RESEARCH ARTICLE

Identification of the First Germline Mutation in the Extracellular Domain of the Follitropin Receptor Responsible for Spontaneous Ovarian Hyperstimulation Syndrome

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The receptors for follitropin (FSHR), thyrotropin (TSHR), and lutropin/chorionic gonadotropin (LHCGR) are the members of the glycoprotein hormone (GPH) receptors (GPHR) family. They present a bipartite structure with a large extracellular amino-terminal domain (ECD), responsible for high-affinity hormone binding, and a carboxyl-terminal serpentine region, implicated in transduction of the activation signal. Spontaneous ovarian hyperstimulation syndrome (sOHSS) is a rare genetic condition in which human chorionic gonadotropin (hCG) promiscuously stimulates the FSHR during the first trimester of pregnancy. Surprisingly, germline FSHR mutations responsible for the disease have so far been found only in the transmembrane helices of the serpentine region of the FSHR, outside the hormone binding domain. When tested functionally, all mutants were abnormally sensitive to both hCG and thyrotropin (TSH) while displaying constitutive activity. This loss of ligand specificity was attributed to the lowering of an intramolecular barrier of activation rather than to an increase of binding affinity. Here we report the first germline mutation responsible for sOHSS (c.383C>A, p.Ser128Tyr), located in the ECD of the FSHR. Contrary to the mutations described previously, the p.Ser128Tyr FSHR mutant displayed increase in affinity and sensitivity toward hCG and did not show any constitutive activity, nor promiscuous activation by TSH. Thus, sOHSS can be achieved from different molecular mechanisms involving each functional domains of the FSHR. Based on the structure of the FSHR/FSH complex and site-directed mutagenesis studies, we provide robust molecular models for the GPH/GPHR complexes and we propose a molecular explanation to the binding characteristics of the p.Ser128Tyr mutant. Hum Mutat 29(1), 91–98, 2008. © 2007 Wiley-Liss, Inc.

KEY WORDS: FSHR; TSHR; GPCR; OHSS; hCG

INTRODUCTION

The human follitropin (FSH) receptor (FSHR; GenBank NM_000145.2 and MIM_136435), like the other members of glycoprotein hormone receptors (GPHR) family, TSHR (GenBank NM_000369.2) and LHCGR (GenBank NM_000233.2) [Ascoli et al., 2002; Dias et al., 2002; Gether, 2000; Szkudlinski et al., 2002], presents a bipartite structure, with a large extracellular domain (ECD) responsible for high affinity hormone binding [Fan and Hendrickson, 2005; Braun et al., 1991; Remy et al., 2001; Schmidt et al., 2001; Smits et al., 2003a; Vischer et al., 2003; Puett et al., 2007], and a carboxyl-terminal serpentine region, shared by rhodopsin-like G-protein-coupled-receptors (GPCR), implicated in transmission of the activation signal [Gether, 2000]. The ECD is structurally related to the family of proteins with leucine-rich repeats (LRR) and a 2.9-Å resolution structure of human FSH complexed with the extracellular hormone binding domain of its receptor was published [Fan and Hendrickson,

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2005]. In this crystal, the hormone is bound in a hand-clasp fashion to an elongated and curved FSHR ectodomain and the authors suggested that all glycoprotein hormones (GPH) would bind to their receptors in this mode. Retrospectively, these structural data validate functional studies generated by extensive site-directed mutagenesis of the thyrotropin (TSH) and FSH receptors, where exchange of specific residues in the LRR by their LHCGR counterparts could switch hormonal specificity [Smits et al., 2003a].

The GPH are heterodimeric proteins made of an alpha (α) subunit common to all four proteins and hormone-specific beta (β) subunits encoded by paralogous genes [Li and Ford, 1998] and sharing about 40% sequence identity as do the ECD of the corresponding receptors. Despite similarity between these paralogous hormones and receptors, coevolution of the hormone-receptor couples resulted in the establishment of tight specificity barriers, preventing promiscuous activation under normal physiological conditions. These barriers can be overruled in diseases, as in severe hypothyroidism, where very high concentrations of TSH have been made responsible for cases of ovarian hyperstimulation due to spillover of TSH activity on the FSH receptor [Anasti et al., 1995; Nappi et al., 1998]. The emergence of chorionic gonadotropin (CG) in primates, which achieves very high concentration during early pregnancy and is 85% identical to lutropin (LH), constituted an evolutionary challenge to the specificity barrier in GPHR couples. In humans, in particular, during the first trimester of pregnancy, human CG (hCG) reaches concentrations at which it displays some thyrotropic activity, bringing most pregnant women to the fringe of hyperthyroidism [Glinoer, 1997]. Overproduction of hCG in molar or twin pregnancies may result in overt gestational hyperthyroidism (MIM_603373) [Hershman, 1999].

However, specific mutations affecting the hormone binding surface of the TSHR or FSHR would be expected to lower the specificity barrier, and cause gestational hyperthyroidism or spontaneous ovarian hyperstimulation syndrome (sOHSS; MIM# 608115), even in the presence of normal levels of hCG. One case of gestational hyperthyroidism was reported with a mutation (p.Lys183Arg, K183R) [Rodien et al., 1998] in the ECD of the human TSHR, but so far and quite unexpectedly, all cases of sOHSS were linked to mutations in the serpentine domain of the FSHR [Smits et al., 2003b; Vasseur et al., 2003; Montanelli et al., 2004a, 2004b; De Leener et al., 2006]. In these reports, a relation was established between constitutive activity and lowering of specificity in the FSHR mutants, suggesting that the gain of sensitivity of the mutants to hCG would be due to lowering of an intramolecular barrier to activation rather than to increase in binding affinity [Vassart et al., 2004].

In the present study, we report the first mutation responsible of sOHSS located in the ECD of the human FSHR (c.383C>A, p.Ser128Tyr, S128Y), in the vicinity of residues implicated in hormone binding. When tested functionally, the p.Ser128Tyr mutant displayed promiscuous activation by hCG but, in contrast with the previous cases, it did not show any constitutive activity or promiscuous activation by TSH. As predicted from its location in the ECD, the p.Ser128Tyr mutant presented an increase of binding affinity for hCG. This finding demonstrates that in sOHSS, promiscuous activation of the FSHR by hCG can be achieved by different molecular mechanisms involving each of the two functional domains of the FSHR.

Additional site-directed mutagenesis demonstrates that some substitutions at this position were less selective than p.Ser128Tyr and could, in addition, open the specificity toward TSH. Serine 128 is located in the vicinity of residues implicated in hormone binding in the crystal structure of the FSHR/FSH [Fan and Hendrickson, 2005]. Molecular models of the illegitimate FSHR/hCG and FSHR/TSH complexes were built by homology with the FSHR/FSH crystal structure. They provided a molecular explanation for the observed increase of affinity of FSHR mutants to hCG, and, for some of them, to TSH.

MATERIALS AND METHODS

Reagents

Plasmid pBluescript SK+ and *Pfu* turbo polymerase were obtained from Stratagene (La Jolla, CA, USA) and plasmid pSVL was obtained from Amersham Pharmacia Biotech (Roosendaal, The Netherlands). Restriction enzymes were obtained from Invitrogen (Merelbeke, Belgium) and New England Biolabs (Beverly, MA). Mouse monoclonal antibody 5B2 was obtained by genetic immunization with the cDNA encoding the human FSHR [Costagliola et al., 1998]. Recombinant human FSH (rhFSH) was from Organon Belge (Brussels, Belgium), recombinant hCG (rhCG) from Sigma Chemical (St. Louis, MO) and recombinant human TSH (rhTSH) from Genzyme (Cambridge, MA). Polyethylenimine (PEI), linear, molecular weight (MW) ~25,000 was from Polysciences, Inc. (Warrington, PA) [Boussif et al., 1995]. [^{125}I]hFSH was from Perkin Elmer (Rodgau-Jügesheim, Germany).

Patient Subjects

The clinical characteristics of the sOHSS patient have been described previously [Cepni et al., 2006]. The proband was a 21-year-old Turkish woman, gravida 1, para 0, presenting with severe sOHSS at week 11 of her first pregnancy. The β-hCG level was normal for a 3-month singleton pregnancy. The TSH level was normal. Symptoms regressed during the second and third trimester with a conservative medical treatment and resolved in postpartum. Her medical and family history was unremarkable.

DNA Sequencing and Mutation Identification

Human DNA was extracted from peripheral blood leukocytes and the sequences of all the exons of the human FSHR gene (GenBank NM_000145.2), together with intron–exon boundaries, were determined as previously described [Smits et al., 2003b]. The patient gave informed consent to participation in this study, which has been approved by the Ethical Committee of Erasme Hospital, Brussels, Belgium. The patient is heterozygote for a C to A transversion (c.383C>A) that substitutes a tyrosine (Tyr, Y) for serine (Ser, S) at position 128 (p.Ser128Tyr, S128Y). The DNA mutation numbering is based on cDNA sequence where +1 corresponds to the A of the ATG initiation codon. The sequence of the segment harboring this mutation has been determined from the product of two independent polymerase chain reactions (PCRs). The presence of the mutation has been confirmed on a second blood sample. The same region was sequenced from 96 control blood samples from the same ethnical origin and the same substitution was not found.

Construction of Human FSHR Mutants

Mutations were introduced in the human wild-type (wt) FSHR by site mutagenesis as previously described [Vlaeminck-Guillem et al., 2002]. The appropriate mutated portions of SK+FSHR mutants were subcloned in the pSVL-FSHR cDNA using natural restriction sites. The constructs were verified by sequencing on both strands.

Transfection Experiments

COS-7 cells were used for all the transient expression experiments. The cells were transfected with PEI [Boussif et al., 1995] (stock 1 μg/μl, pH 7.2, in water, conserved at −80°C) with modified protocol. Briefly, 6 μg of DNA were mixed with 18 μg PEI in NaCl (150 mM, 1,400 μl final volume), then added on 10^6 cells in suspension (in 1,200 μl of culture medium) after 30 minutes of incubation at room temperature. After addition of 6 ml of fresh culture medium, cells were distributed in 48 wells (250 μl per well) for stimulation with hormones or for flow immunocytometry (FACS), 24 wells (500 μl per well) for binding experiments, or six wells (1.5 ml per well) for basal cyclic adenosine monophosphate (cAMP) accumulation. The medium was changed the day after transfection and cells were used 2 days after transfection for functional experiments.

Quantification of Cell Surface Expression by FACS

Expression on the cell surface was assessed by flow immunocytometry (FACScan flow cytofluorometer; Becton Dickinson, Erembodegem, Belgium) with the 5B2 mouse monoclonal antibody as previously described [Costagliola et al., 1998]. Cells transfected by pSVL alone (empty vector) and by pSVL-FSHR wt were always used as negative and positive controls, respectively.

Determination of cAMP Production

At 48 hr after transfection the intracellular accumulation of cAMP was measured by radioimmunoassay (RIA) as described previously [Smits et al., 2003a]. cAMP concentrations were determined in duplicate on extracts from duplicate transfection dishes or wells. Results are expressed as picomoles cAMP per milliliter (pmol/ml), or percentage of maximal cAMP response. The Prism computer program (GraphPad Software, Inc., San Diego, CA) was used for curve fitting and for EC_{50} determination.

Binding Experiment

Ligand binding was measured on COS-7 cells transfected with various constructs with PEI as previously described [Smits et al., 2003a], with minor modifications, when volumes were adjusted to work in 24-well culture dishes. Briefly, 48 hr after transfection, cells were washed twice with 400 μl modified Krebs-Ringer-HEPES buffer (without NaCl, isotonicity maintained with 280 mM sucrose). Thereafter, cells were incubated overnight at room temperature with 200 μl of the same buffer supplemented with 5% low fat milk, [^{125}I]hFSH (100,000 counts per minute [cpm] per well) and graded concentration of cold rhCG or rhFSH. Thereafter, the cells were washed twice with 400 μl of the same ice-cold buffer and solubilized with 200 μl of 1 N NaOH. Radioactivity was measured in a γ counter. All experiments were carried out at least in duplicate and results are expressed as cpm [^{125}I]hFSH bound. The Prism computer program was used for curve fitting.

Construction of Homology Models for wt and Mutant Glycoprotein Hormone Receptors in Complex With Hormones

The crystallographic structure of the extracellular domain of the FSHR in complex with FSH (Protein Databank [PDB] code 1XWD) [Fan and Hendrickson, 2005] has been employed to build homology models of the LHCGR-hCG and TSHR-TSH complexes, and mutants FSHR in complex with hCG and TSH. SCWRL 3.0 was employed to add the side chains of the nonconserved residues based on a backbone-dependent rotamer library [Canutescu et al., 2003]. The resulting structures were placed in a rectangular box (~101 Å × 97 Å × 108 Å in size) containing ~27,000 Monte Carlo–equilibrated TIP3P water molecules. Initially, the system was subjected to 500 iterations of energy minimization and then heated to 300°K in 15 ps. Structures were collected every 10 ps during 1 ns (100 structures per simulation). During the molecular dynamics simulations of mutant FSHR in complex with hCG and TSH, a positional restraint of 1 kcal mol^{-1} $Å^{-2}$ was applied to the C_α atoms of the

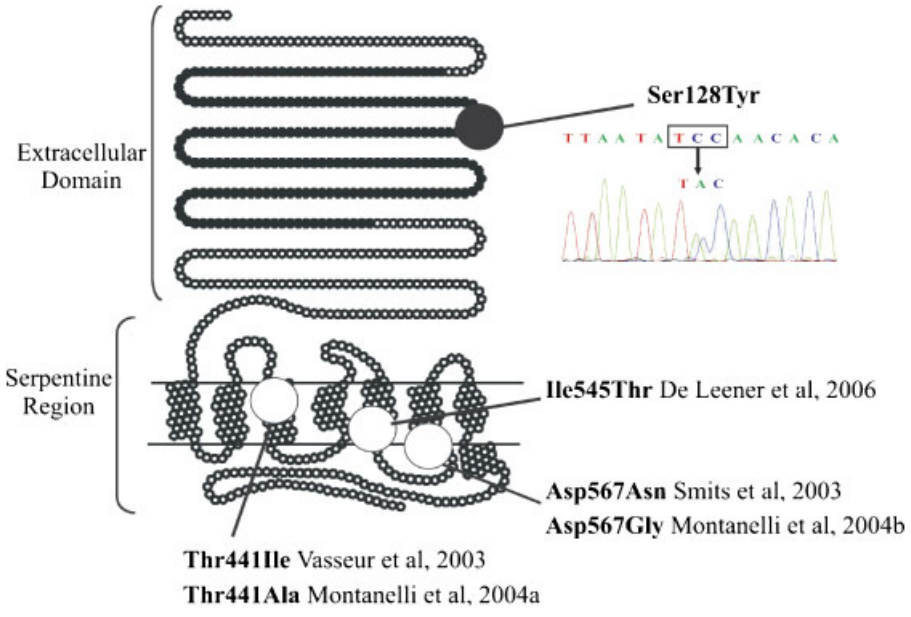

FIGURE 1. **Presentation of the mutation. Left: Locations of the mutations identified to date in the FSHR (GenBank NM_000145.2) of patients presenting with sOHSS. The numbering of amino acids begins at ATG initiating codon. Right: Representation of the nucleotide sequence around codon 128 (exon 5) of the FSHR in the new sOHSS patient. The patient is heterozygote for a c.383C>A mutation encoding a p.Ser128Tyr protein change. The DNA mutation numbering is based on cDNA sequence where +1 corresponds to the A of the ATG initiation codon.**

TABLE 1. Functional Characterization of All the Ser128 Mutants[†]

	FACS % expression of wt		rhCG screening 300 UI/ml			rhTSH screening 100 mUI/ml	
Mutation	Mean	SEM	Mean	Range	rhCG*100/FACS	Mean	Range
FSHR wt	100	0	0.54	0.01	0.54	1.76	1.16
p.Ser128Tyr	35.92	4.39	2.02	0.30	5.61	1.10	0.47
p.Ser128Cys	42.82	5.91	0.37	0.13	0.85	0.60	0.31
p.Ser128Phe	23.11	3.15	0.73	0.15	3.18	0.04	0.04
p.Ser128Ala	103.64	2.82[a]	0.26	0.01	0.25	nd	nd
p.Ser128Asp	12.28	2.04[a]	1.18	0.42	9.62	nd	nd
p.Ser128Glu	26.00	4.00	1.83	0.76	7.04	0.47	0.14
p.Ser128Gly	43.95	2.15[a]	1.19	0.49	2.72	nd	nd
p.Ser128His	127.48	7.15[a]	0.00	0.11	0.00	nd	nd
p.Ser128Ile	38.77	3.86	4.43	1.01	11.43	7.77	0.77
p.Ser128Lys	151.85	10.05[a]	0.00	0.16	0.00	nd	nd
p.Ser128Leu	32.51	0.75[a]	0.50	0.25	1.55	nd	nd
p.Ser128Met	50.56	12.03[a]	0.39	0.29	0.77	nd	nd
p.Ser128Asn	14.77	1.86[a]	0.00	0.10	0.00	nd	nd
p.Ser128Pro	0	0[a]	0	0	0	nd	nd
p.Ser128Gln	106.53	5.50	2.57	0.74	2.41	6.69	1.85
p.Ser128Arg	149.17	19.66[a]	0.00	0.00	0.00	nd	nd
p.Ser128Thr	42.24	1.87[a]	0.56	0.57	1.32	nd	nd
p.Ser128Val	31.77	4.75	2.68	1.22	8.43	3.65	1.23
p.Ser128Trp	55.96	1.46[a]	0.00	0.15	0.00	nd	nd

[†]Column 1: The FSHR mutants characterized in this study. Column 2: Expression of wt or mutant receptors as measured by FACS (expressed in % of the wt FSHR; GenBank NM_000145.2). Sensitivity of individual mutants to rhCG (columns 3 and 4), or rhTSH (column 5). Results are expressed as mean ± standard error of the mean (SEM) pmol/ml of cAMP. Since maximal stimulation could not be achieved (see Fig. 2), sensitivity to rhCG or rhTSH could not be measured as EC_{50}. Hence, cAMP accumulation over basal (empty vector transfected) for a fixed concentration of hormone was used as an index of sensitivity (normalized to surface expression [for hCG, column 4], or not [columns 3 and 5]).
[a]Two measurements were performed. Results are expressed as mean ± range of duplicate.
nd=not done.

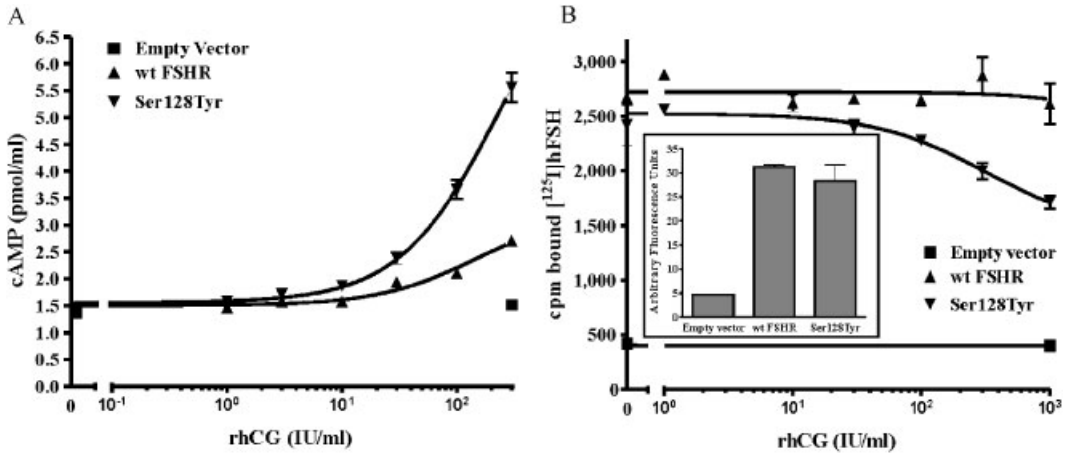

FIGURE 2. **Functional characterization of the p.Ser128Tyr FSHR mutant. Various amount of DNA of the wt FSHR were transfected in COS-7 cells and the conditions were selected giving equal expression of the wt FSHR and the Ser128Tyr mutant (see inset panel B). A: Stimulation of the cells expressing wt FSHR or the Ser128Tyr mutant with rhCG. Results are expressed as mean ± standard error of the mean (SEM) pmol/ml of cAMP. B: Competition between binding of [^{125}I]hFSH and cold rhCG to the wt FSHR and the Ser128Tyr mutant. Results are expressed as mean ± SEM cpm bound [^{125}I]hFSH. Each graph is representative of at least three independent experiments.**

receptor structure. Langevin temperature regulation was used at constant pressure using the particle mesh Ewald method to evaluate electrostatic interactions. The molecular dynamics simulations were run with the Sander module of AMBER 9 [Case et al., 2005], the all-atom force field [Ponder and Case, 2003], SHAKE bond constraints in all bonds, a 2-fs integration time step, and constant temperature of 300°K coupled to a heat bath.

RESULTS

Functional Characterization of the S128Y FSHR Mutant Responsible for sOHSS

We describe here a mutation resulting in the replacement of a Serine (Ser, S) by a Tyrosine (Tyr, Y) at codon 128 (p.Ser128Tyr, c.383C>A) in the FSHR of a patient presenting with sOHSS (see case report in Materials and Methods [Cepni et al., 2006] and Fig. 1). Serine 128 is located in position X_4 of the fifth LRR (LRR_5) in the FSHR ECD. The mutant was analyzed functionally by transient expression in COS-7 cells. Despite reduction of expression at the cell surface (36% of the wt FSHR; Table 1), the p.Ser128Tyr mutant showed a clear lowering of its specificity toward hCG with an increase of sensitivity (Fig. 2A; Table 1) and affinity (Fig. 2B) toward this hormone. In contrast with the results obtained with previously described sOHSS mutations [Smits et al., 2003b; Vasseur et al., 2003; Montanelli et al., 2004a, 2004b; De Leener et al., 2006], neither the basal activity (Fig. 2) nor the sensitivity to TSH (Table 1; Fig. 3) were affected by the mutation (see Supplementary

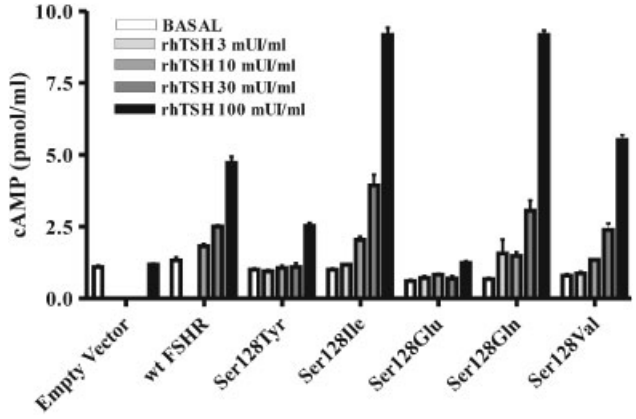

FIGURE 3. **rhTSH response of the Ser128 mutants. COS-7 cells transiently transfected with Ser128Tyr(Y), Ser128Ile(I), Ser128-Glu(E), Ser128Gln(Q), and Ser128Val(V) mutant receptors were stimulated with increasing concentration of rhTSH, expressed in mIU/ml. Intracellular cAMP accumulation was determined by RIA. This graph is representative of at least two independent experiments.**

Fig. S1; available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat; which compares in vitro phenotypes of mutant p.Ser128Tyr and sOHSS mutant p.Asp567Asn previously described). The sensitivity to FSH remained unchanged (data not shown).

Phenotype of Other Ser128 Mutants

To explore the molecular mechanisms of the gain of function caused by substitutions at position 128, a panel of mutants was engineered in which Ser128 was replaced by the 19 other amino acids. None of the expressed mutants presented detectable constitutive activity (data not shown). Differences were observed in the level of expression at the cell surface of individual mutants, ranging from 0% (Ser128Pro) to 150% (Ser128Lys) of the wt FSHR (Table 1). The sensitivity to hCG remained unchanged for mutants Ser128Ala/His/Lys/Pro/Arg/Trp and to a lesser extent mutants Ser128Cys/Met (Table 1). Interestingly the substitution of Ser128 by Cys or Phe (residues located at this position in the ECD of LHCGR and TSHR, respectively) was with minor effect. Despite weak expression (38% of the wt), mutant Ser128Ile was

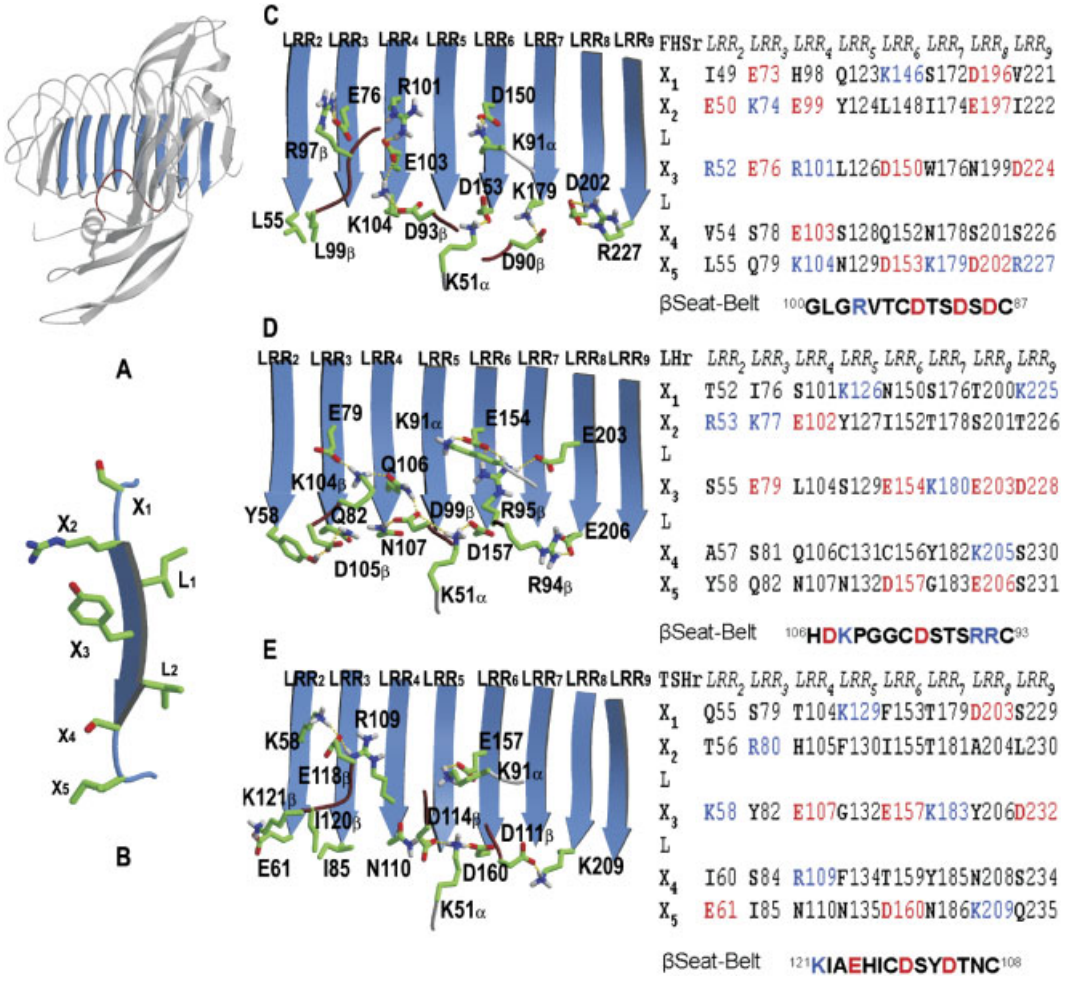

FIGURE 4. **Molecular models of GPHR in complex with hormones. A:** General view of the FSHR-FSH crystal structure (PDB code 1XWD) [Fan and Hendrickson, 2005]. The position of LRR_{2-9} and the seat-belt portion of the β-subunit are shown in blue and dark red. **B:** Structure of a representative LRR showing the positions of $X_1X_2LX_3LX_4X_5$. **C–E:** Detailed view (LRR_{2-9}) of the crystal FSHR-FSH (C) structure and computer models of LHCGR-hCG (D) and TSHR-TSH (E) complexes. Schematic representation of the X_{1-5} side chains in LRR_{2-9} of the GPHR exposed to solvent and predicted to contact the ligands, together with the amino acid side chains of the seat-belt portion of the β-subunit of GPH.

particularly sensitive to hCG (see Table 1) and in addition, together with Ser128Gln and Ser128Val mutants, it displayed also a strong increase of sensitivity to TSH (Table 1; Fig. 3). The Ser128Glu mutants was sensitive to hCG (like Ser128Asp) but without effect regarding TSH (see Table 1).

The only structural data available is from the crystal of the complex between FSHR ECD and FSH [Fan and Hendrickson, 2005]. Therefore, in order to interpret in molecular terms the increase in sensitivity of individual Ser128 mutants to hCG (and/or TSH), we used homology modeling techniques (see Materials and Methods) to build models of LHCGR-hCG and TSHR-TSH complexes (accessible at http://gris.ulb.ac.be), employing the FSHR-FSH crystal structure as a template (Fig. 4A) and the ECD gain of function mutants available in the literature.

Specificity of Recognition of GPHR

Selectivity of FSHR toward hCG. The specificity for the different GPH is guided by the "seat-belt" portion of the β-subunit [Dias et al., 1994; Moyle et al., 1994; Grossmann et al., 1997]. Interestingly, the seat-belt of β-hCG (CGB; GenBank NM_000737.2) has two basic residues (Arg94 [R94β] and Arg95 [R95β]), while β-FSH (FSHB; GenBank NM_000510.2) has acidic residues in this part of the hormone (D88β and D90β) (Fig. 4). Appropriately, X_5 of $LRR_{7,8,9}$ contain G183, E206, and S231 in LHCGR (negative electrostatics in this area of the receptor) and K179, D202, and R227 in FSHR (positive electrostatics), respectively. Thus, similarly to the interaction between D90β of FSHB and K179 of FSHR (Fig. 4C), we propose that the CGB-specific R94β forms a salt bridge with E206 of LHCGR while R95β interacts with both E154 and E203 (Fig. 4D). K179 of FSHR is thus a key determinant of the specificity against hCG because it is repulsive with R94β of CGB [Smits et al., 2003a].

Selectivity of FSHR toward TSH. Both FSHR (K179; Fig. 4C) and TSHR (K209; Fig. 4E) form at the bottom of $LRR_{7,8}$ an ionic interaction with a negatively-charged side chain of FSHB (D90β) or β-TSH (TSHB; GenBank NM_000549.3) (D111β), respectively. The specificity of the FSHR against TSHB resides at $LRR_{2–4}$. TSHR contains K58 and R109, conferring a positive electrostatic surface to this domain, which forms a salt bridge with the TSHB-specific E118β (Fig. 4E). In contrast, E76 confers to FSHR a negative electrostatic region that interacts with the FSHB-specific R97β (Fig. 5A).

Sensitivity of Ser128Gln(Q)/Glu(E)/Tyr(Y) mutant FSHR toward hCG. Mutation of Ser128 to Gln(Q), Glu(E), or Tyr(Y) permits FSHR to hydrogen bond the CGB-specific side chain of R95β, increasing the binding of the mutant FSHR toward hCG (Fig. 5). In particular, the side chain of Q128 bridges R95β and D99β of hCG (Fig. 5D); E128 forms attractive interactions with R95β and repulsive interactions with D99β (Fig. 5E); and Y128 forms a hydrogen bond with R95β of

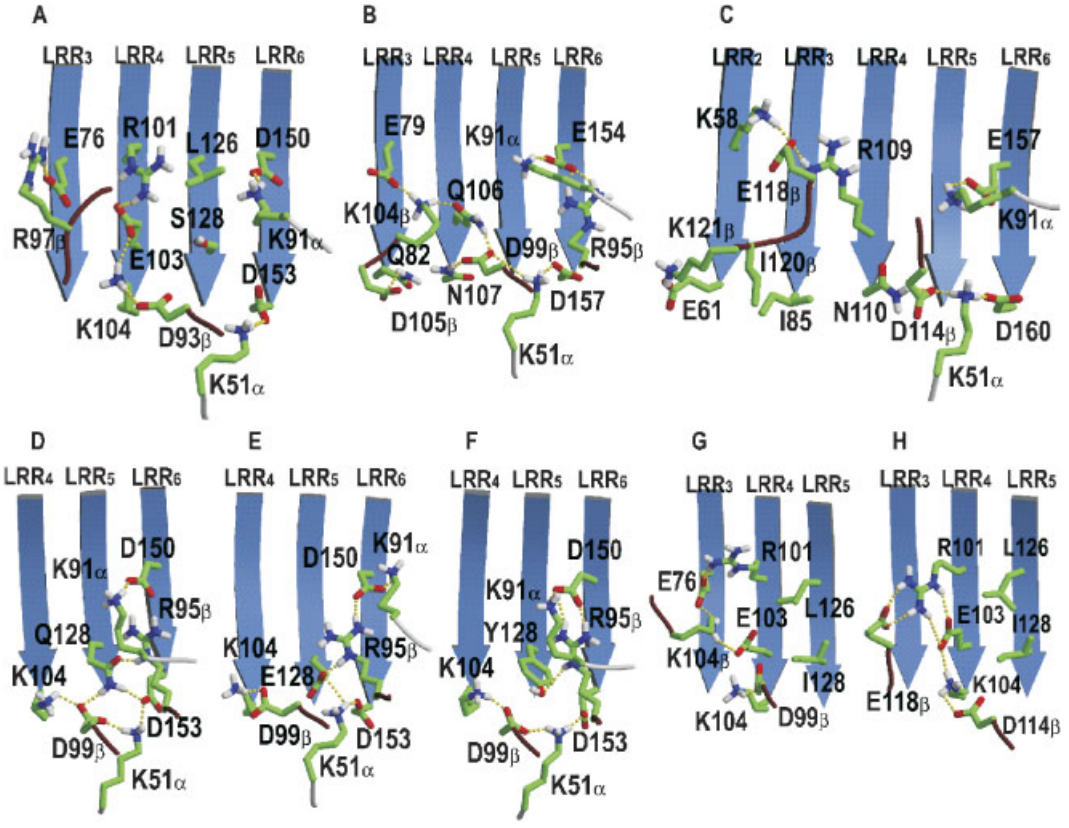

FIGURE 5. **Detailed view of $LRR_{3–6}$ of GPHR in complex with hormones. The crystal FSHR-FSH (A) structure, computer models of LHCGR-hCG (B) and TSHR-TSH (C) complexes, computer models of Ser128Gln(Q)/Glu(E)/Tyr(Y) mutant FSHR in complex with hCG (D–F), and computer models of Ser128Ile(I) mutant FSHR in complex with hCG (G) and TSH (H). The Ser128Gln(Q)/Glu(E)/Tyr(Y) mutation permits FSHR to hydrogen bond the βhCG-specific side chain of R95β (D–F), increasing the sensitivity of the mutant FSHR toward hCG. The bulky and β-branched side chain of Ile(I)128 modifies the conformation of the nearby Glu(E)103 side chain and its ionic Arg(R)101 partner, allowing Lys(K)104β of hCG to interact with Glu(E)103 of FSHR (G) and Glu(E)118β of TSH to interact with Arg(R)101 of FSHR (H).**

hCG (Fig. 5F). The repulsive interaction between E128 and D99β explains the lower effect in hCG binding of the S128E mutation relative to S128Q.

Sensitivity of Ser128Ile(I)/Val(V) mutants FSHR toward hCG and TSH. The Ser128Ile(I)/Val(V) mutation in FSHR adds a bulky and β-branched side chain, which the Cγ moiety modifies the conformation of the nearby E103 side chain and its ionic R101 partner in the wt receptor (Fig. 5A). Thus, S128I/V mutants force R101 and E103 to point toward LLR_3 and allow K104β of hCG to interact with E103 of FSHR (Fig. 5G) and E118β of TSH to interact with R101 of FSHR (Fig. 5H), increasing the sensitivity of the mutant FSHR toward hCG and TSH. Importantly, non-β-branched, more flexible, hydrophobic residues do not elicit this effect due to the possibility of their side chain adopting a conformation distant from E103.

DISCUSSION

In opposition to the five previously described mutations found in sOHSS cases [Smits et al., 2003b; Vasseur et al., 2003; Montanelli et al., 2004a, 2004b; De Leener et al., 2006] (see Introduction), the location of the mutation in the extracellular binding region of the FSHR and the functional characterization of the p.Ser128Tyr mutant clearly suggests that the increase of sensitivity toward hCG observed here is the consequence of an increase of affinity toward this hormone. Interestingly, the p.Ser128Tyr substitution is selective because no increase of sensitivity to TSH was measured. In addition, the absence of constitutive activity of this p.Ser128Tyr mutant demonstrates that this is not a prerequisite condition in vivo to develop OHSS, contrary to what previous natural mutations found in sOHSS had suggested before [Delbaere et al., 2005].

Finally, extensive directed mutagenesis at the position 128, demonstrates that the increase of sensitivity toward hCG is not the consequence of the loss of the Serine at position 128 because some substitutions were neutral (e.g., p.Ser128Ala and p.Ser128His; Table 1). Also, some substitutions were poorly selective, increasing the sensitivity to both hCG and TSH (e.g., p.Ser128Ile and p.Ser128Val).

Previously, one case of gestational hyperthyroidism was reported with a mutation (p.Lys183Arg, K183R) [Rodien et al., 1998] in the ECD of the human TSHR. In vitro characterization demonstrated that mutation of K183 (X_3 of LRR_7) in the TSHR to any amino acid [Smits et al., 2002] or adding a negative charge in X_3 of LRR_8 (the Y206E mutation) [Smits et al., 2003a] increase the sensitivity of TSHR for hCG. Analysis of this mutant in the light of the molecular models provided here, confirms the suggestion that the increase of sensitivity for hCG was due to the release of neighboring residue E157 from a neutralizing interaction with K183 [Smits et al., 2002], permitting the access of R95β of hCG to the acidic E157 of TSHR. Thus, both S128Y of FSHR and K183R of TSHR, the two unique natural mutations in the ECD of a GPHR increasing its sensitivity toward hCG, cause their irregular function by interacting with R95β of hCG.

Together with the crystal structure of the FSH-FSHR complex [Fan and Hendrickson, 2005], natural [Smits et al., 2003b; Vasseur et al., 2003; Montanelli et al., 2004a, 2004b; De Leener et al., 2006; Rodien et al., 1998], or experimental [Smits et al., 2003a] gain of function mutants of the GPHR showing an increase of sensitivity regarding hCG allowed us to build robust models of both legitimate and illegitimate interactions between these families of paralogous gene products. With the increasing availability of animal genome and GPH-GPHR sequences, these models will be of great help to decipher the evolution of ligand binding specificity during the expansion of the GPH-GPHR family.

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