Identification of the growth hormone receptor in an advanced teleost, the tilapia (*Oreochromis mossambicus*) with special reference to its distinct expression pattern in the ovary

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Abstract

There is considerable evidence that the GH/IGF-I axis plays an important role in female reproduction. We report the isolation and characterization of the GH receptor (GH-R) and its gene expression profile during oogenesis in the tilapia, *Oreochromis mossambicus*. cDNA encoding GH-R was cloned and sequenced from the tilapia liver. The predicted GH-R preprotein consisted of 635 amino acids and contained a putative signal peptide, an extracellular region with a characteristic motif, a single transmembrane region, and a cytoplasmic region with conserved box 1 and 2 domains. The tilapia GH-R shared 34–74% identities with known GH-Rs in vertebrates. A binding assay using COS-7 cells showed that the cloned GH-R bound specifically to tilapia GH. Northern blot analysis showed a single mRNA transcript in the liver and ovary. *In situ* hybridization revealed intense signals of GH-R in

the cytoplasm and nucleus of immature oocytes. The granulosa and theca cells surrounding vitellogenic oocytes also contained the GH-R mRNA signals. About a tenfold greater level of GH-R mRNA was found in the immature oocytes versus the mature oocytes, along with high levels of IGF-I mRNA. There were no significant changes in mRNA levels of GH-R and IGF-I in the liver or in plasma IGF-I levels during oocyte development. No correlation was found between hepatic GH-R mRNA and ovarian GH-R mRNA. These results suggest that the GH/IGF-I axis in the ovary may be involved in the early phases of oogenesis, under a different regulatory mechanism of GH-R gene expression from that of the liver.

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Introduction

The interaction between somatic growth and reproduction has been implicated in vertebrates in general. Although follicle-stimulating hormone and luteinizing hormone are the major regulators of gonadal development, there is considerable evidence that the growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis plays an important role in female reproduction, in addition to its metabolic actions for somatic growth (Hull & Harvey 2001). In mammals, GH deficiency caused a delayed onset of puberty, which was restored by exogenous GH treatment (Ramaley & Phares 1980, Advis *et al.* 1981). Co-administration of GH with gonadotropin (GTH) has also been used to induce ovulation in patients who show poor sensitivity to GTH (Homburg 1996). Furthermore, Laron syndrome, GH insensitivity resulting from a variety of GH receptor (GH-R) mutations, shows a hereditary dwarfism, truncal obesity, and delayed puberty, which are restored by IGF-I treatment (Laron & Klingler 1998).

In vitro studies in mammals have shown that GH enhances follicular growth and differentiation of granulosa cells (Jia *et al.* 1986, Yoshimura *et al.* 1994), steroidogenesis (Mason *et al.* 1990), and inhibits follicle apoptosis (Danilovich *et al.* 2000). In teleosts, GH potentiates the actions of GTH on ovarian steroidogenesis in the goldfish (Van der Kraak *et al.* 1990) and directly stimulates steroidogenesis in isolated ovarian follicles of killifish and seatrout (Singh *et al.* 1988, Singh & Thomas 1993). These studies led to attempts to localize the GH-R transcripts or peptides, but the results are still ambiguous, even in mammals. GH-R is localized in a variety of rat ovarian cells including follicles (Lobie *et al.* 1990), granulosa cells, corpus luteum, and germinal epithelium (Carlsson *et al.* 1993), and also in human granulosa cells of dominant and antral follicles, and corpus luteum (Sharara & Nieman 1994). According to Kölle *et al.* (1998), GH-R transcripts are co-localized with GH-R protein in the bovine oocytes of the primordial and primary follicles, in the cells of the cumulus oophorus, large granulosa lutein cells, germinal

epithelium, and endothelial cells of ovarian vessels. In teleosts, there seems to be no report on specific localization of GH-R in the ovary, except for a report on specific binding sites for GH in the ovary of rainbow trout by radioreceptor assay (Gomez *et al.* 1999). On the other hand, localization of IGF-I and IGF-I receptor were reported in the ovarian follicle of tilapia and seabream, suggesting that fish also have an IGF-I system within the ovary (Reinecke *et al.* 1997, Perrot *et al.* 2000). Recently, GH-R cDNA has been isolated in several teleost species (Calduch-Giner *et al.* 2001, Lee *et al.* 2001, Tse *et al.* 2003). However, the mode of actions of GH/IGF-I in ovarian development in the fish remains unknown.

While most of our knowledge regarding hormonal regulation of the ovarian function has been derived from work on a limited number of model systems, fish offer an alternative and excellent model to clarify the functional significance of GH in ovarian development. Among more than 22 000 species of teleosts, the tilapia (*Oreochromis mossambicus*) is one of the most advanced species, and shows asynchronous development of the ovary, where different developmental stages of oocytes are simultaneously present, different from salmonid species which show synchronous ovarian development, thus providing a unique model (Smith & Haley 1988). The aim of the present study was to examine a possible role of the GH/IGF-I axis in the ovarian development of the tilapia. We have isolated and characterized GH-R cDNA, identified the localization of the GH-R transcripts in the ovary, and examined the gene expression profiles of GH-R and IGF-I during ovarian development.

Materials and Methods

Samples

Female tilapia (*Oreochromis mossambicus*), weighing 40–80 g, were reared in circular 700 liter tanks in fresh water under natural condition at the Hawaii Institute of Marine Biology, University of Hawaii, Manoa. Water temperature was maintained at $25 \pm 2^\circ\text{C}$. They were fed twice daily with ProForm (Agro Pacific, Chilliwack, BC, Canada), approximately 2% of body weight per day. The experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii. For gene expression studies, female tilapia, weighing 40–180 g, were sampled at the Ocean Research Institute, University of Tokyo.

cDNA cloning of tilapia GH-R

Total RNA was extracted from the tilapia liver using an RNA extraction solution (ISOGEN; Nippon Gene, To-

kyo, Japan), and poly(A)⁺ RNA was subsequently purified from total RNA with Oligotex-dT 30 (Takara, Shiga, Japan). Double-strand cDNA was reverse transcribed using a Marathon cDNA Amplification Kit (CLON-TECH Laboratories, Palo Alto, CA, USA) according to the manufacturer's instruction. Degenerate primers, F1 (5'-GAY CCI CCM RTD KSI CTI AAC TGG AC-3'), R1 (5'-TYG CTI ACY TGI GYA TAR AAG TC-3'), and R2 (5'-AGC TSD ATG AAC TCN ACC CA-3'), were designed based on the known cDNA sequences of vertebrate GH-R. PCR was performed in a thermal cycler PC-701 (Astec, Fukuoka, Japan) in 20 µl containing 1 × PCR buffer (Takara), 200 µM dNTPs (Takara), 0.5 U Taq DNA polymerase (Takara), 0.5 µM of a primer pair (F1 and R1), and an appropriate amount of the cDNA template. The PCR was performed as follows: 94 °C for 2 min, 40 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and 72 °C for 7 min. Diluted PCR product was used for the nested PCR with F1 and R2 under the same conditions as the first PCR. The PCR products were analyzed on a 2.0% agarose gel and ligated into pBluescript II SK(–) vector (Stratagene, La Jolla, CA, USA). Purified plasmids were sequenced using a DNA sequencer ABI PRISM 310 (Applied Biosystems, Foster City, CA, USA).

After determination of the partial cDNA sequences, rapid amplification of cDNA ends (RACE) was employed. For 3'-RACE, specific primers, F2 (5'-GCT ATT GAA GAA GGG GAA GCT GGA TGA GC-3') and F3 (5'-TAT GCT GAG TGG TGG AGG AAT GGA TGG C-3'), were designed. For 5'-RACE, R3 (5'-AGA CTG TCG CTG AAT GTC CAA TGC TTC C-3') and R4 (5'-CTC ATA CTC CAC ACG CAT CCA TCC CAA CC-3') were also designed and used for primary and nested PCR respectively. PCR was carried out with a specific primer and an adaptor primer under the conditions of 94 °C for 2 min, 5 cycles at 94 °C for 30 s and 72 °C for 2 min, 5 cycles at 94 °C for 30 s and 70 °C for 2 min, 30 cycles at 94 °C for 30 s and 68 °C for 2 min, and 72 °C for 7 min. PCR products were subcloned and sequenced as described above. Sequence data were analyzed by Sequencher software version 3.1.1 (Hitachi, Tokyo, Japan) and SeqEd software version 1.0.3 (Perkin-Elmer, Branchburg, NJ, USA). The nucleotide sequence was determined by analyzing more than six clones from distinct amplifications and reverse transcriptions to avoid errors. Amino acid sequence alignment was performed using the CLUSTAL W multiple sequences alignment program (Thompson *et al.* 1994).

Functional analysis of tilapia GH-R

A cDNA fragment including the open reading frame of GH-R was synthesized with a forward primer (5'-CTT CTC GAG GCC ACC ATG GCT CTC TCG CCC TCC T-3') and a reverse primer (5'-GAT CTC GAG

GTC ATT TCA TTG TGA GAG GTT CCC-3'), and subcloned into the expression vector pcDNA3 (Invitrogen, Carlsbad, CA, USA). The vector with or without GH-R cDNA was transfected into monolayer cultures of COS-7 cells using TransFast transfection reagent (Promega Corp., Madison, WI, USA). COS-7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in 5% CO₂/95% air at 37 °C for 48 h.

Tilapia GH (5 µg) was iodinated by chloramine-T, and used for binding assays as a ligand. After the medium was aspirated, confluent cells in six-well plates were washed in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA; Sigma, St Louis, MO, USA). The binding assays were conducted by adding 1 ml iodinated GH (approximately 20 000 c.p.m.) to each well with or without non-radioactive GH (1 µg), following Zhang *et al.* (2000) with modifications. After incubation for 18 h at 4 °C, the medium was aspirated and the cells were washed in PBS containing BSA, and solubilized in 0.1 M NaOH with 1% SDS. The radioactivities in the solubilized cells were counted in a gamma counter.

For the competitive binding assay, approximately 1×10^6 cells in 300 µl binding assay buffer (40 mM Tris-HCl, 10 mM CaCl₂, and 0.1% BSA, pH 7.4) were incubated with 100 µl iodinated GH (20 000–25 000 c.p.m.) and 100 µl serial dilutions (0.015–1000 ng) of non-radioactive tilapia GH or tilapia prolactins (PRL₁₈₈ and PRL₁₇₇). After incubating overnight at 4 °C, 2 ml ice-cold assay buffer were added to the each tube, and centrifuged at 750 g for 10 min. Subsequently, the tubes were aspirated and then counted in a gamma counter. Non-specific binding were quantified in the presence of 1 µg non-radioactive GH. Assays were conducted in triplicate and repeated at least twice by distinct transfection. Data were analyzed using PRIZM software (GraphPad Software, San Diego, CA, USA).

Tissue distribution analysis of GH-R

Total RNA was extracted from brain, pituitary, gill, liver, spleen, head kidney, body kidney, heart, skeletal muscle, stomach, intestine, ovary, and testis as described above. After treatment with DNase (Invitrogen), the total RNA (2.5 µg) was reverse transcribed with random hexamer primers and SuperScript II reverse transcriptase following the manufacturer's protocol (Invitrogen). RT-PCR was performed with a primer pair (5'-CTG CAG CCA CAA CGG A-3')/(5'-CTT TCC TGG TGA ATC AGC C-3') under the PCR cycles at 94 °C for 2 min, 32 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. PCR products were analyzed on a 2.0% agarose gel and stained with ethidium bromide. A plasmid containing the full length of GH-R cDNA was used as a positive control, and distilled water as a negative control.

Northern blot analysis

A cDNA including the full open frame region of tilapia GH-R was used as a probe. The cDNA was labeled using a random primed DNA labeling kit (Takara) with [α -³²P]dCTP (Amersham Bioscience, Piscataway, NJ, USA). The specific activities were greater than 1×10^9 c.p.m./µg. Four micrograms of poly(A)⁺ RNA purified from the liver and ovary were fractionated on a 1.1% formaldehyde agarose gel and transferred to a Hybond N⁺ nylon membrane (Amersham Bioscience). After prehybridization in hybridization buffer (PerfecHyb; Toyobo, Tokyo, Japan), the membrane was hybridized with DNA probe at 65 °C for 18 h. The membranes were then washed for 10 min in $2 \times$ SSC with 1% SDS at 65 °C, followed by a 10-min wash in $0.2 \times$ SSC with 0.1% SDS at 65 °C. The membrane was exposed to Fuji X-ray film (Fuji Film, Tokyo, Japan) at -80 °C for 1 day.

In situ hybridization

Tilapia ovaries (gonadosomatic index (GSI); gonad weight/body weight $\times 100$: 0.62–4.50%) were embedded in paraplast and cut at 6 µm. Subsequently, sections were deparaffinized by washing in xylene and series of ethanol. An antisense oligonucleotide probe (5'-GTT AAG GAG CAG ACT GTG ATG GCT GTC TAA CTC CTG AAC CAC TG-3'; nucleotide position 1757–1800) or a sense probe (5'-CAG TGG TTC AGG AGT TAG ACA GCC ATC ACA GTC TGC TCC TTA AC-3') were labeled with [α -³⁵S]dATP (Dupont/NEN, Boston, MA, USA) on their 3'-ends and purified using MicroSpin G-50 columns (Amersham Bioscience). After prehybridization in hybridization buffer (1 $\times$ Dehnhardt's solution containing 1 M NaCl, 25 mM Tris-HCl, 7.5 mM EDTA, 12.5 mM dithiothreitol, 10% dextran sulfate, and 150 µg/ml calf thymus DNA), slides were hybridized with labeled antisense probes or sense probes (control) overnight at 45 °C in a humidified chamber. Slides were then washed in $2 \times$ SSC for 10 min at room temperature, followed by two washes in $1 \times$ SSC for 30 min at 45 °C and in $2 \times$ SSC for 30 min at room temperature. Dehydrated sections were then exposed to autoradiographic emulsion (Konica, Tokyo, Japan) for 2 weeks at 4 °C. Sections were counterstained with hematoxylin and eosin after radioautography.

Real-time quantitative RT-PCR

mRNA levels of GH-R and IGF-I in the liver and ovary were determined using the ABI PRISM 7700 Sequence Detector (Perkin-Elmer). Total RNA was extracted from the liver and from the whole ovary. Additionally, using a stereomicroscope, oocytes at the most developed stage found in each ovary were selected, from which total RNA was extracted. They were treated with DNase

(Invitrogen) and then reverse transcribed as described above. For GH-R mRNA, a specific primer pair (5'-CAC AGA CTT CTA CGC TCA GGT CA-3')/(5'-TGA GTT GCT GTC CAG GAG ACA-3') and a Taqman probe (5'-CAAT GTT ATG CCA ACT GGT GGT GTG GTG-3') were designed. IGF-I mRNA levels were determined by amplifications of A, D, and E domains with a specific primer pair (5'-CTG CTT CCA AAG CTG TGA GCT-3')/(5'-GAT CGA GAA ATC TTG GGA GTC TTG-3') and a Taqman probe (5'-CAG CGC CTT GAG ATG TAC TGT GCA CCT-3') as described previously (Kajimura *et al.* 2001). All primer pairs were designed using Primer Express software version 1.0 (Perkin-Elmer). Serial dilutions of the plasmid DNAs (5×10^5 ; 6.4 copies) containing the amplified fragments of target mRNAs were prepared as standard samples. PCR reaction contained 0.2 μ M of each primer and 0.2 μ M Taqman probe, and 12.5 μ l Platinum Quantitative PCR Super-Mix-UDG (Invitrogen). The cycling parameter was as follows: 2 min at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Data were analyzed by Sequence Detector version 1.6.3 (Perkin-Elmer) and expressed as the copy number of the target mRNA per 1 μ g total RNA.

RIA

Plasma levels of estradiol-17 β (E₂) were measured using a commercially available RIA kit (ImmunoChem Coated Tube E₂ ¹²⁵I RIA Kit; ICN Biochemicals, Costa Mesa, CA, USA). Plasma levels of GH and IGF-I were measured by homologous RIAs as described elsewhere (Kajimura *et al.* 2001).

Statistics

Significance of differences was analyzed by one-way ANOVA followed by Fisher's protected least significant difference, using Stat View software (SAS Institute, Cary, NC, USA). Plasma levels of GH, mRNA levels of GH-R and IGF-I in the whole ovary and in the oocytes were first log-transformed, and then applied to one-way ANOVA since S.D. values were not equal. Levels of correlation were determined by calculating the correlation coefficient. Significance was set at $P < 0.05$.

Results

cDNA cloning of tilapia GH-R

PCR amplification of the tilapia liver cDNA with the degenerate primer set yielded a cDNA fragment (599 bp) sharing a high homology with other known GH-R cDNA sequences. Subsequently, tilapia GH-R cDNA was sequenced from 3'- and 5'-RACE products (Fig. 1). The

GH-R cDNA consisted of 2807 bp which encoded 635 amino acids containing a putative signal peptide (27 amino acids), an extracellular domain (225 amino acids), a single transmembrane domain (24 amino acids), and an intracellular domain (359 amino acids). The homology analysis based on the amino acid sequences revealed that the tilapia GH-R showed the highest identity (74%) to the GH-R of turbot, *Scophthalmus maximus* (Calduch-Giner *et al.* 2001). The tilapia GH-R also shared identities with the counterparts of seabream, *Acanthopagrus schlegel* (Tse *et al.* 2003) (73%), goldfish, *Carassius auratus* (Lee *et al.* 2001) (49%), masu salmon, *Oncorhynchus masou* (AB071216) (40%), mouse, *Mus musculus* (AF120489) (34%), and human, *Homo sapiens* (NM-000163) (35%). On the other hand, the tilapia GH-R shared only 21% identity with tilapia PRL receptor (PRL-R) (L34783).

The deduced amino acid sequence had several characteristics conserved in known vertebrate GH-Rs (Fig. 2). The extracellular domain contained a characteristic FGEFS motif. The highly conserved box 1 and 2 regions were found within the intracellular domain of GH-R. The positions of six cysteine residues out of seven in the extracellular domain were highly conserved in the vertebrate GH-R with the exception of masu salmon. Three cysteine residues in the intracellular domain were also well conserved. There were six potential N-linked glycosylation sites, four of which were highly conserved in vertebrates. The nucleotide sequence reported in this paper has been deposited in the DDBJ/EMBL/GenBank DNA databases under Accession No. AB115179.

Radioreceptor assay of tilapia GH-R

The COS-7 cells transfected with the vector containing the GH-R insert showed specific binding to tilapia GH, and the binding was blocked by excess amounts of unlabeled GH (Fig. 3A). Competitive binding assay showed that GH, as compared with PRL₁₇₇ and PRL₁₈₈, bound to the GH-R with the highest sensitivity (ED₅₀ = 0.85 ng). Labeled GH was displaced slightly by PRL₁₇₇ (ED₅₀ = 333.3 ng), but not by PRL₁₈₈ (Fig. 3B).

Tissue distribution of GH-R

RT-PCR showed that the expected size (283 bp) of a single band was obtained in all the tissues examined (Fig. 4A). The highest expression was found in the liver, followed by the spleen and the heart. Northern blot hybridization showed a single transcript around 5.0 kb, and liver expressed higher levels of GH-R mRNA than ovary (pooled sample at the different ovarian development, GSI = 1.67 ± 0.68) (Fig. 4B).

Localization of GH-R in the ovary

In situ hybridization was performed to localize GH-R transcript in the ovary. Ovarian development in the tilapia

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ggattgctggcgatgagggcgcttgatcgctgcgcgaacacactgccacttttttctgt
ttaagcgacgtagcgtgtctctaccctccaagtggtctagacgataagcagcaataaggcaaaacccgaccaggaagcagcatcc
tgagattgagtgatatgttaaaagtcgtcccgaaaggtctgagtcggaccgagcagtcgtgagtgaaagaacgagactcgtaaccggta
gtgcttgcgctggccatcagatgagcaactctgaaaagtaaatctgcgcgctgcttgagcttttcagctccacgcttcggaagaacatc
1 ATGGCTCTCTCGCCTCCTCTAATCTCCTGATCTCTCATCCTTCTCCTGCGGATGGCTGCGCTCTCCAGGATCTACATTTCTCACT 90
M A L S P S S N L L I L L I L S S L D W L P S P G S T F L T
91 GACTGGGACCACAGCATCATCAGCTCTCATTGAGCCTCATTTTACTGAGTGTATCAAGGACCAGGAGACGTTCCACTGCTGGTGG 180
D W D H T T S S A L I E P H F T E C I S R D Q E T F H C W W
181 AGTCCCGGCGAGCTCCACAACCTCTCTCCCCTGGAGCAGCTCCGAGTCTTCTACCTTAAGAAAGAGTCTCTACCAGCCAGTGAAGGAG 270
S P G S F H N L S S P G A L R V F Y L K K E S P T S Q W K E
271 TGTCTGAGTATATCCATTCAAATAGGGAATGTTTCTTGTAGAGCTCACACATCCATATGGATCACTTACTGCATGCAGCTTCGCACT 360
C P E Y I H S N R E C F F D E A H T S I W I T Y C M Q L R T
361 CAAAACAACACTTATTTCAATGAGGATGACTGTTTCAGTGTGGAGAATATGTACGTCCTGACCCGCCAGTGAATTTAAACTGGACC 450
Q N N I T Y K E Y E N E D D C F S V E N I V R P D P V L N N W T
451 CTGCTGAATATAAGTCTTCCGGGTAAATTATGATGTCATGATTAAGTGGAGCCCCACCTACTGCTGATGTTAGGTGGGATGGATG 540
L L N I S P S G L N Y D V M I N W E P P P T A D V R L G W M
541 CGTGTGGAGTATGAGTTGAGTACAGAGAGAGAAATACCACAACTGGGAAGCATGGACATTCAGCGACAGTCTCATCAGACAATCTAC 630
R V E Y E L Q Y R E R N T T N W E A L D I Q R Q S H Q T I Y
631 GGTCTGCGCTTAGGAAAGAATAGAGTACACATCCGCTGAGGATGAGGCTTTCATTAATTTGGCGAGTTCAGTGAATCCATCTTC 720
G L R L G K E Y E V H I R C R M Q A F I K F G E F S E S I F
721 ATTCAGTGACTGAGATTCCTAGCAGAGTCTACTGTCCATCTCACACTGGTCTTGTATTTGGGACTGTGGGTATCCTCATACTATC 810
I Q V T E I P S T E S T V H L T L V L V F G T V G I L I L I
811 ATGCTCATAGTCACTCTCAGCAGAACCGATTAATGATATTCCTGCTGCCGCCTGTTCTGCACCCAAATCAAAGGCATCGATTAGAG 900
M L I V I S Q Q N R L M I F L L P P V P A P K I K G I D S E
901 CTATTGAAGAAGGGGAAGCTGGATGAGCTGAATTTTATGCTGAGTGGTGGAGGAATGGATGGCCTGCCCATTTATGCACAGATTTCTAC 990
L L K K G K L D E L N F M L S G G G M D G L P I Y A P D F Y
991 CAAGACGAGCCGTGGGTGGAGCTCATGGAGGTCGATGAGACAGAGGATGTAGATAATGGAGAGAAGAAGGATAAAGTGGGCTCAGACACC 1080
Q D E P W V E L M E V D E T E D V D N G E K K D N W G S D T
1081 CAGAAACTCCTGGGTGAGTCCCAACCTGTCAGTCAACACATCAACATAAAGTCTCAAATTCAGTCAAGTGGTCCGGATGCTGAGTCATCC 1170
Q K L L G Q S Q P V S Q H I N I N C S N S V S G P D A E S S
1171 CAGGCCACCTGTACAAACACAGATCTCCCTGAAGAAGAAACACAAATGCTGATGGCCACGCTTCTACCAGGACACAGCTGATGAAGAGGAA 1260
Q A T C Y N T D L P E E E T Q M L M A T L L P G Q P D E E E
1261 ACCTCCCTTGATACTGTAGAAGATCCTCAGCCTCTGAGACAGGTGAAAGACAGCTCATCCAAACCCAAACAGAGGGCCCCAGACCTGG 1350
T S L D T V E R S S A S E T G E R Q L I Q T Q T R G P Q T W
1351 GTCAACACAGACTTCTACGCTCAGGTCAGCAATGTTATGCCAACTGGTGGTGGTGGTGTCTCTGACAGCAACTCAGAAATCCAGGAG 1440
V N T D F Y A Q V S N V M P T G G V V L S P G Q Q L R I Q E
1441 AGCATCTCAGCCGCCGAGAAGGAAACAAAAAGAGAGAGTGAAGACAGCGAGGAGCTGAGGACAGCGAAGCAAAAGAGAGCCA 1530
S I S A A E K E T K K K R K E S E D S E E S E E R K Q K E P
1531 CAGTTTCAGTGCTAGTAGTGGATCCAGAAGGAAGTGCCTACGTACAGAGAGCAGTATCCAGCAAATCAGCACTCTCCCCCTAGCTCT 1620
Q F Q L L V V D P E G S A Y S T E S S I Q Q I S T P P P S S
1621 CCCATGCCCGGTGAGGGGTACCACATCATACCTCAGCCAGTGGAGGCCAGACCTGCAGCCACAACGGAGCTTAATCAGTCACCTTAC 1710
P M P G E G Y H I I H P Q P V E P R P A A T T E L N Q S P Y
1711 ATTATTCCTGACTCTCCCAAGTTTTTTGCTCTGTTGCACTACACAGTGGTTCAGGAGTTAGACAGCCATCAGCTCTGCTCCTTAAC 1800
I I P D S P Q F F A P V A D Y T V V Q E L D S H H S L L L N
1801 CCGCCTTGCCACAGACCCCCCTCCCTGCCTGCCACAGCACCACCTCAAGGACCTATGCCTGTGGGTACATTACCCAGACCTGCTG 1890
P P C H Q T P P P C L P Q H P L K A P M P V G Y I T P D L L
1891 GGAACCTCTCACAATGAaatgacaatgacatcagacaccttacctgtgaaggctgattcaccaggaagttaacctacgtcgctcctaccga 1980
G N L S Q *
1981 ttcccggttggaactcccagcacactgggtggctgagatgtgcacgggtgtctatttttcagaggtgctgaaagcgagatataaaaggct 2070
2071 Acaagatactctcttaactcttttgcatctgctaagcagttctttttttaggcagcgaataattaacatcacaaacaaatgcacagtt 2160
2161 tgcctctgttttgcgaacacatcatgcatcattgacagtgagccaagcaaacataatcaatgcttaactgccttggtgtgca 2250
2251 ttgtatctctggcttattgtgtccagagcaatgacgctgttacagctttaatttttacagagtgctttcagtgcatgaccgatgggtgag 2340
2341 tttcacaccaaaacaaagtcatgtgacgggtgatgctgtttaaccacagaggtactttatcagtttatcaatagaccgttccctatctt 2430
2431 tcttaactcatcatatcatattagcacttcaactagatatatttt

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Figure 1 The nucleotide sequence of cDNA encoding tilapia GH-R and the deduced amino acid sequence. Nucleotide numbers are shown on both sides. The FGEFS motif is underlined with a broken line, and the transmembrane domain with a solid line. Box 1 and 2 regions are shown in shaded rectangles. The stop codon is marked with an asterisk.

Figure 2 Alignment of the amino acid sequences of GH-R in vertebrates. The alignment was performed using the CLUSTAL W multiple sequences alignment program (Thompson *et al.* 1994). Identical amino acids are indicated by asterisks above the sequences. Gaps are shown as dashes. The cysteine residues are shown in solid rectangles, and the putative N-linked glycosylation sites in shaded rectangles.

is asynchronous, and thus oocytes at different developmental stages are found simultaneously in a single ovary. In the immature ovary, intense signals were observed in the cytoplasm and nucleus of the oocyte at the perinucleolus stage and the yolk vesicle stage (Fig. 5A). In the follicles surrounding the primary yolk globule stage, both granulosa and theca cells contained GH-R transcripts

(Fig. 5B). Strong signals were found in the somatic cells surrounding the follicles (Fig. 5C). Oocytes at the late yolk globule stage were distinguished by the presence of chorion observed on the vitelline membrane (marked with an asterisk in Fig. 5D and F). GH-R transcripts were also found in the granulosa and theca cells at the secondary yolk globule stage (Fig. 5D). No signal was

Figure 3 Functional analysis of tilapia GH-R cDNA. (A) Binding of ^{125}I -GH to COS-7 cells transfected with vector containing GH-R cDNA (GH-R) or vector only (control) in the absence (open bars) or presence (shaded bars) of 1 μg unlabeled tilapia GH. (B) Competitive binding assay with serial dilutions of unlabeled tilapia GH, PRL₁₇₇, and PRL₁₈₈. Values are means $\pm$ S.E.M.

seen in the control sections incubated with the sense probe (Fig. 5E and F).

Changes in plasma hormone levels during ovarian development

Table 1 shows the changes in hormone levels in the plasma during ovarian development. Fish were divided into three groups based on the developmental stages of the ovaries by histological analysis as follows: immature, ovaries contained mainly oocytes at the perinucleolus stage and the yolk vesicle stage (GSI: $0.52 \pm 0.08\%$); maturing, ovaries contained oocytes at the perinucleolus stage, the yolk vesicle stage, and the primary yolk globule stage (GSI: $1.50 \pm 0.16\%$); matured, ovaries contained various stages of ovarian development including the secondary yolk globule stage (GSI: $2.93 \pm 0.32\%$). Plasma E_2 levels were low in immature fish but increased significantly ($P < 0.05$) in maturing and matured fish. A significantly ($P < 0.05$) higher level of plasma GH was found in the matured fish than in the maturing fish. No significant change was seen in plasma IGF-I level throughout ovarian development.

Gene expression profiles of GH-R and IGF-I during ovarian development

To further quantify the mRNA levels of GH-R and IGF-I at the different ovarian developmental stages, real-time quantitative RT-PCR was employed. As shown in Table 2, there were no significant changes in GH-R and IGF-I mRNA levels in the liver among the three stages. No significant changes were found in GH-R and IGF-I

Figure 4 Tissue distribution and transcripts of GH-R gene in the tilapia. (A) RT-PCR analysis with specific primers. N.C and P.C represent negative control and positive control respectively. The molecular markers are shown on both sides of the gel. (B) Northern blot analysis of tilapia GH-R in the liver and ovary (pooled sample at the different ovarian developments, GSI = 1.67 ± 0.68). The molecular marker is presented on the right.

Figure 5 *In situ* hybridization of GH-R in the tilapia oocytes. (A) Immature oocytes at the yolk vesicle stage (YV). (B) Vitellogenic oocytes at the primary yolk globule stage (YG). Follicles (granulosa and theca cells) contained GH-R transcripts (arrow). (C) Somatic cells surrounding follicles at the primary yolk globule stage (arrowheads). (D) Vitellogenic oocytes at the late yolk globule stage (LYG). Chorion is marked with an asterisk. Follicle is indicated with an arrow. (E) Immature oocytes at the yolk vesicle (YV) stage hybridized with sense probe (negative control). (F) Vitellogenic oocytes at the late yolk globule (LYG) stage hybridized with sense probe (negative control). Chorion is marked with an asterisk. Scale bar=50 μ m.

mRNA levels in the whole ovary. Tilapia ovary simultaneously contains oocytes at different developmental stages. When oocytes at the same developmental stage were selected from each ovary, the mRNA level of GH-R was significantly ($P<0.05$) greater in the immature oocytes, as compared with matured oocytes. Similarly, IGF-I mRNA level was significantly ($P<0.05$) higher in the immature oocytes, and decreased in the maturing and matured oocytes.

Figure 6 shows the correlation analysis between mRNA levels of GH-R and IGF-I in the liver and ovary (GSI: 0.59–4.49%). Hepatic GH-R mRNA showed a significant positive correlation with hepatic IGF-I mRNA (Fig. 6A: $R^2=0.84$, $P<0.001$). In addition, ovarian GH-R mRNA was positively correlated with ovarian IGF-I mRNA (Fig. 6B: $R^2=0.72$, $P<0.001$). However, no significant correlation was found between hepatic GH-R and ovarian GH-R (Fig. 6C: $R^2=0.02$). Similarly, hepatic

Table 1 Changes in GSI, plasma levels of E₂, GH, and IGF-I during ovarian development. Values are means ± S.E.M. (n=4–9)

	Immature	Maturing	Matured
Index			
GSI (%)	0.52 ± 0.08 ^a	1.50 ± 0.16 ^b	2.93 ± 0.32 ^c
Plasma E ₂ (ng/ml)	7.35 ± 2.35 ^a	20.63 ± 4.08 ^b	18.42 ± 5.82 ^b
Plasma GH (ng/ml)	7.18 ± 5.12 ^{a,b}	3.03 ± 0.56 ^a	8.09 ± 2.09 ^b
Plasma IGF-I (ng/ml)	174.7 ± 14.1 ^a	136.4 ± 18.2 ^a	134.7 ± 8.2 ^a

Different letters (a, b, c) indicate significant difference at $P < 0.05$.

IGF-I mRNA was not correlated with ovarian IGF-I mRNA (Fig. 6D: $R^2 = 0.008$).

Discussion

To our knowledge, this is the first report on specific localization of GH-R mRNA in the fish ovary. Intense signals of GH-R transcripts were observed in the cytoplasm and nucleus of the immature oocytes, and in the granulosa and theca cells surrounding vitellogenic oocytes of the tilapia. There was no correlation between hepatic GH-R mRNA and ovarian development. On the other hand, the high level of GH-R mRNA in the immature oocytes was well correlated with the high level of IGF-I mRNA in the oocytes, indicating that the GH/IGF-I axis in the ovary may be involved in the early stages of oocyte development, possibly under a different regulatory mechanism of GH-R gene expression from that of the liver.

The cloned tilapia GH-R cDNA contained several characteristics conserved among vertebrate GH-R cDNAs. These include the FGEFS motif, which replaces the WSXWS motif found in other cytokine receptors including PRL-R, but conserved among all known fish GH-R to date. The intracellular domain has a highly conserved proline-rich motif named box 1, which is known to be important for interacting with the Jak kinase-mediating signals of ligands such as GH and PRL to the signal transducer and activator of the transcription family (Frank *et al.* 1994). The intracellular domain also

contains the box 2 region, involved in the internalization of the receptor (Govers *et al.* 1999). However, the conserved sequence (WVEFI) is changed to WVELM in the tilapia, even though it is a relatively conservative change in terms of polarity of amino acid. In fact, the amino acid sequence in box 2 is less conservative than that in box 1 among the known GH-R sequences in vertebrates, but how the structural changes affect the function of GH-R remains unknown. Comparative analysis of fish GH-R structures and those of tetrapods will further reveal the core sequence essential for GH-R functions.

Binding assays using COS-7 cells were performed to confirm the function of the cloned cDNA. ¹²⁵I-Labeled GH was displaced by homologous tilapia GH and slightly by PRL₁₇₇, one of two homologous tilapia PRLs (PRL₁₈₈ and PRL₁₇₇), but not by PRL₁₈₈. The ED₅₀ of GH in the binding assay using 0.85 ng was similar to the results from membrane fractions of tilapia liver (0.68 ng) (Shepherd *et al.* 1997), suggesting that the cloned tilapia GH-R cDNA is the functional GH-R of the tilapia. Shepherd *et al.* (1997) reported that PRL₁₇₇ exhibits somatotrophic actions by acting as a competitive ligand for GH-Rs on the hepatic membrane and promotes IGF-I gene expression in fresh water, where PRL₁₇₇ reaches sufficient levels to displace GH-Rs (50-fold less than GH). In the present study, PRL₁₇₇ had much lower potency (392-fold less than GH) than the previous report using hepatic membrane fraction (Shepherd *et al.* 1997). The difference in cross-reaction between the two experiments might be due to the different membrane preparations or different batches of PRL₁₇₇.

Tissue distribution studies indicate the substantial amounts of GH-R transcripts in a variety of extrahepatic tissues including the ovary, in agreement with other teleost species (Calduch-Giner *et al.* 2001, Lee *et al.* 2001, Tse *et al.* 2003). Furthermore, a single transcript of GH-R was detected in the liver and ovary by Northern blot analysis. In rodents, an alternative splicing variant of GH-R gene expresses a short form of mRNA encoding GH-binding protein (GHBP) (Smith *et al.* 1989). On the other hand, the human and the rabbit express a single mRNA of GH-R, and the GHBP was produced

Table 2 Changes in mRNA levels of GH-R and IGF-I during ovarian development. Values are means ± S.E.M. ($\times 10^5$ copies/ μ g total RNA; n=4–9)

	Tissue	Immature	Maturing	Matured
Index				
GH-R	Liver	12.26 ± 1.57 ^a	10.13 ± 2.99 ^a	8.45 ± 2.56 ^a
	Whole ovary	3.14 ± 1.32 ^a	3.80 ± 0.40 ^a	4.97 ± 1.36 ^a
	Oocytes	1.62 ± 0.28 ^a	1.09 ± 0.68 ^{a,b}	0.16 ± 0.03 ^b
IGF-I	Liver	15.32 ± 5.42 ^a	10.59 ± 4.05 ^a	10.48 ± 3.36 ^a
	Whole ovary	3.27 ± 0.51 ^a	1.82 ± 0.36 ^a	3.94 ± 1.12 ^a
	Oocytes	1.27 ± 0.35 ^a	0.32 ± 0.15 ^b	0.13 ± 0.03 ^b

Different letters (a, b) indicate significant difference at $P < 0.05$.

Figure 6 Correlation analysis between (A) hepatic GH-R mRNA and hepatic IGF-I mRNA, (B) between ovarian GH-R mRNA and ovarian IGF-I mRNA, (C) between hepatic GH-R mRNA and ovarian IGF-I mRNA, and (D) between hepatic IGF-I mRNA and ovarian IGF-I mRNA at different stages of ovarian development. Data are expressed as $\times 10^5$ copies/ μ g RNA.

byproteolytic cleavage of the GH-R (Sotiropoulos *et al.* 1993). In teleosts, a short form of GH-R, which may encode a membrane-anchored truncated receptor, was cloned in the turbot (Calduch-Giner *et al.* 2001). On the other hand, the goldfish produces multiple forms of GHBP in the circulation (Zhang & Marchant 1999), whereas a single transcript of GH-R was reported (Lee *et al.* 2001). To our knowledge, there seems to be no report on GHBP in the tilapia. The single transcript of the GH-R gene in the tilapia may indicate that GHBP could be produced by proteolytic cleavage of the GH-R.

In situ hybridization in the tilapia ovary revealed intense signals in the cytoplasm and nucleus of small oocytes at the perinucleolus stage and yolk vesicle stage, implying an important role of GH in the early stages of oocyte growth and development. This is supported by the findings that the GH-R/GHBP knockout mouse exhibits a reduced number of follicles (Bachelot *et al.* 2002), and that exogenous GH significantly increased the number of small follicles in the cow (Gong *et al.* 1991). We also found GH-R transcripts in the granulosa and theca cells surrounding vitellogenic oocytes, which is in agreement with other reports in mammals (Lobie *et al.* 1990, Carlsson *et al.*

1993, Sharara & Nieman 1994, Kölle *et al.* 1998, Zhao *et al.* 2002). These results further strengthen the direct role of GH on follicular growth and cell differentiation of granulosa cells (Jia *et al.* 1986, Yoshimura *et al.* 1994), and possibly on steroidogenesis (Singh *et al.* 1988, Mason *et al.* 1990, Van der Kraak *et al.* 1990, Singh & Thomas 1993). Interestingly, strong signals were detected in the somatic cells surrounding the follicles of vitellogenic oocytes. It has been shown that distinct amounts of GH-R mRNA and protein were observed in the cumulus cells of the bovine ovary during all stages of follicular development (Kölle *et al.* 1998) and that GH accelerates oocyte maturation depending on the cumulus cells synthesizing GH-R (Izadyar *et al.* 1997).

To further analyze the GH-R gene expression profile during oogenesis, we have quantified the mRNA levels of GH-R and IGF-I in the liver and ovary at various stages of ovarian development. In the liver, there was no significant change in GH-R or IGF-I mRNA throughout oogenesis. Hepatic GH-R mRNA was positively correlated with hepatic IGF-I mRNA, but not with ovarian GH-R or IGF-I mRNA. It should be noted that we did not find a significant change in plasma IGF-I during ovarian

development. We have previously reported the positive correlation between plasma levels of IGF-I or liver IGF-I mRNA and somatic growth in the tilapia (Kajimura *et al.* 2001). Therefore, liver-derived IGF-I (i.e. circulating IGF-I) may not be a main contributor to ovarian development. To support our data, no significant change in GH-specific binding was reported during oogenesis in rainbow trout (Gomez *et al.* 1999). On the other hand, significantly higher levels of GH-R and IGF-I mRNA were found in the immature oocytes in agreement with our *in situ* hybridization study. A radioreceptor assay in rainbow trout indicated that the concentration of the GH-binding sites was highest during the early phases of follicular development (Gomez *et al.* 1999). We failed to find any significant changes in GH-R or IGF-I mRNA levels in the whole ovary. Since ovarian development in the tilapia is asynchronous and oocytes of different developmental stages are simultaneously found in the matured ovary, mRNA levels of GH-R or IGF-I in the whole ovary may not reflect the physiological status of each oocyte. In fact, we found relatively high error rates for mRNA levels in the whole ovary, which might repudiate what appeared to be potentially valid biological differences. Perrot *et al.* (2000) reported that high levels of IGF-I mRNA were found in the immature gonad; these were decreased along with gonadal development in the hermaphroditic gilthead seabream. Furthermore, IGF-I receptor was detected in the membrane of previtellogenic oocytes of this species. According to Yoshimura *et al.* (1994), an increase in follicle diameter by GH is correlated with intraovarian IGF-I contents in *in vitro* perfused rabbit ovaries, suggesting that the stimulatory effects of GH on follicular growth are mediated by ovarian IGF-I. These results indicate that paracrine/autocrine actions of the GH/IGF-I axis play an important role in the development or maintenance of the ovary in the early phases. In addition, IGF-I has also been known to regulate oocyte maturation and ovarian steroidogenesis in teleosts. Weber & Sullivan (2000, 2001) reported that IGF-I induced oocyte germinal vesicle breakdown (GVBD) in the striped bass (*Morone saxatilis*) in association with phosphatidylinositol 3-kinase activity. However, the source of IGF-I (i.e. circulating IGF-I or intraovarian IGF-I) that induces GVBD is not known.

The different gene expression profiles of GH-R and IGF-I in the liver and in the ovary suggest different regulatory mechanisms of GH-R and IGF-I gene expression in these tissues, possibly by using alternate promoters. In some mammalian species, such as rat and bovine, a splicing variant containing exon 1 (1B) is expressed in hepatic and extrahepatic tissues including the ovary, whereas another variant (1A) is mainly expressed in the liver (Heap *et al.* 1996). According to Baumbach & Bingham (1995), these 1A and 1B variants are differentially regulated by GH and sex steroids in the rat. The tissue-specific regulatory mechanism of

GH-R gene expression may account for the paracrine or autocrine actions of GH/IGF-I in the ovary independent of hepatic-derived IGF-I.

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