Loss-of-Function Mutations in the Human Luteinizing Hormone Receptor Predominantly Cause Intracellular Retention

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Mutations in G protein–coupled receptors (GPCRs) have been identified for many endocrine hormone signaling deficiencies. Inactivating mutations can impair ligand binding, receptor activation/coupling to signaling pathways, or can cause receptor misfolding and consequent impaired expression at the cell membrane. Here we examine the cell surface expression, ligand binding, and signaling of a range of mutant human luteinizing hormone receptors (LHRs) identified as causing reproductive dysfunction in human patients. The data obtained reveal how mutations in GPCRs can have diverse and severely deleterious effects on receptor function. Furthermore, it was found that impaired functionality of the majority of the mutant LHRs was due to reduced expression at the cell surface (14/20) while only two mutations caused impaired binding affinity and two impaired in signaling. An additional two mutations were found to cause no impairment of receptor function. These data demonstrate that the majority of LHR mutations lead to intracellular retention and highlight the potential for novel pharmacological chaperone therapeutics that can "rescue" expression/function of retained mutant GPCRs. (*Endocrinology* 157: 4364–4377, 2016)

The hypothalamic-pituitary-gonadal axis governs the endocrine control of reproduction. GnRH released from the hypothalamus binds the GnRH receptor on gonadotrope cells in the anterior pituitary, stimulating secretion of the gonadotropins, LH and FSH. LH and FSH enter the circulation and activate their cognate receptors LHR/LHCGR and FSHR in the gonads, to stimulate gametogenesis and the production/secretion of the sex steroid hormones. Perturbation of the hypothalamic-pituitary-gonadal axis is associated with reproductive phenotypes, such as Leydig cell hypoplasia (LCH) in males (manifesting as a continuum of disorders from micropenis and hypospadias [Class II LCH], through to pseudohermaphroditism [Class I LCH]), and amenorrhea, and ovarian cysts in females (1). LCH is a 46,XY disorder, characterized by high circulating levels of LH with impaired male gonadal development. In some cases, LCH can be attributed to inactivating mutations in the LHR gene. In 46,XX patients inactivating LHR mutations can result in infertility, oligo-/amenorrhea, and/or empty follicle syn-

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Abbreviations: 7TM, seven transmembrane; CRE, cAMP response element; FCS, fetal calf serum; GPCR, G protein–coupled receptor; HEK, Human embryonic kidney; LCH, Leydig cell hypoplasia; LHR, luteinizing hormone receptor; LRR, leucine-rich repeat; TM, transmembrane domain; WT, wild-type.

Table 1. Naturally Occurring Inactivating Mutations of the LHR: Clinical Phenotypes

Mutation(s)	Phenotype(s)	Reference(s)
Ins(LLKLLLLLQLQ) after Q18 & *W491Stop* (cpd het)	Pseudohermaphroditism (46, XY)	(48)
Ins(LLKLLLLLQLQ) after Q18 & *C545Stop* (cpd het)	Pseudohermaphroditism (46, XY)	(49, 50)
I114F & ?[a] (het)	Pseudohermaphroditism (46, XY)	(34)
C131R (hom)	Hypoplastic phallus with hypospadias (46, XY)	(38)
V144F (hom)	Pseudohermaphroditism (46, XY)	(26)
I152T & [*sp> ± Del Exon7*][b] (cpd het)	Predominantly female genitalia, but with some degree of virilisation (46, XY)	(39)
F194V (hom)	Pseudohermaphroditism (46, XY)	(27)
Del Exon8 & S616Y[c] + [N291S][d] (cpd het)	Micro penis, hypospadias (46, XY)	(51)
Del Exon 10 (hom)	Absence of puberty (46, XY)	(52)
Sp>Del Y317-S324 (hom)	Micro penis, delayed puberty, oligospermia (46, XY), Infertility ± oligomenorrhea (46, XX)	(47)
C343S & C543R (cpd het)	Pseudohermaphroditism (46, XY)	(28)
E354K (hom)	Pseudohermaphroditism (46, XY), Primary amenorrhea (46, XX)	(53)
I374T + T392I (dbl hom)	Pseudohermaphroditism (46, XY)	(32, 54)
N400S (hom)	Infertility, empty follicle syndrome (46, XX)	(55)
N415T *& mutExon6A*[e] *(cpd het)*	Micropenis (46, XY)	(56)
T461I & *mutExon6A*[e] (cpd het)	Pseudohermaphroditism (46, XY)	(57)
L502P (hom)	Pseudohermaphroditism (46, XY)	(33)
R554Stop (hom)	Pseudohermaphroditism (46, XY), Primary amenorrhea (46, XX)	(58)
Ins (>fs with premature Stop) after A589 (hom)	Pseudohermaphroditism (46, XY)	(59)
A593P (hom)	Pseudohermaphroditism (46, XY), Primary amenorrhea (46, XX)	(29, 60)
Del L608-V609 (hom)	Pseudohermaphroditism (46, XY), Oligomenorrhea and infertility (46, XX)	(30)
Y612Stop (hom)	Pseudohermaphroditism (46, XY)	(61)
S616Y[c] (hom)	Micro penis (46, XY)	(58)
I625K (hom)	Micro penis, lack of puberty and infertility (46, XY)	(31, 48)

Abbreviations: Cpd het, compound heterozygote; dbl hom, double homozygote; Del, deletion; Ins, insertion; fs, frame shift; sp, splice site mutation; hom, homozygous; het, heterozygous.

Mutations highlighted in bold type indicate those examined in the present study.

[a] Patient was heterozygous for indicated mutation; therefore, this mutation alone does not account for the observed phenotype.

[b] A splice-acceptor mutation causes abnormal splicing and deletion of Exon 7 in a subset of receptors; therefore, it is only partially deleterious.

[c] The S616Y mutation has been identified in both compound heterozygous and homozygous cases.

[d] This mutation is not deleterious.

[e] Mutations in a cryptic exon (6A) of the LHCGR increase expression of transcripts that trigger nonsense medicated decay of transcribed LHCGR mRNA.

drome. To date, approximately 30 naturally occurring inactivating genetic mutations of the LHR gene have been described in patients suffering from varying degrees of reproductive dysfunction (Table 1).

The LHR is a member of the G protein–coupled receptor (GPCR) superfamily. GPCRs are characterized by a serpentine seven transmembrane (7TM) domain connected by intracellular and extracellular loops, with an extracellular N-terminus and an intracellular C-terminal tail. They are activated by a diverse spectrum of ligands and elicit their intracellular effects through activation of a class of intracellular GTPases, termed G proteins. In addition to the 7TM domain, the glycoprotein hormone receptor subfamily, of which the LHR is a member, have a large extracellular N-terminal ectodomain (organized as 12 leucine-rich repeats [LRRs], stabilized by disulphide bridges) (Figure 1). The extracellular and 7TM domains are joined by a "hinge region" which, in the receptor's inactive state, is believed to confer an inhibitory effect on the 7TM domain. Hormone binding to the large concave surface formed by LRRs 1–8 results in movement of the hinge and release of its inhibitory constraint on the serpentine domain, allowing its activation (2–4).

The central role of GPCR signaling in most human physiological processes is reflected by the number of pathophysiological conditions caused by impaired GPCR signal transduction, and also the large number of therapeutics targeting GPCR signaling pathways [approximately 30% of all marketed drugs (5)]. Classically, GPCR mutations were thought to result in impaired ligand binding or disruption of intracellular signaling, but it is now apparent that inactivating GPCR mutations also result in a failure to reach the cell surface due to misfolding of the nascent receptor protein, and subsequent intracellular re-

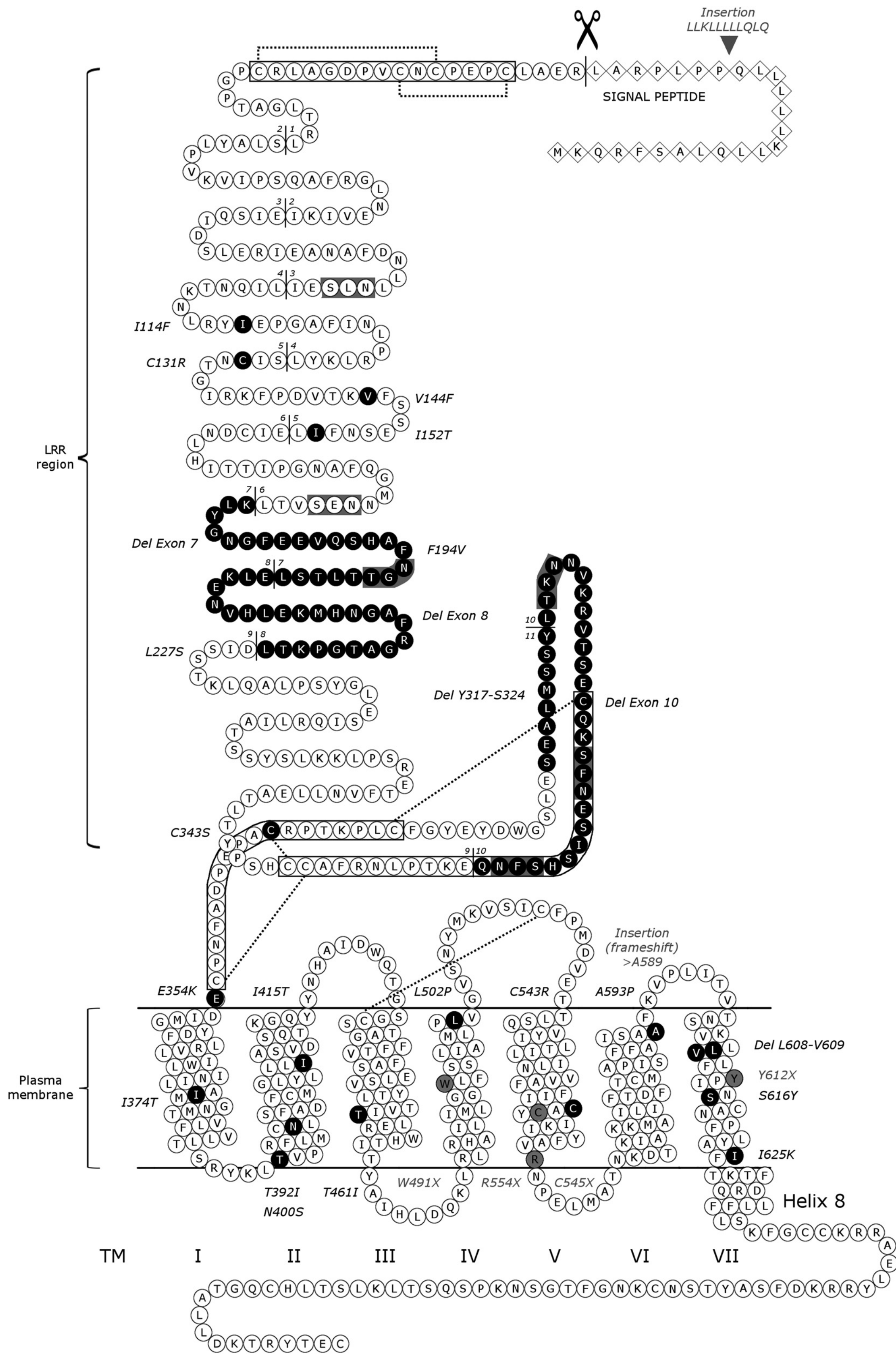

Figure 1. Location of naturally occurring inactivating mutations in the LHR. Schematic of the LHR with deletions and point mutations indicated in black and truncation mutants and insertions in gray. Signal-peptide residues are shown as diamonds with the signal peptide cleavage site indicated. Exon boundaries are indicated by numbered lines. Cysteine-rich domains are outlined with open black boxes and putative N-linked glycosylation sites with filled gray boxes. Disulphide bonds are indicated by dotted lines. Abbreviation: LRR, leucine-rich repeat; TM, transmembrane domain.

tention and degradation. Tao (6) proposed that inactivating GPCR mutations can be classified as: Class I (defective receptor biosynthesis, which includes mutations which truncate the receptor prematurely), Class II (defective trafficking to the cell surface), Class III (defective ligand binding), Class IV (defective receptor activation, which includes those unable to achieve an active conformation and those unable to couple to and/or activate G proteins) and Class V (mutants with no known defects) (6).

The mechanisms of GPCR intracellular retention are only partially understood, and yet a number of diseases are attributable to this phenomenon, including nephrogenic diabetes insipidus (V_2R), retinitis pigmentosa (rhodopsin), congenital hypothyroidism (TSHR), hypogonadotropic hypogonadism (GnRHR), hypergonadotropic ovarian failure (FSHR), familial glucocorticoid deficiency (MC2R), Leydig cell hypoplasia (LHR), and Hirschsprung's disease (ET_BR) (7, 8). Recently, cell-permeant molecules termed pharmacological chaperones/pharmacoperones, have been successfully used to "rescue" the cell surface expression of a number of intracellularly retained mutant GPCRs [reviewed in Tao and Conn (9)]. These molecules are thought to bind misfolded mutant GPCRs and stabilize their structure, preventing recognition by endoplasmic reticulum quality control machinery, and promoting cell surface delivery. These exciting findings lend credence to the possibility of treating disorders arising from retained GPCR mutants with cell-permeant compounds; therefore, understanding the mechanisms behind functional inactivation of GPCR mutants is of great importance.

We recently described a novel pharmacological chaperone able to rescue function of two intracellularly retained mutant LHRs (10), a compound that shows promise for future therapeutic development. However, of the disease-causing LHR mutations described, functional analyses are often incomplete and, in some cases, inconclusive. In the current study we perform comprehensive and comparative functional analyses of inactivating disease-causing mutations of the LHR, using a combination of in vitro signaling assays, ligand-binding assays, and by determining cell surface expression of wild-type and mutant receptors. These analyses provide an insight into the mechanisms underlying functional inactivation of mutant LHRs, and identify a spectrum of retained LHR mutations that may be receptive to pharmacological chaperone rescue.

Materials and Methods

Reagents

Recombinant hCG (Pregnyl) was obtained from NV Organon. Myo Inositol-[2-^{3}H] (^{3}H-myoinositol) was obtained from PerkinElmer. pcDNA3.1(-) mammalian expression vector containing cDNA encoding the wild-type (WT) human LHR (LHCGR, NM_000233.3) with an N-terminal FLAG epitope tag downstream of a signal peptide cleavage site (pcDNA-WT) was generously donated by Professor Ilpo Huhtaniemi (Imperial College, London). Empty pcDNA3.1(-) vector was obtained from Invitrogen. Mammalian expression vector containing cDNA encoding human $G\alpha_{16}$ was a generous gift from Dr Anna Aragay Combas (Institut de Biologia Molecular de Barcelona) and expression vectors encoding Renilla luciferase and cAMP response element (CRE)-linked firefly luciferase (CRE-luciferase) were obtained from Promega and Clontech, respectively.

Plasmid Mutagenesis

Mammalian expression vectors encoding mutant LHRs with N-terminal epitope tags were produced by site-directed mutagenesis of the pcDNA-WT vector, using a QuikChange site-directed mutagenesis kit (Stratagene). Exceptions were mutants I152T, Del Exon8, Del Exon10, and Del Y317-S324, which were produced using 5′ phosphorylated primers and a Phusion site-directed mutagenesis kit (Finnzymes). For mutagenesis primer sequences see Supplemental Table 1. All inserts were sequence verified prior to use.

Cell Culture and transfection

Maintenance of cell lines

Human embryonic kidney (HEK) 293-T cells were maintained in complete media (DMEM supplemented with 10% fetal calf serum (FCS) and 4mM L-glutamine), at 37°C in a humidified 5% CO_2 atmosphere. All cell culture plates and dishes were coated with a 1:30 dilution of Matrigel Matrix, Growth Factor Reduced (BD Biosciences) prior to cell seeding.

Transient transfection

Cells were transiently transfected with wild-type or mutant LHRs using Fugene HD (1:3 ratio) or X-tremeGENE HP (1:2 ratio) DNA transfection reagent (Roche).

Quantification of Receptor Expression by ELISA

Expression of FLAG-tagged LHRs in intact or permeabilized cells was measured by ELISA [an established method for measurement of GPCR expression (11–14)], as a ligand-independent method for determining total and cell surface receptor expression, respectively. In each case, expression of the mutant LHRs was assessed and compared with that measured for the wild-type receptor in the same experiment. Cell surface expression levels determined by this method correlate with maximal [^{125}I]-hCG binding measured to intact cells, verifying its suitability for quantification of receptor expression ($R^2 = 0.98$; slope = 1.06; Supplemental Figure 1).

Cells were seeded in 48-well tissue culture plates and were transiently transfected with mammalian expression vectors encoding wild-type or mutant LHRs (0.3 μg DNA/well). To measure cell surface LHR expression, 48-hour post-transfection cells were washed with Dulbecco's PBS supplemented with 0.5mM $MgCl_2$ and 0.9mM $CaCl_2$ (PBS+) before incubation with primary antibody (mouse anti-FLAG M2 [Sigma] 1:1000 in DMEM supplemented with 10% FCS) for 2 hours at 37°C. Cells were then washed three times with PBS+ and incubated with

secondary antibody (horseradish peroxidase–conjugated goat anti-mouse [Bio-Rad] 1:1000 in DMEM supplemented with 10% FCS) for 1 hour at 37°C. Cells were then washed an additional three times with PBS+ and twice with PBS (to remove any metal ions that may interfere with detection) before incubation with 0.1 mg/mL 3,3′,5,5′-Tetramethylbenzidine (prepared in phosphate-citrate buffer, pH 5.0 supplemented with 0.006% H_2O_2) for 20 minutes in the dark followed by addition of sulfuric aid (1M final concentration). Samples were then diluted 1/3 in 1M sulfuric acid and 150μL transferred to a clear 96-well plate prior to measurement of absorbance at 450 nm using an Anthos 2001 spectrophotometer. To measure total cellular LHR expression, cells were permeabilized by incubation in methanol for 10 minutes at −20°C followed by three 10-minute washes with PBS+ and incubation with blocking solution (DMEM supplemented with 10% FCS and 5% BSA) prior to incubation with primary antibody.

Nonspecific antibody binding (measured in the presence of cells transfected with empty vector) was subtracted from all samples and absorbance values normalized to the mean values measured for cells transfected with wild-type LHR in the same assay. Data are presented as mean ± SEM of at least three independent experiments, in which each data point was performed in triplicate.

Radioligand Binding

Radiolabeled hormone binding assays were used to determine the effects of mutations on hormone binding. Maximal radioligand binding to cells expressing wild-type or mutant receptors was first determined using a fixed concentration of radioligand. Specific binding to cells expressing the mutant LHRs was assessed and compared with that measured for the wild-type receptor in the same experiment. For those mutant receptors that displayed measurable levels of maximal binding (>10% of wild type), competition binding assays were then performed to determine any effects of the mutations on hormone affinity.

[^{125}I]-hCG was iodinated in house using hCG (Ovidrel; Merck Serono), Iodine-125 radionuclide (PerkinElmer), and Pierce Iodination Reagent (Thermo Fisher Scientific). Cells were seeded in 24-well tissue culture plates and were transiently transfected with mammalian expression vectors encoding wild-type or mutant LHRs (0.5 μg DNA/well). 48 hours post-transfection, cells were incubated in the absence (measurement of maximal cell surface binding) or presence (radioligand competition binding assays) of competing ligand (0–100 nM hCG) prepared in binding buffer (HEPES-DMEM supplemented with 0.1% BSA) for 1 hour at 4°C before addition of [^{125}I]-hCG (50 000 cpm/well) and incubation for a further 4 hours at 4°C. Cells were then washed with PBS (4°C) before lysis by incubation for 15 minutes with 0.1M NaOH at room temperature. Radioactivity (cpm) of the lysates was measured using a Berthold LB211 gamma counter.

Nonspecific radioligand binding (measured in the presence of cells transfected with empty vector and incubated in the absence of competing ligand) was subtracted from all samples. Specific binding (B_{max}) was then normalized to the mean values measured for cells transfected with wild-type LHR and incubated in the absence of competing ligand in the same assay. For competition radioligand binding assays, data were analyzed by nonlinear regression using GraphPad Prism and were fitted to sigmoidal dose-response equations to generate values for IC_{50}. Data are presented as mean ± SEM of at least three independent experiments, in which each data point was performed in triplicate.

Receptor Activation by $G\alpha_{16}$-linked inositol phosphate accumulation assay

A $G\alpha_{16}$-linked inositol phosphate accumulation assay, which measures LHR-induced activation of the promiscuous $G\alpha_{16}$ G protein (15), was used to determine the effects of mutations on receptor signaling in response to hormone stimulation. For each mutant receptor, hormone-induced activation was measured to determine effects on maximal stimulation (E_{max}) and hormone potency (EC_{50}), which were compared with that measured for the wild-type receptor in the same experiment. The $G\alpha_{16}$-linked inositol phosphate accumulation assay was selected for these experiments as it resulted in good correlation (R^2:0.86) between cell surface receptor expression (ie, density of agonist-accessible receptors) and functional response, indicative of little/no "receptor reserve" (Supplemental Figure 2), which can result in submaximal receptor occupancy eliciting maximal responses, and observed hormone potencies being affected by receptor density (16).

Cells were seeded in 24-well tissue culture plates and were transiently transfected with mammalian expression vectors encoding wild-type or mutant LHRs (0.25 μg DNA/well) and $G\alpha_{16}$ (0.25 μg DNA/well). 24 hours post-transfection, media was aspirated, cells were washed with IP media (Media 199 supplemented with 2% FCS) and then incubated with [^{3}H]-myoinositol (0.5 μCi/well) prepared in IP media for 24 hours at 37°C. The media was then aspirated and cells incubated for 30 minutes with buffer I (140mM NaCl, 4mM KCl, 20mM HEPES, 8mM glucose, 0.1% BSA, 1mM $MgCl_2$, 1mM $CaCl_2$) supplemented with 10mM LiCl (Buffer I+) followed by incubation for 1 hour at 37°C in the presence of ligand (0–100nM hCG) prepared in Buffer I+. Cells were then lysed by incubation for 1 hour with 10mM formic acid. Radiolabeled inositol phosphates were separated by ion-exchange chromatography using Dowex 1 × 8200 resin (Sigma) (17) and radioactivity (cpm) of the eluates measured by liquid scintillation counting using a Packard Tricarb 2100TR liquid scintillation analyzer.

Accumulation measured in the presence of cells transfected with wild-type LHR and incubated in the absence of stimulating ligand was subtracted from all samples. Radioactivity measurements were normalized to the maximal response measured for cells transfected with wild-type LHR in the same assay. Data were analyzed by nonlinear regression using GraphPad Prism and were fitted to sigmoidal dose-response equations to generate values for EC_{50} and E_{max} Data are presented as mean ± SEM of at least three independent experiments, in which each data point was performed in triplicate.

Determining residue conservation

To examine residue conservation between the glycoprotein hormone receptors and LHRs of different species, sequence alignments were produced with the human LHR (NP_000224.2) and FSHR (NP_000136.2)/TSHR (NP_000360.2) or murine (NP_038610.1)/ovine (NP_001265495.1)/bovine (NP_776806.1)/porcine (NP_99 9614.1) LHRs (18). To examine the conservation of residues within the Class A GPCR family, a GPCRs Motif Searcher tool was used (http://lmc.uab.cat/gmos/).

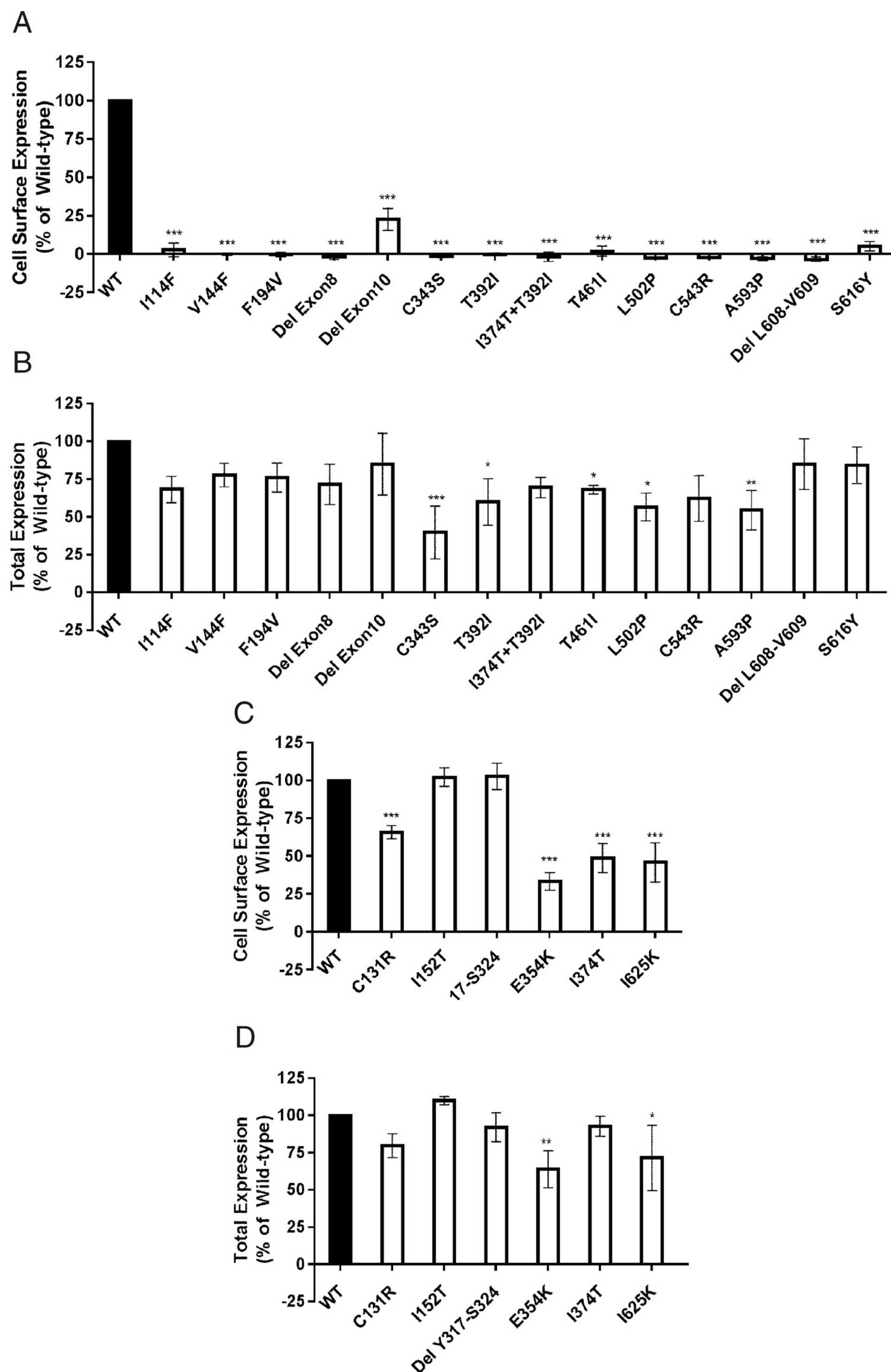

Figure 2. Cell surface and total cellular expression of LHR mutants. Receptor expression was measured in intact cells (A and C) (cell surface expression) or permeabilized cells (B and D) (total cellular expression) expressing wild-type (WT) LHRs (filled bars), A and B retained mutant LHRs or C and D partially/nonretained mutant LHRs (open bars), using an ELISA assay targeting N-terminal FLAG epitope tags of the receptors. Data are presented as percentage of the maximal expression measured for the WT receptor (set at 100%) after subtraction of nonspecific signal (measured in the presence of cells transfected with empty vector) and are mean ± SEM from at least three independent experiments. ***, $P < .001$; **, $P < .01$; *, $P < .05$, by one-way ANOVA followed by Dunnett's multiple comparison test, for comparison with WT.

Results

Naturally occurring LHR mutations

A review of the literature identified all reported naturally occurring inactivating mutations of the human LHR (Figure 1 and Table 1). Mutations of LHRs, which cause reduced mRNA transcript, alterations in the signal peptide, frame shifts, premature termination, or severe truncation of the receptor protein are clearly explainable (marked in italics in Table 1). We therefore focused on the identified missense and in-frame deletion mutations in the processed receptor protein (highlighted in bold text in Table 1 and in black in Figure 1). Two of the cases with point mutations (N400S [hom] and N415T combined with mutExon6A [cpd het]) were published after initiation of these studies and therefore have not been included. When studying the effects of homozygous double mutations (I374T in combination with T392I), each was assessed individually and in combination.

Most LHR mutations cause severe intracellular retention

Measurement of receptor cell surface expression revealed that the majority (13/20; 65%) of mutations (I114F, V144F, F194V, Del Exon 8, C343S, T392I, T461I, L502P, C543R, A593P, Del L608-V609, and S616Y) resulted in severe receptor intracellular retention, with little (<10% of wild-type) or no cell surface expression measured (Figure 2A). Total receptor cellular expression (measured in permeabilized cells) also seemed affected; however, these differences were only significant in a subset of the severely intracellularly retained mutant receptors (C343S, T392I, T461I, L502P, and A593P; Figure 2B), and were modest in comparison with the effects on cell surface expression. Nonquantitative fluorescent confocal microscopy of a representative severely retained mutant, C543R, confirmed its intracellular retention (Supplemental Figure 3).

In the case of the double homozygous mutations, the T392I mutation caused severe intracellular retention alone and in combination with its I374T counterpart. Total expression of T392I was also reduced, but this reduction was not observed when the I374T mutation was also present.

To exclude the possibility that the differences in wild-type and mutant LHR expression measured in the LHR ELISA assay may be due to induction of apoptosis, quantification of cell number was performed 48 hours after transfection with the wild-type and all of the examined mutant LHRs, and no difference was observed (Supplemental Figure 4).

As expected, functionality of the intracellularly retained mutants is severely impaired due to lack of receptor accessibility to LH/hCG. Maximal hCG binding to intact cells mirrored cell surface expression (<10% of wild-type binding; Figure 3A) and little/no hormone stimulation was measured in cells expressing most of these intracellularly retained mutant receptors (Figure 4A and Table 2). Where measurable, hormone affinity and potency of many of these mutant receptors were similar to the wild-type receptor (Table 2). The exceptions were, I114F and T392I, which both elicited a greater-than-expected response based on their cell surface expression levels and had increased hCG potencies of 5-fold and 8-fold, respectively (Figure 4A and Table 2), suggestive of conformational changes which facilitated increased receptor activation in addition to decreased cell surface expression, although this was not sufficient to overcome the impaired hormone responses resulting from their intracellular retention. Interestingly, no increase in hCG potency was observed for the double mutant, I374T+T392I, sug-

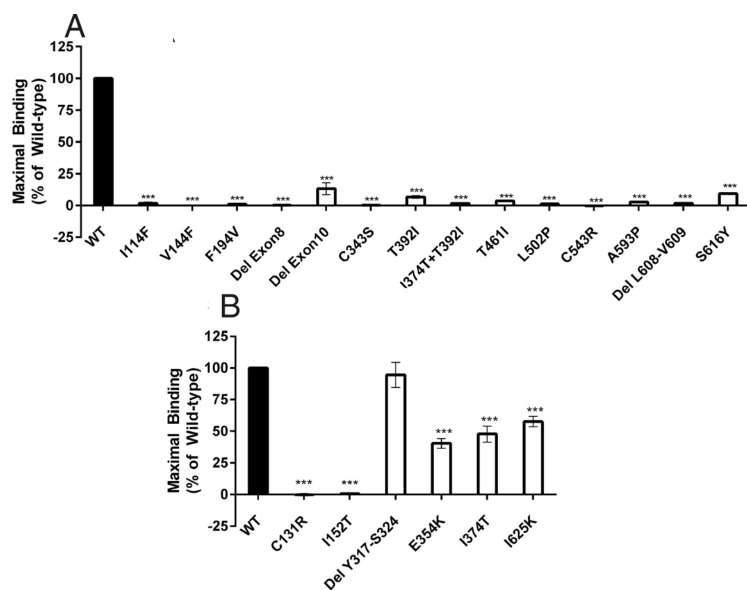

Figure 3. Hormone binding to cells expressing mutant LHRs. Intact cells expressing wild-type (WT) LHRs (filled bars), (A), retained mutant LHRs or (B) partially/nonretained mutant LHRs (open bars), were incubated for 4 h in the presence of [^{125}I]-hCG (50 000 cpm/well) and maximal binding measured after washing out of unbound ligand. Data are presented as percentage of the maximal binding measured for the WT receptor (set at 100%) after subtraction of nonspecific signal (measured in the presence of cells transfected with empty vector) and are mean ± SEM from at least three independent experiments. ***, $P < .001$, by one-way ANOVA followed by Dunnett's multiple comparison test, for comparison with WT.

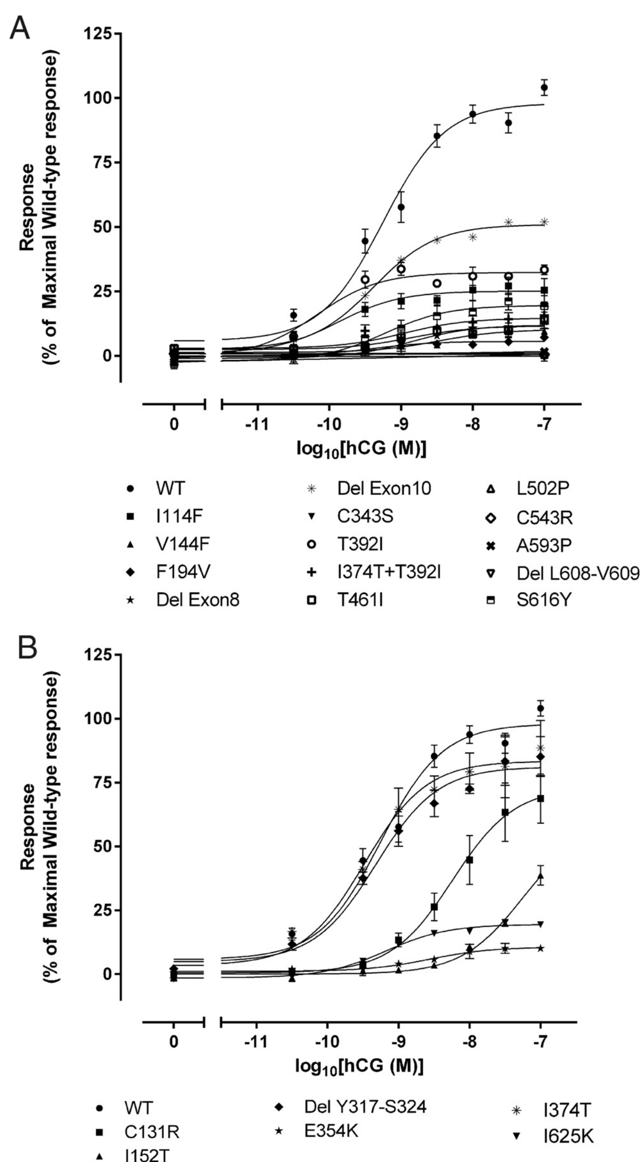

Figure 4. Hormone stimulation of inositol phosphate production in cells expressing mutant LHRs. Stimulation of inositol phosphate production in cells coexpressing wild-type (WT), (A) retained mutant LHRs or (B) partially/nonretained mutant LHRs, and $G\alpha_{16}$ G proteins was determined, over a range of concentrations of hCG. Data were fitted by sigmoidal dose response curves with Hill coefficients of unity. Data are presented as percentage of the maximal response obtained at the WT receptor (set at 100%) and are mean ± SEM from three independent experiments.

gesting that the presence of I374T negates the positive effects of T392I on hormone-binding affinity.

With the exception of the Del L608-V609 and S616Y mutants, examination of basal (ligand-independent) signaling, revealed that most of the severely intracellular retained mutants had lower basal activity than the wild-type receptor, correlating with their low levels of cell surface expression (Supplemental Figure 5).

Sequence alignments suggest that majority of the severely retained (Class II) mutations are in residues conserved within the glycoprotein hormone receptor family and also across species, with identical residues, or conservative substitutions occurring at positions corresponding to I114, V144, F194, C343, T392, T461, L502, C353, A593, Del V608-L609, and S616. Several of the Class II single-point mutations located within the 7TM domain region are also conserved within the wider rhodopsin-like GPCR family. At positions L502 (4.61), A593 (6.59), and S616 (7.46), leucine, alanine, and serine, respectively, are the most commonly occurring residues within this receptor family [parentheses refer to Ballesteros-Weinstein numbering (19)]. In the case of Exon 8, sequence alignments suggest that many residues are conserved within in the glycoprotein hormone receptor subfamily (32% identity/44% similarity) and between different species (60% identity/72% similarity).

Del Exon 10 mutants are retained but have enhanced signaling activity

Cell surface expression of the Del Exon 10 mutant was 23% of the wild-type receptor (Figure 2A), with reduced levels of maximal hormone binding (Figure 3A), However, although affinity and potency of hCG for this mutant receptor is not different from wild-type (Table 2), upon hCG stimulation a response measuring 51% of the wild-type response (Figure 4A) was measured, and basal activity of this mutant receptor also seems slightly elevated (Supplemental Figure 5). Previous studies have shown that deletion of LHR Exon10 impairs signaling of LH but not hCG (36, 37). However, for those extracellular mutants with measurable signaling, there was a strong correlation between the potency measured for both ligands, suggesting that none of the mutants (including Del Exon 10) displayed bias for either hormone (Supplemental Figure 6).

LHR mutants displaying impaired hormone binding/signaling

Not all of the mutants tested displayed severe intracellular retention. Partially retained mutants (C131R, E354K, I374T, and I625K) had cell surface expression levels ranging from 33–66% of wild-type levels (Figure 2C). Visualization of two representative examples of partially retained mutants (C131R and I625K) by confocal microscopy supports a combination of both cell surface and intracellular localization of these mutant receptors (Supplemental Figure 3). As a large proportion of these mutants are successfully trafficked to the cell surface, other receptor defects may be contributory to reduced receptor function. In addition to cell surface expression, total expression of some (E354K and I625K) of the partially retained mutants was also reduced (Figure 2D). Other, mutants (I152T and Del Y317-S324) had cell surface and

Table 2. Summary of Expression and Pharmacological Parameters for LHR Mutants

		Radioligand Binding		Functional Response ($G\alpha_{16}$ Activation)		
	Cell Surface Expression (% of WT)	Maximal Binding (% of WT)	pIC_{50} (IC_{50}; nM)	E_{max} (% of WT)	pEC_{50} (EC_{50}; nM)	Primary Cause of Nonfunctionality
Wild-type	100	100	8.34 ± 0.15 (4.6)	100	9.22 ± 0.11 (0.60)	
I114F	nm	nm	nm	25 ± 3	9.89 ± 0.12 (0.13)[a]	Severe intracellular retention (Class II)
V144F	nm	nm	nm	nm	nm	
F194V	nm	nm	nm	nm	nm	
Del Exon 8	nm	nm	nm	12 ± 2	8.89 ± 0.12 (1.3)	
C343S	nm	nm	nm	nm	nm	
T392I	nm	nm	nm	33 ± 1	10.13 ± 0.17 (0.074)[b]	
I374T+T392I	nm	nm	nm	15 ± 4	9.01 ± 0.32 (0.98)	
T461I	nm	nm	nm	11 ± 1	8.96 ± 0.11 (1.1)	
L502P	nm	nm	nm	nm	nm	
C543R	nm	nm	nm	nm	nm	
A593P	nm	nm	nm	nm	nm	
Del L608-V609	nm	nm	nm	nm	nm	
S616Y	nm	nm	nm	20 ± 4	9.20 ± 0.18 (0.63)	
Del Exon10	23 ± 7	13 ± 5	8.16 ± 0.36 (6.9)	51 ± 3	9.39 ± 0.13 (0.41)	Moderate intracellular retention (Class II)[†]
C131R	66 ± 4	nm	nm	76 ± 11	8.15 ± 0.10 (7.1)[a]	Hormone-binding deficiency (Class III)
I152T	102 ± 6	nm	nm	62 ± 9[x]	7.25 ± 0.10 (56)[ax]	
E354K	33 ± 6	40 ± 4	8.20 ± 0.21 (6.3)	12 ± 0	8.32 ± 0.34 (4.8)[b]	Receptor-signaling deficiency (Class IV)
I625K	46 ± 13	58 ± 4	8.36 ± 0.11 (4.4)	20 ± 0	9.17 ± 0.12 (0.67)	
I374T	49 ± 10	48 ± 6	8.68 ± 0.17 (2.1)	83 ± 9	9.49 ± 0.04 (0.32)	None[†Φ] (Class V)
Del Y317-S324	103 ± 9	95 ± 10	8.18 ± 0.09 (6.6)	82 ± 6	9.31 ± 0.11 (0.49)	

Abbreviations: nm, not measurable: expression/binding/response ≤ 10% of WT; [x], plateau not achieved so parameter calculation may not be accurate; [†], Further investigation may be required; [Φ], I374T mutation displays constitutive activity.

Data are mean ± SEM.

[a] $P < .001$; [b] $P < .01$, by one-way ANOVA followed by Dunnett's multiple comparison test, for comparison of pIC_{50} or pEC_{50} with that of the wild-type receptor.

total cellular expression levels not significantly different from the wild-type LHR (Figure 2, C and D). Confocal microscopy confirmed the cell surface localization of a representative nonretained mutant (Del Y317-S324) (Supplemental Figure 3).

C131R and I152T mutants have impaired hormone binding

For most of the partially/nonretained mutants, maximal hormone binding correlated with cell surface expression levels (Supplemental Figure 1), and hormone-binding

Table 3. Antibody Table

Peptide/Protein Target	Antigen Sequence (if known)	Name of Antibody	Manufacturer, Catalog Number, and/or Name of the Individual Providing the Antibody	Species Raised in; Monoclonal or Polyclonal	Dilution Used
FLAG	DYKDDDDK	anti-FLAG Clone M2	Sigma No. F1804	Mouse monoclonal	1:1000 (ELISA) 1:2000 (Confocal)
Mouse IgG	N/A	Goat Anti-Mouse IgG (H + L) HRP Conjugate	Bio-Rad No. 1721011	Goat polyclonal	1:1000
Mouse IgG	N/A	Goat Anti-Mouse IgG (H + L) Alexa Fluor 488 Conjugate	Invitrogen No. A-11001	Goat polyclonal	1:400

affinities were not different from that measured at the wild-type receptor (Table 2). Exceptions were the C131R and I152T mutants, which displayed 66% and 102% of wild-type cell surface expression, respectively, but for which no measurable radioligand binding was detected (Figure 3B and Table 2). This is indicative of impaired hormone binding, reflected by a significant reduction (C131R: 12-fold and I152T: 93-fold) in the potency of hCG stimulation of these mutant receptors (Figure 4B and Table 2). Although hormone-stimulated signaling of these mutants is impaired by their poor hormone-binding affinity, basal stimulation does not seem to be affected (Supplemental Figure 5). Sequence alignments suggest that residues C131 and I152 are both conserved between LHRs of different species, but, whereas residue I152 is conserved between the different glycoprotein hormone receptors (which have identical or similar residues at the equivalent positon), C131 is not conserved between the different receptor types.

E354K and I625K mutants have impaired hormone signaling

Maximal hormone binding for the E354K and I625K mutants correlated with their cell surface expression and hormone binding affinity was not different from wild-type (Figure 3B, Figure 4 and Table 2). However, the signaling response was severely attenuated, indicative of an impairment in the signaling ability of these mutant receptors. Despite I625K cell surface expression levels of approximately 50% of wild-type, maximal response generated was only 20% of that measured at the wild-type receptor (Figure 4B). Similarly, cell surface expression of the E354K mutant was 30–40% of the wild-type receptor but the maximal response generated was attenuated (12%). In this case, the potency of hormone stimulation was also reduced (8-fold; Figure 4B and Table 2) suggesting a marked reduction in the ability of the ligand to activate the receptor. For both the I625K and E354K mutants, basal signaling in the absence of hormone stimulation was also impaired (Supplemental Figure 5). Sequence alignments suggest that both residues E354 and I625 are highly conserved (although the murine LHR contains conservative substitution of valine at position I625).

I374T mutants are constitutively active

The I374T mutation also caused partial retention, resulting in cell surface expression (Figure 2C) and maximal hormone binding (Figure 3B) of approximately half of that of the wild-type receptor. Binding affinity and hormone potency for this mutant was not different from that of the wild-type receptor (Table 2). However, the functional response generated by this mutant upon hormone stimulation is greater than expected (83% of the wild-type response; Figure 4B), suggesting that this mutation may promote an "active" conformation or enhanced coupling to intracellular signaling partners. Indeed, this mutant exhibited significantly increased basal activity compared with the wild-type receptor, indicative of "constitutive activity" (Supplemental Figure 5). Residue I374 is highly conserved, with 74% of rhodopsin-like GPCRs containing isoleucine or the similar leucine/valine residues at the equivalent position (1.47).

Del Y317-S324 mutants have no functional impairments

Interestingly, the Del Y317-S324 mutation studied did not seem to cause any significant impairment of the receptor expression/localization or function. In this case, cell surface expression (Figure 2C and Supplemental Figure 3), maximal hormone binding (Figure 3B), and maximal response (Figure 4B) were very similar to those of the wild-type receptor. Hormone binding affinity/potency were also not different from those measured at the wild-type receptor (Table 2).

Discussion

With two exceptions, all of the mutant receptors had impaired functionality. For the majority, this was due to reduced expression at the cell surface (Class II mutations), whereas two mutations caused a decrease in binding affinity (Class III mutations) and two a decrease in signaling (Class III mutations).

Class II mutations

Our finding that Class II mutations are the most common defect in the LHR is in agreement with a study demonstrating that up to 70% of inactivating vasopressin V2 receptor mutations caused intracellular retention (20). Unsurprisingly, the male patients harboring homozygous/compound heterozygous Class II mutations present with severe LCH/pseudohermaphroditism. The exceptions being homozygous S616Y mutations or S616Y in combination with Del Exon8. In these cases, male patients present with milder phenotypes (micropenis with/without hypospadias) (Table 1). This is surprising given the level of retention observed in the current study. However, previous studies using stably transfected cells have demonstrated only partial retention of this mutant receptor (10), indicating that different in vitro systems may have differential receptor processing, expression, or trafficking properties.

One of the mutations (I114F) was identified in a heterozygous patient. As LCH is an autosomal-recessive disorder, this is suggestive of the presence of additional gene mutation(s) which may account for the severe phenotypes observed. Alternatively I114F may act in a

dominant negative manner, resulting in retention of the wild-type receptor in this patient. Indeed, intracellular entrapment of wild-type TSH receptors been implicated in dominant transmission of partial TSH resistance in heterozygous patients (21). Furthermore, LHR has been shown to dimerise/oligomerise [eg, Guan et al(22)], with dominant negative effects of intracellularly retained mutants observed (23). However, the contribution of this phenomenon to heterozygous patient phenotypes has yet to be explored.

In contrast, with the high sequence conservation within their TM domains (±70% identity), glycoprotein hormone receptor ectodomains have greater sequence divergence (±40% identity) (24). The prevalence of inactivating mutations in conserved ectodomain residues implies functional and/or structural importance. Indeed, from the crystal structure of FSH in complex with the ectodomain of the FSHR it can be inferred that mutations in residues I114, F194, and C343 may disrupt the densely packed hydrophobic core of the LRR region (4). In particular, C343 is believed to form part of the β-sheet structure of LRR12 (Figure 1) (2, 4). Exon 8 also includes several conserved hydrophobic residues several of which are postulated to be involved in β-sheet-forming regions of LRRs 8 and 9 (Figure 1) (4). Nonconservative substitutions in these positions might disrupt the LRR superstructure, with subsequent detection and retention by the cellular quality control system. Furthermore, residue C343 forms a disulphide bond (Figure 1) (25) important for conferring stability to the ectodomain structure (4). Therefore, it is not surprising that its mutation might lead to disruption of receptor folding.

Within the helical regions of the 7TM domain, substitutions with charged residues (eg, C543R), may be predicted to affect membrane insertion/conformation leading to receptor trafficking/localization defects. The presence of proline residues within TM helix regions (eg, A593P and L502P) could also result in helix "kinks," again disrupting the structure/correct folding of the 7TM domain, whereas substitution of residues with a small side chains to ones with bulkier side chains (eg, S616Y) could also disrupt helix packing.

Our conclusions that the impaired functions of mutants V144F, F194V, C343S, C543R, A593P, Del L608-V609, S616Y, T391I, and I374T+T392I, are primarily due to severe intracellular retention are in agreement with previous reports showing their reduced cell surface expression (or reduced hormone binding to intact cells (10, 26–32). Conversely, previous studies of the I114F and L502P mutants concluded that receptor dysfunction was due to impaired hormone binding, not cell surface expression (33, 34). In these studies, reduced radioligand binding to detergent solubilized cells expressing the mutant receptors was observed, suggestive of impaired hormone binding. However, reduced total expression (as noted for the L502P mutant in the present study) could also be causative. Furthermore, other studies noted similarly reduced radioligand binding of receptor mutants despite ligand affinity and total receptor expression levels being unaffected (32), indicating that this experimental approach may not always be appropriate/accurate. Previous studies on the L502P and I114F mutants also reported no differences between fluorescently-labeled wild-type and mutant receptor expression in contrast with our findings. However, the fluorescent images obtained were of limited resolution and lacked quantification (33, 34). Flow cytometry analyses also showed similar levels of expression of the wild-type and I114F mutant receptors. However, only a very small subpopulation (6%) of the transfected cells were positively stained, indicative of impaired transfection/expression of both the wild-type and mutant receptors, which may have confounded analysis of the effects of the mutation (34).

We found the Del Exon10 mutant to be strongly intracellularly retained and have therefore provisionally classified it as a Class II mutation. Zhang et al (35) also observed retention of LHRs in which Exon 10 had been deleted; however, given that we have found enhanced signaling activity of this mutant receptor, this may somewhat negate the effects of retention. Thus, retention is unlikely to be the sole cause of nonfunctionality. Indeed, previous studies have attributed nonfunctionality of Del Exon10 to an impaired responsiveness to LH, but not hCG (36, 37), supported by the fact that LHRs of the New World monkey lineage, in which CGβ has functionally replaced LHβ, do not contain Exon 10. Furthermore, a homozygous Del Exon10 patient seemed to have normal hCG-mediated fetal gonadal development, but lacked LH-mediated pubertal development. In contrast, we observed no hormone-specific effects of the Del Exon10 mutation. We used measurement of a $G\alpha_{16}$-mediated signaling output while previous studies have examined receptor mediated cAMP accumulation (by $G\alpha_s$). It is therefore tempting to speculate that this discrepancy is due to differential signaling by the two hormones, with activation of $G\alpha_s$ by LH being more sensitive to the absence of Exon 10 than the activation of $G\alpha_{16}$, whereas hCG activation of both pathways is unaffected. Further examination of these effects may reveal interesting insights into the differential activation of the LHR and its downstream-signaling pathways by these two ligands.

Class III Mutants

Although they had similar cell surface expression as the wild-type receptor, hormone binding to the C131R or I152T mutants was severely impaired, resulting in a 12-fold and 93-fold decrease in hormone potency, respectively. We are therefore able to characterize these as Class III mutants. Our conclusions that these residues are important for hormone binding are in agreement with pre-

vious studies (38, 39). Both residues are located within the LRR domain of the receptor (Figure 1) and are conserved across species, emphasizing their functional importance. Given that residue C131 is not conserved in the FSHR or TSHR, it is likely involved in the specific binding of LH/hCG. Furthermore, conservative mutation of the equivalent residue in the rat LHR to serine (40) or substitution with alanine (41) have also been shown to impair hormone binding without affecting cell surface localization. The presence of a cysteine in this position therefore seems important for hCH/LH binding, possibly due to disulphide bridge formation, which might play a role in defining the binding site for these hormones. Conversely, the conservation of residue I152 between the different glycoprotein hormone receptors suggests that it may be involved in receptor binding interactions common to all of the glycoprotein hormones. Indeed, the crystal structure the FSHR ectodomain demonstrates that the equivalent FSHR residue is buried at the ligand-receptor interface by the common hormone α subunit (2). The I152T mutation was identified in a heterozygous patient who also harbored a splice-site mutation in intron 6 which results in skipping of Exon 7 in a subset of transcribed receptors. As expected, the combination of this severely binding-impaired mutation with the splicing mutation results in a more severe phenotype than that seen with the patient homozygous for the C131R mutation (Table 1).

Class IV Mutants

Although mutants E354K and I625K are retained to some extent, substantial cell surface expression was observed. Partial retention alone cannot therefore account for the severe loss of function and we have been able to characterize them as Class IV (defective receptor activation) mutations.

Residue E354 is in the "hinge" region of the receptor between the ecto- and serpentine domains, and is located just prior to TMI (Figure 1) within a highly conserved motif (FNPCEDIMGY) of the glycoprotein hormone receptors (24). The E354K mutation has no effect on hormone binding affinity, but reduces hormone potency and maximal response (Figure 4B and Table 2). Previous studies in rat LHR have also noted that substitutions of the glutamic acid of this motif results in impaired signal transduction without affecting hormone binding (42, 43). Furthermore, in the TSHR, this residue has been identified as important for stabilization of the active conformation of the receptor (44). Therefore, this residue/region seems important for transducing hormone binding in the ectodomain into activation of the serpentine domain, explaining the severe phenotype noted in the patient homozygous for the E354K mutation.

The I625K mutation (located in TMVII; Figure 1) also causes reduced hormone responsiveness, without affecting hormone binding affinity, although only maximal response and not hormone potency was affected. This mutation may not disrupt hormone activation of the receptor per se, but might promote an active conformation that is not as effective at coupling to downstream signaling pathways. Although a previous study observed a decrease in hormone potency at the I625K mutant receptor (31), their study used a different method of measurement of receptor activation (CRE-luciferase-reporter gene assay to measure $G\alpha_s$ activation). We cannot rule out the possibility that this mutation has differential effects on $G\alpha_{16}$ and $G\alpha_s$ activation; however, high levels of receptor reserve in the CRE-luciferase assay may explain the higher EC_{50} measured for the mutant receptor in this earlier study. Residue I625 is just two residues downstream of the NPxxY motif, which is highly conserved in Rhodopsin-like GPCRs and is believed to act as a molecular switch during GPCR activation (45). Substitution of I625 with a basic lysine reside might change the charge and ionic environment proximal to this motif, altering the conformational changes that occur upon receptor activation. As intracellular retention and signaling deficiency of this mutant receptor are not as severe as that of the E354K mutation, these patients display a milder phenotype.

Class V Mutants

Interestingly, some of the LHR mutations studied did not cause inactivation of the receptor and therefore can be classified as Class V mutants (mutants with no known defects). Although the mutation I374T, located in TMI, caused partial retention of the receptor, it also resulted in constitutive activation and, thus, increased basal activity and increased hormone-induced response. However, the patient was homozygous for both I374T and T392I mutations and the constitutive activation seems to be negated by the T392I mutation. Interestingly, a mutation of the alanine immediately preceding this residue identified in a patient with male precocious puberty (A373V), has also been shown to cause constitutive activation of the LHR (46) suggesting that this region of TMI is important for maintaining interactions that keep the receptor in an inactive conformation in the absence of ligand.

For the Del Y317-S324 mutant, no functional impairment was observed, with expression, hormone binding and hormone-signaling properties not different from the wild-type receptor. Unlike the other deletion mutations studied, this deletion is caused by a point mutation at the intron-10-exon-11 boundary that results in altered splicing. This altered splicing has been shown to occur less efficiently (47), leading us to postulate that reduced LHR expression, due to altered processing during biosynthesis (a Class I mutation), results in the relatively mild repro-

ductive dysfunctions of these patients, or that there is another, as yet, undiscovered, contributing factor.

Conclusion

These data reveal how genetic mutations in the LHR can have diverse and often severely deleterious effects on receptor function with consequential effects on sexual development and reproductive competence. Most inactivating LHR mutations studied caused intracellular retention of the mutant receptors (Class II mutations), supporting the premise that development of pharmacological chaperone agents able to rescue the cell surface expression/function of retained mutant GPCRs [such as a cell-permeant small molecule LHR agonist previously described by us (10)] is a valid and important therapeutic avenue.

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