIMAEE-----KAKKELQ-	479
sbGHR1	QLLVVDPEGSGYTTESNARQISTPPSTPMPSGCMQTIHPQPVETKPAATAENNQSPVILPDSPQS	579
sbGHR2	-LLVVNADQGGVYSELNAGKMS-----PRLSIGDQSEPCLTGDLSPLPFA-----SPVHESDTAAV	535
sbGHR1	QFFAPVADYTVVQEVDSQHSLLLNPFRQSPPPCLPHHPAKALPAMPVGVYTPDLLGNLSP	640
sbGHR2	SPLPPAPVYTVVEGVDRQNSLLLTNPSTPAPQLIIP---KTMT-PPGVLTPLDGLSGITP	591

Figure 2 Alignment of sbGHR1 and sbGHR2 aa sequence. The conserved cysteine residues in the extracellular domain and conserved tyrosine residues in the intracellular domain are shaded in dark. The FGEFS motif is boxed by an open rectangle. The transmembrane domain is underlined with two solid lines. The Box 1 and Box 2 regions are shaded in gray. Gaps are introduced for maximal similarity.

sbGHRs, characteristic motifs of GHR could also be identified in the scGHRs and ntGHRs. Sequence information of sbGHR2, scGHR1, scGHR2, ntGHR1 and ntGHR2 can be viewed in the NCBI GenBank database with accession numbers AY662334, AY336104, AY973231, AY973232 and AY973233 respectively.

Phylogenetic analysis of GHRs from various vertebrates indicated that the fish GHRs could be clustered into two clades (Fig. 3). The GHR1 clade encompasses the sequences previously reported in a number of fish species including the goldfish (Lee *et al.* 2001), grass carp (GenBank accession number AY283778), black seabream (Tse *et al.* 2003), gilthead seabream (Calduch-Giner *et al.* 2003), Mozambique tilapia (Kajimura *et al.* 2004), halibut (GenBank accession number AB058418),

turbot (Calduch-Giner *et al.* 2001) and eel (isoform 1: GenBank accession number AB180476; isoform 2: GenBank accession number AB180477), as well as the Southern catfish and Nile tilapia GHR1 sequences obtained in the present study. The GHRs within this clade are structurally more homologous to GHRs found in the non-teleost vertebrates. The GHR2 clade, which is unique to teleosts, encompasses the previously reported GHR sequences in salmonid species (Fukada *et al.* 2004, Very *et al.* 2005), and gilthead seabream (Saera-Vila *et al.* 2005) as well as the black seabream, Southern catfish and Nile tilapia GHR2 sequences obtained in the present study. Structural homology among all the so far known members of the fish GHR2 clade is shown in Fig. 4. They exhibit higher aa sequence similarity to each other (Table 3).

Figure 1 Nucleotide and deduced aa sequences of the sbGHR2. Nucleotides (upper sequence) and aa (lower sequence) are numbered on the right hand side. The following are highlighted: conserved cysteine residues in the extracellular domain (circled), FGEFS motif (underlined with a dotted line), transmembrane domain (underlined with two solid lines), Box 1 and Box 2 regions (boxed in shaded rectangles), and the stop codon (marked with an asterisk).

Table 2 Amino acid similarity between the fish GHR1s and GHR2s

	GHR1									
	B_seabream	Catfish	N_tilapia	Turbot	Goldfish	Grass carp	Halibut	M_tilapia	Eel isof1	Eel isof2
GHR2										
B_seabream	33.7	35.5	28.8	33.7	35.5	37.1	33.2	29.4	35.5	38.7
Catfish	26.4	31.6	25.7	25.9	31.8	32.7	26.0	26.2	34.5	32.3
N_tilapia	31.8	33.2	29.4	31.0	34.6	34.8	31.1	29.4	35.5	38.1
Cherry salmon	32.3	35.8	31.8	34.3	40.3	41.0	36.0	31.8	42.4	42.9
Coho isof1	34.9	33.1	33.2	34.7	39.3	41.0	33.9	33.2	37.8	41.0
Coho isof2	32.1	35.6	31.6	33.9	40.8	41.3	35.5	31.6	43.0	41.9
Atlantic salmon	33.0	33.2	32.5	33.0	39.6	41.2	35.5	32.5	41.2	40.8
Trout isof1	32.7	32.2	33.1	34.6	39.1	40.8	34.1	33.1	39.3	40.4
Trout isof2	33.3	34.0	32.8	34.2	40.7	41.6	33.8	33.0	43.1	42.1
G_seabream	32.2	35.5	29.2	36.0	36.5	37.4	31.0	30.9	36.7	38.1

The Clustal method of the DNASTAR software was employed to compare the fish GHR1 and GHR2 aa sequences. Data are expressed as percentage aa identity. B_seabream, black seabream GHR; Trout isof1, rainbow trout GHR isoform 1; isof2, isoform 2; Coho isof1, coho salmon GHR isoform 1; isof2, isoform 2; M_tilapia, Mozambique tilapia; N_tilapia, Nile tilapia.

Different expression patterns of sbGHR1 and sbGHR2 in seabream tissues

Similar to that of sbGHR1, expression of sbGHR2 could be detected in all the seabream tissues tested, albeit at different levels. The expression level of sbGHR2 appeared to be higher than sbGHR1 (Fig. 5). The highest expression of sbGHR2 was found in the liver, followed by muscle and pituitary. In the gonad, kidney, muscle, pituitary and spleen, the expression of sbGHR2 was found to be significantly higher than that of sbGHR1.

Functional expression of sbGHR2 in CHO-K1 cells

As shown in Fig. 6, when sbGHR2 was expressed in cultured CHO-K1 cells, receptor activation by sbGH could stimulate both the Spi 2·1 promoter (Fig. 6A) and the β -casein promoter (Fig. 6B) but not the c-fos promoter (Fig. 6C), while sbGHR1 could stimulate all the three promoters. A much bigger stimulation of

promoter activities by sbGHR1 than sbGHR2 was also observed. This could not be attributed to the difference in the expression levels of the two receptors in the transfected cells since determination of receptor levels in the cultured cells by RT-PCR (Fig. 6D) indicated that the two receptors had very similar expression levels in the transfected cells. Ligand specificity of receptor interaction was tested by stimulating the receptor transfected cells with sGH, salmon prolactin (sPRL) or salmon somatolactin (sSL). It was shown that either sbGH or sGH could stimulate the Spi 2·1 promoter (Fig. 7A), the β -casein promoter (Fig. 7B) and the c-fos promoter (Fig. 7C) in CHO cells expressing sbGHR1. On the other hand in CHO cells expressing sbGHR2, the promoter activities could be stimulated by sbGH for the Spi 2·1 promoter (Fig. 7A), and by sbGH or sGH for the β -casein promoter (Fig. 7B). In contrast to the sbGHR1, no stimulation of the c-fos promoter could be observed for the sbGHR2 (Fig. 7C). Neither sPRL nor sSL could trigger any detectable activation of the Spi 2·1

Figure 3 Phylogenetic analysis of aa sequences of GHRs from different vertebrates. The default settings of the ClustalX protein alignment program was employed using the goldfish PRLR (NCBI accession no. AF144012) as the outgroup, and visualized by the NJPLOT program. The values represent bootstrap scores out of 1000 trials, indicating the credibility of each branch. The two fish GHR clades are named as GHR1 and GHR2. The GHR sequences used in the present study are: Southern catfish (*Silurus meridionalis*), AY336104; goldfish (*Carassius auratus*), AF293417; grass carp (*Ctenopharyngodon idella*), AY283778; black seabream (*Acanthopagrus schlegelii*), AF502071, bastard halibut (*Paralichthys olivaceus*), AB058418; cherry salmon (*Oncorhynchus masou*), AB071216; gilthead seabream (*Sparus aurata*), AF438176; turbot (*Scophthalmus maximus*), AF352396; coho salmon (*Oncorhynchus kisutch*) isoform 1, AF403539; coho salmon isoform 2, AF403540; Japanese eel (*Anguilla japonica*) isoform 1, AB180476; Japanese eel isoform 2, AB180477; Mozambique tilapia (*Oreochromis mossambicus*), AB115179; Nile tilapia (*Oreochromis niloticus*), AY973233; black seabream, AY662334; Southern catfish, AY973231; rainbow trout (*Oncorhynchus mykiss*) isoform 1, AY861675; rainbow trout isoform 2, AY751531; Atlantic salmon (*Salmo salar*), AY462105; gilthead seabream, AY573601; domestic pigeon (*Columba livia*), D84308; rabbit (*Oryctolagus cuniculus*), AF015252; Bolivian squirrel monkey (*Saimiri boliviensis*), AF339061; soft-shelled turtle (*Pelodiscus sinensis japonicus*), AF211173; Norway rat (*Rattus norvegicus*), NM_017094; olive baboon (*Papio anubis*), AF150751; gray short-tailed opossum (*Monodelphis domestica*), AF238491; house mouse (*Mus musculus*), NM_010284; human (*Homo sapiens*), NM_000163; silver-gray brushtail opossum (*Trichosurus vulpecula*), AF467545; domestic guinea pig (*Cavia porcellus*), AF247665; African clawed frog (*Xenopus laevis*), AF193799; cow (*Bos Taurus*), NM_176608; dog (*Canis familiaris*), AF133835; pig (*Sus scrofa*), X54429; sheep (*Ovis aries*), M82912; chicken (*Gallus gallus*), NM_001001293; Rhesus monkey (*Macaca mulatta*), U84589; giant panda (*Ailuropoda melanoleuca*), AF395535.

B_seabream	MYLLQDQMTAASLTLLFFLY-IFTSSALES-TSEQVHPQRDPQLTG	VSANMETFR	RWNVGTQLQNLKSGPGLRLFYINK	78
G_seabream	M-----AAALTLLFCLY-LITSSALES-ASEQVHPQRDPQLTG	VSANMETFR	RWNVGTQLQNLKSGPGLRLFYINK	70
Catfish	M-----GIHSIFICLFLVAVASAEVQASVQAQNKSLPFLIG	YSREFTVTR	NWNAGSFWNLTPEGDLRLFYMHK	71
Tilapia	M-----APTVTMLLF-VLHILTALVLQS-ASEFVLSERRPHLTS	ASPDMMTFR	RWNVGFQNLSDPRALRLFYFRH	71
Coho 1	M-----ATSHILFICLVLILPVLVSQEPPTSKQALPQIRPOITG	VSHDMNTFR	RWNVGVFQNLTEPRDLRFYYIN	72
Coho 2	M-----ATSRILFICLVLILPVLVSQEPPTSEQALPQIRPOITG	VSHDMNTFR	RWNVGTQNLTEPRDLRFYYIN	72
Cherry	M-----ATSYILFICLVLILPVLVSQEPPTSEQALPQIRPOITG	VSHDMNTFR	RWNVGTQNLTEPRDLRFYYIN	72
Trout 1	M-----ATSHILFICLVLILPVLVSQEPPTSKQALPQIRPOITG	VSHDMNTFR	RWNVGVFQNLTEPRDLRFYYIN	72
Trout 2	M-----NSMATSHILFICLVLILPVLVSQEPPTSEQALPQIRPOITG	VSHDMNTFR	RWNVGTQNLTEPRDLRFYYIN	75
A_salmon	M-----ATSHILFICLVLILAVLSQEPPTSKQALSQIRPQITG	VSHDMNTFR	RWNVGVFQNLTEPRDLRFYYIN	72
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B_seabream	LSPLDSPKEWTECPHYSTDRPNE	FFNENHTSIWTFYRVQLRSRDESTVYDENKFTVEAIVQDPDPFDLTWTTLNESLSG		158
G_seabream	LSPLDSPKEWTECPHYSIDRPNE	FFNKNHTSVWTFYRVQLRSRDESTLYDENTFTVDAIVQDPDPVDLTWTTLNESLSG		150
Catfish	ETKNDG--KWQECPSYSSAVENE	YFDANHTAVWFQYAIQLRSRTND-VYDEMYFSLEEIVFPDPKVLNWTLLSLGPTG		148
Tilapia	VPPPLSSKNWTECPHYSTETPNE	FFDQNHNTSIWTFYRVQLRSRDETILYDEKGFVNDIVQDPDPVSLNWTLLNVSSTG		151
Coho 1	-DRNISPKWESECPRY-ADRTNE	FFNESYTKVMWTSVQLRSGDQDILYXEVIFTVEDIVPEPDPPIALNWTLLNVGLTG		150
Coho 2	-NRNISPKWESECPNYMADRIDE	FFNESYTKVMWTSVQLRSGDQDILYXEVIFTVEDIVPEPDPPIALNWTLLNVGLTG		151
Cherry	-NRNISPKWESECPNYMADRIDE	FFNESYTKVMWTSVQLRSGDQDILYXEVIFTVEDIVPEPDPPIALNWTLLNVGLTG		151
Trout 1	-DRNISPKWESECPRYV-DRTNE	FFNESYTKVMWTSVQLRSGDQDILYXEVIFTVEDIVPEPDPPIALNWTLLNVGLTG		150
Trout 2	-NRNISPKWESECPNYMADRIDE	FFNESYTKVMWTSVQLRSGDQDILYXEVIFTVEDIVPEPDPPIALNWTLLNVGLTG		154
A_salmon	-DKNISPKWESECPHY-ADRTNE	FFNESYTKVMWTSVQLRSGDQDILYXEVIFTVEDIVPEPDPPIALNWTLLNVGLTG		150
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B_seabream	AYYDVLSSWKPPQSA--DVATGWMTLQYEVQYRSVSSDQWKVMDPVISTHRSYGLKHNVNNEHVVR	RLAGK	FGFES	236
G_seabream	TYYDILSSWKPPQSA--DVAMGWMTLQYEVQYRSASDLWHAVEPVTVTQSLFGLKHNVNNEHVVR	RLAGK	FGFES	228
Catfish	LFCDVMVSDIPSSAASDVKGWMTLWHTQYRERGSDWKSLDNGKDTQANIYGLRSNTEYEVVRVRSK-MRGVYFGDPS			227
Tilapia	IHYDILSSWKPPSS--DVETGWMKLQYEVQHRDSSSVWEMVDPVSSTQCSLYGLQTNINHEIVR	KLGGK	FGFES	229
Coho 1	SHFDIMLSWEPHSA--DVSMSGWMTLQYEVQYREVNSTLWRTVDLEKGMQRSLYGLRNTDNEVVR	KTLASR	FGFES	228
Coho 2	SHFDIMVSWEPHSA--DVSMSGWMTLQYEVQYREVNSTLWRMVNLEKGRQSLYGLRNTDNEVVR	KTLASR	FGFES	229
Cherry	SHFDIMVSWEPHSA--DVSMSGWMTLQYEVQYREVNSTLWRMVNLEKGRQSLYGLRNTDNEVVR	KTLASR	FGFES	229
Trout 1	SHFDIMLSWEPHSA--DVSMSGWMTLQYEVQYREVNSTLWRTVDLEKGMQRSLYGLRNTDNEVVR	KTLASR	FGFES	232
Trout 2	SHFDIMVSWEPHSA--DVSMSGWMTLQYEVQYREVNSTLWRMVNLEKGRQSLYGLRNTDNEVVR	KTLASR	FGFES	228
A_salmon	SHFDIMLSWEPHSA--DVSMSGWMTLQYEVQYREVNSTLWRTVDLEKGMQRSLYGLRNTDNEVVR	KTLASR	FGFES	228
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B_seabream	DSIFIHIPAKVSSFPVVALLLFGALCLVAIIMLVIIISQKEKLMFILL	PPVPGPKIRGIDPELLKGGKRLRELTSLILGGPPN		316
G_seabream	DSIFVHIIPAKVSSFPVVALLLFGALCLVAIIMLVIIISQKEKLMFILL	PPVPGPKIRGIDPELLKGGKRLRELTSLILGGPPN		308
Catfish	DSIFILVFSKGRSIPISAVFILMGIAIGIMLILIVLSRKQKLMVILL	PPVPGPKIKGIDPVLVQLQGQLTFTSLILGAHPD		307
Tilapia	ESVFIHIPSYSRFPVVALLLFGALCLVIGIMLVIIISQKEKLMVILL	PPVPGPKIKGIDPELLKGGKRLRELTSLILGGPPN		309
Coho 1	DSIFIHIPKESRIPVTVLLVFAALGLAVILMLVIYSQQQKLMVILL	PPVPGPKIKGIDPELLFKKGLAELTSLILGGIPD		308
Coho 2	DPVIVHIPKESRFPVTVLLVFAALGLAVILMLVIYSQQQKLMVILL	PPVPGPKIKGIDPELLFKKGLAELTSLILGDHPD		309
Cherry	DPVIVHIPKESRFPVTVLLVFAALGLAVILMLVIYSQQQKLMVILL	PPVPGPKIKGIDPELLFKKGLAELTSLILGDHPD		309
Trout 1	DSIFIHIPKESRIPVTVLLVFAALGLAVILMLVIYSQQQKLMVILL	PPVPGPKIKGIDPELLFKKGLAELTSLILGGIPD		308
Trout 2	DPVIVHIPKESRFPVTVLLVFAALGLAVILMLVIYSQQQKLMVILL	PPVPGPKIKGIDPELLFKKGLAELTSLILGDHPD		312
A_salmon	DSIFIHIPKESRIPVTVLLVFAALGLAVILMLVIVYSQQQKLMVILL	PPVPGPKIKGIDPELLKGGKLAELTSLILGGIPD		308
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B_seabream	LRPELYNNDPWWVEFIDLDIE	QSKDKLTDLDTCLMHRSLSSNCTPISIGFRDDDSGRASCDDPDLDPDEASFPFH--LI		394
G_seabream	LRPELYNNDPWWVEFIDLDIE	QSKDKLTDLDTCLMHRSLSSNCTPVSIGFRDDDSGRASCDDPDLDPDEASFPFH--LI		386
Catfish	LRPELYNNDPWWVEFIDLDIE	QSDRVTLDTCLMHRSLSSNCTPVSIGFRDDDSGRASCDDPDLDPDEASFPFH--LI		387
Tilapia	LRPELYNNDPWWVEFIDLDIE	QSDRVTLDTCLMHRSLSSNCTPVSIGFRDDDSGRASCDDPDLDPDEASFPFH--LI		387
Coho 1	LRPELYSDPWWVEFIDLDIE	QSDRVTLDTCLMHRSLSSNCTPVSIGFRDDDSGRASCDDPDLDPDEASFPFH--LI		384
Coho 2	LRPELYSDPWWVEFIDLDIE	QSDRVTLDTCLMHRSLSSNCTPVSIGFRDDDSGRASCDDPDLDPDEASFPFH--LI		386
Cherry	LRPELYSDPWWVEFIDLDIE	QSDRVTLDTCLMHRSLSSNCTPVSIGFRDDDSGRASCDDPDLDPDEASFPFH--LI		386
Trout 1	LRPELYSDPWWVEFIDLDIE	QSDRVTLDTCLMHRSLSSNCTPVSIGFRDDDSGRASCDDPDLDPDEASFPFH--LI		384
Trout 2	LRPELYSDPWWVEFIDLDIE	QSDRVTLDTCLMHRSLSSNCTPVSIGFRDDDSGRASCDDPDLDPDEASFPFH--LI		385
A_salmon	LRPELYSDPWWVEFIDLDIE	QSDRVTLDTCLMHRSLSSNCTPVSIGFRDDDSGRASCDDPDLDPDEASFPFH--LI		385
	▲			
B_seabream	PNQTLSEKVSQCTASEPSSPVQSPASGEPPFAAPGREAMYTVQSEVRSSGKVL	LSPEEQTEVEQTTGDKTEKDIAEKEK		474
G_seabream	PNQTLSEKVSQCTASEPSSPVQSPASGEPPFAAPGREAMYTVQSEVRSSGKVL	LSPEEQTEVEQTTGDKTEKDIAEKEK		466
Catfish	PNANLGAQSQSVVSHAQ-----EPAW-----QNSYSQVAEVMPCGETLLCPEQNVTDCCINQDKTSEY-----K			466
Tilapia	PNALSQETLCAATSEPPSIQSCNSGELPSFVTGRDLYTQVTEVRSSGKVL	LSPEEQTEVEQTTGDKTEKDIAEKEK		467
Coho 1	PNTSHALEPSCLASTKASSPVQPTTTEDSPWAAAGREDLYTQVNEVGFTGEVLLTNEEQNRNVEENSSEKDEKEN---EKEK			461
Coho 2	SNTSPSLVPSG-ASTEASSPVQPTTTGMLMAVPGREDLYTQVSEVRPTGEVLLMPEEQSKVEKDAEKAKEEM---GKKK			463
Cherry	SNTSPSLVPSG-ASTEASSPVQPTTTGMLMAVPGREDLYTQVSEVRPTGEVLLMPEEQSKVEKDAEKAKEEM---EKEK			463
Trout 1	PNTSHALEPSCLASTKASSPVQPTTTEDSPWAAAGREDLYTQVNEVRLTGEVLLTNEEQNRNVEENSSEKDEKE---KEKEK			461
Trout 2	SNTSPSLVPSG-ASTEASSPVQPTTTGMLMAVPGREDLYTQVSEVRPTGEVLLMPEEQSKVEKDAEKAKEEM---EKEK			462
A_salmon	SNTSCALEPSCLASTKASSPVQPTTTEDSPWAAAGREDLYTQVNEVRLTGEVLLTPEEQNRNVEENAEKDAKE---EKEKEK			462
	▲			
B_seabream	AKKELQLLVNADGGYTTSELNAGKMSPRLSIGDQSEPLTGDLSPPLPASPYHE-----SDTAAVSPPLPPA			541
G_seabream	AKKEFQLLVNADHGGYTTSELNAGKMSPRLSIGDQSEPLTGDLSPPLPASPYHE-----SDTAAVSPPLPPA			533
Catfish	EKRPLWMLVTN-----GYSVN-EPSSLIENNSTSQ-----QCCNPPIKQPTSTVSPAFFILMTPTP			501
Tilapia	---KEFQLLVNADHRRDYTTSELNAGKMSPELSTGDLTEPCKT-----FP---SPHHE-----SNTTTPPPPIA			524
Coho 1	KKKEFQLLVNADGGYTTSELNAGKMSARLPTGRASQAPATED-SSLVQGPFFGEYQSLYFAEAMPPIPPASPVSPPLPV			540
Coho 2	PRKEFQLLVNADGGYTTSELNAGKMSARLPTGRASQAPATED-SRLVQGPFFGEYQSLYFAEAMPPIPPASPVSPPLPV			542
Cherry	PRKEFQLLVNADGGYTTSELNAGKMSARLPTGRASQAPATED-SRLVQGPFFGEYQSLYFAEAMPPIPPASPVSPPLPV			542
Trout 1	KRNEFQLLVNADGGYTTSELNAGKMSARLPTGRASQAPATED-SSLVQGPFFGEYQSLYFAEAMPPIPPASPVSPPLPV			540
Trout 2	PRKEFQLLVNADGGYTTSELNAGKMSARLPTGRASQAPATED-SRLVQGPFFGEYQSLYFAEAMPPIPPASPVSPPLPV			541
A_salmon	KRKEFQLLVNADGGYTTSELNAGKMSARLPTGRASQAPATED-SSLVQGPFFGEYQSLYFAEAMPPIPPASPVSPPLPV			541
	▲			
B_seabream	PVYTVVEGVDRQNSLLLTNPSTPAQQLIIPK---TMPTPGGYLTDPDLGSIPT--			591
G_seabream	PVYTVVEGVDRQNSLLLTNPSTPAQQLIIPK---TMPTPGGYLTDPDLGSIPT--			583
Catfish	PEYTMVGVDRQNSLLLTNPSTPAQQLIIPK---SGTPEGYLTDPDLGSIPT--			553
Tilapia	PVYTVVEGVDRQNSLLLTNPSTPAQQLIIPK---NMPTPDGYLTADLLGSIPT--			574
Coho 1	SAYTMVEGVDRQNSLLLTNPSTPAQQLIIPK---NMPTPDGYLTADLLGSIPT--			593
Coho 2	SVYTMVEGVDRQNSLLLTNPSTPAQQLIIPK---NMPTPDGYLTADLLGSIPT--			595
Cherry	SVYTMVEGVDRQNSLLLTNPSTPAQQLIIPK---NMPTPDGYLTADLLGSIPT--			595
Trout 1	SAYTMVEGVDRQNSLLLTNPSTPAQQLIIPK---NMPTPDGYLTADLLGSIPT--			593
Trout 2	SVYTMVEGVDRQNSLLLTNPSTPAQQLIIPK---NMPTPDGYLTADLLGSIPT--			594
A_salmon	SAYTMVEGVDRQNSLLLTNPSTPAQQLIIPK---NMPTPDGYLTADLLGSIPT--			594

Table 3 Amino acid sequence similarities among teleostean GHR2s

	GHR2								
	N_tilapia	Catfish	Cherry salmon	Coho isof 1	Coho isof2	B_seabream	Atlantic salmon	Trout isof 1	Trout isof2
GHR2									
N_tilapia	100								
Catfish	37.3	100							
Cherry salmon	53.6	40.7	100						
Coho isof 1	49.4	37.1	84.0	100					
Coho isof2	53.4	40.5	97.1	83.8	100				
B_seabream	68.7	38.0	51.9	54.1	51.1	100			
Atlantic salmon	54.1	41.4	86.0	94.6	85.7	56.9	100		
Trout isof 1	49.0	40.1	83.8	96.0	83.5	53.5	93.9	100	
Trout isof2	52.3	36.7	97.0	83.5	96.3	54.7	85.2	83.3	100
G_seabream	69.4	37.6	53.0	54.7	52.0	93.1	57.5	54.0	55.7

The Clustal method of the DNASTAR software was employed to compare the fish GHR2 aa sequences. Data are expressed as percentage aa identity. B_seabream, black seabream GHR; Trout isof 1, rainbow trout GHR isoform 1; isof2, isoform 2; Coho isof 1, coho salmon GHR isoform 1; isof2, isoform 2; M_tilapia, Mozambique tilapia; N_tilapia, Nile tilapia.

promoter, the β -casein promoter or the c-fos promoter activities via either sbGHR1 or sbGHR2.

Differential regulation of sbGHR1 and sbGHR2 expression in seabream liver *in vivo*

In vivo expression of sbGHR1 and sbGHR2 in seabream liver under various hormonal stimulations was investigated (Fig. 8). A significant increase of sbGHR1 expression in the liver was found when the fish was injected with cortisol. The same treatment with cortisol, however, could not elicit any changes in sbGHR2 gene expression in the fish liver. On the other hand, testosterone significantly decreased sbGHR2 gene expression in the fish liver but not sbGHR1. Expression of both sbGHR1 and sbGHR2 was suppressed by estradiol and ipamorelin administration, but not by sbGH.

Discussion

The present study provides evidence for the concomitant existence of two GHR subtypes in a single fish species in different teleost lineages. Both GHR subtypes in fish were shown, for the first time, to be functionally active. In seabream the two GHR subtypes were found to exhibit different patterns of tissue distribution and post-receptor signaling events. They are also subjected to

different transcriptional regulation by various hormone treatments.

Structural analysis of the GHR2 sequences reported herein and those reported in salmonid species and gilthead seabream reveal that GHR2 in fish possesses structural features distinct from the conventional GHR1 which is more akin structurally to the non-teleost GHR. Notably, GHR2 contains 4 to 5 extracellular cysteines instead of 6 to 7 in GHR1. In sbGHR1, disulfide bonds could form between the following pairs of cysteine residues: C54/C64, C97/C107, and C121/C138 (Fig. 2), according to their homologous positions on human GHR (Fuh *et al.* 1990). Cysteine residues corresponding to C54, C64, C97 and C107, but not C121 and C138 of sbGHR1, could be found in fish GHR2 (Fig. 4). Although it is not known at the present stage if this extra pair of cysteine in GHR1 accounts for any variance between GHR1 and GHR2 in terms of the topology of their extracellular domains, its absence in all GHR2 sequences appears to be a characteristic feature. A highly conserved intracellular motif is found between D367 and L381 of sbGHR2. This motif, which is identical in all fish GHR2 sequences known to date, is not present in GHR1. Further studies on the biological significance of this unique GHR2 motif are highly warranted.

Expression of the cloned receptors in cultured eukaryotic cells followed by functional assays using

Figure 4 Multiple alignment of all the fish GHR2 sequences known to date. Identical aa sequences are indicated by asterisks above the sequences. The conserved cysteine residues in the extracellular domain are shaded in dark. The FG(E/D)FS motif is boxed by an open rectangle. The transmembrane domain is underlined with two solid lines. The Box 1 and Box 2 regions are shaded in gray. The conserved tyrosine residues in the intracellular domain are highlighted by dark triangles underneath the sequences. Gaps were introduced for maximal similarity. The fish species included in this comparison are: black seabream (B_seabream), gilthead seabream (G_seabream), Southern catfish (Catfish), Nile tilapia (Tilapia), coho salmon isoform 1 (Coho 1), coho salmon isoform 2 (Coho 2), cherry salmon (Cherry), rainbow trout isoform 1 (Trout 1) and isoform 2 (Trout 2), Atlantic salmon (A_salmon).

Figure 5 Distribution of sbGHR1 and sbGHR2 in seabream tissues. Results are normalized against β -actin expression and are expressed as mean values $\pm$ S.E.M. from six fishes. Significant difference revealed by unpaired *t*-test between sbGHR1 and sbGHR2 expression in each tissue type is marked by asterisks (*, $P < 0.05$).

specific promoter-driven reporter gene expression in the present study indicated that sbGHR2 is indeed a functional entity capable of transducing post-receptor signaling events. Both sbGHR1 and sbGHR2 could activate the Spi 2.1 and β -casein promoters upon receptor stimulation. It has been reported previously that association of JAK2 to the Box1 motif as well as the presence of the C-terminal portion of the intracellular tail are essential in mediating Spi 2.1 promoter activation by mammalian GHR (Goujon *et al.* 1994, Sotiropoulos *et al.* 1994, Dinerstein *et al.* 1995, Gong *et al.* 1998). Moreover, rat GHR was able to activate the β -casein promoter by stimulating the phosphorylation of the signal transducers and activators of transcription-(STAT)1 and STAT5 proteins (Smit *et al.* 1997, Gerland *et al.* 2000). In view of the fact that both sbGHR1 and sbGHR2 are able to activate these promoters, a similar signal transduction pathway is probably conserved in fish. Nevertheless, the efficacies of the receptor-mediated promoter activation for sbGHR2 are much lower than sbGHR1. This could be attributed to the following possibilities. First, a reduced number of disulfide linkages in the extracellular domain of sbGHR2 might result in a less rigid ligand binding domain essential for maximal interaction with the ligand. Second, the missing of some intracellular tyrosine residues in sbGHR2 might explain the reduced efficiency of signal transduction, as these

intracellular tyrosine residues have been reported to be involved in the GH-dependent phosphorylation of STAT5 (Herrington *et al.* 2000).

Interestingly, only sbGHR1 but not sbGHR2 could trigger the c-fos promoter activation. The significance of this difference in signal transduction between the two receptor subtypes remains to be elucidated. GH-induced tyrosyl phosphorylation of STAT1 and STAT3 in mammalian GHR is followed by the binding of these STATs to the sis inducible element (SIE) of c-fos. The phosphorylated tyrosines in the N-terminal half of the cytoplasmic domain of the rat GHR are thought to contribute towards maximal activation of STAT1 and STAT3 in response to GH stimulation (Herrington *et al.* 2000). In comparison with sbGHR1, two tyrosine residues (Y331 and Y400 of sbGHR1, Fig. 2) are absent in the same region in sbGHR2, and this might account for the inactivity of sbGHR2 towards the c-fos promoter.

Recently, it has been reported that the sSL receptor bears a relatively high sequence homology to GHR in teleosts (Fukada *et al.* 2005). The possibility therefore arises that this sbGHR2 could be the SL receptor in seabream. This is however unlikely. Amino acid sequence comparison indicated that a higher sequence identity in fact exists between sbGHR1 and sSL receptor (59%) than that between sbGHR2 and sSL receptor (46.8%), suggesting that the sbGHR2 reported herein is

Figure 6 Transactivation of the different promoters in CHO-K1 cells expressing sbGHR1 and sbGHR2. CHO-K1 cells were co-transfected with a pcDNA3.1 vector containing the entire coding region of sbGHR1 or sbGHR2 together with a luciferase reporter plasmid driven by the Spi 2.1 promoter (Panel A), the β -casein promoter (Panel B), or the c-fos promoter (Panel C). An empty vector transfection was performed in parallel as the negative control. The transfected cells were subsequently stimulated by different concentrations of sbGH as indicated on the x-axis. The expression levels of the two receptor subtypes in the transfected cells (from Panels A, B and C) were determined by RT-PCR (Panel D). Results are expressed as mean values $\pm$ S.E.M. ($n=6$). (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ as compared with the respective control by one-way ANOVA.)

unlikely to be a SL receptor. Moreover, we have tested its ligand specificity towards sGH, sPRL and sSL. Neither sPRL nor sSL could elicit any activation of the Spi 2.1 promoter or the β -casein promoter via sbGHR2. Similar to sbGH, sGH was able to activate sbGHR2. However, it should be noted that the use of heterologous hormones in this ligand specificity study is not the most ideal, and further confirmation awaits

the availability of homologous hormones from black seabream.

Although sbGHR1 and sbGHR2 are present in all the tissues examined, the expression of sbGHR2 is significantly higher than sbGHR1 in many tissues examined including the gonad, kidney, muscle, pituitary and spleen. The physiological significance of the higher expression of sbGHR2 is not known at present but we

postulate that sbGHR2 might serve as a modulator to GH signaling since a lower efficacy of sbGHR2-activated signal transduction is invariably observed. This phenomenon of a receptor being attenuated by another related receptor subtype has been observed in a number of cases. These include the estrogen receptor (ER) in which ER β was demonstrated to play a role in modulating the effects of ER α (Zhang *et al.* 2000), the interleukin (IL)-6 receptor in which the $\delta 4$ isoform acts as a competitor of ligand binding terminating the cytokine-induced signal transduction (Bihl *et al.* 2002), and the growth hormone secretagog receptor (GHSR) in which the GHSR-1b subtype attenuates the signaling of the GHSR-1a subtype (Chan & Cheng 2004). Whether the same principle applies to sbGHR1 and sbGHR2 here awaits further studies. However, the possibility that sbGHR2 might activate other signaling pathways could not be excluded. Further investigations on the functional significance of possessing two receptor subtypes at the same time are highly warranted. Interestingly, the relative expression level of sbGHR1 and sbGHR2 in the liver and muscle is different from that reported in gilthead seabream (Saera-Vila *et al.* 2005). In gilthead seabream, the expression of GHR2 in the liver is lower than that of GHR1 whereas GHR2 expression in the muscle is similar to that of GHR1. In our case, we have observed a very different expression pattern of these two receptor subtypes in black seabream in which the expression of sbGHR2 is much higher than sbGHR1 in many tissues examined. An explanation for this apparent dichotomy in receptor subtype expression in the two seabream species remains elusive. The time of tissue sampling could be a possible reason since temporal variation in GHR expression has been reported in some fish species (Wargelius *et al.* 2005). The extent of feeding is another possibility since satiety and starvation could affect GHR expression (Deng *et al.* 2004, Fukada *et al.* 2004).

To date, information on the hormonal regulation of GHR expression in teleosts is very limited despite the cloning of the GHR sequences from a number of fish species. For example, information regarding cortisol regulation of hepatic GHR expression is scarce.

Figure 7 Transactivation of the different promoters in CHO-K1 cells expressing sbGHR1 or sbGHR2 under various hormone stimulation. CHO-K1 cells were co-transfected with a pcDNA3-1 vector containing the entire coding region of sbGHR1 or sbGHR2 together with a luciferase reporter plasmid driven by the Spi 2-1 promoter (Panel A), the β -casein promoter (Panel B), or the c-fos promoter (Panel C). The transfected cells were subsequently stimulated by different hormones as indicated on the x-axis at 300 ng/ml. Results are expressed as mean values $\pm$ s.e.m. ($n=6$). (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ as compared with the respective control by one-way ANOVA.) (sbGH: seabream GH; sGH: salmon GH; sPRL: salmon PRL; sSL: salmon SL.)

Figure 8 *In vivo* regulation of sbGHR1 and sbGHR2 expression in seabream liver by hormone treatment. Results are expressed as mean values $\pm$ S.E.M. from six independent fishes. (**, $P < 0.01$; ***, $P < 0.001$ as compared with the respective control by one-way ANOVA.) The hormone concentrations used were: 5 μ g/g cortisol, 2 μ g/g estradiol, 2 μ g/g testosterone, 1 μ g/g sbGH and 10 ng/g ipamorelin. Olive oil (for the control group in Panel A) and 0.8% w/v NaCl solution (for the control group in Panel B) were used as the vehicle control.

In mammals, GHR mRNA level was reported to be increased in the fetal ovine liver by cortisol (Li *et al.* 1999). On the other hand, administration of cortisol to coho salmon did not increase hepatic GH binding (Gray *et al.* 1992). In the present study, cortisol was found to upregulate sbGHR1 expression but not sbGHR2 in black seabream. It has been reported recently that cortisol could elicit metabolic changes in seabream liver by decreasing the glucose-6-phosphate dehydrogenase (G6PDH) activity (Laiz-Carrión *et al.* 2003). The enhanced expression of sbGHR1 by cortisol might provide a possible explanation for this report since GH has been shown to reduce hepatic G6PDH activity in fish (Leena *et al.* 1999).

Reports on the actions of androgens on growth in teleosts are controversial. On one hand, methyltestosterone was reported to increase growth in tilapia (Riley *et al.* 2002, Sparks *et al.* 2003). On the other hand, both testosterone and methyltestosterone were demonstrated to decrease growth in perch (Mandiki *et al.* 2004, Mandiki *et al.* 2005). These ambivalent actions of androgens are reflected in the effects of the steroids on the GH level in fish. For example, testosterone was demonstrated to exhibit dose-dependent stimulation of the pituitary GH mRNA level in goldfish (Huggard *et al.* 1996). Also, plasma GH level was increased after testosterone administration in fasted rainbow trout (Holloway & Leatherland 1997). In salmon, however, both testosterone and 11-ketotestosterone were demonstrated to increase plasma insulin-like growth factor

(IGF)-I level without affecting the GH level (Larsen *et al.* 2004). So far no information is available on whether testosterone affects the expression of GHR in teleosts. From our *in vivo* studies, testosterone was found to decrease hepatic sbGHR2 expression but not sbGHR1 in black seabream. If our postulation that sbGHR2 could act as a negative modulator of GH signaling prevails, then this downregulation of sbGHR2 in the liver of the seabream by testosterone would bear important physiological implications. The decreased sbGHR2 level would release the negative modulatory action on sbGHR1 thereby enhancing the biological action of GH.

There is also no report so far on the regulation of GHR expression in teleosts by estradiol. In the present study, the expression of both sbGHR1 and sbGHR2 was found to be decreased by estradiol treatment in black seabream. This decreased receptor expression in the seabream liver by estradiol in fact provides an explanation to a recent report that estradiol decreases GH-stimulated expression of IGF-I mRNA in the seabream liver (Carnevali *et al.* 2005). In mammals, rather diverse observations on estradiol regulated GHR expression have been reported. It has been shown that estradiol caused a reduction of GHR expression in the rabbit liver (Domene *et al.* 1994) but an elevated GHR mRNA was detected in the rat liver by estradiol (Carmignac *et al.* 1993, Gabriellsson *et al.* 1995). The estradiol-induced GHR expression in mouse was found to be GH dependent in which only a combination of

estradiol and GH could upregulate hepatic GHR expression (Contreras & Talamantes 1999). It is not known whether the species variation observed in mammals is also seen in teleosts. In this context, it is noteworthy to mention the results of Kajimura *et al.* (2004) that a high plasma estradiol level did not correlate with an elevation of hepatic GHR expression in tilapia. Nonetheless, the present study is the first report demonstrating that GHR expression in fish could be regulated by both estradiol and testosterone, constituting a new target for the actions of steroid hormones on the growth axis in teleosts.

Another interesting observation is that ipamorelin, a synthetic growth hormone secretagogue (GHS) which has been shown to exert potent stimulatory effect on GH secretion in seabream (Chan *et al.* 2004), downregulates both sbGHR1 and sbGHR2 in seabream liver. Since GHSR is also present in the seabream liver (Chan & Cheng 2004), this decreased expression of sbGHRs could be a direct action of ipamorelin on the seabream liver. In line with this, treatment by sbGH alone did not cause any significant alteration in the expression of either sbGHR1 or sbGHR2 in the seabream liver. In mammals, it has been reported that GHS could induce an elevation of GHR in the liver of uremic rats (Krieg *et al.* 2002). However, no studies have been performed so far on GHS-regulated GHR expression in teleosts or in normal mammals. Our observation reported herein thus opens up new avenues in studying the regulation of GHR expression in fish.

Genomic data indicate that the genomes of most vertebrates (including human) had undergone two whole genome duplications in their evolutionary history, i.e. the 2 round (2R) hypothesis (Ohno 1970), while the genomes of most bony fishes had undergone an additional duplication (the fish-specific genome duplication (FSGD) or the 3R duplication) at the origin of modern fish (Amores *et al.* 1998, Hoegg *et al.* 2004, Meyer & Van de Peer 2005). FSGD probably occurred after the bichirs (Polypteriformes), sturgeons (Acipenseriformes), gars and bowfins (Semionotiformes) branched off from the fish stem lineage (Hoegg *et al.* 2004). In the present study, two GHR subtypes encoded by two different genes were found in three different teleost species. Phylogenetic analysis of all known GHR sequences revealed that the occurrence of two GHR subtypes exists only in teleost fish. We have also searched the genomes of *Xenopus*, chicken, mouse, rat and human but failed to obtain any GHR2-like sequences by *in silico* analysis. Hence we propose that the GHR2 sequences probably originated from the FSGD event associated with the emergence of modern teleosts, and therefore most teleosts with the possible exception of bichirs, sturgeons, gars and bowfins should possess both GHR subtypes unless they had experienced a secondary loss after the FSGD event, pending experimental proof. This

notion of two GHR subtypes is entirely different from the reported GHR isoforms in eel and in salmonids. As can be viewed from our phylogenetic tree (Fig. 3), both the two GHR isoforms in eel belong to the GHR1 clade and both the two GHR isoforms in coho salmon belong to the GHR2 clade. However, the two GHR subtypes from seabream, catfish and tilapia reported herein were segregated into the two separate clades. Salmonids have undergone a 4R duplication, which is supported by the fact that these fishes possess close to 16 genes of a conserved gene family (i.e. the *Hox* genes) (Moghadam *et al.* 2005). Thus there might be multiple GHRs in salmonids. The GHRs cloned in salmonids so far all belong to the GHR2 subtype. We postulate that GHR1s can also be found in salmonids, again pending actual experimental evidence. The story in eel might be more complicated. Eels diverged earlier than most other teleosts (Colbert & Morales 1991). They are more akin to the primitive bony fishes (Suzuki *et al.* 1999). Till now, studies on eel genome or gene duplication are very scarce so that the significance and implication of having two GHR1s in eel is not understood at the moment. Further studies are needed to unravel these intricacies.

Duplicated genes in a genome can only be maintained if they acquire a new function or maintain only some functions of the ancestral gene (Force *et al.* 1999). In this study, we have demonstrated the co-existence of both GHR1 and GHR2 in black seabream, Southern catfish and Nile tilapia. Based on the gene expression results and the post-receptor signal transduction studies in seabream, it is likely that GHR2 plays a different biological role from GHR1. These functional differences probably explain and justify the preservation and co-existence of both GHR subtypes during teleostean evolution after genome duplication. Further dissection of such functional differences between GHR1 and GHR2 in fish is highly warranted.

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