Production of recombinant channel catfish (*Ictalurus punctatus*) FSH and LH in S2 *Drosophila* cell line and an indication of their different actions

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Abstract

Due to the lack of purified, native gonadotropins (GtH) for almost all species of fish, we designed a system for the production of recombinant bioactive luteinizing hormone (LH) and follicle stimulating hormone (FSH) using the channel catfish (*Ictalurus punctatus*) as a model animal. The strategy was to produce the three subunits composing FSH and LH, i.e. the common α -subunit (α -glycoprotein hormone (α -GP)), β -FSH, and β -LH subunit, individually in stable recombinant insect cells (S2) with C-terminal His-tag. This expression system was also used to co-express the α -subunit without the His-tag with each of the His-tagged β -subunits. The recombinant S2 cells were capable of secreting FSH and LH heterodimers and α -GP in abundance; however, expression of the individual β -subunits was much less successful. The recombinant GtHs were partially purified from the cell

medium by immobilized metal affinity chromatography to ~15% purity with a yield of 7 and 4 mg per liter of medium for FSH and LH respectively. These recombinant GtHs activated their receptors *in vitro*, enhanced estrogen secretion, up-regulated several steroidogenic enzyme genes in channel catfish ovarian follicles, and increased androgen secretion from African catfish testis. Interestingly, the FSH and LH dose-response curves for each of these biological activities clearly demonstrate differences in their cellular action and physiological roles. This expression system may be an important development for the production of species-specific GtHs so that FSH- and LH-specific mechanisms of actions within the reproductive endocrine processes can finally be examined with homologous, albeit recombinant, hormones.

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Introduction

In fish, as in other vertebrates, gonadal steroidogenesis is controlled by the pituitary gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH; Gharib *et al.* 1990). FSH and LH belong to the glycoprotein hormone family, which consists of FSH, LH (and CG in certain mammals), and thyroid-stimulating hormone (TSH). They are all characterized by a heterodimeric structure, consisting of a common α -subunit (α -glycoprotein hormone (α -GP)) and a hormone specific β -subunit. The α - and β -subunits associate non-covalently and their assembly is obligatory for the biological activity of the hormones (Pierce & Parson 1981).

Although teleostean LH has been isolated from quite a few species, the identification of FSH in teleosts is a relatively recent advancement and has been isolated from only a few fish

species (Itoh *et al.* 1988, Suzuki *et al.* 1988, Swanson *et al.* 1991, Van Der Kraak *et al.* 1992, Copeland & Thomas 1993, Koide *et al.* 1993, Tanaka *et al.* 1993, Garcia-Hernandez *et al.* 1997). Unlike LH, teleostean FSH displays high diversity between species and therefore suggests that the use of heterologous FSH for studying its function may not be appropriate (Swanson & Dittman 1997). Although well studied in mammals, the precise role of each GtH in teleosts is not yet fully clear. As demonstrated with the salmonid model, it is believed that FSH plays a primary role in early stages of gonadal development (Swanson 1991, Campbell *et al.* 2003), whereas LH is linked to oocyte maturation and ovulation and to spermiation (Swanson *et al.* 1991, Slater *et al.* 1994, Kusakabe *et al.* 2006).

Mammalian recombinant GtHs have been successfully produced and are extensively used for endocrine studies

(Amano & Kobata 1993, Szkudlinski *et al.* 1996, Ben-Menahem *et al.* 1997) as well as for pharmacological applications (Lathi & Milki 2001). Although the cDNAs encoding the α -GP, β -FSH, and β -LH have been isolated from a growing number of teleosts (Schulz *et al.* 2001), the production of recombinant fish GtH has been far less successful. There are reports of the production of biologically active recombinant fish GtHs (Kamei *et al.* 2003, Vischer *et al.* 2003, Aizen *et al.* 2007, Cui *et al.* 2007, Ko *et al.* 2007); however, an efficient expression technology that addresses all aspects, such as i) biological activity of the resultant recombinant GtHs, ii) productivity in high abundance, and iii) easy protocol of purification, has not been developed for teleosts GtHs.

We have addressed these problems by producing recombinant channel catfish (*Ictalurus punctatus*; *Ip*) FSH and LH in a modification of an insect cell (S2) expression system (Drosophila Expression System; Invitrogen). This recombinant protein expression system is based on cells derived from a higher eukaryotic (albeit invertebrate); therefore, they possess the capacity for appropriate post-translational modifications, such as glycosylation.

Materials and Methods

Construction of expression vectors

The cDNAs encoding the entire subunits (secretory signal peptide and the mature peptide) of GtHs, α -GP, β -LH, and β -FSH were amplified by PCR from reverse-transcribed RNA isolated from a channel catfish (*I. punctatus*) pituitary. GtH-specific primers (based on the sequence published in Liu *et al.* 1997; GenBank #AF112191 and AF112192) were designed to eliminate the termination codon and insert *Age*I restriction sites. Table 1 provides the sequence of all primers used in this report including the primers for the α -GP (primers 1 and 2), β -LH (primers 3 and 4), and β -FSH (primers 5 and 6). PCR amplification was performed using the Advantage 2 PCR kit (BD Biosciences, Palo Alto, CA, USA) under the following conditions: 3-min denaturation at 94 °C followed by 32 cycles of 94 °C for 30 s, 55 °C for 30 s,

and 72 °C for 30 s, and 10-min elongation at 72 °C. Each amplicon was ligated into a pDrive vector (Qiagen), released by *Eco*RI and *Age*I restriction digest, and subcloned into a similarly digested pMT/V5-His (pMT) expression vector (DES kit, Invitrogen), thereby generating an in-frame hexahistidiny fusion (His-tag) at the C-terminus (Fig. 1A). These constructs were labeled pMT α -His, pMT β FSH-His, and pMT β LH-His. Expression by pMT vectors is driven by the divalent metal inducible metallothionein promoter.

The expression of α -GP/ β -FSH and α -GP/ β -LH heterodimeric hormones was encoded by a modified pMT/V5-His vector engineered to contain two inducible promoters for the simultaneous expression of the two subunits (pMT α / β FSH and pMT α / β LH). In order to avoid potential steric hindrance by the His-tag, the α -GP amplicon (using primers 1 and 7; Table 1) for these constructs was generated with its native stop codon intact thus lacking the His-tag fusion. This α -GP amplicon was inserted, as described above, into a pMT vector in order to generate the pMT α construct. In another PCR amplification using primers 8 and 9 (Table 1), a high-fidelity DNA polymerase (Herculase, Stratagene, La Jolla, CA, USA) and pMT β FSH-His or pMT β LH-His as template, an amplicon was generated containing the sequence for the metallothionein promoter, the β -subunit, the His-tag, and the termination signal. After digestion with *Nar* I and *Aat* II, these amplicons were ligated separately into a similarly digested pMT α vector constructs (Fig. 1B).

Production and purification of recombinant proteins

Transfection, selection, maintenance, and induction of expression of the Schneider's S2 cell line were performed according to the manufacturer's protocol (Drosophila Expression System, DES, Invitrogen). Described in brief, each of the GtH constructs were co-transfected with the pCoBlast selection vector (included in the kit) into S2 cells using Effectene transfection reagent (Qiagen). Transfected cells were selected for a period of 2 weeks in a 'complete medium' composed of Schneider Insect Medium (Sigma Chemicals) supplemented with Yeastolate 10 000 molecular weight cut-off (MWCO) Ultrafiltrate, chemically defined

Table 1 Oligonucleotide primers used for PCR amplification of the three *Ip* gonadotropin subunits and for real-time quantitative RT-PCR assays. Introduced restriction sites are underlined. F, sense primer; R, antisense primer

Primer no.	Name and direction	Sequence
1	<i>Ip</i> - α -F	5' GAGGAAGAAGAGAAGACTGTCT 3'
2	<i>Ip</i> - α -R	5' <u>GAACCGGT</u> AAATTTATGATAGTAACAAGTGCTGCAAT 3'
3	<i>Ip</i> -LH-F	5' CA AA ATCTCTAAAGATGTCAGTGCC 3'
4	<i>Ip</i> -LH-R	5' GAACCGGTGTAATCCAGGATGTA CT CTTTCTG 3'
5	<i>Ip</i> -FSH-F	5' GAATTCGGCAGCAGGGTGAT 3'
6	<i>Ip</i> -FSH-R	5' <u>CTTACCGGT</u> GTAGTAGGCGTGTGTGTGG3'
7	<i>Ip</i> - α stop-R	5' <u>GAACCGGT</u> CAAAATTTATGATAGTAACAAGTGC 3'
8	pMT-F	5' GAGGCGCGGTTCAGGACAGGATGT 3'
9	pMT-R	5' <u>CTGACGTC</u> TGTGTGTGTTAACTTGTT 3'

Figure 1 Schematic representation of a portion of the expression vector constructs. (A) Expression elements of the pMT α -His, pMT β FSH-His, and pMT β LH-His vectors. (B) Expression elements of the dual promoter pMT α / β FSH and pMT α / β LH transfer vectors. Hatched box, metallothionein promoter; solid box, gonadotropin subunit cDNA; gray box, His-tag fusion; and open box, termination signal.

Lipid Concentrate (both from Life Technologies, Gaithersburg, MD, USA), blasticidin S (25 μ g/ml; Invitrogen), and 10% heat-inactivated fetal calf serum (FCS). After selection and expansion, the cells were transferred to a baffled shake flask in complete medium containing a reduced concentration of blasticidin (10 μ g/ml) and grown to a density of $20\text{--}30 \times 10^6$ cells/ml in a volume of 500 ml. At this point, the complete medium was replaced with 1 l serum-free, antibiotic-free medium (complete medium lacking the FCS and Blasticidin). After an adaptation period of 3 days (cell density of 15×10^6 cells/ml), protein expression was induced by the addition of CuSO_4 (500 μ M). After 4 days, the medium was harvested, clarified by centrifugation, sequentially filtered through 100 000 MWCO and 10 000 MWCO ultrafiltration membranes (Millipore Corporation, Bedford, MA, USA). The retentates from both ultrafiltration steps were passed through an immobilized metal affinity chromatography (IMAC) column (Talon metal affinity; Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. In order to produce highly purified *Ip* LH and FSH, IMAC-purified *rec*-GtHs were further subjected either to size-exclusion HPLC using Superdex 75 column equilibrated with 250 mM Tris-HCl, pH 7.2 (AKTA 10S, Amersham) or were subjected to ethanol precipitation for preparation of the glycoprotein fraction as described by Mananos *et al.* (1997).

Gel electrophoresis and western blot analysis

PAGE was performed using 4–20% gradient denaturing gels or 12% non-denaturing gels (Bio-Rad). Tris/glycine/SDS running buffer (Bio-Rad) was used for denaturing electrophoresis, and the same buffer lacking the SDS was used for non-denaturing electrophoresis. Abundant proteins were visualized with gelCode Blue stain (Pierce, Rockford, IL, USA).

Proteins in non-stained gels were electrophoretically transferred onto Nytran membrane (Schleicher & Schuell, Keene, NH, USA) in preparation for immunoblotting. The membranes were incubated with either rabbit anti-African

catfish α -GP (1:10 000) or with rabbit anti-striped bass β -LH in TBS-T buffer containing 5% skimmed milk powder, washed, and incubated with 1:5000 goat anti-rabbit IgG-HRP conjugate (Bio-Rad). The *rec*-GtHs were also analyzed by western blot using His-tag-specific INDIA-HisProbe-HRP (Pierce) following the manufacturer's instruction. The luminescent signal emitted by the Super Signal West Pico substrate (Pierce) was detected with X-ray film.

In order to estimate the purity of IMAC-purified *rec*-FSH or *rec*-LH, the proteins were resolved on a non-denaturing PAGE (see Fig. 3 Ba), stained, and scanned using an Epson perfection 3200 scanner (Epson, Long Beach, CA, USA). The bands were quantified densitometrically using a PC-based image analysis system and a program developed in the KS400 version 3.0 software package (Carl Zeiss Vision, Göttingen, Germany). The bands were delimited using a threshold-dependent gray level of the local neighborhood area, before the integrated optical density of each band of interest was determined separately, and then expressed as percentage of the total optical density of the respective lane.

Receptor binding and activation

Recombinant *Ip* FSH receptor (*rec*-FSH-R) and LH receptor (*rec*-LH-R) transiently expressed in COS cells were previously demonstrated to respond to heterologous FSH and LH (Kumar *et al.* 2001a,b). Using this system, COS cells, expressing *rec*-FSH-R and *rec*-LH-R along with a cAMP-responsive luciferase reporter system, were incubated for 6 h with IMAC-purified *Ip* *rec*-LH and *rec*-FSH (0–200 ng/ml) in order to determine their ability to bind and activate the receptors. Activation of the receptors was indirectly quantified by luciferase activity (Luciferase Assay kit; Promega Corporation) using a MicroLumat LB96P luminometer (EG&G Berthold, Stammwerk Wilbad, Germany).

In vitro hormonal treatment of channel catfish ovarian follicles with *rec*-GtH

The biological activity of the *rec*-LH and *rec*-FSH was further tested for their ability to induce E_2 secretion from *Ip* ovarian follicles *in vitro*. Ovarian follicles (100 mg) from a catfish ovary with a GSI of 8 (i.e. in late or post-vitellogenic growth phase) were incubated in 1 ml of 75% L-15 medium supplemented with HEPES (15 mM; pH 7.4) and various concentrations of IMAC-purified *rec*-LH and *rec*-FSH (0.2–150 ng/ml) in triplicates. After 15 h of incubation, the E_2 concentration in the medium was determined by specific RIA (DPC, Los-Angeles, CA, USA) and the abundance of the ovarian aromatase (CYP19, P450arom) transcript in the tissues was also determined by real-time quantitative RT-PCR (rtqRT-PCR; Kumar *et al.* 2000). In order to further clarify the bioactivity of *Ip* *rec*-GtHs and to compare the activity with that of hCG that has often been used to investigate the action of GtH in fish, ovarian follicles at the post-vitellogenic stage

(GSI=12) were incubated with either *Ip rec*-GtHs (100 ng/ml) or human chorionic gonadotropin (hCG; 30 IU/ml). Changes in the expression of genes associated with reproduction, i.e., four steroidogenic enzymes (P450_{scc}, P450_{c17}, P450_{arom}, and 3 β -HSD; Kumar *et al.* 2000) and two GtH receptors (FSH-R and LH-R; Kumar *et al.* 2001a,b) were determined by rtqRT-PCR. Ovarian tissues were flash frozen and stored at -80°C until analyses. The procedures for the care and use of the catfish were approved by the Center's and University's IACUC.

In vitro hormonal treatment of African catfish testis with Ip rec-GtH

Using the established method in African catfish (Vischer *et al.* 2003), the bioactivity of GtH in testicular steroidogenesis was examined. In brief, for each experiment testis, tissue was collected from three fully mature males of 12–14 months of age. The tissue was pooled in a Petri dish containing Dulbecco's Modified Eagle's medium (DMEM)–HEPES (Sigma) supplemented with L-glutamine (8 mM), and penicillin G and streptomycin (100 U/ml each; all from Invitrogen). While submerged in medium, the tissue was minced into fragments of 2–3 mm³. The fragments were filtered over two layers of cheesecloth, pre-incubated in fresh medium in a shaking incubator at 25 °C for 30 min, and filtered again over two layers of cheesecloth, to remove suspended sperm and tissue debris. Four or five randomly selected fragments (total wet weight 20–30 mg) were placed per well of a 24-well culture plate containing 0.2 ml medium. Six replicates per ligand concentration were tested in dose–response experiments. The tissue fragments were incubated at 25 °C for 18 h in the presence of varying concentrations of IMAC-purified recombinant hormones as indicated. The incubation medium was then collected, heated at 80 °C for 1 h, and centrifuged at 16 000g at room temperature for 30 min. The supernatants were stored at 20 °C until the secreted androgen (4-androsten-11 β -ol-3, 17-dione; OHA) levels were determined by RIA as described previously (Schulz *et al.* 1996). OHA is the quantitatively dominating androgen secreted by African catfish testis (Vermeulen *et al.* 1993). The results are expressed as nanograms of OHA secreted per milligram of testis tissue incubated.

Measurement of transcript abundance of genes in i.p. ovarian follicles

One microgram of total RNA was extracted from ovarian samples using Trizol Reagent (Life Technologies) and reverse transcribed using random hexamer primers. Analyses by rtqRT-PCR assays (Kumar *et al.* 2000, 2001a,b) were performed in duplicate 20 μl PCRs using SYBR Green Core Reagents (PE Applied Biosystems, Foster city, CA, USA) and an ABI Sequence Detector 7700 to determine the transcript abundance of target genes, specifically, four steroidogenic enzymes and the two GtH receptors. Abundance of the gene transcript was normalized to that of β -actin (Kumar & Trant 2004) and

reported as a fold change in abundance relative to the sample with the lowest level of expression.

Statistical analysis

All numerical data are presented as means $\pm$ S.E.M. Statistical differences to control were determined by one-way ANOVA followed by Fisher's PLSD *post hoc* test.

Results

The following results clearly show that biologically active recombinant catfish FSH and LH can be produced in abundance using a modification of a commercially available *Drosophila* cell expression system. The resultant recombinant proteins were significantly purified by IMAC and size-exclusion chromatography, and biological activity of IMAC-purified *Ip rec*-GtHs suggests important endocrine differences in their mode of action and physiological roles.

Production of Ip α -GtH, β -LH, β -FSH subunits

The cDNAs encoding the three different GtH subunits were initially introduced individually into the pMT/V5-His vector and expressed in S2 cells. During the selection period, cells seemed to grow normally and were capable of expressing the appropriate subunits as determined by western blot analysis using a specific anti-His probe and/or specific antibodies to

Figure 2 4–20% SDS-PAGE analysis of IMAC purified *Ip* GtH subunits (β -FSH, β -LH, α -GP) isolated from conditioned medium of S2 cells transfected with vectors encoding individual subunits. Molecular weight markers (kDa) and the expected location of each subunit (arrows) are indicated.

subunit proteins, i.e. anti-African catfish α -GP (1:10 000) and anti-striped bass β -LH (data not shown). However, upon continued culture of the recombinant cells expressing the β -LH and β -FSH subunits alone, significant cell death resulted and marginal amounts of recombinant protein were detected. SDS-PAGE analysis of the IMAC-purified proteins revealed that α -GP, at the expected size of ~ 15 kDa, abundantly accumulated in the medium, while the β -LH and β -FSH subunits, at the size of 21 and 20 kDa respectively, were poorly produced (Fig. 2). The β -subunits were undetectable in cell extracts (data not shown), suggesting that the β -subunits were either intrinsically poorly expressed, the β -monomers were unstable, or the β -peptides were toxic to the cell.

The strategy of producing both α - and β -subunits simultaneously in the same cell as mediated by the dual promoter vectors, i.e. pMT α / β FSH and pMT α / β LH, significantly improved yields. Two major bands matching the expected sizes of α -GP and β -FSH or β -LH were visualized by protein stain after SDS-PAGE of IMAC-purified GtHs (Fig. 3Aa). It has been confirmed that these bands were not observed in the IMAC-purified protein from culture media of S2 cells transfected with the empty vector (data not shown). Western blot analysis of the proteins using antiserum raised against the α -GP of the African catfish confirmed the identity of the abundant 15 kDa protein as recombinant α -GP subunit (Fig. 3Ab). The identity of the 20 and 21 kDa protein signals were confirmed for FSH and LH respectively by western blot for detection of His-tagged protein (Fig. 3Ac). Furthermore, an antiserum against the β -LH of the striped bass also clearly recognized the 21 kDa protein (data not shown), while, due to the lack of antiserum cross-reacting with catfish β -FSH, the immunochemical identification of the 20 kDa protein was not confirmed as β -FSH.

Non-denaturing acrylamide gel electrophoresis was used to evaluate the association of the native recombinant proteins. The most abundant stained protein was the larger of the two and one major bands in the *rec*-FSH and *rec*-LH sample respectively (Fig. 3Ba). Western blot analysis revealed that the anti- α -GP recognizes the largest major band of *rec*-FSH and LH preparations (Fig. 3Bb). Furthermore, the identical larger protein of the *rec*-LH was labeled with the anti- β -LH, indicating that the protein is a heterodimer consisting of both α -GP and β -LH subunits (Fig. 3Bc). The smaller protein may be free β -LH (Fig. 3Bc). It should be noted that the anti-striped bass LH β reacted with a protein pituitary extract of catfish in a native PAGE, at a position similar to that of the higher molecular weight band of *Ip rec*-LH, the putative heterodimeric LH (supplementary data; see supplementary figure in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol194/issue2>). There was no evidence of free α -GP in either the *rec*-LH or the *rec*-FSH preparations (Fig. 3Bb). Western blot analysis using His-probing was also carried out for the *rec*-GtHs under native conditions; however, specific signals could not be visualized (data not shown).

The purity of the IMAC prepared *rec*-FSH and *rec*-LH was densitometrically determined to be 15 and 16% respectively (native PAGE analysis). In combination with a total protein Lowery assay, the yield of IMAC-purified, heterodimeric hormone from each of two production trials was estimated to be 6 and 7 mg/l of culture medium for *rec*-FSH and 4 and 3 mg/l for *rec*-LH. Additionally, a small portion of the IMAC-purified hormone preparations was further purified by size-exclusion chromatography with Superdex 75 or ethanol precipitation of the glycoprotein fraction. SDS-PAGE followed by gelCode blue stain of the resultant *rec*-GtHs visualized only two protein bands corresponding to

Figure 3 PAGE and immunoblot analyses of the IMAC-purified *rec*-FSH and *rec*-LH isolated from conditioned medium of S2 cells transfected with the dual promoter vectors encoding both the α - and the respective β -subunit. (A) Denaturing SDS-PAGE; (B) native PAGE. a: Total proteins (protein stain); b: immunoblot with anti-African catfish α -GP; c: western blot with His-probe; d: immunoblot with anti-striped bass β -LH. Asterisks point to the putative subunits (A) or the heterodimers (B) of *rec*-GtHs.

α - and β -subunits (data not shown). The resultant yield of the highly purified *rec*-GtHs was below the detection limit of the protein assay; therefore, the readily quantifiable IMAC-purified preparations were used to demonstrate bioactivity of these preparations (see below).

Receptor binding and activation of cAMP production in vitro

An efficient functional expression system in COS cells for channel catfish *rec*-FSH receptor (FSH-R) and *rec*-LH-R was previously developed (Kumar *et al.* 2001a, b). In these earlier studies, it was shown that heterologous FSH and LH activated the respective catfish GtH receptors when produced in COS cells. We used this system to verify the biological activity of *Ip rec*-FSH and *rec*-LH preparations. As seen in Fig. 4A, *rec*-FSH efficiently activated FSH-R in a dose-dependent manner. A concentration of 50 ng/ml total protein of the IMAC-purified preparation (an estimated 7.5 ng/ml *rec*-FSH) was sufficient to induce cAMP-dependent luciferase expression by sixfolds and 100 ng/ml total protein (estimated 15 ng/ml *rec*-FSH) resulted in a maximal induction of 7.6-fold above control. As found by us before, *rec*-LH also activated

the FSH-R; however, it was 1.8- to 2.8-fold less potent than *rec*-FSH at all concentrations tested (Fig. 4A). Again, as found by us before, *rec*-LH, but not *rec*-FSH, activated the LH-R. A concentration of 50 ng/ml total protein of the IMAC preparation (estimated 7.5 ng/ml *rec*-LH) induced a 1.3-fold increase in the activity of the reporter gene and 100 ng/ml total protein (15 ng/ml *rec*-LH) resulted in a maximal stimulation of 2.5-fold (Fig. 4B).

Bioactivity of *rec*-LH and *rec*-FSH in catfish gonads

In order to determine whether *rec*-GtH preparations were also active when incubated with their homologous target tissue and to verify possible differential bioactivity of FSH and LH, we tested their ability to induce steroidogenesis in *Ip* ovarian follicles and African catfish testis. The dose-dependent effect of the *rec*-FSH and *rec*-LH preparations on steroidogenesis in vitellogenic *Ip* follicles was determined by the changes in E₂ secretion and P450arom gene regulation (transcript abundance). As seen in Fig. 5A, both LH and FSH induced E₂ secretion in a dose-dependent manner. In follicles of this

Figure 4 Activation of *Ip* FSH-R (A) and LH-R (B) expressed in COS cells by the *rec*-GtHs. An increase in the relative light units (means $\pm$ S.E.M.) produced by the cells that were transiently co-transfected with a cAMP responsive reporter gene (luciferase) and *Ip* FSH-R or *Ip* LH-R constructs is due to a GtH-mediated increase of cAMP. Statistical analysis: one-way ANOVA followed by Fisher's PLSD *post hoc* test. * $P < 0.05$, ** $P < 0.01$.

Figure 5 Concentration response of *Ip* oocytes in the mid-vitellogenic growth phase to *Ip rec*-FSH and *Ip rec*-LH. Changes in estradiol (E₂) concentration (A) and aromatase gene expression (B); transcript abundance) are presented as mean $\pm$ S.E.M. of triplicate observations. Statistical analysis: one-way ANOVA followed by Fisher's PLSD *post hoc* test. * $P < 0.05$, ** $P < 0.01$.

reproductive stage, *rec*-LH was more potent than *rec*-FSH. A dose of 10 ng/ml total protein (1.5 ng/ml *rec*-LH) significantly increased E_2 secretion by 150% and a maximal increase of 280% was obtained with 120 ng/ml total protein (18 ng/ml *rec*-LH; Fig. 5A). The *rec*-FSH significantly induced E_2 secretion by 240% at a relatively high dose of 120 ng/ml total protein (18 ng/ml *rec*-FSH; Fig. 5A). P450arom expression in the vitellogenic ovarian tissues was readily up-regulated threefold at a dose of 10 ng/ml total protein (1.5 ng/ml *rec*-LH); however, only moderate increases in the transcription was evident with further increases of *rec*-LH concentration (Fig. 5B). P450arom was significantly up-regulated with a minimum concentration of 60 ng/ml IMAC preparation (9 ng/ml *rec* FSH) and could be maximally induced to 720% above control (Fig. 5B).

Both IMAC-purified *rec*-GtHs induced the release of OHA (the primary testicular androgen) from African catfish

testis in a dose-dependent manner (Fig. 6). At concentrations lower than ~15 ng GtH/ml (100 ng/ml IMAC protein), the steroidogenic activity of *rec*-FSH seems to be higher than that of *rec*-LH; however, these *rec*-GtHs behaved similarly at the higher doses (> 45 ng GtH/ml; 300 ng/ml IMAC protein).

For further assurance of the bioactivity of the *rec*-GtHs, the regulation of the genes associated with ovarian reproductive physiology, i.e. four key steroidogenic enzymes and the two GtH receptors, were examined in ovarian follicles at or near the post-vitellogenic stage of gametogenesis (Fig. 7). Treatment with *rec*-LH up-regulated the expression of all genes except FSH-R, whereas hCG induced only P450scc, P450arom, and 3 β -HSD (Fig. 7). In contrast, *rec*-FSH treatment enhanced transcriptional activity for 3 β -HSD gene only, although the transcript abundance of P450scc and P450arom tended to be higher than that of the control group. It also should be noted that the average Ct values (number of PCR cycles required for the signal to surpass a set threshold) for actin transcript abundance were not

Figure 6 Concentration response of African catfish testis to *lp rec*-FSH and *lp rec*-LH. Changes in OHA concentration in the media are presented as mean $\pm$ S.E.M. of triplicate observations. Note the log scale of the axis for GtH concentration. Statistical analysis: one-way ANOVA followed by Fisher's PLSD *post hoc* test. * $P < 0.05$, ** $P < 0.01$.

Figure 7 Hormonal regulation of gene expression of four steroidogenic enzymes and the two GtH-Rs by *lp rec*-GtHs and hCG. Changes in transcript abundances of the genes are presented as mean $\pm$ S.E.M. of triplicate observations. Statistical analysis: one-way ANOVA followed by Fisher's PLSD *post hoc* test. * $P < 0.05$, ** $P < 0.01$.

statistically different between the experimental groups compared here.

Discussion

In this study, an efficient protocol for the abundant production of biologically active recombinant channel catfish gonadotropins was developed utilizing a modification of a commercially available eukaryotic expression cell system. The recombinant *Drosophila* S2 cells were grown to high density in a suspension culture of a relatively inexpensive, supplemented Schneider's Medium. Expression was induced in a similarly formulated serum-free medium. Fortunately, fusion of the His-tag onto the β -subunits (but not the α -subunit) did not sterically hinder dimerization or receptor binding yet it remained available for IMAC purification. The insect cells presumably recognized the teleostean secretory signal of the GtH subunits since the GtHs were found exclusively in the medium. Although we have no data demonstrating the type or degree of glycosylation of *rec*-GtHs, the structural features of recombinant proteins were compatible with heterodimerization of the α - and β -subunits in a manner that allowed the formation of proteins recognized by and able to activate their cognate receptors. Although both recombinant GtHs stimulated ovarian steroidogenesis, there appear to be important differences in the intrinsic response elicited by both hormones. Due to the lack of contamination of a recombinant GtH with the reciprocal GtH or TSH, which is problematic with purified preparations of native GtH derived from pituitaries (Pierce & Parsons 1981), these GtHs and this methodology will provide the required reagents to address questions about hormone-specific actions throughout a reproductive cycle in specific target tissues.

Of the three subunits expressed independently, only the α -GP subunit was successfully produced in abundance as a single subunit (Fig. 2). However, co-expression of the α -GP subunit with a significant β -FSH or β -LH subunit resulted in the significantly greater yield. Similarly, it has been demonstrated that transfected CHO cells readily secreted ovine α -GP but not the β -FSH subunit and co-expression of both subunits led to heterodimer assembly and efficient secretion (Mountford *et al.* 1994).

Densitometric analysis showed that hormone contents in the IMAC-purified *rec*-GtHs were $\sim 15\%$, resulting in a recovery of 7 mg *rec*-FSH and 4 mg *rec*-LH per liter of medium by a simple three-step purification system (i.e. two ultrafiltration steps for buffer exchange followed by passage through a single IMAC column). These results are significantly greater than the yield from other heterologous expression systems such as the soil amoeba (< 80 ng/ml; Vischer *et al.* 2003) for GtH production of another catfish species, African catfish. The only expression system of fish GtH with comparable yield and purity to S2 cell system seems to be silkworm larvae (60–180 μ g/larvae; Ko *et al.* 2007). Further optimization of purification protocol will certainly

lead to enhance hormone recovery and purity. Although CHO cells were reported to be relatively efficient when expressing rat FSH (2 μ g/ml; Hakola *et al.* 1996) and LH (1 μ g/ml; Hakola *et al.* 1997), this expression system has proven to be much less efficient with regards to the production of fish GtHs (Kumar & Trant, unpublished data).

Immunoblots of electrophoresed proteins clearly showed that both α - and β -subunits were simultaneously produced by the S2 cells transfected with the dual promoter vectors (Fig. 3). In addition, the formation of LH heterodimer was demonstrated by the recognition of the same proteins by both anti- α -GP and anti- β -LH antibodies in blots of the native PAGE gels. Even without an antibody against the β -FSH subunit, indirect proof for the heterodimerization of FSH is provided by the immunovisualization of the proteins (the one upper band in Fig. 3Ba) in the IMAC-purified *rec*-FSH preparation by the anti- α -GP antibody. Since the *rec*- α -GP encoded by this set of dual-promotor vectors does not contain a His-tag (and therefore is unlikely to bind to the IMAC matrix), the retention of the visualized α -GP protein is likely due to its association with the His-tagged β -FSH subunit.

In the established reporter assay using recombinant catfish FSH-R and LH-R, both *rec*-GtHs were shown to activate their corresponding receptor. In addition, it should be noted that the FSH-R was activated by both *rec*-FSH and *rec*-LH (Fig. 4) as previously reported using mammalian GtHs (Kumar *et al.* 2001a,b). The possibility that His-tagging with the β -subunit interfered with the recombinant GtH's receptor specificity and/or affinity could not be ruled out. However, LH-mediated activation of the FSH-R by the recombinant protein without His-tag or by native pituitary LH has been reported in several fish species including the zebrafish (So *et al.* 2005), African catfish (Vischer *et al.* 2003), and salmon (Swanson & Dittman 1997). We have also confirmed that the IMAC-purified protein from culture media of control cells (S2 cells stably transfected with the empty pMT) is incapable of activating either FSH-R or LH-R (Japanese eel receptors; data not shown).

To verify the biological activity of *rec*-GtHs in their natural target tissue, their ability to induce E_2 secretion and gene expression of P450arom was examined using the ovarian follicles in mid-vitellogenic growth. Both parameters were significantly induced essentially in a dose-dependent manner. These data suggest that enhanced steroid production is due to the up-regulation of P450arom gene and possibly other steroidogenic enzymes. However, the dose-response curves, the potency, and the maximal induction with *rec*-FSH and *rec*-LH varied, thereby suggesting a differential action of FSH and LH on steroidogenesis in catfish ovary at least in the vitellogenic stage of development. Interestingly, stimulation of androgen release by the African catfish testis also showed differential effects, particularly in the lower doses range where *rec*-FSH was more potent than *rec*-LH. Although no information is available yet on the molecular and cell biological basis for this strong steroidogenic activity of FSH, this aspect of piscine FSH bioactivity has been described

previously in other teleost species (e.g. Planas & Swanson 1995). As a further support, FSH was shown to have a role in moderating basal and LH-stimulated androgen production, where circulating androgen levels and the responsiveness to exogenous LH were reduced due to a loss of FSH receptor function in mice (Krishnamurthy *et al.* 2001). Furthermore, it has also been confirmed that the concentration of eel FSH to activate the FSH receptor is much lower than that of eel LH to activate the LH receptor using homologous recombinant GtHs produced by S2 cells (data not shown), which reinforces the hypothesis of differential actions of FSH and LH on the gonadal steroidogenesis in fish.

Further examination of the hormonal regulation of the genes encoding the steroidogenic enzymes and the GtH receptors in ovarian follicles in the post-vitellogenic stage of development using the *rec*-GtHs and a heterologous GtH, hCG, revealed differential actions of these gonadotropic reagents on the ovary. The effects of *rec*-LH and hCG were similar but with important differences, such as P450c17 and LH-R genes were up-regulated by the *rec*-LH but not hCG. It was demonstrated in African catfish that recombinant LH, but not hCG, activated both FSH-R and LH-R (Bogerd *et al.* 2001). In addition, even though hCG activated FSH-R in channel catfish (Kumar *et al.* 2001b), the concentration for FSH-R activation was over 100 IU/ml (~10 g/ml) that is much higher than the effective concentration of *Ip rec*-LH, 50 ng/ml total protein of the IMAC-purified *rec*-LH (an estimated 7.5 ng/ml *rec*-LH). Therefore, at least, the affinity and specificity of the *rec*-LH to the GtH-Rs appear to be different from that of hCG. This finding suggests that studies using hCG with fish models may have yielded inappropriate conclusions. This conclusion is significant because hCG is often used as a 'standard' LH in fish systems.

In contrast to *rec*-LH, treatment with *rec*-FSH was essentially incapable of modulating transcriptional activity of these genes (with the exception of 3 β -HSD) in post-vitellogenic follicles. Since P450arom expression was stimulated with *rec*-FSH in follicles in the vitellogenic growth stage (Fig. 5), it is clear that the FSH-mediated regulation of P450arom (and likely the other genes as well) is dependent on the developmental stages of the ovarian follicles. It has been demonstrated in adult catfish that the FSH-R expression is elevated in association with the onset of ovarian recrudescence and decreases prior to spawning (Kumar *et al.* 2001b). Therefore, the stage-dependent modulation of P450arom gene by FSH may be partially due to the abundance of the receptor.

In summary, a method was developed to abundantly produce bioactive recombinant channel catfish FSH and LH that has been engineered to facilitate purification. Presumably, the same strategy can be used with other species to generate wild-type GtHs (for endocrine studies, aquacultural manipulations, or antibody production) and GtHs with site-specific mutations (for structure-function studies). As with many species of fish, isolation of native FSH from pituitaries has been unsuccessful, therefore this methodology may be the sole mechanism to generate useful quantities of this hormone.

Furthermore, the ability to generate *rec*-LH and *rec*-FSH for fish species in which these GtHs are not available will enable precise examinations of the mechanisms by which target tissues respond and dissect the functional duality of the FSH and LH system. The function and interaction of GtHs in more fish models can finally be examined with species-specific reagents.

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