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.


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 & Klinger 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 sea trout (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 ± 2°C. They were fed twice daily with ProForm (Agro Pacific, Chilliwaec, 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, Tokyo, 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 (CLONTECH 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 (Strategene, 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 ATG GGT GTC TGC TGC TGC TCC CAC ACC CC-3’) and R4 (5’-CTC ATA CTC CAC AGT 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 72°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’-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 x 10^6 cells in 300 µl binding assay buffer (40 mM Tris–HCl, 10 mM CaCl_2 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 (PRL-188 and PRL-175). 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-35S]dCTP (Amersham Bioscience, Piscataway, NJ, USA). The specific activities were greater than 1 x 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 x SSC with 1% SDS at 65 °C, followed by a 10-min wash in 0·2 x 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 x 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’-GGT AAG GAG CAG ACT GTG ATG GCT GTC TAA CTC CTG CAG TGG TTC AGG AGT TAG ACA -3) nucleotide position 1757–1800) or a sense probe (5’- CAG TGG TTG AGG AGT TAG ACA GCC ATC ATC 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 x 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 humified chamber. Slides were then washed in 2 x SSC for 10 min at room temperature, followed by two washes in 1 x SSC for 30 min at 45 °C and in 2 x 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 GTC GTA CA-3′/5′-TGA GTT GCT GTC CAG GAG ACA-3′) and a Taqman probe (5′-CAAT GTT ATG CCA ACT GGT GTG GTC 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′-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 × 10−3; 6·4 copies) containing the amplified fragments of target mRNAs were prepared as standard samples. PCR reaction contained 0·2 µM of each primer and 0·2 µM Taqman probe, and 12·5 µl 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 µg total RNA.

**RIA**

Plasma levels of estradiol-17β (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 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 schlegelii (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 FGEGS 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...
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.
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
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 ± 0.08%); maturing, ovaries contained oocytes at the perinucleolus stage, the yolk vesicle stage, and the primary yolk globule stage (GSI: 1.50 ± 0.16%); matured, ovaries contained various stages of ovarian development including the secondary yolk globule stage (GSI: 2.93 ± 0.32%). Plasma E2 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
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
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Table 1 Changes in GSI, plasma levels of E \(_2\), GH, and IGF-I during ovarian development. Values are means ± s.e.m. (n=4–9)

<table>
<thead>
<tr>
<th>Index</th>
<th>Immature</th>
<th>Maturing</th>
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</tr>
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<tr>
<td>GSI (%)</td>
<td>0.52 ± 0.08(^a)</td>
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<td>Plasma E (_2) (ng/ml)</td>
<td>7.35 ± 2.35(^a)</td>
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<td>Plasma GH (ng/ml)</td>
<td>7.18 ± 5.12(^{1,2b})</td>
<td>3.03 ± 0.56(^a)</td>
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<tr>
<td>Plasma IGF-I (ng/ml)</td>
<td>17.47 ± 14.1(^a)</td>
<td>136.4 ± 18.2(^a)</td>
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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 of the tilapia. There was no correlation between hepatic IGF-I mRNA (Fig. 6D; \(R^2=0.008\)).

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

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\(^{1,2b}\) 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\(^a\) 0.13 ± 0.03\(^b\)

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

Table 2 Changes in mRNA levels of GH-R and IGF-I during ovarian development. Values are means ± s.e.m. (× 10\(^5\) copies/μg total RNA; n=4–9)

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GSI (%) 0.52 ± 0.08\(^a\) 1.50 ± 0.16\(^b\) 2.93 ± 0.32\(^c\)
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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 (VWEFI) 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. \(^{125}\)I-Labeled GH was displaced by homologous tilapia GH and slightly by PRL\(_{177}\), one of two homologous tilapia PRLs (PRL\(_{188}\) and PRL\(_{177}\)), but not by PRL\(_{188}\). The ED\(_{50}\) 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 PRL\(_{177}\) exhibits somatotropic actions by acting as a competitive ligand for GH-Rs on the hepatic membrane and promotes IGF-I gene expression in fresh water, where PRL\(_{177}\) reaches sufficient levels to displace GH-Rs (50-fold less than GH). In the present study, PRL\(_{177}\) 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 PRL\(_{177}\).

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...
by proteolytic 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, Carlson 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

![Figure 6](https://www.endocrinology.org/journals/journals/content/181/1/65/article-fig6.png)
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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