The Cloned Equine Thyrotropin Receptor Is Hypersensitive to Human Chorionic Gonadotropin; Identification of Three Residues in the Extracellular Domain Involved in Ligand Specificity

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The receptors for TSH, LH/chorionic gonadotropin (CG), and FSH belong to the same subfamily of G protein-coupled receptors. The specificity of recognition of their cognate hormone involves a limited number of residues in the leucine-rich repeats present in the N-terminal ectodomain of the receptor. It is admitted that receptors of this subfamily coevolved with their respective ligands. The secretion of CG is restricted to gestation of primates and *Equidae*. We hypothesized that, facing the challenge of a new hormone, the glycoprotein hormone receptors would have evolved differently in *Equidae* and human so that distinct residues are involved in hormone specificity. In particular, it is known that equine CG has a dual (FSH and LH) activity when administered to other species. In the present work, we cloned and characterized functionally the equine TSH receptor (TSHR), which shares 89% homology with the human TSHR. The equine TSHR is not responsive to equine CG but is more sensitive to human CG than the human TSHR. Three residues, at positions 60, 229, and 235 of the ectodomain, are responsible for this difference in sensitivity as shown by modelization and targeted mutagenesis, followed by *in vitro* functional characterization. The phylogenetic approach is a suitable approach to identify determinants of specificity of receptors. (*Endocrinology* 149: 5088–5096, 2008)

THE TSH receptor (TSHR) (1–3), a rhodopsin-like G protein-coupled receptor (GPCR), together with the LH/chorionic gonadotropin (CG) receptor (4, 5) and the FSH receptor (FSHR) (6, 7), belongs to the glycoprotein hormone receptor (GPHR) family. GPHRs exhibit a bipartite structure with a large N-terminal extracellular domain (ECD), responsible for the specific binding of hormones (8–13), and a C-terminal portion with a rhodopsin-like serpentine structure, built of seven transmembrane α-helices, responsible for transduction of the signal (14, 15). The crystal structures of the FSHR-FSH complex (12) and the TSHR in complex with a thyroid-stimulating autoantibody (16) have confirmed that the ECD of GPHRs belongs to the family of proteins with leucine-rich repeats (LRRs) (17), as was previously suggested by sequence analysis and homology modeling (18–20). The ECD of GPHRs also contains, flanking the LRR region, two cysteine-rich clusters.

Glycoprotein hormones, TSH, LH, and FSH are dimers composed of an α and β-subunit. Whereas the α-subunit is common to the three hormones, their β-subunits have only 40% homology. Similarly, the GPHRs are extremely conserved in the serpentine domain, whereas the ectodomains also have only 40% homology (21). This leads to the hypothesis that selective binding of the hormones to their respective receptors is the result of a coevolutionary mechanism (22).

During evolution, a fourth hormone, the CG, has appeared in primates (23) and *Equidae* (24), secreted only during gestation. The high concentration, at least in humans, of CG constitutes a challenge to the specificity of the TSH and the FSHRs (21). This is demonstrated by the frequent mild hyperthyroidism during early gestation (25, 26). It is also illustrated by the only case of familial gestational thyrotoxicosis (27) or the few cases of gestational ovarian hyperstimulation (28–33) due to illegitimate stimulation by CG of mutated TSHR and FSHR, respectively. Characterization of these mutants and targeted mutagenesis have helped in the identification of certain "hot spot" positions both at the hormone and the concave inner surface of the receptor that play attractive and repulsive interactions for binding and specificity, respectively (34, 35). The challenge of specificity due to the late appearance of CG may have altered the pattern of evolution of GPHR as well as of the hormones themselves, in a few species. We hypothesized that evolution would have selected different residues in *Equidae* and human to achieve specificity. Interestingly, the equine gonadotropin, when used

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in other species as close as the donkey, display LH and FSH activity, whereas it has a strict LH activity in the horse (24, 36). This indicates species-restricted exquisite selection of determinants for specificity of gonadotropin receptors. In this study we have cloned the horse (*Equus caballus*) TSHR cDNA and show here that the equine TSHR (eTSHR) is hypersensitive to human CG (hCG) when compared with human TSHR (hTSHR). This has permitted to identify three additional residues involved in the selectivity of TSHR toward hCG, acting synergistically and independently of previously identified key residues.

Materials and Methods

Cloning of the eTSHR

Total RNA was isolated from a batch of thyroid glands of horses obtained in a slaughterhouse, using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD). Ten micrograms of RNA were reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Clontech Laboratories, Inc., Mountain View, CA), according to the manufacturer's guidelines. This cDNA was subsequently used as template for PCR amplification with primers designed in the most conserved regions of the sequences of TSHR already published (list and sequences available on request). Step-by-step PCRs generated a series of overlapping fragments that encompassed the full-length of horse TSHR cDNA. The 3' end of the cDNA was isolated using a primer diverted from the bovine TSHR sequence. The 5' end was isolated by the T_4 RNA ligase (Promega Corp., Madison, WI) method (37). The full-length cDNA was amplified with primers encompassing the ATG (5'-GGTACCGGATC-CGGAAAAATGAGGCCGACAC-3') and the stop codon (5'-AAAAGT-GTATAAGCAAACAGTTTTGTAATCT-3'), introducing restriction enzyme sites on both extremities (respectively, *Bam*HI and *Xba*I) and subcloned in the eukaryotic expression vector pcDNA3.1 (Invitrogen Corp., Carlsbad, CA). All the constructs were amplified in chemically competent *Escherichia coli* TOP 10 (Invitrogen), purified using the QIA-GEN maxiprep plasmid kit (QIAGEN, Inc., Valencia, CA), and sequenced. Sequencing was performed with CEQ Dye Terminator Cycle Sequencing (Beckman Coulter, Inc., Fullerton, CA) on a CEQ 2000 DNA Analysis System. Sequence analyses were performed on the Infobiogen web site (http://www.infobiogen.fr). Multiple protein sequence alignments were done using ClustalW, and a dendrogram was constructed with multiple TSHR sequences obtained from GenBank using the Protein Sequence Parsimony Method.

Transfection experiments

COS-7 cells were grown in DMEM supplemented with fetal bovine serum (10%), sodium pyruvate (0.1 mg/ml), penicillin (100 IU/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). A total of 300,000 cells were seeded in 3.5-cm culture dishes. Two days later, they were transfected with 600 ng plasmid coding for the different receptors by the diethylaminoethyldextran method, followed by a dimethylsulfoxide shock (38). Two days after transfection, cells were used for cAMP determinations and flow im-

munocytofluorometry. Triplicate dishes were used for each assay, and each experiment was repeated at least twice. Cells transfected with the empty pcDNA3.1 vector were always used as controls.

Quantification of cell surface expression of TSHR constructs by flow immunocytofluorometry

Cells were detached from the plates with PBS containing EDTA (5 mM). After pelleting, they were incubated for 30 min at room temperature with the monoclonal antibodies (mAbs) 3G4, 74, or BA8 (39–41) (generous gifts of Dr. Sabine Costagliola, IRIBHM, ULB, Brussels, Belgium) in PBS-BSA (0.1%). The mAb 3G4 recognizes a linear epitope of the TSHR ectodomain, conserved in many species, including the horse. After washing, cells were incubated for 30 min with fluorescein-conjugated γ-chain-specific goat antimouse IgG (DakoCytomation, Glostrup, Denmark) in the same buffer, on ice, in the dark. Propidium iodide (10 μg/ml) was used for detection of damaged cells that were excluded from the analysis. Cells were washed and suspended in PBS-BSA (0.1%). The fluorescence of 10,000 cells per tube was assayed by a FACScan flow cytofluorometer (BD Biosciences, San Jose, CA).

cAMP determination

For cAMP determinations, 48 h after transfection, cells were washed then incubated for 60 min in fresh Krebs-Ringer-HEPES buffer [Krebs-Ringer-HEPES isotonic (pH 7.4)] supplemented with Rolipram 25 μM (Sigma-Aldrich, St. Louis, MO), in the absence or presence of various concentrations of bovine TSH (bTSH) (Sigma-Aldrich), highly purified human or equine CG (eCG) (Sigma-Aldrich), or recombinant human TSH (hTSH) (Genzyme Corp., Cambridge MA). The supernatant was harvested for cAMP measurements by a cAMP enzyme immunoassay (EIA) (cAMP Biotrak EIA system: Amersham Biosciences Inc., Piscataway, NJ). Each sample was assessed in duplicate in all experiments; results are expressed as picomoles of cAMP per milliliter. Basal cAMP was normalized to cell surface expression for each construct. To this end, receptor-dependent cAMP accumulation (*i.e.* cAMP in cells transfected with receptor cDNA − cAMP in cells transfected with vector) was divided by the receptor-dependent fluorescence measured by flow immunocytofluorometry (fluorescence of cells transfected with receptor cDNA − fluorescence of cells transfected with empty vector). The values were then normalized to the basal activity of the wild-type (wt) hTSHR, arbitrarily set to one. The validity of this method of normalization was assessed by the observation that, for the range of constitutive activity investigated, a linear relation exists between cAMP accumulation and receptor expression at the cell surface. Concentration-action curves were fitted with Prism version 4.00 (GraphPad Software Inc., San Diego, CA) and EC_{50} calculated. When a plateau was not attained, the cAMP value obtained with the highest dose of hormone was arbitrarily set as the plateau to allow for calculation of EC_{50}. The EC_{50} values presented are the arithmetic mean of two EC_{50} values experimentally obtained.

Construction of human and eTSHR mutants

The cDNA coding for the wt hTSHR, the mutant K183R hTSHR subcloned in pcDNA3.1, and the wt human lutropin receptor (hLHR)

FIG. 1. Alignment of hTSHR and eTSHR sequences. Nonconserved amino acids are boxed in *black*. LRRs$_{1–9}$ at the ECD of the receptors, responsible for selective binding of the corresponding hormones, the seven transmembrane domains TMD$_{1–7}$ and the cytoplasmic α-helix H$_8$ parallel to the cell membrane, characteristic of the GPCR family, involved in transduction of the activation signal to the G protein(s), the SHCCAF motif in the hinge region (boxed in *red*), and the YDY motif involved in O-sulfation of the first tyrosine residue (boxed in *red*) are shown. The generic numbering scheme of Ballesteros and Weinstein (45) that allows easy comparison among residues in the 7TM segments of different receptors is also shown.

subcloned in the expression plasmid pSVL were kindly provided by Dr. Sabine Costagliola. Artificial mutations were introduced in the wt hTSHR, the K183R hTSHR, or the eTSHR by site-directed mutagenesis based on the PCR as described previously (42). TSHR mutants were sequenced for confirmation of the nucleotide sequences.

Construction of chimeric TSHR

cDNA for chimeric receptors were obtained by fusion PCR.

For e*ext*/h*tmd*TSHR (chimeric receptor with equine ectodomain linked to human serpentine domain of TSHR), two amplicons encoding eTSHR ectodomain and hTSHR serpentine domain, overlapping in the conserved CEDIM motif (nucleotide 1222–1236), were annealed and amplified by PCR using the two external primers, T7 and BGH (pcDNA3.1 nucleotide 863 and 1036, respectively), used for the starting PCR.

The same method was used for the chimera h*ext*/e*tmd*TSHR by fusing the cDNA coding the hTSHR ectodomain to the cDNA coding the eTSHR serpentine domain, and for h*ext*/e*tmd*TSHR triple mutant.

Construction of homology models

The previously reported molecular models of the hLHR-hCG and hTSHR-hTSH complexes (33), constructed from the crystal structure of the FSHR-FSH complex (Protein Data Bank code 1XWD) (12), are used. The model of eTSHR in complex with hCG was constructed from the hLHR-hCG and hTSHR-hTSH models. Molecular dynamics simulations, in an explicit water environment, of the hormone-receptor complexes were performed using the protocol previously described (33).

Results

eTSHR sequence

A step-by-step process allowed for the cloning of a full-length cDNA of 2292-bp long (accession no. EU784446). The nucleotide sequence contains an open reading frame encoding a protein of 764 amino acids. The eTSHR sequence shows 89.4% homology with the hTSHR sequence (Fig. 1). Several sequence patterns of the GPCR and GPHR families and of the TSHR were recognized: the SHCCAF motif in the so-called hinge region; Y(D/E)Y motif located 27 residues upstream from transmembrane helix 1, involved in O-sulfation of the first tyrosine residue (43); (N/S)LxxxD motif in TM2; (D/E)R(Y/W) motif in TM3, engaged in an ionic interaction with D/E6.30 (position 619 in the TSHR) near the cytoplasmic end of TM6 (31); FTD and CxxP motifs in TM6; NPxxY motif in
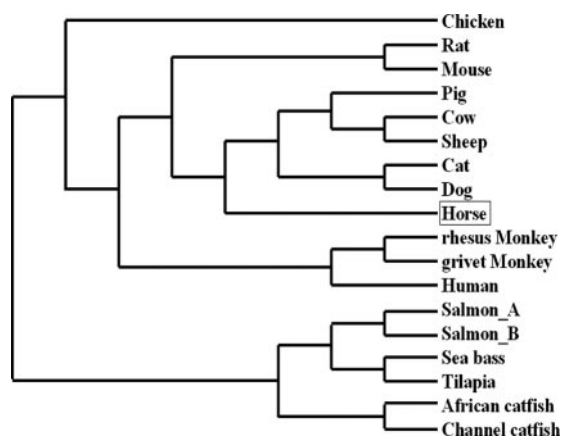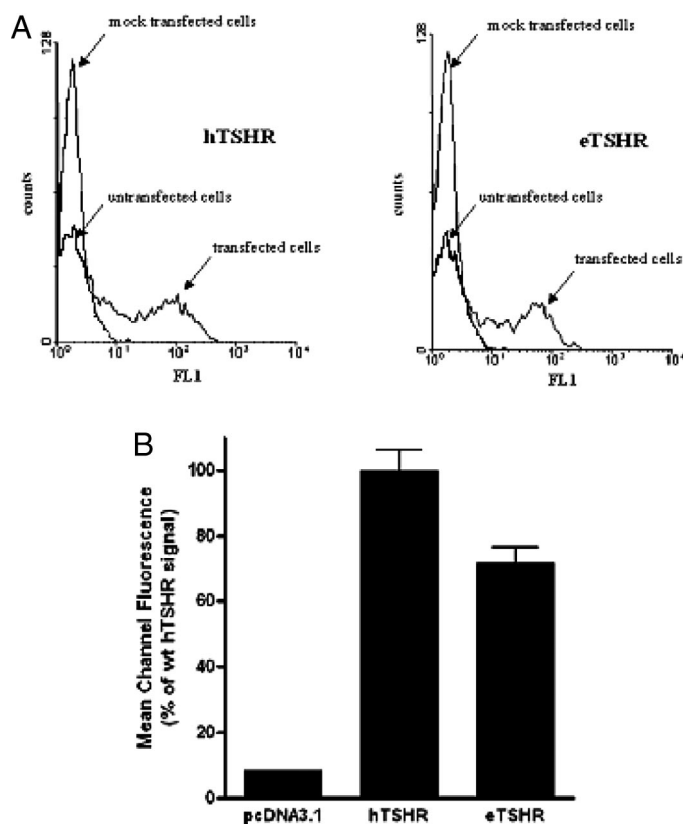

FIG. 3. Cell surface expression of the hTSHR and eTSHR. A, Membrane expression was determined by flow immunocytometry using the mAb 3G4. COS-7 cells were transfected with an empty vector (mock transfected cells) or a plasmid encoding the hTSHR or eTSHR. B, A Representative assessment of cell surface expression of hTSHR and eTSHR expressed as percentage of expression of hTSHR-pcDNA3.1 (set at 100%). Results are expressed as mean ± range of duplicates. FL1, Fluorescence channel 1.

TM7, acting as an on/off switch by adopting two different conformations in the inactive and active states (44); and the 50 amino acids insertion in the ECD (1, 2). We identified two variants, the most frequent with a valine in position 758 (Fig.

FIG. 2. Phylogenic relationship between 18 TSHR peptidic sequences. These sequences (excepted eTSHR) were obtained on GenBank, and the consensus tree was obtained using the Protein Sequence Parsimony Method.
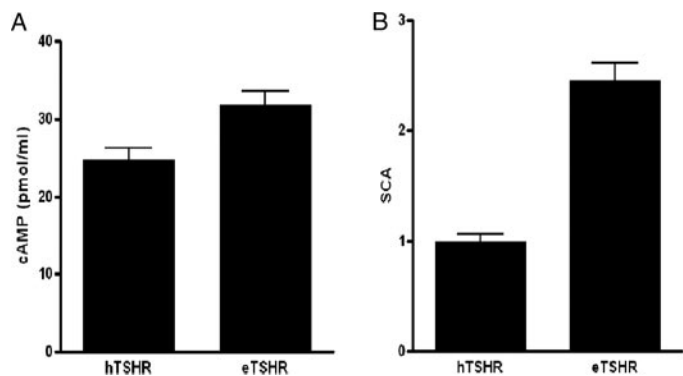

FIG. 4. Constitutive activity of hTSHR and eTSHR after transfection in COS-7 cells. A, A representative total basal extracellular cAMP accumulation measured in COS-7 cells transiently transfected with hTSHR or eTSHR. Extracellular cAMP was determined by EIA. B, Constitutive activity normalized to cell surface expression according to the SCA calculation (see *Materials and Methods*). Basal activity of the hTSHR was arbitrarily set to one.

FIG. 5. Response of eTSHR to bTSH, hTSH, or hCG. COS-7 cells transiently transfected with the various constructs were stimulated by increasing concentrations of hormone and extracellular cAMP were determined by EIA. Each curve is representative of at least two separate experiments, and data are presented as means ± SEM of triplicate dishes. A, Stimulation of the cells expressing hTSHR or eTSHR with bTSH. B, Stimulation of the cells expressing hTSHR or eTSHR with human recombinant TSH. C, Stimulation of the cells expressing hTSHR or eTSHR or hLHR with hCG. D, Stimulation of the cells expressing hTSHR or eTSHR or hLHR with eCG.
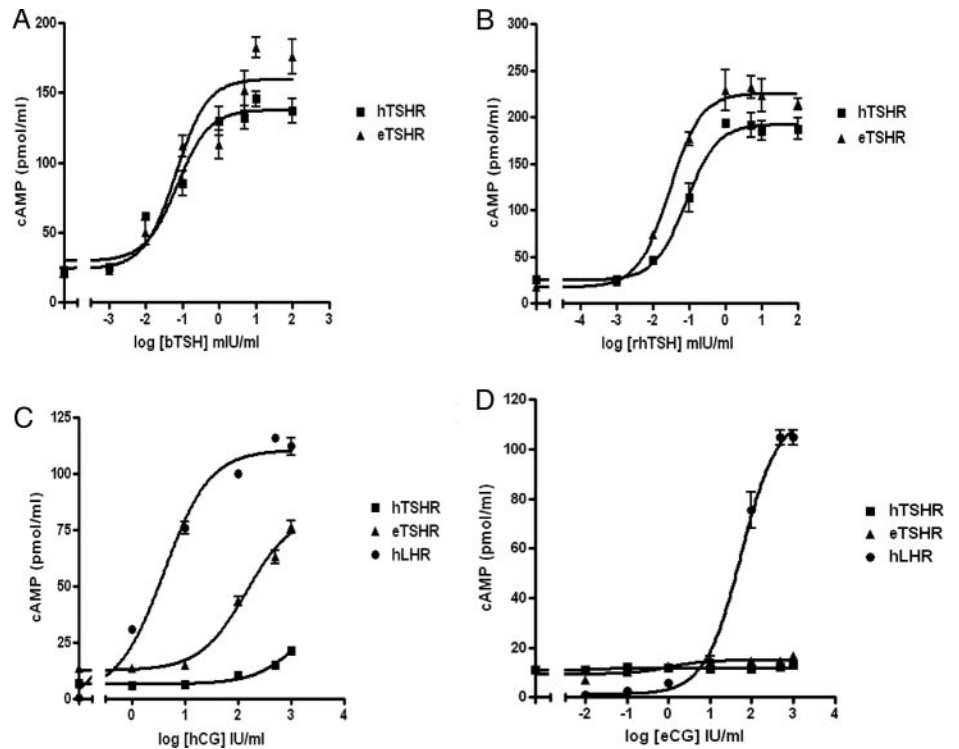
1) and the other with a glutamic acid in the same position (data not shown). A phylogenetic dendrogram was made using ClustalW showing the closest homologies of the eTSHR with the swine, feline, and canine TSHR (Fig. 2).

Functional characterization of the eTSHR

COS-7 cells were transiently transfected with the eTSHR and the wt hTSHR. Membrane expression of the eTSHR was only 70% of the hTSHR (Fig. 3) when assessed by fluorescence-activated cell sorter analysis using the mAb 3G4 (39). Similar results were obtained with mAb 74 (40) (data not shown), whereas the hTSHR-specific mAb BA8 (41) did not recognize the eTSHR (data not shown). Basal cAMP produced by COS cells expressing the eTSHR was higher (Fig. 4A), and specific constitutivity (after normalization to cell surface expression) was about 2.5-fold higher than the hTSHR (Fig. 4B). bTSH, as well as hTSH, was able to stimulate cAMP production of the eTSHR in a dose-dependent manner. When compared with the hTSHR, the eTSHR sensitivity to bTSH and hTSH appeared slightly higher (Fig. 5, A and B), but EC_{50} values were not significantly different (0.071 ± 0.004 mIU/ml and 0.065 ± 0.009 mIU/ml with bTSH for the human and the equine receptor, respectively, and 0.080 ± 0.007 mIU/ml and 0.029 ± 0.011 mIU/ml with hTSH). No response of the eTSHR to human FSH (hFSH) was observed (supplemental data, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org), either to eCG or of the hTSHR to eCG (Fig. 5D). In contrast, the eTSHR displayed increased responsiveness to hCG, when compared with the minimal response of the hTSHR (Fig. 5C). The response of the eTSHR to 1000 IU/ml hCG was about 6-fold higher than that of the hTSHR, and close to that of the previously described

human mutant K183R hypersensitive to hCG (27) (See Fig. 7C). The EC_{50} of eTSHR and K183R hTSHR were 105.2 ± 1.22 IU/ml and 71.8 ± 1.36 IU/ml, respectively.

To determine which part of the eTSHR is responsible for increased sensitivity to hCG, the ECDs of the equine and human receptors were swapped, generating two chimeric receptors, e*ext*/h*tmd*TSHR and h*ext*/e*tmd*TSHR. Cell surface expression of the e*ext*/h*tmd*TSHR was identical to that of the hTSHR, whereas h*ext*/e*tmd*TSHR had a lower expression close to that of the eTSHR (Fig. 6). The response to bTSH of
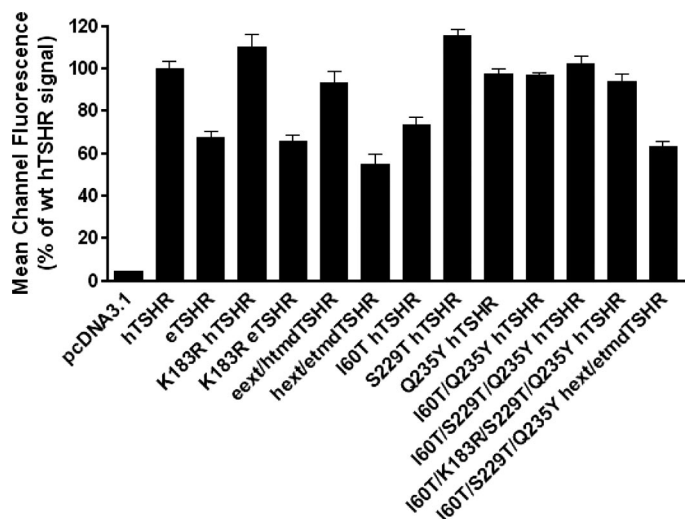

FIG. 6. Cell surface expression of hTSHR, eTSHR, hTSHR mutants, eTSHR mutant, and chimeric receptors transfected in COS-7 cells. Cell surface expressions after transfection of COS-7 cells determined by fluorescence-activated cell sorter using the mAb 3G4. Results are expressed as percentage of expression of hTSHR (set at 100%).
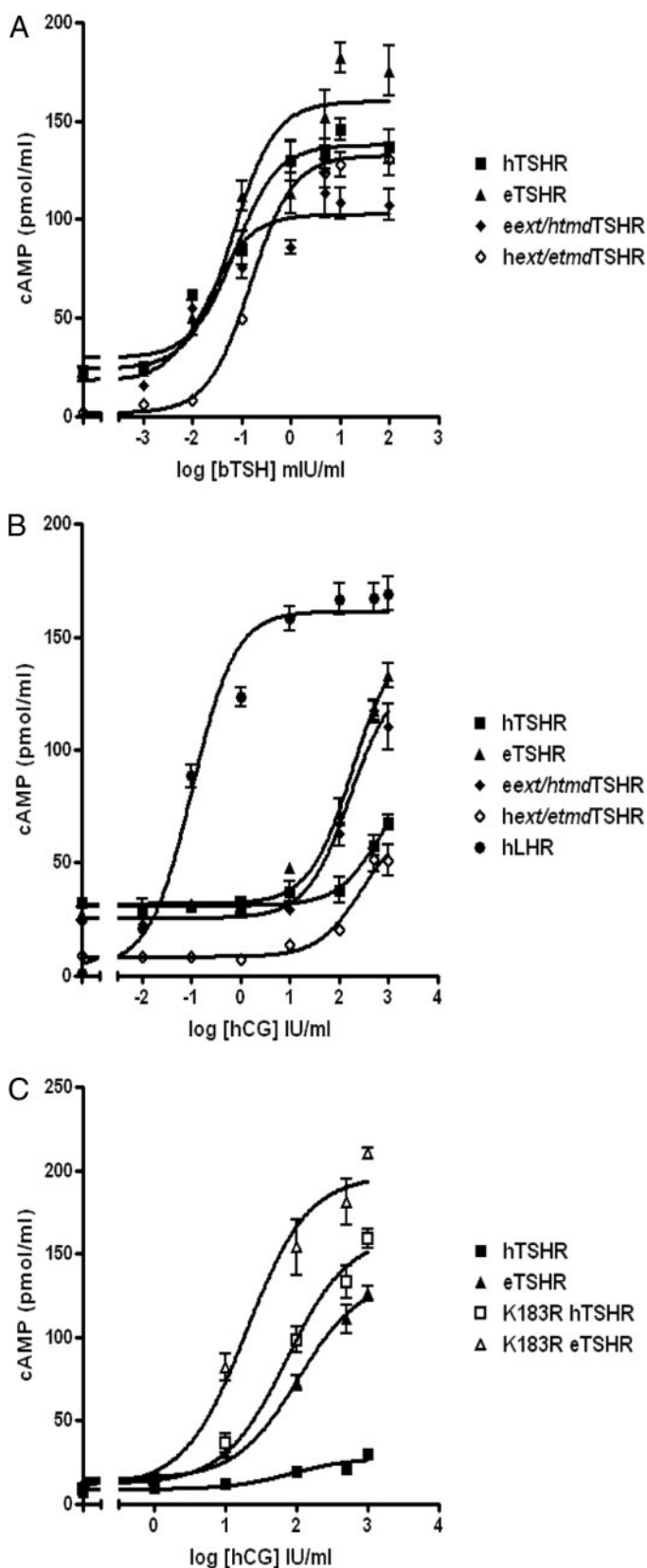
FIG. 7. Response of the e*ext*/h*tmd*TSHR, h*ext*/e*tmd*TSHR, and K183R eTSHR to bTSH or hCG. COS-7 cells transiently transfected with the various constructs were stimulated by increasing concentrations of hormone and extracellular cAMP were determined by EIA.

the two chimeric receptors was similar and close to that of hTSHR and eTSHR (Fig. 7A). The sensitivity to hCG of the e*ext*/h*tmd*TSHR was close to that of the eTSHR, whereas the sensitivity of h*ext*/e*tmd*TSHR was close to that of the hTSHR (Fig. 7B). This locates the responsiveness to hCG in the ECD of the eTSHR.

To investigate whether the same mechanisms of ligand specificity are operating in the eTSHR and hTSHR, the mutation K183R was introduced in the eTSHR. The mutated K183R eTSHR exhibited a dramatic increase in sensitivity to hCG when compared with K183R hTSHR and to wt eTSHR with an EC_{50} of 19.6 ± 1.49 IU/ml (Fig. 7C).

The residues responsible for the increased sensitivity of the eTSHR to hCG

To identify residues putatively involved in the different sensitivity to hCG, the coding sequence of the eTSHR and hTSHR were compared, especially in the LRRs at privileged locations according to the proposed model for hormone binding (Fig. 8). In the LRR positions, hTSHR differs from eTSHR only in X_4 of LRR_2 (I60 in human and T in equine), X_1 of LRR_9 (S229 and T), and X_5 of LRR_9 (Q235 and Y). All these dissimilar positions between human and eTSHR are supposed to contact the hormone β-subunit (see *Discussion*). Several mutants of the hTSHR were built, with replacement of the wt residues by the corresponding equine residues. These mutant receptors had membrane expression and sensitivity to bTSH similar to the wt hTSHR (data not shown).

Single I60T hTSHR and S229T hTSHR mutants did not exhibit any significant increase in sensitivity to hCG. In contrast, Q235Y hTSHR displayed a mild response to hCG. However, this increase in sensitivity was much lower than that of the wt eTSHR (Fig. 9A). Surprisingly, the double I60T/Q235Y hTSHR mutant and the triple I60T/S229T/Q235Y hTSHR mutant displayed a response to hCG higher than that of Q235Y hTSHR, the triple mutant being endowed with the same sensitivity to hCG as the eTSHR (Fig. 9B). This suggests a synergistic contribution of the three amino acids to the gain in sensitivity to hCG.

Because introducing the K183R mutation in the eTSHR increased the sensitivity to hCG, we also introduced it in the triple hTSHR mutant, generating a quadruple I60T/K183R/S229T/Q235Y hTSHR mutant. The sensitivity to hCG was increased to the same extent it was in the K183R eTSHR (Fig. 9C).

Finally, to determine the role of the serpentine domain in specificity, we engineered a triple mutant chimeric receptor I60T/S229T/Q235Y h*ext*/e*tmd*TSHR. Cell surface expression and response to bTSH were the same as the hTSHR. Surprisingly, it displayed an even greater responsiveness to hCG, beyond the sensitivity of the triple mutant hTSHR or the wt eTSHR and close to the mutant K183R eTSHR or the

Each curve is representative of at least two separate experiments, and data are presented as means ± SEM of triplicate dishes. A, Stimulation of the cells expressing hTSHR or eTSHR or e*ext*/h*tmd*TSHR or h*ext*/e*tmd*TSHR with bTSH. B, Stimulation of the cells expressing hTSHR or eTSHR or e*ext*/h*tmd*TSHR or h*ext*/e*tmd*TSHR or hLHR with hCG. C, Stimulation of the cells expressing hTSHR or eTSHR or K183R hTSHR or K183R eTSHR with hCG.

FIG. 8. Homology models of GPHRs in complex with hormones. A, Schematic representation of the X_{1-5} side chains in LRR$_{2-9}$ of hTSHR (*left panel*), eTSHR (*middle*), and hLHR (*right*) exposed to solvent and predicted to contact the hormones, together with the amino acid side chains of the "seat-belt" portion of the β-subunit of hTSH (*left*) and hCG (*middle and right*). B, Detailed view of the interaction between LRR$_{2-9}$ of the receptor and the C-terminal segment of the α-subunit and the "seat-belt" segment of the β-subunit of the hormone in the computer models of hTSHR-hTSH (*left*), eTSHR-hCG (*middle*), and hLHR-hCG (*right*) complexes.
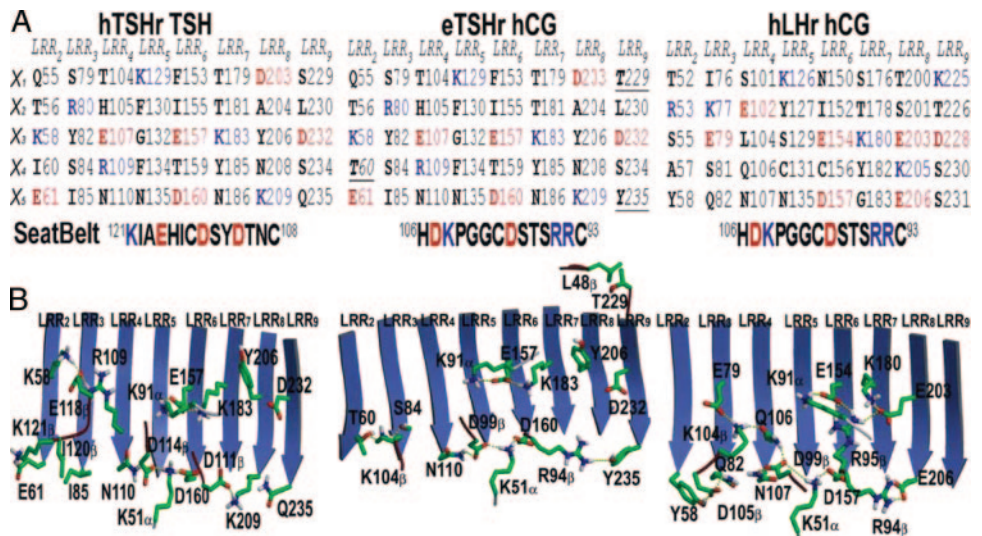
quadruple mutant. EC$_{50}$ for the chimeric mutant was 18.81 ± 1.38 IU/ml *vs.* 105.2 ± 1.22 IU/ml for eTSHR (Fig. 9C).

Molecular models of the hormone-receptor complexes

It has been proposed that the mode of binding observed between FSH and the FSHR also pertains to the other members of the GPHR family (12). This has been corroborated by the fact that ligands of different origin like FSH or a thyroid-stimulating autoantibody show almost identical receptor-binding features (16). Thus, homology models of GPHRs in complex with different hormones (33), using the structure of the FSHR-FSH complex as a template (see *Materials and Methods*), are a useful tool for understanding the key structural determinants of hormone specificity.

Key structural determinants of the selectivity of TSHR toward hCG. Figure 8B shows the molecular models of the hTSHR-hTSH and hLHR-hCG complexes. The key important discriminating determinants for selective binding are the following (see Fig. 8A for notation). First, the "seat-belt" of hTSH contains a positive K121 amino acid to form an ionic interaction with E61, at the bottom X_5 part of LRR$_2$, of hTSHR; whereas hCG contains a negative D105 amino acid at this part of the β-subunit to form polar hydrogen bond interactions with Y58 (X_5 of LRR$_2$) and Q82 (X_5 of LRR$_3$) of hLHR. Thus, E61 of hTSHR is a key determinant of the specificity against hCG because it is repulsive with D105 of hCG. In agreement, the substitution of E61 of hTSHR by Tyr (the hLHR counterpart) gave a gain of sensitivity toward hCG (35). Second, X_3 of LRR$_2$ is positive in hTSHR (K58), whereas X_3 of LRR$_3$ is negative in hLHR (E79). Thus, the positive electrostatics in this domain of the hTSHR interact with the hTSH-specific E118; whereas the negative electrostatics in hLHR interact with the hCG-specific K104 of the "seat-belt." Accordingly, substitution of Y82 of hTSHR by glutamic acid (the hLHR counterpart) increases the sensitivity toward hCG (35). The importance of the loss of basic and gain in acidic character in this region of the hTSHR to increase sensitivity to hCG is also shown by K58 and R80 mutations in hTSHR. When K58 was mutated to

Ser, Ala, or Asp, and R80 to Ala, a steady increase in sensitivity of the mutants to hCG was observed (35). Third, the X_5 amino acid in LRR$_{7,8}$ is positive in hTSHR (K209) and negative in hLHR (E206) to interact appropriately with a negative amino acid at the "seat-belt" of hTSH (D111) and positive in hCG (R94). Therefore, K209 in hTSHR plays a key role in the repulsion against the R94 side chain of hCG. Accordingly, the K209E mutation in hTSHR causes an important gain of function toward hCG, while reducing strongly responsiveness to TSH (35). Thus, these hormone-receptor pairs have evolved in such a manner that certain "hot spot" positions both at the "seat-belt" domain of the hormone and the concave inner surface of the receptor play attractive and repulsive interactions for binding and specificity, respectively.

The gain of function of eTSHR toward hCG. hTSHR differs from eTSHR, in the LRR domains, only in X_4 of LRR$_2$ [I60 in human and threonine (Thr) in equine], X_1 of LRR$_9$ (S229 and Thr), and X_5 of LRR$_9$ (Q235 and Tyr). The exchange of I60 in hTSHR by Thr in eTSHR creates, together with the nearby S84 in X_4 of LRR$_3$, a polar hydrogen bond acceptor area. These amino acids, in the computational model of the eTSHR-hCG complex, can form a polar hydrogen bond interaction with the K104 "hot spot" position at the "seat-belt" domain of hCG (Fig. 8, *middle panel*). Y235 of eTSHR maintains, relative to Q235 of hTSHR, the hydrogen bond acceptor/donor capabilities but at a longer distance. Thus, the longer Y235 of eTSHR is predicted to interact with the R94 "hot spot" position at the "seat-belt" domain of hCG (Fig. 8, *middle panel*). Finally, T229 of eTSHR adds, relative to S229 of hTSHR, a methyl group. The computational model locates the methyl group of T229 in the environment of the hydrophobic V48 and L49 of the β-subunit of hCG (Fig. 8, *middle panel*).

Discussion

In the present study, we report the cloning, from thyroid gland RNA, of the cDNA coding for the eTSHR. Because CG is present only in primates and *Equidae*, we thought that cloning the eTSHR would allow us to identify determinants selected during evolution of species to avoid illegitimate
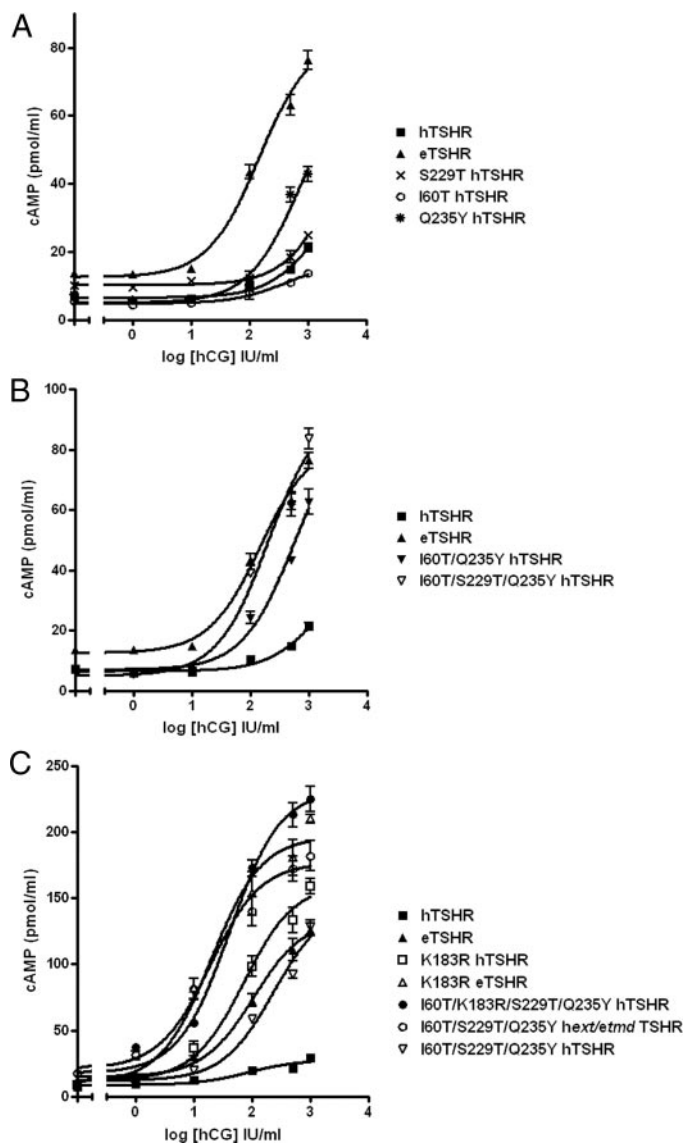
FIG. 9. Response of the hTSHR mutants and the h*ext*/*etmd* TSHR triple mutant to hCG. COS-7 cells transiently transfected with the different constructs were stimulated by increasing concentrations of hormone and extracellular cAMP were determined by EIA. Each curve is representative of at least two separate experiments, and data are presented as means ± SEM of triplicate dishes. A, Stimulation of the cells expressing hTSHR or eTSHR or S229T hTSHR or I60T hTSHR or Q235Y hTSHR with hCG. B, Stimulation of the cells expressing hTSHR or eTSHR or I60T/Q235Y hTSHR or I60T/S229T/Q235Y hTSHR with hCG. C, Stimulation of the cells expressing hTSHR or eTSHR or K183R hTSHR or K183R eTSHR or I60T/K183R/S229T/Q235Y hTSHR or I60T/S229T/Q235Y h*ext*/*etmd* TSHR or I60T/S229T/Q235Y hTSHR with hCG.

stimulation. The coding sequence of the eTSHR consists of a 2292-bp open reading frame, which encodes a 764-amino acid seven transmembrane G protein-coupled glycoprotein. The overall cDNA homology with TSHR sequence of *Homo sapiens* (NM_000369) is 89%. Although cDNA sequences of several mammalian species and some fish species have already been determined, among mammals, this is the first *Equidae* TSHR to be cloned. The isolated eTSHR contains all the structural features that characterized GPHRs, (21) such as

a large ECD that represents more than half of the total length of the protein, seven transmembrane stretches of largely hydrophobic amino acids connected by three intracellular and three extracellular loops, and an intracellular C-terminal tail containing an α-helix parallel to the cell membrane (15) (Fig. 1). Nine putative LRRs were recognized in the ECD of the eTSHR (12, 16). The LRRs of GPHRs contain the structural determinants involved in ligand recognition specificity, mainly by establishing specific hormone-receptor electrostatic interactions (35).

A functional analysis was performed to determine whether the cloned cDNA encoded a functional receptor. The recognition in transfected COS-7 cells by mAb 3G4, which is specific of an epitope present only in TSHR in different species, as well as dose-dependent response to bTSH and hTSH, absence of response to eCG or hFSH, clearly identifies this cDNA as the eTSHR cDNA. The CG hormone is secreted in the maternal placenta, and because its gestational secretion is limited to primate (23) and equine (24) species, challenging the eTSHR with this hormone appeared to be potentially an interesting experience. Whereas the equine receptor did not respond to eCG, it displayed a significant sensitivity toward hCG. This responsiveness to an illegitimate hormone, despite strong specificity barriers between hormone-receptor couples, points to the potential vulnerability of the specificity of receptors for glycoprotein hormones. This has been illustrated in two pathological situations in human resulting from spontaneous mutations in TSHR and FSHR. In the case of hTSHR, only one mutation was identified, leading to gestational hyperthyroidism (27), whereas in the case of FSHR, six mutations were identified, leading to spontaneous gestational ovarian hyperstimulation syndrome (28–33), in both cases through promiscuous activation of receptors by hCG. This subsequently permitted the identification by targeted mutagenesis and modelization of several residues of the hTSHR ectodomain involved in TSHR specificity. In contrast, all but one FSHR mutation were located in the serpentine domain. This indicated that the two brother receptors, hFSHR and hTSHR, had evolved through distinct ways to acquire and maintain the specificity to their respective ligands. It appeared that the specificity of TSHR was strictly encoded in the LRRs, whereas the specificity of FSHR, although encoded in LRRs also, could be strongly altered by modifications of the serpentine domain. Here, the study of chimeric TSHRs swapping human and equine ectodomains clearly demonstrates that the increased sensitivity of eTSHR to hCG is due to the ectodomain.

Homology models, using the crystallographic structure of the ECD of the FSHR in complex with FSH as a template, of GPHRs in complex with different hormones allowed us to identify three amino acids putatively responsible for the gain of sensitivity of the eTSHR toward hCG. The exchange of S229 in hTSHR by Thr in eTSHR adds a methyl group that can favorably interact with the hydrophobic V48 and L49 of the β-subunit of hCG (Fig. 8). The other two substitutions of eTSHR, relative to hTSHR, Thr instead of I60 and Tyr instead of Q235 are in the proximity of the "seat-belt" portion of the β-subunit of hCG. The presence of a Thr in position 60 creates a polar hydrogen bond acceptor area together with the nearby S84, which is predicted to interact with the K104 of hCG (Fig. 8). The

Y235 maintains the hydrogen bond acceptor capability but at a distance larger than with Q235. Thus, the longer Y235 is predicted to interact with the basic residue R94 of hCG (Fig. 8). These additional interactions of eTSHR, relative to hTSHR, cause the gain of function of eTSHR toward hCG. By targeted mutagenesis of these three residues, alone or in combination, we could confirm their involvement in sensitivity to hCG, and show they were acting synergistically. We cannot determine why the I60T or S229T single point mutations do not exhibit any significant increase in sensitivity to hCG, whereas the triple I60T/S229T/Q235Y hTSHR mutant is necessary to achieve the same sensitivity to hCG as the eTSHR. Interestingly, in a previous work, replacement of these three residues in the hTSHR by the hLHR counterparts (I60A, S229K, and Q235S mutants) did not show any particular phenotype or sensitivity toward hCG (35). Clearly, the I60A substitution did not add the hydrogen bond acceptor group to interact with K104 of hCG; S229K did not add the methyl group to form hydrophobic-hydrophobic interactions, and the shorter S235, relative to Q235, in the Q2^{35}S substitution could not reach R94 of hCG. Thus, surrounding neutral residues may participate in the appropriate positioning of the residues interacting with the hormone. In addition, the fact that the eTSHR is not sensitive to eCG indicates that the receptor is not permissive but rather that evolution has selected negative determinants targeting the coevolving hormone, in agreement with the known strict LH activity of eCG in the horse (24, 36). eCG has replaced K104 of hCG by arginine that cannot optimally interact with T60 and S84, and has replaced R94 of hCG by glutamine that cannot reach Y235. These replacements in the hormone explain the unresponsiveness of eTSHR to eCG. The hTSHR does not respond to eCG either, which is unlikely to result from a selection during evolution because the receptor is not exposed to eCG. Similarly, it is probable that the response of eTSHR to hCG is not resulting of any selection because an evolutionary event common to human and horse would have affected other species as well. When comparing residues at positions 60, 229, and 235 in nonprimate nonequine mammalian species (Fig. 10), it appears that T229 and Y235 are present in many species deprived of CG. Thus, the T229 and Y235, although participating in selectivity toward hCG, probably do not participate in the barrier against eCG. The I60 is present in all but the equine species. Because we show here that it is involved in specificity, it may be hypothesized that its replacement by a Thr in the eTSHR was related to the appearance of eCG rather than being the result of evolutionary drifting. Cloning and comparison of TSHR sequences in other primates and *Equidae* completed by targeted mutagenesis would help to answer this question.

The introduction of the K183R mutation in the eTSHR or in the human triple I60T/S229T/Q235Y mutant increased the sensitivity to hCG to the same extent. This purely additive effect suggests independent interactions between hCG and the three residues identified here on one hand and K183 on the other hand. The positive side chain of K183 is a key determinant of the specificity of the TSHR against hCG due to its repulsion with R95 of hCG (Fig. 8). In agreement with this hypothesis, mutation of K183 to any amino acid or the K183R mutation (34), releasing the nearby E157 to interact with R95 of hCG, increases the binding of hCG to TSHR.

Unexpectedly, the chimeric I60T/S229T/Q235Y h*ext/*

FIG. 10. Alignment of LRR$_2$ and LRR$_9$ of mammal TSHR sequences. Sequence alignment of LRR$_2$ and LRR$_9$ of the eTSHR and the hTSHR with nonprimate and non-*Equidae* mammal orthologs performed with ClustalW. LRR$_2$ and LRR$_9$ contain amino acids in positions 55–61 and 229–235, respectively. Alignment of the seatbelt of hCG and eCG. The key positions 60 (in LRR$_2$), 229, and 235 (in LRR$_9$) of TSHR and 94 and 104 in gonadotropins are highlighted in *gray boxes*.

e*tmd*TSHR was found to be more sensitive to hCG than the eTSHR and I60T/S229T/Q235Y hTSHR. At variance with the proposed model for hTSHR, the serpentine domain of the eTSHR appears to participate in the specificity barrier.

In conclusion, we have cloned the eTSHR that appeared more sensitive to hCG, but not to eCG, than the human receptor. We identified three residues located in the LRRs of the ectodomain, implicated for this enhanced sensitivity. Because evolution apparently selected different mechanisms in different species and different receptors to avoid the promiscuous stimulation by CG, the phylogenetic approach, complementary to modelization, may be useful for a better understanding of the ways of controlling specificity for the GPHRs.

Acknowledgments

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