Differential ligand binding and agonist-induced regulation characteristics of the two rainbow trout GH receptors, Ghr1 and Ghr2, in transfected cells

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Abstract

Previously, we isolated and characterized two distinct GH receptor (GHR)-encoding mRNAs, *ghr1* and *ghr2*, from rainbow trout. In this study, Chinese hamster ovary-K1 cells were individually transfected with plasmids that contained cDNAs encoding rainbow trout *ghr1* or *ghr2*. High affinity binding of ^{125}I -salmonid GH (sGH) by the expressed receptors was saturable, displaceable, and ligand selective. Whole-cell binding analysis revealed a single class of binding site; for Ghr1 $K_d=8\text{ nM}$, for Ghr2 $K_d=17\text{ nM}$. While salmonid prolactin (sPrl) displaced ^{125}I -sGH from both Ghr1 and Ghr2, the affinity of either receptor subtype for sPrl was substantially less than for sGH; salmonid somatolactin, another member of the

GH-PRL family, did not displace labeled sGH except at pharmacological concentrations. ^{125}I -sGH was internalized by Ghr1- and Ghr2-expressing cells in a time-dependent manner; the maximum internalization reached was 71% for Ghr1 and 55% for Ghr2. Long-term exposure (24 h) of transfected cells to sGH up-regulated surface expression of both Ghr1 and Ghr2; however, sGH induced surface expression of Ghr1 to a greater extent than that of Ghr2. These results indicate that rainbow trout ghrs display both overlapping and distinct characteristics that may be important for ligand selection and differential action in target organs.

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Introduction

GH is a structurally diverse member of the GH/prolactin (PRL)/placental lactogen/somatolactin (SL) family that regulates numerous physiological processes in vertebrates, including growth, metabolism, reproduction, osmoregulation, immune function, and behavior (Forsyth & Wallis 2002, Bjornsson *et al.* 2004, Norrelund 2005). GH has been isolated from the pituitary of representatives of every extant class of vertebrate (Kawauchi *et al.* 2002). Extrapituitary production of GH also has been noted in fish and other vertebrates (Harvey *et al.* 2000, Biga *et al.* 2004). The structural heterogeneity of GH in mammals arises from alternate splicing of mRNA precursors or from proteolysis of GH (Forsyth & Wallis 2002). Two GHs were characterized in chum salmon (Kawauchi *et al.* 1986), and subsequently duplicate GH-encoding genes were described for many salmonids, including rainbow trout (Agellon *et al.* 1988, Devlin 1993, Du *et al.* 1993). The sequence identity between the two salmonid GHs (sGHs) is quite high (95% or greater), which explains the inter-species validity of RIAs (Bjornsson 1997), and no differences in biological activity have been reported so far.

GH initiates its actions by binding to GH receptors (GHR) present on the plasma membrane of target cells. GHRs belong to the class I cytokine receptor family, which consists of a single transmembrane protein with extracellular, transmembrane, and intracellular domains; the extracellular domain is important for ligand binding, whereas the intracellular domain is important for signal transduction (Argetsinger & Carter-Su 1996). GHRs have been characterized from over 100 species, including 25 species of fish. In mammals, there are two forms of GHR that result from differential post-transcriptional/post-translation processing; the short, soluble GHR may serve as a GH binding protein to extend the half-life of circulating GH (Talamantes & Ortiz 2002). Multiple GHRs derived from distinct mRNAs have been described in many species of fish, including Atlantic salmon, coho salmon, and rainbow trout (Benedet *et al.* 2005, Very *et al.* 2005). The use of the terms 'Ghr1' and 'Ghr2' for naming of the multiple GHR subtypes was first adopted in the tetraploid salmonids, and this convention continued for other teleosts (e.g. eel, grouper, tilapia; Ozaki *et al.* 2006, Li *et al.* 2007, Ma *et al.* 2007). There has emerged two distinct clades, sometimes referred to as a GHR 'type 1' (sometimes written type I) and GHR 'type 2' (sometimes written type II)

respectively (cf. Saera-Vila *et al.* 2005, Ozaki *et al.* 2006), with both of the original salmonid Ghr1 and Ghr2 subtypes contained within the type 2 clade. The nomenclature became more complex following the characterization of what appeared to be a distinct SL receptor (SLR) from masu salmon based on ^{125}I -SL binding (Fukada *et al.* 2005) that claded with type 1 GHRs. Recently, Fukamachi & Meyer (2007) re-analyzed fish GHR phylogeny and suggested that one clade (the former type 1 clade) represents SLRs and that the other major clade (the former type 2) represents GHRs (which includes the Ghr1 and Ghr2 of salmonids) and that SLR is a teleost-specific paralog of GHR that arose during the fish-specific genome duplication event some 350 million years ago. The two GHRs of salmonids most likely arose during a more recent tetraploidization event associated with the evolution of this group. GHRs are most abundant in the liver of mammals and fish; however, they also are expressed in brain, cardiac muscle, skeletal muscle, adipose tissue, kidney, spleen, and intestine, as well as in the gills of fish and mammary glands of mammals (Kopchick & Andry 2000, Very *et al.* 2005, Pierce *et al.* 2007). The wide-spread distribution of GHRs is consistent with the pleiotropic actions of GH.

Binding data of fish GHRs/SLRs are limited (Fukada *et al.* 2004, 2005, Ozaki *et al.* 2006) and the functional significance of the multiple subtypes of receptors in fish is unclear. The objective of this study was to determine the ligand binding characteristics of the two rainbow trout GHRs, Ghr1 and Ghr2. To this end, Chinese hamster ovary (CHO)-K1 cells were individually transfected with plasmids that contained cDNAs encoding full-length rainbow trout Ghr1 or Ghr2. Agonist-induced regulation features of the two GHR subtypes also were investigated.

Materials and Methods

Cell culture

The CHO cell line (CHO-K1 wild type) was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in 5% CO₂ and 95% relative humidity at 37 °C in Ham's F-12K nutrient mixture supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.1% (v/v) fungizone (base medium). For passage, the cells were detached from the culture flasks by washing with PBS followed by brief incubation (5 min) in trypsin (0.5 mg/ml)/EDTA (0.2 mg/ml). The cells were split every 3 days. For storage, the cells were resuspended in a medium containing dimethyl sulfoxide (10% v/v final concentration) and frozen in liquid nitrogen.

Plasmid construction

Juvenile rainbow trout (*Oncorhynchus mykiss*) were obtained, housed, and their tissues harvested as described previously (Very *et al.* 2005). Total RNA was extracted from rainbow

trout liver using TRI Reagent as specified by the manufacturer (Molecular Research Center, Inc., Cincinnati, OH, USA), quantified by u.v. (A₂₆₀) spectrophotometry, then diluted with RNase-free deionized water to 100 ng/µl, and stored at -80 °C until use. RNA quality was examined with the Agilent 2100 Bioanalyzer (Santa Clara, CA, USA) prior to use. First-strand cDNA was prepared from total RNA with the Clontech SMART RACE cDNA amplification kit (Clontech Laboratories, Inc.) and was then used as template for PCR using specific primers for each trout GHR cDNA previously characterized (Very *et al.* 2005; accession numbers for *ghr1* and *ghr2* respectively, are NM001124535 and NM001124731). The two primers used to obtain the complete coding region of *ghr1* were 5'-CATGGATCCTT-CAGCCTAGACGACGTTTC-3' (forward primer) and 5'-CATGCGGCCGCGCCCCCTCTGATCTGTGTC-CAA-3' (reverse primer). The two primers used to obtain *ghr2* were 5'-CATGGATCCGGTTGAGACGAGTGTTC-CGCTTGAT-3' (forward primer) and 5'-CATGCGGCCG-CCCATTTGGCACCTACTCTTTGATT-3' (reverse primer). The PCRs were performed using the Clontech Advantage 2 PCR kit under the following conditions: 94 °C for 5 min, followed by 35 cycles at 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min, and finally ended at 70 °C for 10 min. The resulting PCR products were visualized by electrophoresis on agarose gels containing 2% (w/v) OmniPur agarose (EM Science, Gibbstown, NJ, USA) in 1 × Tris-borate-EDTA buffer followed by ethidium bromide staining and u.v. transillumination. PCR products were cloned directly into the pcDNA3.1(+) mammalian cell expression vector (Invitrogen Corporation), which contains the neomycin phosphotransferase gene that confers resistance to the neomycin analogue, geneticin (G418). Positive colonies were identified by agarose gel electrophoresis of restriction enzyme digests (EcoRI; Promega) of purified plasmid preparations (Wizard Plus SV Minipreps, Promega Corporation). Plasmid DNA (75 fmol) was sequenced with the CEQ 2000 sequencer using the Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter; Fullerton, CA, USA) according to the manufacturer's protocol.

Stable transfection

Stable transfection of CHO-K1 cells was accomplished by an electroporation procedure using the BTX ECM399 system (Harvard Apparatus, Inc., Holliston, MA, USA). CHO-K1 cells were grown to 70% confluence in base medium. Cells were harvested with trypsin, washed twice with ice-cold electroporation medium (Ham's F-12K without FCS or antibiotics) and then resuspended in the same medium to ca. 1.0×10^6 cells/ml. Ten micrograms of purified plasmid and 0.4 ml of cell suspension were placed in a 0.4 cm gene pulse cuvette, mixed thoroughly by pipetting, and incubated on ice for 10 min. The cuvette containing the cells and DNA was

placed in an ECM399 pulse chamber, and pulsed once for 30 ms at 250 V. The cuvette was returned to the ice and incubated for an additional 10 min before plating. The cells were diluted with an appropriate volume of base medium, then plated and allowed to grow for 48 h before applying selection medium. The selection medium consisted of base medium with 500 µg/ml (final concentration) G418, and the medium was replaced every 48–72 h. After 10–14 days of growth in the selection medium, the G418-resistant clones were sub-cultured for binding analysis. Four different subclones were typically used for binding studies.

Analysis of [125 I]-GH binding

Initial screening of [125 I]-sGH binding was performed on microsomes isolated from clonal lines of CHO-K1 cells transfected with *ghr1* or *ghr2* mRNAs. Cells were collected by centrifugation (500 *g* for 5 min at 14 °C), then lysed with 100 µl 1× cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) with 1 mM phenylmethylsulphonyl fluoride (PMSF). Lysates were incubated on ice for 5 min, refluxed 20 times with a micropipette, then centrifuged at 1000 *g* for 30 min at 4 °C. The supernatant was centrifuged at 15 000 *g* for 30 min at 4 °C, and the subsequent supernatant was centrifuged at 110 000 *g* for 90 min at 4 °C. The resulting pellet was resuspended in 25 mM TRIS-HCl with 0.1 TIU aprotinin/ml and 1 mM PMSF, pH 7.5. The protein concentration of the microsomal preparation was determined using the Bio-Rad dye-binding method for microplates. Binding assays were conducted using the method described by Gray *et al.* (1990). sGH was obtained from GroPep, Ltd (Adelaide, Australia) and iodinated with [125 I]-sodium iodide (ICN Biomedicals, Irvine, CA, USA) as specified by the sGH supplier.

Detailed kinetic analysis was performed using whole-cell binding assays in a manner similar to that described by Gong *et al.* (2004). Cells (ca. 1.5×10^5 cells) successfully transfected with *ghr1* and *ghr2* were seeded in each well of a 24-well culture plate and incubated (37 °C, 5% CO₂, 95% humidity) until the cells attached firmly to the plate and reached 90% confluency. The cells were then washed thrice with binding medium (Ham's F-12K without FCS or antibiotics). Routine binding was analyzed by adding 200 µl binding medium, 50 µl radioactive ligands (25 pM final concentration), and 50 µl cold ligand (ranging in concentration from 0 to 1000 nM) to each well and mixed thoroughly. For the saturation assay, 200 µl binding medium was added to each well with 50 µl of eight different concentrations (0–250 pM) of [125 I]-sGH in the presence or absence of 1000 nM cold sGH and incubated (37 °C, 5% CO₂, 95% humidity) for 1 h. Binding in the presence of 1000 nM sGH was taken as nonspecific binding. Cell monolayers were then washed thrice with binding medium to remove unbound peptides. Trypsin (0.5 mg/ml)/EDTA (0.2 mg/ml) was then added to the wells and the detached cells were transferred to gamma tubes (12×75 mm) for determination of bound radioactivity.

For internalization experiments, CHO-K1 cells expressing Ghr1 and Ghr2 were incubated overnight at 4 °C with [125 I]-sGH (25 pM final concentration) in the presence or absence of 1000 nM sGH. After removal of pulse medium, the cells were washed thrice with binding buffer and the incubation was continued at 37 °C. At various times, surface-bound radioligands were removed by treatment with 1 ml of acidic glycine-buffered saline (100 mM glycine, 50 mM NaCl, pH 3.0) for 20 min at 37 °C. Internalized radioligands were measured as acid resistant counts in 0.1 M NaOH extracts of acid-washed cells (Hipkin *et al.* 2000).

For up-regulation experiments, CHO-K1 cells expressing Ghr1 and Ghr2 were incubated with various concentrations of sGH for up to 24 h at 37 °C. Immediately after removal of surface-bound sGH with acidic glycine-buffered saline, whole-cell binding assays with [125 I]-sGH were performed as described above. To investigate the fate of the up-regulated membrane receptors, cells were first cultured for 24 h with 100 ng/ml sGH at 37 °C to induce up-regulation, then residual surface binding was analyzed at various times following removal of surface-bound sGH. Binding data for long-term agonist regulation experiments were normalized to total protein content; protein was measured by the Bio-Rad dye-binding method for microplates and was typically 50 µg/sample.

Data analysis

Quantitative data are expressed as means $\pm$ S.E.M. Ligand binding characteristics were calculated with the SigmaPlot Ligand Binding Module (SPSS, Inc., Chicago, IL, USA) using the one-site model, which gave the best fit for the data (assessed by F-ratio) and was used for interpretation of results. Other statistical differences were evaluated by ANOVA followed by the Student–Newman–Keuls multiple range test or by a two-tailed *t*-test, as appropriate, using SigmaStat (SPSS, Inc.); α was set at 0.05.

Results

Expression of two subtypes of rainbow trout GHR in CHO-K1 cells

Two full-length cDNAs containing the entire coding regions of *ghr1* and *ghr2* were obtained by reverse transcription-PCR for use in the stable transfection of CHO-K1 cells (Fig. 1). The size of the cDNAs (1910 bp for *ghr1* and 1946 bp for *ghr2*) matched values predicted from primer locations. Nucleotide sequencing verified that no random mismatching of bps or point mutations occurred during plasmid construction. The *ghr1* cDNA possessed an open reading frame with 1779 nucleotides that encodes a 593-amino acid protein and the *ghr2* cDNA possessed an open reading frame with 1782 nucleotides that encodes

Figure 1 Reverse transcription-PCR amplification of rainbow trout GH receptor subtypes 1 (*ghr1*) and 2 (*ghr2*) in stably transfected CHO-K1 cells. Total RNA was extracted from cells and subjected to reverse transcription PCR as described in Materials and Methods; negative controls were as follows: 1) *ghr2* primer sets were used to amplify cDNA obtained from cells transfected with *ghr1* (*ghr1* negative control) and 2) *ghr1* primer sets were used to amplify cDNA obtained from cells transfected with *ghr2* (*ghr2* negative control). PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining and u.v. transillumination. M, marker (showing 500, 750, 1000, and 2000 bp standards); lane 1, *ghr1*; lane 2, *ghr1* negative control; lane 3, *ghr2*; lane 4, *ghr2* negative control.

a 594-amino acid protein; observations that were identical to those reported previously (Very *et al.* 2005).

To verify the functional expression of trout GHRs, specific binding of [125 I]-sGH to microsomes isolated from transfected cells was assessed. CHO-K1 cells individually transfected with plasmids containing *ghr1* and *ghr2* cDNAs displayed significantly higher specific binding than non-transfected cells (Fig. 2). However, a comparable level of expression was achieved in *ghr1*- and *ghr2*-transfected cells. Typically, 25 000 c.p.m. of labeled sGH was added to each reaction containing 50 μ g of microsomal protein. Under these conditions, specific binding ranged from 5.0 to 5.4% of total radioactivity added and up to 60–80% of total [125 I]-sGH bound; nonspecific binding averaged $28.4 \pm 8.6\%$.

Figure 2 Specific binding of [125 I]-salmonid GH (sGH) to microsomes isolated from non-transfected CHO-K1 cells and from CHO-K1 cells individually transfected with plasmids containing *ghr1* or *ghr2* cDNAs. Data are expressed as means $\pm$ S.E.M. from four independent assays with different batches of cells; groups with different letters are significantly different from each other ($P < 0.05$).

Figure 3 Saturation of [125 I]-salmonid GH (sGH) binding to CHO-K1 cells expressing rainbow trout GH receptor 1 (Ghr1) or GH receptor 2 (Ghr2). Data are expressed as means $\pm$ S.E.M. from four independent assays with different batches of cells.

Binding characteristics of expressed rainbow trout Ghr1 and Ghr2

Whole-cell binding analysis enabled detailed kinetic analysis of expressed GHRs and revealed that the expression products of the two rainbow trout GHR-encoding cDNAs were correctly targeted to the plasma membrane of the cell. Binding of [125 I]-sGH to Ghr1 and Ghr2 on CHO-K1 cell membranes was saturable and of limited capacity (Fig. 3). No specific binding was detected in either non-transfected CHO-K1 cells or in cells transfected with vector alone (data not shown). Scatchard analysis indicated a single class of high affinity binding site for each GHR subtype (Fig. 4). While the binding capacity of the expressed receptors was the same (Ghr1 $B_{\max} = 0.52 \pm 0.04$ nM; Ghr2 $B_{\max} = 0.54 \pm 0.03$ nM), there was a significant difference ($P < 0.05$) in their affinity (Ghr1 $K_d = 8.1 \pm 1.6$ nM; Ghr2 $K_d = 16.9 \pm 2.3$ nM).

The binding specificity of Ghr1 and Ghr2 was evaluated by displacing [125 I]-sGH with a variety of GH family proteins, including sGH, salmonid PRL (sPRL), and sSL (Fig. 5). The relative displacement profiles for GH family proteins to each of the expressed GHRs was similar, with the rank order of displacing [125 I]-sGH, from high affinity to low affinity, as follows sGH > sPRL >> sSL. For Ghr1, the predicted K_d for sGH was ca. 8 nM, while the K_d for sPRL was ca. 22 nM and the K_d for sSL was ca. 44 nM. For Ghr2, the predicted K_d for sGH was ca. 17 nM and that for sPRL was ca. 48 nM; however, sSL was an extremely weak competitor of GH binding sites and had a predicted K_d of ca. 114 nM.

Agonist-induced regulation of Ghr1 and Ghr2 surface expression

[125 I]-sGH was internalized in a time-dependent manner (Fig. 6). The rate of internalization was initially rapid, with 42–54% of the bound hormone partitioning with the internalized fraction within 15 min. Maximum internalization reached 55% for Ghr2 and 71% for Ghr1 after 90 min at 37 $^{\circ}$ C.

Figure 4 Scatchard plots of the binding of [125 I]-salmonid GH (sGH) to CHO-K1 cells expressing rainbow trout GH receptor 1 (Ghr1) or GH receptor 2 (Ghr2). Binding in the presence of 1000 nM sGH was taken as nonspecific binding. Data are expressed as means from four independent assays with different batches of cells; error bars were omitted for clarity.

Long-term exposure of transfected CHO-K1 cells to sGH induced up-regulation of Ghr1 and Ghr2. Exposure of transfected cells to sGH for 24 h at 37 °C increased surface expression of Ghr1 to a greater extent than of Ghr2 (Fig. 7A). Up-regulation of GHRs was time- and temperature-dependent. Surface binding to both GHRs increased 24–33% with 48 h exposure to sGH at 37 °C, while up-regulation was virtually abolished when cells were incubated at 4 °C (data not shown). Up-regulation of GHRs also was dependent on ligand concentration (Fig. 7B). sGH was more effective in up-regulating surface expression of Ghr1 than of Ghr2; surface expression of GHRs was maximal at a sGH concentration of 1000 ng/ml, increasing 82% for Ghr1 and 32% for Ghr2. When cells

Figure 5 Competitive inhibition of binding of [125 I]-salmonid GH (sGH) to CHO-K1 cells expressing rainbow trout GH receptor 1 (Ghr1) or GH receptor 2 (Ghr2). Receptor-transfected cells were incubated with [125 I]-sGH and the indicated concentration of sGH (●), salmonid prolactin (sPRL; ○), or salmonid somatotactin (sSL; ▲). Data are expressed as means $\pm$ S.E.M. from four independent assays with different batches of cells.

Figure 6 Internalization of [125 I]-salmonid GH (sGH) by CHO-K1 cells expressing rainbow trout GH receptor 1 (Ghr1) or gh receptor 2 (Ghr2). Data are expressed as means $\pm$ S.E.M. from four independent assays with different batches of cells; *represents significant difference between Ghr1- and Ghr2-expressing cells at a given time ($P < 0.05$).

expressing *ghr1* and *ghr2* were first treated with sGH for 24 h to induce up-regulation and then the ligand was removed, there was a slow loss of surface GHR expression from initial levels at time 0 (ligand removal; Fig. 7C).

Discussion

The analysis of GH binding in tissues and native cells (e.g. hepatocytes) of fish is complicated by the existence of multiple GHRs (cf. Fukamachi & Meyer 2007). Notably, studies of GH binding in tissues or hepatocytes in primary culture appear to indicate the presence of only a single class of binding site (Gray *et al.* 1990, Very & Sheridan 2007). This study made use of transfected cells in order to pharmacologically characterize the two GHRs of rainbow trout, Ghr1 and Ghr2. The individual expression of rainbow trout GHRs in a cell line enabled the characterization of receptor features not otherwise possible in native fish cells that endogenously express both receptor forms (Very *et al.* 2005). Stably transfected CHO-K1 cells synthesized and translated trout *ghr* mRNAs and correctly targeted the receptors to the surface of the cell. Although plasmids containing cDNAs encoding fish GHRs have been transfected into eukaryotic cells previously (Lee *et al.* 2001, Tse *et al.* 2003, Benedet *et al.* 2005), to our knowledge, this is the first report of the ligand binding features of expressed full-length GHRs from a nonmammal. The findings indicate that rainbow trout Ghr1 and Ghr2 possess both distinct and overlapping ligand binding and agonist-induced regulation features.

Ghr1 and Ghr2 expressed on CHO-K1 cells displayed saturable and displaceable high affinity binding of 125 I-sGH. Scatchard analysis revealed a single class of binding site for both Ghr1 and Ghr2. Linear Scatchard plots are typical for GHR binding in mammals and also have been observed in fish preparations examined to date, including 125 I-sGH binding to native hepatocytes isolated from rainbow trout

Figure 7 Agonist-induced surface GH receptor (GHR) expression in stably transfected CHO-K1 cells. Receptor-transfected cells were incubated with [125 I]-salmonid GH (sGH) under the conditions specified. (A) sGH (100 ng/ml) induced surface expression of rainbow trout Ghr1 and Ghr2 in CHO-K1 cells incubated for 24 h at 37 °C. (B) Agonist-induced expression of Ghr1 and Ghr2 in CHO-K1 cells incubated for 24 h at 37 °C depends on sGH concentration. (C) Removal of sGH (100 ng/ml) following a 24 h induction period (37 °C) reduced surface expression of Ghr1 and Ghr2 in CHO-K1 cells. Data were normalized to protein content and are expressed as means $\pm$ S.E.M. from four independent assays with different batches of cells; for a given GHR subtype, groups with different letters are significantly different; *represents significant difference between induced and noninduced (control) groups ($P < 0.05$) at a given time period.

(Fukada *et al.* 2004, Ozaki *et al.* 2006, Very & Sheridan 2007). The affinity of the expressed rainbow trout GHRs was in the range of that observed previously in native hepatocytes (Very & Sheridan 2007); interestingly, however, the affinity of Ghr1 for labeled sGH was significantly greater than that of Ghr2. Binding studies also have been performed using purified recombinant receptor protein in eels and masu salmon (Fukada *et al.* 2004, Ozaki *et al.* 2006). The masu

salmon preparation was based on the extracellular domain of the receptor and yielded an affinity similar to that of trout Ghr2. Although only one GHR has been reported for masu salmon (Fukada *et al.* 2004), it appears to be homologous to trout Ghr2; most likely a second masu GHR homologous to trout Ghr1 remains to be characterized. Of the two GHRs isolated from eel (Ozaki *et al.* 2006), only the type 1 form (SLR in the nomenclature proposed by Fukamachi & Meyer (2007)) was subjected to binding analysis and, interestingly, it displayed significant specific binding of DIG-labeled GH, but the K_d was not determined.

Competitive inhibition studies revealed several binding features of the expressed rainbow trout GHRs. While the rank order of the different ligands to displace radiolabeled sGH was the same for Ghr1 and Ghr2, the specific potency for a given ligand differed between the receptor forms. For example, the rank order for displacing labeled sGH for the ligands tested was sGH > sPRL $\gg$ sSL for both Ghr1 and Ghr2. Interestingly, sPRL was a relatively effective competitor of sGH for both Ghr1 ($K_d = 22$ nM) and Ghr2 ($K_d = 48$ nM). sSL did not displace labeled sGH from either receptor form except at pharmacological concentrations (cf. Rand-Weaver *et al.* 1995 for plasma SL levels in rainbow trout). These observations contrast with those of masu salmon GHR (which we presume to be homologous to trout Ghr1) from which neither sPRL nor sSL were effective at displacing labeled sGH (Fukada *et al.* 2004). The extent to which differences in the preparations (complete receptors expressed on CHO cells for trout versus purified recombinant extracellular domain for masu salmon) may have influenced observed binding properties is not known. It also should be noted that eel GH specific binding of the type 1 eel GHR (presumed to be an SLR under the nomenclature system of Fukamachi & Meyer (2007)) could not be displaced with physiological concentrations of eel PRL or eel SL (Ozaki *et al.* 2006). On the other hand, the putative masu salmon SLR specifically bound labeled sSL with high affinity, and this binding could be displaced by sSL and, at a 10-fold higher concentration, sGH; sPRL could displace label only at pharmacological concentrations (Fukada *et al.* 2005).

Rainbow trout GHRs also display unique agonist-induced regulation features. Short-term exposure of transfected cells to sGH induced rapid internalization of surface-expressed GHRs; agonist-induced internalization of Ghr1 was significantly more pronounced than that of Ghr2. Prolonged exposure of transfected cells to sGH up-regulated the expression of both GHR forms; however, the up-regulating effect was greater in *ghr1*-transfected cells than in *ghr2*-transfected cells. Interestingly, long-term induction of both GHRs required the continued presence of GH, for removal of GH resulted in reduced surface expression of GHRs. The contrast between short-term down-regulation and long-term up-regulation of GHRs by GH is intriguing. GH-induced internalization of GHR has been previously described in mammals, and appears to occur via two

mechanisms: an ubiquitin-dependent system involved with internalization of receptor dimers and an ubiquitin-independent system involved with endocytosis of non-dimerized GHRs (Strous & Gent 2002). Although internalization does not appear to relate directly to GH signal transduction (Argetsinger & Carter-Su 1996), it may represent a means to remove ligand and cease cellular responses to 'old' information and position the cell to respond to 'new' information. The long-term effect of GH in up-regulating surface-expressed GHRs is consistent with previous observation in mammals and rainbow trout in which GH increases transcription of *ghr* mRNA (Schwartz *et al.* 2002, Very & Sheridan 2007). The significance of such a contrasting long-term response is not clear, but given the nature of the actions of GH, many of which are anabolic (cf. Forsyth & Wallis 2002, Bjornsson *et al.* 2004, Norrelund 2005), it seems reasonable that the presence of GH would increase its tissue sensitivity as a means of heightening its action.

The differences in binding characteristics and agonist-induced regulation features between rainbow trout Ghr1 and Ghr2 most likely result from structural differences between the receptors. Despite the high amino acid similarity between rainbow trout Ghr1 and Ghr2 (85.5%, Very *et al.* 2005), several differences exist between the two forms in their extracellular and cytoplasmic domains. Substitution studies in mammals have shown that ligand binding, receptor dimerization, and signal propagation depend on N-linked glycosylation sites, four segments of a cysteine-rich region, and the WSXWS motif (Y/FGXFS in fish; Kopchick & Andry 2000). While the number of N-linked glycosylation sites and the FGEFS motif are identical between trout Ghr1 and Ghr2, there are numerous differences between the first four Cys residues (cf. position 55/58 to position 96/100) as well as between the last Asp and the last Cys residue (cf. position 184/188 to position 216/220) of the ligand-binding domain (Very *et al.* 2005). Mammalian GHR also are known to dimerize, in part due to an unpaired Cys in their extracellular domain (Argetsinger & Carter-Su 1996). Although fish GHRs also possess an unpaired Cys, there is no direct evidence of dimerization; however, GH stimulates cell proliferation in CHO cells transfected with fish GHRs in a manner similar to the dimerization-dependent proliferation observed with mammalian GHRs (Lee *et al.* 2001, Tse *et al.* 2003, Benedet *et al.* 2005). Differences in ligand binding characteristics between and among GHRs, PRL receptors (PRLRs), and putative SLRs probably also have a structural basis. Fukamachi & Meyer (2007) compared several critical regions of GHR family members in fish and tetrapods, but it was not clear if there were sufficient differences in the regions examined to explain the observed patterns of ligand selectivity. More binding studies in fish are needed to functionally characterize GHRs/SLRs/PRLRs.

Although the functional significance of multiple GHRs in fish is not clear, there are several lines of evidence to suggest distinct roles. First, several studies indicate tissue-specific

expression of GHRs forms. This is manifested as different levels of a particular subtype among tissues as well as differences in the relative abundance of subtypes within a particular tissue (Fukamachi *et al.* 2005, Saera-Vila *et al.* 2005, Very *et al.* 2005, Ozaki *et al.* 2006, Li *et al.* 2007, Ma *et al.* 2007, Pierce *et al.* 2007). Second, the pattern of expression of GHRs subtypes changes over the course of embryonic development and sexual maturation (Ma *et al.* 2007, Raine *et al.* 2007, Malkuch *et al.* 2008). Third, numerous perturbations, including fasting and seawater transfer, alter the differential expression profiles of GHR subtypes (Saera-Vila *et al.* 2005, Norbeck *et al.* 2007, Pierce *et al.* 2007, Poppinga *et al.* 2007). It is not clear if the different GHRs link to the same or different signaling systems, although differences between GHR forms in the box 1 region of the intracellular domain, the critical segment for signal transduction in mammals, exist (Argetsinger & Carter-Su 1996, Very *et al.* 2005). Interestingly, using reporter constructs, GH induced the activation of Spi 2.1 promoter through both seabream Ghr1 and Ghr2; whereas, Ghr1 but not Ghr2 mediated the activation of the c-fos promoter (Jiao *et al.* 2006). It should be noted that the reported patterns of expression for non-salmonid species may reflect differences in GHR and SLR under the nomenclature system suggested by Fukamachi & Meyer (2007). Regardless, despite ligand selectivity of fish GHRs/SLRs/PRLRs, the potential for cross binding of ligands exists. Therefore, the actions of GH/PRL/SL may depend on the distribution and abundance of receptor subtype, as well as on the signal pathways in target cells to which they link, under a given developmental/physiological state.

In summary, the two GHRs of rainbow trout, Ghr1 and Ghr2, stably expressed in CHO-K1 cells display both distinct and overlapping ligand binding and agonist-induced regulation features. Both Ghr1 and Ghr2 preferentially bound GH, but the affinity of Ghr1 for GH was twice that of Ghr2. Ghr1 and Ghr2 also bound PRL, and both receptors had extremely low affinity for SL. Short-term exposure of GHR-expressing cells to sGH induced rapid internalization of surface expression of both Ghr1 and Ghr2; however, Ghr2 was internalized to a greater extent than Ghr1. By contrast, long-term exposure to GH up-regulated the expression of GHRs, an effect that was more pronounced on surface expression of Ghr1 than on that of Ghr2. Differences in the ligand binding and agonist-induced regulation features between GHRs and between the GHRs and SLRs/PRLRs may be attributable to structural differences among the receptors. Such differences in binding may form the basis of ligand-selection and have important consequences on target organ responsiveness to GH and related hormones.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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