T222P mutation of the insulin-like 3 hormone receptor LGR8 is associated with testicular maldescent and hinders receptor expression on the cell surface membrane

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Bogatcheva NV, Ferlin A, Feng S, Truong A, Gianesello L, Foresta C, Agoulnik AI. T222P mutation of the insulin-like 3 hormone receptor LGR8 is associated with testicular maldescent and hinders receptor expression on the cell surface membrane. J Physiol Endocrinol Metab 292: E138–E144, 2007. First published August 22, 2006; doi:10.1152/ajpendo.00228.2006.—Insulin-like 3 (INSL3) hormone plays a crucial role in testicular descent during embryonic development. Genetic ablation of Insl3 or its G protein-coupled receptor (GPCR) Lgr8 causes cryptorchidism in mice. Previously, we identified a nonfunctional T222P mutation of LGR8 in several human patients with testicular maldescent. Using a large population of patients and healthy controls from Italy, we have demonstrated that T222P LGR8 mutation is present only in affected patients (19 T222P/+ of 598 vs. 0/450, P < 0.0001). We have also identified a novel allele of LGR8 (R223K) found in one patient with retractile testes. Both mutations are located in the leucine-rich repeats (LRRs) of GPCR ectodomain. The expression analysis of T222P mutant receptor transfected into 293T cells revealed that the mutation severely compromised GPCR cell membrane expression. The substitution of Thr222 with the neutral Ser or Ala, or the R223K mutation, did not alter receptor cell membrane expression or ligand-induced cAMP increase. Additional mutations, affecting first leucine in a signature LxxLxxLxNCxLx stretch of LRR (L283F), or the amino acid residues, forming the disulfide bond or coordinating calcium ion in the LDLa module (C71Y and D70Y), also rendered proteins with reduced cell surface expression. The structural alterations of both LRRs and LDLa of the ligand-binding part of LGR8 cause the inability of receptor to express on the cell surface membrane and might be responsible for the abnormal testicular phenotype in patients.

insulin-like 3; cryptorchidism; G protein-coupled receptor

NORMAL TESTICULAR POSITION in the scrotum sac in males depends on the interplay between several anatomic and endocrine factors. Insulin-like 3 hormone (Ins3l) and its leucine-rich G protein-coupled receptor (GPCR) 8 (Lgr8) were shown to play a pivotal role in testicular descent in mice during development (9, 18, 19, 27). Both genes are believed to play a similar role in humans. Several allelic variants in INSL3 (Chr. 19) and LGR8 (Chr. 13) genes were found exclusively in patients with maldescended testes, suggesting that some cases of human cryptorchidism are associated with the genetic aberrations in INSL3/LGR8 signaling (3, 7, 9). Previously, we have identified LGR8 missense mutation T222P in five patients with testicular maldescent from Southern Europe (7, 9). Analysis of other European and American populations failed to identify the T222P allele (3, 6). Stimulation of LGR8 receptor with INSL3 causes an activation of adenylyl cyclase and accumulation of intracellular cAMP in 293T cells expressing recombinant receptor (12, 15). Analysis of INSL3/LGR8-mediated cAMP production in cells transfected with mutant receptors revealed that T222P mutation renders the protein functionally inactive (9). However, the molecular cause of the observed phenomenon remained unknown.

LGR8 is a member of the glycoprotein hormone receptor superfamily, which includes FSHR, LHR, TSHR, and a recently identified relaxin receptor, LGR7 (12). These receptors are characterized by the presence of a large extracellular NH2-terminal part (ectodomain), the seven-helix transmembrane part, characteristic for G protein-coupled receptors, and intracellular COOH-terminal part (Fig. 1). The ectodomain, in turn, contains several imperfect Leu/Ile-rich repeats, believed to form a horseshoe-like structure, where β-strands are connected to the parallel α-helices (14). The NH2 terminus of LGR7 and LGR8 contains an LDL receptor class A (LDLa) module, stabilized by internal disulfide bonds and coordinating calcium ion (5). Both ectodomains and exoloops of the transmembrane region of LGR7/LGR8 were shown to interact with the ligand (10, 23); however, whereas the LRRs’ involvement in ligand binding was actively studied (4), the role of LDLa module is less clear.

It has been shown that changes caused by mutations in the different parts of the glycoprotein hormone receptors and LGR7 may affect membrane surface expression, ligand binding, and signal transduction (1, 4, 13, 20, 25). Subsequently, the functional deficiency of the receptor may lead to decreased hormonal response and cause the clinically recognized phenotype.

We show here that the mutant LGR8 allele T222P is exclusively associated with the abnormal testicular phenotype. Functional analysis of mutant T222P protein action showed severely reduced receptor surface expression, suggesting the mechanism of its compromised function. Recognition of such
patients would permit family screening and potential early therapeutic intervention.

RESEARCH DESIGN AND METHODS

Subjects. Five hundred ninety-eight subjects (age 20–47 yr) with a history of maldescended testes were recruited in the Centre for Male Gamete Cryopreservation, Padua, Italy. Two hundred forty-one had bilateral cryptorchidism, and 357 had unilateral cryptorchidism. In 504 patients, orchidopexy was performed at the ages ranging from 1 to 27 yr, whereas in the remaining 94 subjects spontaneous descent occurred during the first five years after birth. All 598 men were Caucasian, from different regions of Italy. All men had a normal 46,XY karyotype, and mutations in INSL3 and androgen receptor genes (8) were excluded. A total of 450 Italian men without clinical history of maldescended testes were used as the controls. Informed consent was obtained from each subject; the study conformed to the standards set by the Declaration of Helsinki and was approved by the University of Padova Institutional Review Board.

Mutation analysis of LGR8. The genomic DNA was extracted from peripheral blood obtained from the participants of this study. Analysis was focused on exon 8 of LGR8, where the T222P mutation was localized. This exon was PCR amplified using previously reported conditions (9). Denaturing high-performance liquid chromatography (DHPLC) was carried out on a 2100 WAVE DNA fragment analysis system (Transgenomics, Omaha, NE) as described before (9). For each abnormal elution profile and/or abnormal electrophoretic migration pattern, genomic DNA was reamplified and the PCR products were directly sequenced in both directions. The wild-type and heterozygous for T222P mutation genomic DNA were used as a control in DHPLC analysis.

Expression vectors. The LGR8 cDNA expression vector, containing a prolactin (PRL) signal peptide and the FLAG epitope at the 5′ end of the LGR8 cDNA in pcDNA3.1 vector was kindly provided by Dr. Sheau Yu Hsu (Stanford University) (12). The 1.3-kb 5′ KpnI/BglII fragment of this construct was used to substitute the 5′ end of the LGR8 expression construct in the pcR3.1 vector described previously (9). This construct was used in site-specific mutagenesis (9) to produce various nonsense mutations within the LGR8 cDNA sequence. To ensure an absence of additional nucleotide substitutions, the mutated cDNA constructs were recloned into an intact pcR3.1 vector (Invitrogen, San Diego, CA) and fully sequenced. All plasmid DNAs were isolated using the High Purity Plasmid System (Marigen Biosciences, Ijamsville, MD).

Cell culture and transfection. The 293T HEK cells were cultured in DMEM, 10% fetal calf serum, and 100 μg/ml penicillin-streptomycin. For transfections, 60–80% confluent cells plated into T-25 flask were transfected with 1.6 μg of DNA, using FuGENE 6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer’s instruction.

Total and surface expression of LGR8 mutants. The expression of LGR8 mutants was assessed by cell surface ELISA as described previously (16) with modifications. Briefly, cells plated in the T-25 flask were split after 24-h transfection into 24-well plates coated with 0.1 mg/ml polylysine. Twenty-four hours later, cells were washed with TBS (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM CaCl2) and fixed with 3.7% formaldehyde with or without 0.25% Triton X-100 for the total FLAG or surface FLAG expression analysis. Cells were washed twice with TBS, incubated for 45 min with TBS containing 1% BSA, and then incubated with M1 anti-FLAG (Sigma, 1/5,000 dilution) antibody for 1 h at room temperature. Cells were washed twice with TBS, reblocked with TBS-BSA for 15 min at room temperature, and incubated with secondary antibody [goat anti-mouse antibody conjugated with horseradish peroxidase (HRP), 1/5,000 dilution; Bio-Rad] for 1 h at room temperature. Cells were washed four times with TBS, and then colorimetric HRP substrate TMB (Amersham, Piscataway, NJ) was added. When adequate color was achieved, the reaction was stopped by placing cells on ice, and the supernatants were read at 655 nm with a microplate reader (Bio-Rad). Preliminary calibration experiments with the increasing amount of plasmid used for transfection ensured that surface FLAG expression measurements were conducted in the linear range. The value obtained from vector-transfected cells (nonspecific binding) was subtracted from the values obtained from cells transfected with wild-type or mutant receptor. For each well analyzed for FLAG expression, two parallel measurements were performed. When normalized to the wild-type FLAG expression, the differences in mutant FLAG expressions between independent transfection experiments were no more than 10–15%.

Intracellular cAMP determination. Cells plated into a T-25 flask were split after 24-h transfection into 24-well plates coated with 0.1 mg/ml polylysine. Twenty-four hours later, cells were pretreated with 250 μM IBMX for 10 min and stimulated with different INSL3 (Phoenix Pharmaceuticals, Belmont, CA) concentrations for 10 min. Duplicate wells were used for each INSL3 concentration. The reaction was stopped by placing cells on ice. Cells were washed with PBS and lysed with the extraction buffer from the cAMP determination kit (Amersham) according to the manufacturer’s instructions. Each sample was analyzed in duplicate. To normalize cAMP accumulation, cells from the same transfection were used to assess surface LGR8 expression. For each mutant, at least three individual experiments were performed. The data presented in Figs. 2–4 were obtained from one experiment, when all the mutants were analyzed simultaneously for cAMP response (duplicate well for each concentration point) and surface FLAG expression (triplicate well).

Deconvolution microscopy. The immunostaining protocol was described previously (21). The 293T cells were transfected with FLAG/ LGR8 cDNA expression constructs encoding wild-type and mutant receptors. Cells grown on glass coverslips were fixed with cold 4% paraformaldehyde and permeabilized with 0.25% Triton X-100. Non-specific binding sites were blocked for 1 h with blocking medium prior to incubation with anti-FLAG polyclonal antibody (Sigma, 1:100 dilution in blocking medium), followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen). Coverslips were mounted with SlowFade Anti-Fade Kit (Invitrogen). Images were collected with a DeltaVision (Deconvolution) Restoration Microscope at the Integrated Microscopy Core, Baylor College of Medicine.
Statistical analysis. Fisher’s exact test was used to compare the frequency of T222P mutation in patient and control populations. An ANOVA test was used to analyze the FLAG expression data. cAMP accumulation curve fitting was performed using Origin software.

RESULTS

Mutation analysis of LGR8 exon 8. Mutation analysis of LGR8 exon 8 identified two alleles resulting in amino acid changes. A total of 20 of 598 (3.3%) patients with abnormality of testicular position were heterozygous for missence mutations in exon 8, whereas no mutations were found in this exon in 450 healthy controls. The first mutation was a previously described Thr-to-Pro substitution at the 222-amino acid position (T222P) (Table 1). It was detected in 19 subjects, which included 7 men with bilateral cryptorchidism and 12 men with unilateral cryptorchidism. The severity of the disease in patients with bilateral and unilateral cryptorchidism was highly variable from intra-abdominal, intracanalicular, or suprascrotal testicular position. The T222P mutation was detected exclusively in the affected patient group, which renders highly significant difference of mutation frequency compared with the control population (19/598 vs. 0/450, \( P < 0.0001 \)). We were able to analyze the genomic DNA from parents of nine affected T222P/ heterozygous probands. In all cases, the same mutant allele was detected in maternal but not paternal genomic DNA.

An additional mutation in exon 8 was detected in one subject with bilateral cryptorchidism and spontaneous descent between 2 and 3 yr of age and was characterized by a G-to-A transition at codon 223 resulting in an Arg-to-Lys change (R223K). The subject was heterozygous for this allele. Seminal analysis of this patient revealed a moderate oligozoospermia (sperm concentration between 5.0 and \( 6.7 \times 10^6 \)/ml) with testes of normal volume and highly mobile through the inguinal canal. No cases of cryptorchidism were reported in his family.

Functional analysis of T222P, T222S, T222A, and R223K mutants. To analyze the effect of detected missense mutations, LGR8 cDNA constructs containing T222P and R223K substitutions were transiently expressed in 293T cells (Fig. 2). Previously, we reported an almost complete absence of hormone-induced cAMP synthesis when the expression of T222P

Table 1. T222P LGR8 mutation in patients with testicular maldescent

<table>
<thead>
<tr>
<th>Testicular Position</th>
<th>Subjects with T222P Mutation</th>
<th>Frequency, %</th>
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</thead>
<tbody>
<tr>
<td>Total</td>
<td>598</td>
<td>3.2*</td>
</tr>
<tr>
<td>Bilateral Cryptorchidism</td>
<td>177</td>
<td>3.4*</td>
</tr>
<tr>
<td>Orchidopexy</td>
<td>12</td>
<td>6.6*</td>
</tr>
<tr>
<td>Spontaneous descent</td>
<td>1</td>
<td>1.6*</td>
</tr>
<tr>
<td>Unilateral Cryptorchidism</td>
<td>327</td>
<td>3.7*</td>
</tr>
<tr>
<td>Orchidopexy</td>
<td>12</td>
<td>3.7*</td>
</tr>
<tr>
<td>Spontaneous descent</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total patients</td>
<td>598</td>
<td>3.3*</td>
</tr>
<tr>
<td>Controls</td>
<td>450</td>
<td>0</td>
</tr>
</tbody>
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*Difference with control is significant, \( P < 0.0001 \)

Fig. 2. Functional analysis of T222P, T222S, T222A, and R223K LGR8 variants. A: expression analysis of 2 natural LGR8 mutant receptors, T222P and R223K. Total (filled bars) and surface (open bars) binding of anti-FLAG antibodies is expressed in arbitrary units (AU) as a ratio of specific horseradish peroxidase (HRP) activity displayed by the mutant to the specific HRP activity displayed by the wild-type (WT) receptor. Results are presented as means ± SE (n = 6). There is a significant reduction of T222P surface expression (\( P < 0.05 \)). B: stimulated dose-dependent cAMP production in 293T cells transfected with WT LGR8 (■), T222P (●), and R223K (●) mutant expression constructs. INSIL3, insulin-like 3. Results of 1 representative experiment in which duplicate wells were used are shown as mean ± SE. C: expression analysis of T222S and T222A mutant receptors. Total (filled bars) and surface (open bars) binding of anti-FLAG antibodies is expressed in AU as a ratio of specific HRP activity displayed by the mutant to the specific HRP activity displayed by the WT receptor. Results are presented as means ± SE (n = 3). D: stimulated dose-dependent cAMP production in 293T cells transfected with WT LGR8 (■), T222S (●), and T222A (●) mutant expression constructs. Results of 1 representative experiment in which duplicate wells were used are shown as mean ± SE.
construct was driven by LGR8 intrinsic signal sequence (6, 9). Here, we examined the cAMP response of T222P constructs containing a PRL signal sequence. The limited accumulation of cAMP (~50% of maximal wild-type response) was observed upon INSL3 stimulation in cells expressing T222P mutant. The analysis of the surface expression of T222P mutant revealed that membrane delivery of this mutant was severely compromised (Fig. 2A). When the cAMP production was normalized to receptor surface expression, the maximal cAMP response of mutant receptor was even higher than that of a wild-type protein (Fig. 2B). However, the T222P mutant showed an increased EC50 value (2.69 ± 0.38 nM) compared with that of wild-type LGR8 (1.61 ± 0.21 nM, P < 0.05), indicative of compromised receptor activation.

To analyze the nature of the deleterious effects of T222P mutation, we created two additional mutants containing T222S and T222A substitutions. Both of these mutants displayed only slightly decreased membrane presentation (Fig. 2C). When normalized to the surface expression level, the concentration-response curves for the cells expressing T222S and T222A variants maintained characteristics similar to those of wild type (Fig. 2D).

The novel R223K mutant, detected in patients with retractile testes, rendered the INSL3-dependent cAMP activation curve with EC50 close to that of the wild-type receptor (1.59 ± 0.38 nM; Fig. 1B). Surface expression of this mutant was not compromised (Fig. 1A); maximal cAMP response after normalization to surface expression was only ~20% lower than that of the wild type.

**Functional analysis of the L283F mutant.** To further analyze the role of LRRs in LGR8 biological properties, we mutated one of the conservative residues in core Leu LRR stretch LxxLxLxxN/CxL. For the substitution of the first Leu of one of the conservative residues in core Leu LRR stretch LxxLxLxxN/CxL. For the substitution of the first Leu of

Functional analysis of the L283F mutant. To assess the importance of the LDLa module for LGR8 function, we mutated residues known to stabilize the tertiary structure of the LDLa module for LGR8 (5, 8). Naturally occurring LDL receptor mutations in these sites impaired the protein function and were associated with the hypercholesterolemia (PubMed accession no. P01130, variants 005353 and 005333). Two point mutations were produced (D70Y and C71Y) in the LDLa module of LGR8, in sites homologous to the positions of deleterious LDL receptor mutations. As shown by the antibody surface binding experiments and cAMP accumulation analysis, the C71Y and D70Y variants of the LGR8 receptor rendered the receptor completely nonfunctional, with drastically reduced level of cell surface expression (Fig. 4, B and C).

**Subcellular localization of mutant receptors.** We used anti-FLAG antibody to visualize the localization of the recombinant LGR8 receptors in 293T cells transiently transfected with corresponding LGR8 cDNA expression constructs, encoding the FLAG epitope at the NH2 terminus of the protein. In the absence of agonist, the wild-type receptor was clearly present on the cellular surface membrane, although some staining was also observed in the cytoplasm (Fig. 5). Cells transfected with the mutant T222P, D70Y, and L283F receptors demonstrated homogeneous FLAG/LGR8 fluorescence indicative of cytoplasmic localization of the proteins with little, if any, surface expression (Fig. 5).

**DISCUSSION**

Cryptorchidism, affecting 3–4% of newborn boys, can cause infertility in adulthood and is a known risk factor for developing a testicular cancer. It was suggested that some cases of isolated cryptorchidism can have a genetic cause. The mutations of *Insl3* or *Lgr8* genes in mice lead to high intra-abdominal cryptorchidism, which inspired mutational analysis of the *INSL3* and *LGR8* genes in humans. Screening of homologous human *INSL3* and *LGR8* genes in patients with testicular maldescent identified several mutant alleles (3). Surprisingly, all of them were present in the heterozygous state, raising the question about the causative link between the presence of rare mutant alleles and cryptorchidism. The T222P missence mutation of LGR8 renders a protein nonfunctional and was detected previously in five patients with testicular maldescent (7, 9). Here, we report an extensive screening for T222P mutation in a large patient population. The analysis of almost 600 patients with testicular maldescent and 450 healthy controls from Italy showed that the T222P mutation was present exclusively in patients with a history of cryptorchidism. In nine studied cases, the mutant allele
was transmitted to the probands through their mothers. In vitro analysis shows that this mutation causes a drastic reduction of the receptor surface membrane expression. An additional mutation, R223K, found in a single patient, had little effect on the receptor functions in vitro and most probably could not have been responsible for the abnormal phenotype. Further analysis of artificial mutations compromising structural integrity of LRRs or the LDLa module underlined the significance of these domains in receptor cell surface expression and signaling.

Fig. 4. Functional analysis of C71Y and D70Y LGR8 mutants. A: consensus sequence of LDLa module. Alignment of LDL module of LGR8, 5th LDLa module of the LDL receptor (LR5), and LDLa consensus sequence. Shown are positions of the disulfide bonds (loops) and the residues coordinating calcium ion (*). Boldface letters show the conserved amino acids of the consensus sequence. Underlined are the Cys and Asp residues, mutated to Tyr in LDLa module of LGR8. B: expression analysis of LDLa LGR8 mutants. Total (filled bars) and surface (open bars) binding of anti-FLAG antibodies is expressed in AU as a ratio of specific HRP activity displayed by the mutant to the specific HRP activity displayed by the WT receptor. There is a significant reduction of mutant receptor surface expression ($P < 0.05$). Results are presented as means ± SE ($n = 6$). C: stimulated dose-dependent cAMP production in 293T cells transfected with WT LGR8 (■), C71Y (●), and D70Y (▲) mutant expression constructs. In both mutants there is no response. Results of 1 representative experiment in which duplicate wells were used are shown as mean ± SE.

Fig. 5. T222P, L283, and D70Y LGR8 receptor variants failed to express on the cell surface membrane. 293T cells were transiently transfected with FLAG/LGR8 cDNA constructs followed by immunofluorescent anti-FLAG detection of the protein localization. WT receptor is expressed on the cell surface membrane, whereas mutant receptors are located mainly in the cytoplasm. Representative images are shown. Bar, 10 μm.
So far, the T222P mutant remains the only naturally occurring variant that severely compromises the INSL3 signaling in vitro. Mutation analysis of the LGR8 gene performed in our and other laboratories indicates that the T222P mutation is specific for the South European populations (France and Italy) (7, 9). The haplotype analysis indicated a common origin of this mutation (7). It is possible, therefore, that this allele originated somewhere in this geographic area. Although the frequency of mutation detected in the current screening was relatively low, all T222P individuals had some forms of testicular maldescent; thus the presence of this mutation appears to be a strong risk factor for the disease. As only heterozygous patients were detected in our screen, we hypothesize that the testicular maldescent may be caused by LGR8 haploinsufficiency. Importantly, cotransfection experiments with wild-type and mutant receptor did not show any effect on the function of the wild-type receptor, suggesting a true loss-of-function nature of the mutation (9). It is interesting to note that the recent estimates indicate that only a very limited number of endogenous LGR8 receptor molecules are expressed in adult Leydig cells (2) and in embryonic gubernaculum (S. Feng and A. I. Agoulnik, unpublished data). Significantly, the presence of only one-half of the functional receptors in heterozygotes appears to be detrimental for INSL3 signaling in humans, contrary to the situation in mice, where the null allele of Lgr8 is fully recessive.

To examine the effect of T222P substitution, two variants of mutant expression construct were created. In earlier studies, we used LGR8 constructs with intrinsic LGR8 signal sequence (6, 9). In this study, we created the FLAG-tagged LGR8 constructs, whose expression was driven by PRL signal sequence. Analogous constructs were successfully used by other researchers to assess LGR7/LGR8 expression and signaling (15, 23). The presence of the FLAG epitope in the receptor construct allowed us to demonstrate that T222P mutation dramatically impairs LGR8 targeting toward the cell membrane. We conclude that the undetectable cAMP response of T222P receptor to hormone stimulation shown in our previous studies (6, 9) is likely to be the result of poor receptor surface expression rather than impaired signaling, as was assumed before (9). Although the T222P mutant shows defective activation (EC50 for INSL3 of the mutant is higher than that of the wild-type LGR8), to our surprise, the mutant possessed even greater activity per receptor expressed on the cell surface. We hypothesize that, in vivo, when the T222P cell surface expression is driven by a “weaker” endogenous signal peptide, the T222P mutant does not contribute to INSL3 signaling, due to its negligible surface presentation. This mechanism appears to be responsible for the abnormal phenotype associated with the presence of this mutation in humans.

Two mutant LGR8 variants detected in humans (T222P, R223K) are located within the ectodomain of LGR8. The fact that one of the substitutions, T222P, leads to a severe impairment of surface expression, suggests a significant role for LRR in the receptor folding and targeting. One can speculate that weakened membrane delivery of LGR8 is caused by altered polypeptide chain processing, implying a critical part taken by Thr222 residue in the structural organization of the receptor. The Thr substitution to Pro is likely to impair polypeptide chain flexibility; in our experiments, we tried to determine whether less dramatic changes in this position would affect functional properties of the receptor. We produced mutant receptors with substitutions of the 222nd Thr with conservative Ser and less conservative Ala. In contrast to T222P substitution, both T222S and T222A only moderately affected LGR8 surface expression, with no distortion of the hormone-dependent receptor activation. We assume that the ability of polypeptide chain to acquire certain conformation, rather than hydrophilic nature of the 222nd residue, is important for the LGR8 proper folding and delivery. As Thr222 is positioned in the region linking the β-strand to the adjacent α-helix (14), the extreme flexibility of the region may be of particular importance for the ectodomain structure. Not surprisingly, the mutation of the neighboring residue, R223K, does not alter receptor properties, as it does not change either flexibility or the charge of the region.

The mutations of LGR8 that apparently result in intracellular retention are not limited to the region connecting LRR β-strands to the α-helices. To further investigate the role of LRR elements in the receptor function, we mutated the first Leu in the highly conservative stretch L/I XX L/I X L/I. The position homologous to Leu283 (7th LRR in LGR8) is occupied by Leu in mouse and human LGR8, LGR7, and several other related receptors, including snail, nematode, and sea anemone LGRs and all human glycoprotein hormone receptors. We assumed that this position may be highly sensitive to the substitutions. Indeed, a replacement of hydrophobic Leu with also hydrophobic but more voluminous Phe led to the impaired expression of L283F mutant compared with the wild-type receptor. Certain variations in the total expression were experienced with all mutants (Fig. 2) and could be attributed to the variations in transient transfection efficiency. However, significant reduction in total expression of L283F might be associated with mutant protein instability or lower synthesis rate. Importantly, we demonstrated severe impairment of both receptor plasma membrane presentation, detected by cell surface ELISA and deconvolution microscopy, and signal transduction, detected by cAMP response to INSL3. It was reported earlier that the mutation of the first Leu of the L/I XX L/I X L/I stretch in the 4th LRR of gonadotropin receptor results in the dramatic reduction of receptor surface expression and hormone-induced cAMP accumulation (22). Thus the proper folding of LRRs appears to be crucial for the receptor targeting to the cell surface. The dramatic effects of L283F and T222P mutations prove that both hydrophobic core and hydrophilic phase of LRR play an important role in this process. Interestingly, the minor splice variants of LGR7 missing some of the LRRs also failed to be expressed on cell surface membranes in vitro (17).

Next, we focused on the possible role of LDLa module, located at the NH2-terminus of the mature receptor. This module, repeatedly presented in the structure of non-GPCR LDL receptor, is unique to GPCRs LGR8, LGR7, and the orphan snail LGR (3). The role of LDLa module in these GPCRs remains undefined; however, some data indicate that it might be involved in ligand binding (26). LDL receptor modules are thought to be cross-linked with three disulfide bonds and organized around calcium ion, with several residues stabilizing such conformation (5). Naturally occurring substitutions of these residues were linked to the cases of familial hypercholesterolemia, proving the critical role of LDLa modules for LDL receptor function (5). We have created mutations,
disrupting the coordination of calcium (D70Y) and disulfide bond formation (C71Y) in LDLa module of LGR8. Our results showed that such LGR8 mutants had drastically reduced surface expression and severely blunted hormone-induced cAMP response. We assume that, similarly to the LDLa module of LDL receptor, LDLa module of LGR8 is stabilized by the disulfide bonds and the coordination of calcium ion, with D70 and C71 residues actively involved in such stabilization.

Intracellular retention of the membrane receptors is a well-known cause of several diseases (11, 20, 24). It is not surprising to see that certain LGR8 mutations also lead to the intracellular trapping of the receptor and the consequent impairment of INSL3/LGR8 signaling. For those naturally occurring mutations that are not affecting hormone binding or coupling with G proteins, a therapeutic intervention facilitating protein folding may be recommended. Consequently, it will be important to understand the process of LGR8 posttranslational processing and the mechanisms of receptor membrane delivery.

In conclusion, we have shown that the T222P mutation is caused by poor membrane presentation of mutated receptor rather than impaired signal transduction. A novel mutation of LGR8 receptor has been identified (R223K); however, it does not have significant functional consequences for receptor expression or signaling. We have established that the tertiary organization of both LRRs and LDLa module is critical for the LGR8 expression on the cell surface and its signal transduction.

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GRANTS

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