Cloning and functional characterization of a testicular TSH receptor cDNA from the African catfish (Clarias gariepinus)

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Abstract
A cDNA encoding a putative thyroid-stimulating hormone receptor (cfTSH-R) was cloned from the testis of the African catfish (Clarias gariepinus). The cfTSH-R showed the highest amino acid sequence identity with the TSH-Rs of other fish species. In addition, an insertion of approximately 50 amino acids, specific for the TSH-R subfamily, was also present in the carboxy terminus of the amino-terminal extracellular domain of the cfTSH-R. Next to the testis and thyroid follicles, abundant cfTSH-R expression was detected in cerebellum, brain, ovary, seminal vesicles and pituitary, while weaker expression was found in muscle, stomach, intestine, head-kidney, liver, kidney and heart. HEK-T 293 cells, transiently expressing the cfTSH-R, significantly increased intracellular cAMP levels in response to human TSH. Catfish LH, human choriogonadotropin and human FSH were also able to induce this cfTSH-R-mediated response, although with considerably lower efficiency than human TSH. These results indicated that a functional cfTSH-R had been cloned from the testis of African catfish.

Journal of Molecular Endocrinology (2003) 30, 227–238

Introduction
In fish, as in other vertebrates, the pituitary-derived thyroid-stimulating hormone (TSH) and gonadotropins – luteinizing hormone (LH) and follicle-stimulating hormone (FSH) – are essential for differentiation, growth and functional regulation of thyroid follicles and gonads respectively. These glycoprotein hormones act on their target tissues via their respective cell membrane receptors. The receptors for TSH, FSH and LH (TSH-R, FSH-R and LH-R respectively) belong to the superfamily of G protein-coupled receptors, and constitute, together with a restricted number of structurally homologous, invertebrate and vertebrate orphan receptors, the subfamily of leucine-rich repeat-containing G protein-coupled receptors (LGR) (Hsu et al. 2000). LGRs are characterized by the presence of multiple leucine-rich repeat (LRR) motifs in their relatively large N-terminal extracellular domains, which have been postulated to adopt a horseshoe-shaped conformation to which their respective (glycoprotein) hormones can bind (Kajava et al. 1995, Bhowmick et al. 1996).

In fish, the biological activity of TSH is primarily directed to thyroid follicles, which are dispersely situated in the basibranchial region – within the connective tissue on the surface of the ventral aorta – in contrast to the situation in mammals, where the thyroid follicles are encapsulated into a gland. Moreover, extra-thyroidal TSH-R expression has been reported for several species (e.g. Kumar et al. 2000, Crisanti et al. 2001). In our attempts to clone the testicular cDNA coding for the catfish LH-R (cfLH-R; Vischer & Bogerd 2003), we unexpectedly isolated a catfish TSH-R (cfTSH-R) cDNA fragment.

Here we report the isolation and functional characterization of the full-length cfTSH-R cDNA and demonstrate its extra-thyroidal expression in numerous tissues of the African catfish.
Materials and methods

Animals

African catfish used in the cloning and tissue distribution experiments were bred and raised in the laboratory as previously described (De Leeuw et al. 1985), except that catfish pituitary extract instead of human chorionic gonadotropin (hCG) was used to induce ovulation. All tissue and sperm samples used in the present study for RNA and genomic DNA isolation were collected from (10- to 12-month-old) sexually mature catfish as regards spermatogenesis (i.e. spermatozoa present in many tubuli). Fish raised in captivity do not differ in testis size or histology from wild fish collected from spawning grounds (Van Oordt et al. 1987, Schulz et al. 1994). However, the captive fish are in a pre-spawning condition; in natural habitats, the full spawning condition is triggered by environmental stimuli (i.e. rainy season, resulting in flooded meadows used as spawning grounds). Ovaries were collected from preovulatory, postvitellogenic adult females. Animal culture and handling was consistent with the Dutch national regulations; the Life Science Faculties Committee for Animal Care and Use approved the experimental protocols.

Glycoprotein hormones

Catfish LH (cfLH) was isolated from pituitaries of mature catfish as described earlier (Schulz et al. 2001). Human recombinant FSH (hFSH) and hCG were kindly provided by Dr W G E J Schoonen (Organon, Oss, The Netherlands). Human TSH (hTSH) was purchased from Sigma (St Louis, MO, USA).

Total RNA, poly(A)* RNA and genomic DNA isolation

Total RNA was isolated from various tissues (for each tissue, n=3 animals) of mature African catfish using the guandinium isothiocyanate method (Chirgwin et al. 1979). Poly(A)* RNA was isolated using Dynabeads-oligo dT25 (Dynal AS, Oslo, Norway), according to the manufacturer’s instructions. Genomic DNA was isolated from African catfish sperm according to Strauss et al. (1998).

Primers and PCR

One microgram testicular RNA was reverse transcribed using oligo-dT12–18 and the Superscript II preamplification system (Invitrogen, Breda, The Netherlands), according to the manufacturer’s instructions. Catfish genomic DNA and testicular cDNA were used as templates in two independent PCR amplifications with degenerate primers 598 and 599 (Table 1) corresponding to highly conserved segments unique for the mammalian glycoprotein hormone receptor family. PCRs were carried out in 50 µl volumes containing 50 mM KCl, 10 mM Tris–HCl (pH 8·3), 1·5 mM MgCl2, 0·01% gelatin, 200 µM each dNTP, 50 pmol primers and 100 ng DNA template in a Perkin-Elmer Cetus cycler (Applied Biosystems, Foster City, CA, USA), using 1 U SuperTag (HT Biotechnologies Ltd, Cambridge, Cambs, UK). PCR was performed under the following cycling conditions:
conditions: denaturation at 94 °C for 5 min followed by the addition of Super Taq polymerase at 75 °C, followed by 10 cycles of 94 °C for 45 s, 37 °C for 30 s and 72 °C for 1 min, and then 25 cycles of 94 °C for 45 s, 55 °C for 30 s and 72 °C for 1 min. DNA fragments of approximately 0·4 kb were amplified in the two types of PCRs and subcloned into pGEM-T (Promega, Madison, WI, USA) for sequence analysis.

**Rapid amplification of cDNA ends**

To isolate the 5′- and 3′-ends of the presumptive cfTSH-R cDNA, rapid amplification of cDNA ends (RACE) reactions were performed using the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA). To this end, African catfish testis poly(A)+ RNA was reversed transcribed to mRNA (see above) using the Advantage-HF PCR method (Saitou & Nei 1987). The putative signal peptide cleavage site was predicted using SignalP V1·1 software (Nielsen et al. 1997), from the CBS prediction server (http://www.cbs.dtu.dk/services).

**DNA sequence analysis and phylogenetic analysis**

DNA sequence analyses were performed on automated ABI PRISM 310 and 377 DNA sequencers, using Dye Terminator cycle sequencing chemistry (all from Applied Biosystems). A homology search was performed using the BLAST 2·0·12 program (Altschul et al. 1990) and alignment of multiple protein sequences using the Megalign program of the Lasergene software package (DNASTAR Inc., Madison, WI, USA) with the Clustal (PAM250) algorithm (Higgins & Sharp 1990). A phylogenetic tree was constructed from the aligned sequences using the neighbor-joining method (Saitou & Nei 1987). The putative signal peptide cleavage site was predicted using SignalP V1·1 software (Nielsen et al. 1997), from the CBS prediction server (http://www.cbs.dtu.dk/services).

**Transient expression of the putative cfTSH-R in HEK-T 293 cells**

Human embryonic kidney (HEK-T) 293 cells (DuBridge et al. 1987) were maintained under 5% CO₂ in culture medium (Dulbecco’s modified Eagle’s medium (DMEM) containing 2 mM glutamine, 10% fetal bovine serum and 1× antibiotic/antimycotic solution; all from Invitrogen). For colorimetric detection of cfTSH-R-mediated ligand-induced cAMP production (see below), transient transfections were performed in a 10 cm dish, containing approximately 5 × 10⁶ cells, with 1 µg cfTSH-R expression vector construct in the presence of 10 µg of a pCRE/β-gal plasmid, using the modified bovine serum transfection method, according to the instructions of the manufacturer (Stratagene, La Jolla, CA, USA). The pCRE/β-gal plasmid consists of a β-galactosidase gene under the control of a human vasoactive intestinal peptide promoter containing five cAMP-response elements (Chen et al. 1995). Five micrograms cfTSH-R expression construct, in the absence of the pCRE/β-gal plasmid, was transfected for determining ligand-induced inositol phosphate production (see below). ‘Empty’ pcDNA3·1/V5-His vector was used for mock transfections.
Colorimetric detection of ligand-induced cAMP production

The β-galactosidase activity was measured according to Chen et al. (1995) with minor modifications as described previously (Bogerd et al. 2001). Briefly, 16–18 h after co-transfection of the receptor expression vector construct (1 µg) and the pCRE/β-gal plasmid (10 µg), cells were collected and split into 96-well plates (~2·5 × 10^5 cells/well). The next day, cells were stimulated for 6 h with different concentrations of various glycoprotein hormones in 25 µl Heps-modified DMEM containing 0·1% bovine serum albumin and 0·1 mM 3-isobutyl-1-methylxanthine (all from Sigma). Ligand-induced changes in absorbance were related to the forskolin-induced changes (10 µM) on each 96-well plate. The results are therefore expressed as arbitrary units, related to the forskolin-induced cAMP-mediated reporter gene activation. The agonist concentrations inducing half-maximal stimulation (EC_{50}) were calculated using the GraphPad PRISM3 software package (GraphPad Software, Inc., San Diego, CA, USA). All experiments were repeated at least three times using cells from independent transfections, each performed in triplicate.

Detection of ligand-induced inositol phosphate production

The ligand-induced hydrolysis of [^3H]-phosphatidylinositol was assayed essentially according to Millar et al. (1995). Briefly, 24 h after transfection with cfTSH-R expression vector construct (5 µg) only, the cells were seeded into 48-well plates (~2·5 × 10^4 cells/well) in 0·5 ml DMEM supplemented with 10% dialyzed fetal calf serum containing 1 µCi/ml[^3H]-inositol (Amersham International plc, Little Chalfont, Bucks, UK). The next day, cells were washed and preincubated for 10 min with assay medium (Hes-modified DMEM containing 20 mM LiCl). After removing the assay medium, the cells were incubated in 200 µl assay medium containing hTSH (1 or 10 µg/ml) at 37 °C for 45 min. The assay medium was aspirated and cellular lipids were extracted from the cells by 10 mM formic acid at 4 °C for 90 min. Total inositol phosphates were separated on Dowex (AG 1X8–200) anion-exchange columns and counted with a scintillation counter. As a positive control for the procedure, HEK-T293 cells, transiently transfected with a catfish gonadotropin-releasing hormone receptor 1 (cfGnRH-R1; Tensen et al. 1997), were stimulated with 10 µM chicken GnRH-II (cGnRH-II).

Analysis of cfTSH-R mRNA tissue distribution by real-time, quantitative PCR

The relative cfTSH-R mRNA levels in different tissues were determined in a way similar to that described previously (Bogerd et al. 2001). Specific primers and fluorogenic probes for the cfTSH-R mRNA and the endogenous control RNA (i.e. catfish 28S (cf28S) rRNA) are shown in Table 2. Optimization and validation were performed according to the manufacturer’s guidelines (Applied Biosystems) and as previously described (Bogerd et al. 2001).

### Table 2 Primers and TaqMan fluorogenic probes

<table>
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<th>Target</th>
<th>GenBank accession number</th>
<th>Primers</th>
<th>Sequence (5’→3’)</th>
<th>bp</th>
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<tr>
<td>cfTSH-R</td>
<td>AY129556</td>
<td>cfTSH-R-Fw</td>
<td>CCACCATGACTTCTTAAGTAACCTACA</td>
<td>108</td>
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<td>cfTSH-R-Pr</td>
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<tr>
<td>cf28S</td>
<td>AF323692</td>
<td>cf28S-Fw</td>
<td>TCTCGGAAGTCGGGTGGTTTG</td>
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<td></td>
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<td></td>
<td>cf28S-Pr</td>
<td>TTTACCACCAGCTTTGGGCTGCA</td>
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</table>

*Sequences for the sense (-Fw) and antisense (-Rv) primers and the TaqMan probe (-Pr) as well as the size of the amplicon (base pairs, bp) are shown.
Statistics

All results are shown as the mean ± s.e.m. Statistical analyses to confirm significant hormone-induced over basal signaling were performed using a t-test (Statview 4·5; Abacus Concepts, Berkeley, CA, USA). P values <0·05 were considered statistically significant.

Results

Isolation and characterization of the cfTSH-R cDNA

Degenerate oligonucleotide primers (598 and 599), flanking transmembrane helices 1 and 3 (TM 1 and 3) respectively, and derived from conserved amino acid sequences of vertebrate glycoprotein hormone receptors, were used in two different PCRs with African catfish genomic DNA and testis cDNA respectively as template. Approximately 0·4 kb PCR products were generated in both types of PCRs, and subsequently subcloned and sequenced. The sequences of these PCR products displayed highest identity to other TSH-Rs. Next, four new cfTSH-R-specific primers were designed based on the sequence of the presumptive cfTSH-R cDNA fragment, and used in RACE reactions in order to isolate the 5’- (primer 1043 and nested primer 1044) and 3’-ends (primer 1041 and nested primer 1042) of the cfTSH-R cDNA. The combination of 5’- and 3’-RACE reaction products yielded a 2927 bp cDNA sequence (Fig. 1), consisting of an open-reading frame of 2334 nucleotides that was flanked by leader and trailer sequences of 209 and 384 nucleotides respectively. The cfTSH-R open-reading frame of ~2·3 kb was PCR amplified using primers 1299 and 1300, and subcloned into the pcDNA3·1/V5-His-TOPO expression vector. Sequence analysis of several clones revealed identical sequences to those obtained in the 5’- and 3’-RACE clones.

A putative translation initiation codon was identified starting at nucleotide position 210. Conceptual translation of the open-reading frame following this initiation codon predicted a 777 amino acid protein (Fig. 1) of which the first 18 amino acids were predicted to constitute the putative signal peptide (Nielsen et al. 1997). The mature receptor protein displayed typical features of members of the glycoprotein hormone receptor family and consisted of a large extracellular LRR-containing amino-terminal domain (Ji et al. 1998), followed by a seven TM domain – typical for all members of the rhodopsin-like G protein-coupled family – and an intracellular carboxy-terminal domain.

Amino acid sequence alignment of the cfTSH-R with 54 other LRR-containing G protein-coupled receptors was performed using the Clustal method (Higgins & Sharp 1990). The cfTSH-R protein had the highest amino acid identity to TSH-Rs of other fish species (61–62%), followed by mammalian TSH-Rs (53–54%), vertebrate LH-Rs (40–53%) and FSH-Rs (40–45%). The cfTSH-R shared only 14–27% amino acid identity with vertebrate and invertebrate LGRs. A phylogenetic tree constructed from the aligned amino acid sequences using the neighbor-joining method (Saitou & Nei 1987) revealed that, next to all LGRs, the glycoprotein hormone receptors were divided into two major groups (Fig. 2). One group consisted of all TSH-Rs, while the other group was further subdivided into two subgroups: all FSH-Rs form one subgroup, whereas the LH-Rs form another subgroup. The cfTSH-R was clearly clustered with all other fish TSH-Rs.

PCR amplification using primers 1041 and 1300 (covering the cDNA region between nucleotides 1444 and 2673; Fig. 1) on genomic DNA of the African catfish yielded an ~1·2 kb PCR product, indicating that no introns were present in the transmembrane and intracellular carboxy-terminal region of the cfTSH-R gene (data not shown).

Functional characterization of the cfTSH-R

HEK-T 293 cells, transiently co-transfected with the cfTSH-R expression vector (pcDNA3·1/V5-His) construct and the pCRE/βGal reporter plasmid, were challenged with various purified and recombinant glycoprotein hormones, followed by the measurement of intracellular cAMP levels using an indirect colorimetric reporter gene assay (Chen et al. 1995). All tested ligands were able to increase the intracellular cAMP levels significantly in a dose-dependent manner. However, hTSH had by far the highest efficacy (EC50 = 25 ± 3·4 ng/ml) in activating the cfTSH-R, whereas at least 1 µg/ml hFSH and 10 µg/ml hCG or cfLH were required to enhance significant cfTSH-R-mediated cAMP production (Fig. 3). The glycoprotein hormones tested had no effect on mock-transfected cells (data...
not shown). Measurement of intracellular inositol phosphate formation in cfTSH-R-expressing HEK-T 293 cells, revealed no significantly increased phospholipase C activity upon stimulation with hTSH (data not shown), whereas HEK-T 293 cells transiently transfected with cfGnRH-R1 showed a clear inositol phosphate response to cGnRH-II, as shown previously (Tensen et al. 1997).

Tissue distribution of the cfTSH-R mRNA

Tissue-specific expression of the cfTSH-R gene was determined by sensitive real-time, quantitative PCR, and revealed that cfTSH-R mRNA is abundantly present in cerebellum, brain and ovary (Fig. 4). In addition, testis, seminal vesicles, pituitary and conus arteriosus (i.e. the ventral aorta, delivering blood to the gills and surrounded by thyiodal follicles in bony fish) were positive, while weaker expression was detected in all other tissues analyzed (i.e. muscle, stomach, intestine, head-kidney, liver, kidney and heart).

Discussion

In our attempt to clone cfLH-R DNA fragments (Vischer & Bogerd 2003) from gonadal tissues and genomic DNA of the African catfish, we unexpectedly PCR amplified a cfTSH-R DNA fragment. The full-length cfTSH-R cDNA was subsequently generated using RACE technology. Analysis of its deduced amino acid sequence revealed that this receptor had the highest identity to other fish TSH-Rs. Interestingly, however, all other fish TSH-Rs had a higher identity to each other (61–62%) than each of them to the cfTSH-R receptor had the highest identity to other fish (77–90%) than each of them to the cfTSH-R (E.19–R.417), connected to a rhodopsin-like seven TM domain (residues V.418–T.681) and followed by an intracellular carboxy-terminal domain (residues K.682–C.777). Nine putative LRRs were recognized in the extracellular domain of the cfTSH-R (residues T.33–H.276), most likely consisting of alternating parallel-arranged β strands and α helices (Fig. 1) (Kajava et al. 1995, Bhowmick et al. 1996).

In most vertebrate glycoprotein hormone receptor genes, the region for the transmembrane and intracellular carboxy-terminal domains is encoded by a single exon, namely by exon 10 in FSH-R and TSH-R genes and by exon 11 in LH-R genes (Bogerd et al. 2001, Kumar & Trant 2001). Similar to most other vertebrate glycoprotein hormone receptor genes, the cfTSH-R gene is intronless in this region, which is in contrast to the situation for the salmon glycoprotein hormone receptor (Oba et al. 1999a,b, 2000) and the invertebrate LGR genes (Hauser et al. 1997, Kudo et al. 2000) that all contain introns in this region.

Similar to all other glycoprotein hormone receptors, the cfTSH-R contained a relatively large amino-terminal extracellular domain (residues E.19–R.417), connected to a rhodopsin-like seven TM domain (residues V.418–T.681) and followed by an intracellular carboxy-terminal domain (residues K.682–C.777). Nine putative LRRs were recognized in the extracellular domain of the cfTSH-R (residues T.33–H.276), most likely consisting of alternating parallel-arranged β strands and α helices (Fig. 1) (Kajava et al. 1995, Bhowmick et al. 1996).

The LRR domain of the cfTSH-R is flanked by N- and C-terminal cysteine-rich regions (Fig. 1). The cfTSH-R contains four N-terminal cysteines (i.e. residues 24, 28, 30 and 40) that were found to form two disulfide bonds in most glycoprotein hormone receptors, and in such a conformation are important for cell-surface expression (Zhang et al. 1996, Chen et al. 2001). Although the N-terminal cysteine cluster was found not to be involved in TSH binding, it appeared to form an epitope for the binding of thyroid-stimulating autoantibodies (TSAb; the cause of Graves’ disease), and was found to be important for TSAb-induced receptor activation (Chen et al. 2001).

Figure 1  Nucleotide sequence and deduced amino acid sequence of the cfTSH-R cDNA. Numbers on the right refer to position of the nucleotides (top) and the amino acid residues (bottom). Amino acid numbering begins with the proposed initial methionine. The predicted signal peptide is indicated in bold italics. Conserved cysteine residues in the N- and C-terminal cysteine-rich regions of the extracellular domain are indicated by black boxes. The nine identified β strand motifs (X–X–L–X–L–X–X) of the leucine-rich repeats, each consisting of a parallel arranged β strand and helical segment, are underlined in grey, of which the conserved hydrophobic ‘L’ residues within these motifs are underlined in black. Potential N-linked glycosylation sites are indicated by grey boxes. The positions of the seven putative transmembrane α helices of the receptor are underlined in black. A conserved, presumably palmitoylated Cys residue in the intracellular carboxy-terminal domain is indicated by a grey box. Potential sites for cAMP/cGMP-dependent protein kinase and protein kinase C phosphorylation are indicated by solid diamonds and open boxes respectively. The nucleotide sequence has been submitted to the GenBank and is available under accession number AY129556.
Figure 2. Phylogenetic tree of the leucine-rich repeat-containing, G protein-coupled receptors. The deduced amino acid sequences of 55 LGRs from invertebrate, fish, avian and mammalian species were analyzed using the Clustal method (PAM250) of the Megalign program of the Lasergene software package (DNASTAR). LGRs used: sheep FSH-R (accession number: L07302), bovine FSH-R (L22319), pig FSH-R (AF025377), horse FSH-R (S70150), donkey FSH-R (U73659), human FSH-R (M95489), macaque FSH-R (X74454), guinea pig FSH-R (AY082514), mouse FSH-R (AF095642), rat FSH-R (L02842), newt FSH-R (AB005587), chicken FSH-R (D87871), African catfish FSH-R (AJ012647), channel catfish FSH-R (AF285182), zebrafish FSH-R (AW174149), amago salmon FSH-R (AB030012), tilapia FSH-R (AB041762), sheep LH-R (L36329), pig LH-R (M29525), bovine LH-R (U20504), human LH-R (M63108), marmoset monkey LH-R (U80673), rat LH-R (M26199), mouse LH-R (M81310), chicken LH-R (AB009283), quail LH-R (S75716), turkey LH-R (U92082), amago salmon LH-R (AB030005), African catfish LH-R (AF324540), tilapia LH-R (AB041763), channel catfish LH-R (AF285181), dog TSH-R (P14763), cat TSH-R (AF218264), pig TSH-R (AF338249), bovine TSH-R (U15570), sheep TSH-R (Y13434), human TSH-R (M32215), rat TSH-R (M34842), mouse TSH-R (U02602), amago salmon TSH-R (AB030954), amago salmon TSH-Rb (AB030955), striped bass TSH-R (AF239761), tilapia TSH-R (AB047390), African catfish TSH-R (AY129556), Drosophila melanogaster glycoprotein hormone receptor GPHR-I (U47005), Anthopleura elegantissima LGR (Z28332), human LGR5 (AF061444), mouse FEX (AF110818), human LGR6 (AF190501), rat LGR4 (AF061443), Drosophila melanogaster GPHR-II (AF142343), Drosophila melanogaster LGR2 (AF274591), human LGR7 (AF190500), Lymnaea stagnalis GRL101 (Z23104), and Caenorhabditis elegans LGR (AF224743). The scale beneath the tree measures the distance between the sequences, and units indicate the number of substitution events.
The C-terminal cysteine-rich region – also known as the hinge region – of the cfTSH-R contains six cysteine residues (i.e. residues 282, 283, 300, 389, 397 and 407) similar to the situation in all glycoprotein hormone receptors. Despite the presence of two highly conserved amino acid domains (i.e. \textit{TYPSHCCAF} and \textit{FNPCEDIMG}) in the C-terminal cysteine-rich region of the cfTSH-R (see below), the complete C-terminal cysteine-rich region is the least conserved domain between the three glycoprotein hormone receptors (i.e. the LH-R, FSH-R and TSH-R) in both amino acid identity and number. With respect to the latter, the section between the conserved \textit{[T/S]YPSHCCAF} and \textit{FNPCEDIMG} regions is the shortest in fish FSH-Rs (44–47 residues), followed by LH-Rs (38–97 residues) and tetrapod FSH-Rs (66–78 residues), whereas this region is the longest in TSH-Rs (117–155 residues; 118 residues in the cfTSH-R). A stretch of approximately 50 amino acids (relative to FSH-Rs and LH-Rs, and corresponding to cfTSH-R segment \textit{V}^{316}–\textit{H}^{361}) is often excised from a variable percentage of human TSH-Rs (hTSH-R) that are present on the cell surface as a result of intramolecular cleavage (Chazenbalk \textit{et al}. 1997). The resulting cleaved N-terminal extracellular domain (the so-called A subunit), however, remained linked to the seven transmembrane domain (the so-called B subunit) due to presumed disulfide bonding between the six C-terminal Cys residues (Tanaka \textit{et al}. 1999). Hitherto, no functional significance for the TSH-R-specific intramolecular cleavage was identified (Chazenbalk \textit{et al}. 1999).

The amino acid motif \textit{[T/S]YPSHCCAF} (corresponding to residues 277–285 in the cfTSH-R) is conserved in all members of the glycoprotein hormone receptor family, and constrains the transmembrane domain of unliganded receptors in the inactive conformation, via an interaction with exoloop 2 of the TM domain (Nishi \textit{et al}. 2002). In particular, a somatic mutation of \textit{SYPSHCCAF} to \textit{SYPIHCCAF} (Ser/Thr Ile) in the hTSH-R causes hyperfunctioning thyroid adenomas as a result of a constitutively activated TSH-R (Kopp \textit{et al}. 1997, Ho \textit{et al}. 2001).

The conserved \textit{FNPCEDIMG} (corresponding to residues 404–412 in cfTSH-R), located just upstream of TM 1, was shown to be important for receptor cell-surface expression as well as ligand-mediated signaling (Alvarez \textit{et al}. 1999).

Three potential N-linked glycosylation sites are present in the extracellular domain of the cfTSH-R.
(i.e. $^{76}$NIS$^{78}$, $^{197}$NGT$^{199}$ and $^{301}$NLT$^{303}$), whereas four and five of these sites are present in perciform and most mammalian TSH-Rs respectively. The first glycosylation site is unique to TSH-Rs, while the other two sites are also found in other members of the glycoprotein hormone receptor family.

Glycoprotein hormone binding to the N-terminal extracellular domain leads to conformational changes resulting in the activation of the seven TM domain – by means of rotating or tilting helices – and the subsequent recruitment of G proteins that, in their turn, modulate the activity of intracellular effector pathways (Gether 2000, Nishi et al. 2002). Residues important for TM domain functioning as well as its conformation were conserved in the cfTSH-R. Related to its presumed identical function in signaling, the TM domain of the cfTSH-R shares a higher degree of amino acid sequence identity to other TSH-Rs (59–67%) and both FSH-Rs and LH-Rs (53–60%), than its hormone specificity conferring N-terminal extracellular domain to other TSH-Rs (47–60%), FSH-Rs (28–33%) and LH-Rs (29–41%).

As in most G protein-coupled receptors, a fourth intracellular loop is introduced into the carboxy-terminal domain of the cfTSH-R by presumed palmitoylation of Cys$^{698}$, which is secured in the cell membrane and has been shown to be important for efficient cell-surface targeting of hTSH-Rs (Tanaka et al. 1998). Four consensus phosphorylation sites were recognized in the carboxy-terminal cytoplasmic tail of the cfTSH-R: $^{711}$STK, $^{714}$SSR, $^{737}$TSR and $^{750}$KKD are potential protein kinase C phosphorylation sites, and $^{746}$KKD$^{749}$ is potentially phosphorylated by cAMP- and cGMP-dependent protein kinases. However, although TSH-Rs have been shown to desensitize after TSH binding (Nagayama et al. 1994), probably via receptor phosphorylation, studies using truncated hTSH-Rs revealed that the carboxy-terminal region was not involved in this ligand-dependent desensitization (Haraguchi et al. 1994).

Challenging cfTSH-R-expressing HEK-T 293 cells with a panel of glycoprotein hormones (i.e. cFLH, hFSH, hCG and hTSH) revealed that the cfTSH-R displays a higher ligand selectivity than the other African catfish glycoprotein hormone receptors (cfFSH-R and cfLH-R), as it is only activated by physiological concentrations of hTSH. Promiscuous hormone binding was most obvious in the cfFSH-R (Bogerd et al. 2001), which was equally responsive to cFLH but not to hCG, as the cfLH-R (Vischer & Bogerd 2003). However, both the cfFSH-R and the cfLH-R were responsive to hFSH. Nevertheless, definitive conclusions regarding cfTSH-R ligand selectivity awaits the availability of the endogenous catfish TSH. In contrast to the situation for striped bass (Kumar et al. 2000) and mammalian (Allgeier et al. 1994) TSH-Rs, cfTSH-R signaling is only through the adenylyl cyclase second messenger system. The cfTSH-R does not couple to the inositol phosphate pathway.

Besides the testis, from which the cfTSH-R cDNA was cloned, and the conus arteriosus which is surrounded by thyroid follicles in bony fish and in which tissue one expects its expression, cfTSH-R mRNA expression was detected in all tissues analyzed. Abundant extrathyroidal tissue TSH-R mRNA expression was also observed in the striped bass (Kumar et al. 2000), in which the striped bass TSH-R (sbTSH-R) mRNA was detected in brain, heart, muscle, ovary and testis. In situ hybridizations revealed that the gonadal sbTSH-R mRNA expression was confined to the gametes in both testis and ovary, suggesting a direct role for TSH in gametogenesis, not mediated via surrounding somatic cells. In concert with the above finding, steroid production by African catfish testis tissue fragments was found to be insensitive to TSH stimulation (Bogerd et al. 2001). In contrast to the situation in striped bass and African catfish, salmon TSH-R expression was only observed in the basibranchial region but not in gonads, liver, kidney and brain (Oba et al. 2000). Moreover, extrathyroidal TSH-R expression in mammals has not been reported in the gonads, but was observed in various tissues including fat cells (Endo et al. 1995), lymphocytes (Pekonen & Weintraub 1978) and brain (Crisanti et al. 2001). However, the physiological relevance of TSH stimulation of the extrathyroidal TSH-R expressing tissues remains to be elucidated.

In conclusion, we have cloned a functional cDNA coding for the cfTSH-R from African catfish testis. Hence, TSH may regulate testis physiology in the African catfish, although TSH did not stimulate steroidogenesis. Future analysis of cfTSH-R spatio-temporal expression may shed light on such a potential physiological role of TSH on the regulation of African catfish testis function.
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Journal of Molecular Endocrinology (2003) 30, 227–238

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Received in final form 8 November 2002
Accepted 24 November 2002