*Proc. Natl. Acad. Sci. USA*
Vol. 92, pp. 1906–1910, March 1995
Genetics

# Genetic heterogeneity of constitutively activating mutations of the human luteinizing hormone receptor in familial male-limited precocious puberty

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*Communicated by Earl W. Davie, University of Washington, Seattle, WA, December 1, 1994*

**ABSTRACT** **Genomic DNA from 32 unrelated families with male-limited precocious puberty was examined for the previously described Asp-578 → Gly, Met-571 → Ile, and Thr-577 → Ile mutations in transmembrane helix 6 of the human luteinizing hormone receptor (hLHR). Twenty-eight families had the inherited form of the disorder, and of these, 24 were found to have the Asp-578 → Gly mutation. Four additional mutations were found among the remaining four families with the inherited form and in four sporadic cases of the disorder: an A → C transversion resulting in substitution of leucine for Ile-542 in the fifth transmembrane helix, an A → G transition resulting in substitution of glycine for Asp-564 in the third cytoplasmic loop, a G → T transversion resulting in substitution of tyrosine for Asp-578 in the sixth transmembrane helix, and a T → C transition resulting in substitution of arginine for Cys-581 in the sixth transmembrane helix. Human embryonic kidney cells transfected with cDNAs for each of the mutant hLHRs, created by PCR-based mutagenesis of the wild-type hLHR cDNA, exhibited increased levels of basal cAMP production in the absence of agonist, indicating constitutive activation of the mutant hLHRs. Three of the additional mutations had specific features: Ile-542 → Leu and Cys-581 → Arg appeared ligand-unresponsive, whereas Asp-578 → Tyr appeared to correlate genotype with phenotype. We conclude that the region spanning nt 1624–1741 of exon 11 is a hotspot for heterogeneous point mutations that constitutively activate the hLHR and cause male-limited precocious puberty.**

Familial male-limited precocious puberty (FMPP) is a form of isosexual precocious puberty in boys in which testosterone levels are elevated independent of changes in luteinizing hormone-releasing hormone and serum luteinizing hormone levels (1–3). Signs of puberty usually appear by 3–4 yr of age, and testicular biopsy specimens show Leydig cell hyperplasia (1, 2). The pattern of inheritance is autosomal dominant, although sporadic cases occur.

Three mutations spanning amino acid residues 571–578 of the human luteinizing hormone receptor (hLHR) have been described among 17 families with FMPP (4–8). Substitution of glycine for Asp-578 in the sixth transmembrane helix of the hLHR was found in nine different families with the dominantly inherited form (4, 5) and in five sporadic cases of FMPP (7, 8). This mutant receptor produced increased levels of basal cAMP, consistent with constitutive activation of the hLHR (4). Substitution of isoleucine for Met-571 and isoleucine for Thr-577 in the sixth transmembrane helix was found in three additional FMPP kindreds; however, expression studies to confirm constitutive activation of these mutant hLHRs have not been reported (5, 6).

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These five studies have collectively shown that three activating mutations at different sites between residues 571 and 578 of the hLHR cause FMPP. However, these studies have left the following unanswered questions. (*i*) Are these the only mutations that activate the hLHR, or is there additional genetic heterogeneity of the hLHR gene in FMPP? (*ii*) Does the Asp-578 → Gly mutation account for all the sporadic cases of FMPP (7, 8), or are there other undiscovered sporadic mutations? (*iii*) While the Asp-578 → Gly mutation currently accounts for 82% of all reported FMPP families, is this the true frequency of this mutation in this disorder, and, if so, do the affected families share a common ancestral origin? (*iv*) Are phenotypic variations of FMPP, such as pubertal onset before age 1 yr, associated with novel mutations? (*v*) Are all activating mutations of the hLHR constrained within the narrow region between residues 571 and 578, or are there additional hotspots in exon 11 that can produce even greater degrees of receptor activation? (*vi*) Do only point mutations within exon 11 produce receptor activation, or can microdeletions, insertions, chain terminations, translocations, or mutations in other regions of the hLHR gene produce similar effects and cause FMPP?

To address these further questions, genomic DNA from 32 unrelated families affected with FMPP was examined for mutations of the hLHR gene. In addition to the Asp-578 → Gly mutation, four additional point mutations were identified in eight families (four inherited and four sporadic cases) within transmembrane helices 5 and 6, and the intervening third cytoplasmic loop of the hLHR, spanning residues 542–581. Each of these hLHR mutants produced elevated levels of cAMP when expressed in 293 cells. These four additional activating mutations included two with distinctive patterns of receptor activation that yield insights into mechanisms of receptor signal transduction.

## MATERIALS AND METHODS

Genomic DNA was isolated from blood samples obtained from 32 unrelated families with FMPP. Twenty-eight families had a family history of FMPP extending over several generations. There was no family history of precocious puberty or adult male short stature in four cases. Twenty-nine families originated in the United States, six of which have been reported (9, 10); three families originated in Hong Kong (11), Canada, and Scotland (12), respectively. The study was approved by the Institutional Review Board of the National Institute of Child Health and Human Development, and informed consent was obtained from all patients.

Abbreviations: FMPP, familial male-limited precocious puberty; hLHR, human luteinizing hormone receptor; WT, wild type; hCG, human chorionic gonadotropin.

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With PCR, primers 5′-CACTGCTGGCTTTTTCACTGTATT-3′ and 5′-TGAAGGCAGCTGAGATGGCAAAAA-3′ were used to amplify nt 1320–1783 of exon 11 of the hLHR gene, encoding aa 440–594. This fragment includes most of transmembrane helices 3 to 6, the second and third cytoplasmic loops, and the second extracellular loop of the hLHR. Each PCR mixture contained 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 25 pM of each primer, 2.0 mM $MgCl_2$, 200 μM of each dNTP (Boehringer Mannheim), 2.5 units of *Taq* polymerase (Perkin–Elmer/Cetus), and 2 μg of genomic DNA. PCR conditions were as follows: 5 min at 95°C, 30 cycles of 1 min at 57°C, 30 s at 72°C, and 1 min at 95°C, with a final extension of 3 min at 72°C. To test for the Asp-578 → Gly mutation PCR products were digested for 4 hr with 40 units of *Msp* I (New England Biolabs), separated on a 6% polyacrylamide gel, and visualized after staining with ethidium bromide.

**Sequencing of Genomic DNA.** For sequencing reactions, 3 μl of PCR product was used for a 28-cycle asymmetric PCR. Forty picomoles of primer was added to 100 μl of reaction mixture containing 0.2 mM of each dNTP and 2 units of *Taq* polymerase in 1× PCR buffer (13). The composition of the PCR buffer and conditions were as described (14). After the reaction, excess primers and salts were removed, and the amplified product was concentrated by using a Centricon-100 microcentrator (Amicon). The nucleotide sequence of the amplified DNA was determined by a modified dideoxynucleotide chain-termination method using Sequenase (United States Biochemical), as described (15). All mutations were determined by sequencing both strands generated by asymmetric PCR. Templates for asymmetric PCR were derived from two or more independent PCR products.

**Construction of cDNA Clones for Expression Studies of Mutant Receptors.** All four receptor mutations were located between a unique *Xba* I site (nt 1383–1388) in the hLHR gene (16) and a *Bam*HI site in the cloning vector (pCMX). Thus, two oligonucleotide primers flanking these sites (primer A, corresponding to nt 1375–1393, 5′-GTCATCACTCTAGAAAGAT; primer B, corresponding to nt 2097–2080 plus a 9-nt 5′ extension carrying a *Bam*HI restriction site, 5′-CGGGATCCTTTAACACTCTGTGTAGCG) were used with primers incorporating specific mutations for PCR amplification to obtain the mutant cDNA fragments. For all mutations, primers containing the mutated nucleotide were flanked on the 5′ end by specific restriction enzyme sites. For mutation 542, primers A and 542W [wild type (WT), nt 1619–1598, 5′-AAGAAGGCCACCACATTGAGAA], and primers 542M (mutant, nt 1606–1632; A1624C, 5′-GTGGTGGCCTTCTTCATACTTTGTGCT) and B, respectively, were used to generate two cDNA fragments. After restriction enzyme digestion the two cDNA fragments were joined at the *Hae* III restriction site, together with the flanking sequences of the WT hLHR cDNA, to replace the *Xba* I–*Bam*HI fragment of the WT hLHR cDNA in the cloning vector. With the same method, the other three mutations were introduced by using primers 564M (nt 1667–1694; A1691G, 5′-CAGAATTAATGGCTACCAATAAAGGTAC), 578M (nt 1755–1727; G1732T, 5′-GATAGGTGCCATGCAGGTGAAATAGGTGA) and 581M (nt 1760–1733; T1741C, 5′-AAAGAGATAGGTGCCATGCGGGTGAAAT). A *Ban* I restriction site is present in primers 578M and 581M, whereas an *Asn* I site is present in the 564M primer.

Individual cDNA fragments were amplified in a 50-μl reaction mixture containing 20 mM Tris (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 200 μM of each dNTP, 1 μM of each primer, and 2.0 units of Vent DNA polymerase (New England Biolabs). Using the WT hLHR cDNA cloned into the pCMX vector (pCMXhLHR) as template, samples were subjected to 10 cycles of amplification, followed by incubation for 10 min at 72°C. Each pair of the cDNA fragments for a specific mutation was digested with *Xba* I, *Bam*HI, and the indicated restriction enzymes, before ligation into a truncated pCMXhLHR vector which had the corresponding *Xba* I–*Bam*HI fragment removed. The ligation mixtures were used to transform *Escherichia coli* strain JM109. Ligated clones for mutations Asp-564 → Gly and Cys-581 → Arg were screened by enzyme digestion with *Rsa* I and *Aci* I, respectively. All mutants were sequenced (Sequenase, United States Biochemical) to confirm the presence of the intended mutation and the absence of unintended PCR-generated mutations.

Exponentially growing human embryonic kidney (293) cells in Dulbecco's modified Eagle's medium/5% calf serum/penicillin at 100 μg/ml/streptomycin at 100 μg/ml gentamycin at 100 μg/ml were plated ($2 \times 10^6$ cells) in 100-mm dishes. Cells were transfected by the calcium phosphate precipitation method with 15 μg of DNA per dish (17). Equivalent transfection efficiency was confirmed by cotransfecting pRSV-β-gal and measuring β-galactosidase activity in the cells.

The signal transduction activity of expressed receptors was estimated by determining cAMP production with and without exposure to human chorionic gonadotropin (hCG). Forty-eight hours after transfection, 293 cells were washed, transferred to 24-well tissue culture plates ($2 \times 10^6$ cells per well), and treated with 0.1–1000 ng of hCG per ml (CR-127; 14,900 international units/mg) in the presence of 0.25 mM methyl isobutylxanthine and 5% calf serum for 16 hr at 37°C. Cyclic AMP accumulation was measured by RIA (18).

## RESULTS

*Msp* I restriction digests of PCR product encoding residues 441–594 indicated the previously described Asp-578 → Gly mutation in 24 of the 32 families. These 24 families had a family history of FMPP. PCR product from the eight families that did not cut with *Msp* I revealed four unreported mutations. (*i*) An A → C transversion at nt 1624 in codon 542 was found in four unrelated families (Fig. 1*A*). This mutation causes substitution of leucine for isoleucine in the fifth transmembrane helix (Fig. 2) and creates a recognition site for the enzyme *Tsp*5091. Three of these families had a history of FMPP extending over multiple generations. The fourth boy was a sporadic case whose mother and father had only the WT sequence.

(*ii*) A second mutation, an A → G transition at nt 1691 in codon 564, was found in one affected boy (Fig. 1*B*). This mutation causes substitution of glycine for aspartate in the third cytoplasmic loop (Fig. 2) and creates a recognition site for *Rsa* I (19). Maternal PCR product did not cut with *Rsa* I. Paternal DNA could not be obtained; however, the father's height was 188 cm, and the onset of puberty was said to be normal. No other male family members had a known history of precocious puberty or short stature.

(*iii*) The third mutation, a G → T transversion at nt 1732 in codon 578, was found in two affected boys (Fig. 1*C*). This mutation causes substitution of tyrosine for aspartate in the sixth transmembrane helix (Fig. 2). There was no family history of precocious puberty, and direct sequencing of PCR product from the mother and father of both boys did not reveal the mutation.

(*iv*) The fourth mutation, a T → C transition at nt 1741 in codon 581, was found in one family in which FMPP extended over two generations (11) (Fig. 1*D*). This mutation causes substitution of arginine for cysteine in the sixth transmembrane helix (Fig. 2) and creates a recognition site for the enzyme *Aci* I.

To assess the functional effects of these four mutations directly, WT and mutant hLHRs were transiently expressed in 293 cells, and basal and hCG-stimulated cAMP accumulations were measured. All results of cAMP production were normalized for transfection efficiency based on β-galactosidase activity. Cells transfected with WT hLHR DNA had the same

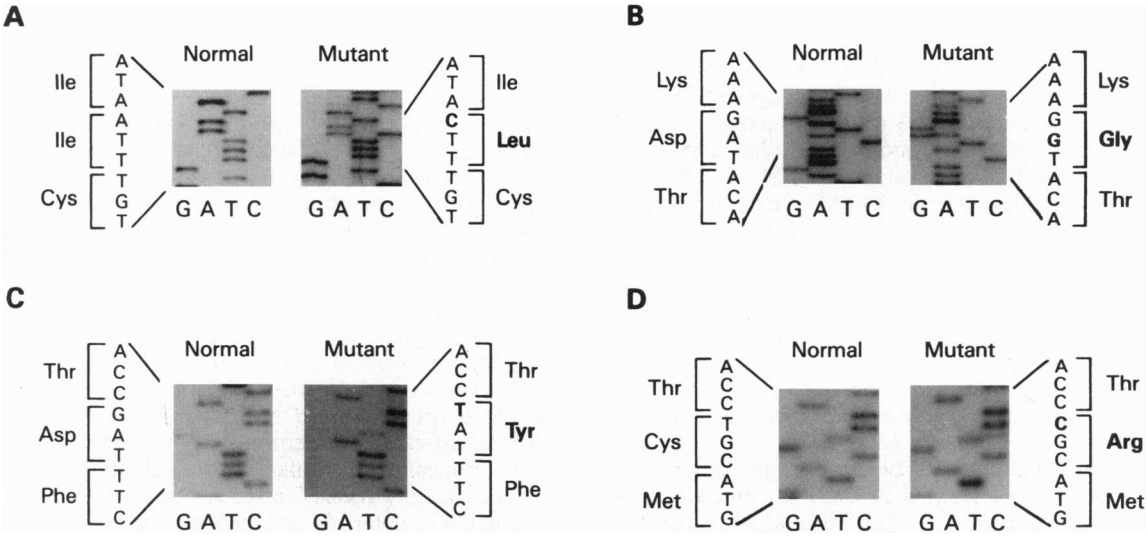


FIG. 1. (*A–D*) PCR product from normal controls and from four patients with FMPP showing WT sequence from the controls (*Left*) and heterozygous mutant sequence from the families with FMPP (*Right*). (*A*) A → C transversion at nt 1624 results in Ile-542 → Leu. (*B*) A → G transition at nt 1691 results in Asp-564 → Gly. (*C*) G → T transversion at nt 1732 results in Asp-578 → Tyr. (*D*) T → C transition at nt 1741 results in Cys-581 → Arg.

basal cAMP production as cells transfected with vector DNA. hCG (0.1–1000 ng/ml) produced a concentration-dependent increase in cAMP production in cells expressing the WT hLHR with an $EC_{50}$ of 25 ng/ml and mean stimulation of 25-fold (range 19- to 33-fold, $n = 5$) (Fig. 3). In contrast, the Asp-564 mutant hLHR had elevated basal cAMP production that was further stimulated by hCG in a dose-dependent manner ($EC_{50}$ of 6.6 ± 1.8 ng/ml, $n = 4$). Maximum cAMP production of this mutant was lower than that of the WT hLHR. We further tested cAMP production by all mutants. Compared with the WT hLHR, all the mutant hLHRs produced higher basal levels of cAMP in 293 cells, consistent with constitutive activation (Fig. 4). The highest basal cAMP production of two of the four mutations (Asp-564 → Gly and Asp-578 → Tyr) could be further increased by hCG stimulation (Fig. 4). However, maximal hCG-stimulated (1000 ng/ml) cAMP levels in the Ile-542 → Leu and Cys-581 → Arg mutant hLHR-transfected cells did not significantly exceed the basal cAMP levels in the absence of hormone (Fig. 4).

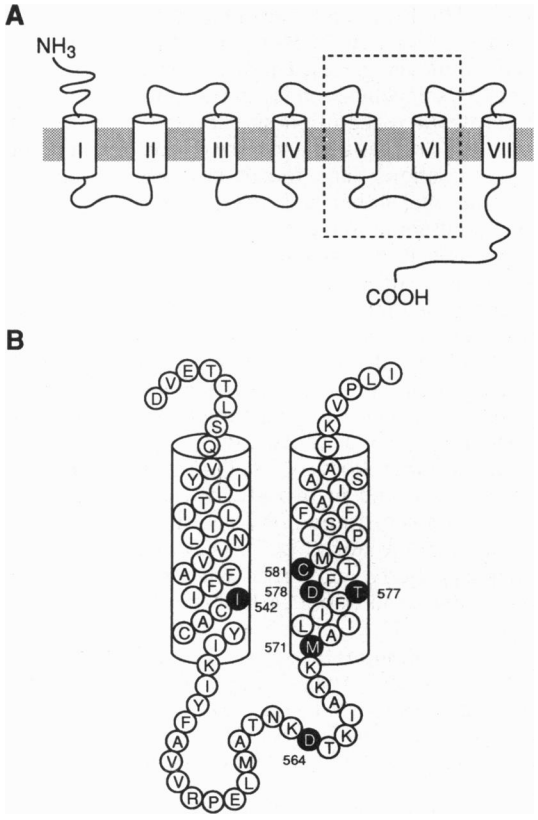


FIG. 2. (*A*) Schematic representation of the membrane topology of the hLHR. (*B*) Position of known constitutively activating mutations causing FMPP: Ile-542 in the fifth transmembrane helix, Asp-564 in the third cytoplasmic loop, and Met-571, Thr-577, Asp-578, and Cys-581 in the sixth transmembrane helix.

## DISCUSSION

This comprehensive study of 32 unrelated families with FMPP from the United States, Hong Kong, Canada, and Scotland resulted in six unreported findings. (*i*) Four additional muta-

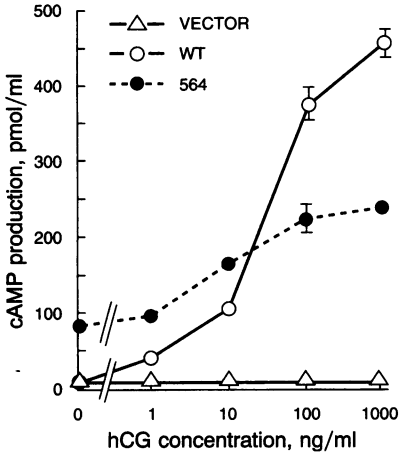


FIG. 3. Dose-dependent increase of cAMP production in 293 cells transfected with vector (pCMX), WT, or Asp-564 mutant hLHR cDNAs. Transfected 293 cells were treated with hCG at 1–1000 ng/ml for 16 hr at 37°C. Basal and hCG-stimulated cAMP production was normalized for the amount of β-galactosidase activity by the same cell preparation to correct for transfection efficiency. Data are means ± SEMs from triplicate observations from one experiment; similar results were found in three additional experiments.

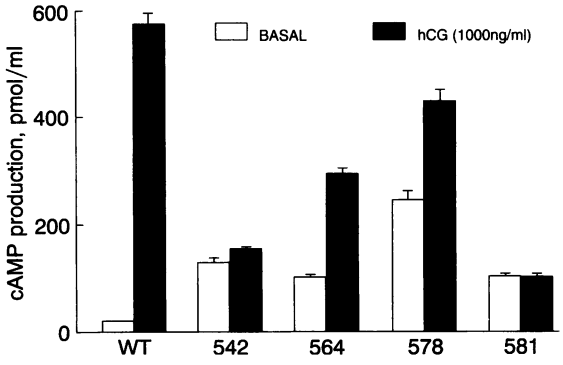


FIG. 4. Basal and maximal hCG-stimulated cAMP accumulation in 293 cells transfected with WT or four mutant 542, 564, 578, and 581 hLHR cDNAs. Basal and hCG-stimulated cAMP production was normalized for the amount of β-galactosidase activity of the same cell preparation to correct for transfection efficiency. Data points are means ± SEMs from triplicate observations from one experiment. Similar results were found in two to four additional experiments.

tions that constitutively activate the hLHR and cause FMPP have been described. These four mutations represent 57% of the seven known mutations and, therefore, extend the concept of genetic heterogeneity of activating hLHR mutations in this disorder.

(*ii*) In contrast to previous reports in which 100% of sporadic cases of FMPP were due to Asp-578 → Gly (7, 8), none of our sporadic cases had this mutation; all had other mutations. Therefore, among the four mutations now associated with all the known sporadic cases, the relative frequency of the Asp-578 → Gly mutation is only 56%. Consequently, sporadic cases of FMPP should be viewed as having a high likelihood of being associated with other mutations. Pedigree analysis of our 24 families with the Asp-578 → Gly mutation did not reveal any discernible relationships among the families. Because these 24 families, when added to the 14 previously reported cases with Asp-578 → Gly (4–8), comprise 38 kindreds from widely disparate geographic regions, it is unlikely that they all share a common ancestral origin.

(*iii*) The comprehensive nature of our study now permits the relative frequency of the seven different constitutively activating mutations of the hLHR to be calculated. The Asp-578 → Gly mutation is by far the most common; however, its overall incidence in FMPP is 78%, rather than 100% as found initially (4). The next most common mutation is Ile-542 → Leu with an overall incidence of 8%. Of the four families with this mutation, three had the inherited form of FMPP, whereas one case was sporadic. Thus, for the inherited form of FMPP, Asp-578 → Gly followed by Ile-542 → Leu were the most common mutations, accounting for 36 of 40 kindreds (90%). The three mutations (Met-571 → Ile, Thr-577 → Ile, and Cys-581 → Arg) found in four families collectively account for the remaining 10% of inherited cases of FMPP.

(*iv*) Differences in phenotypic expression of FMPP may be explained by differences in basal hLHR activity. A previously reported case from Scotland presented at 1 yr of age with signs of pubertal development (12). This case was found to have the Asp-578 → Tyr mutation, which produced the highest basal level of cAMP in transfected cells. The clinical presentation of the second sporadic case with this mutation has not been reported. Thus, classes of activating mutations of the hLHR gene may exist for which genotype correlates with the phenotype of an unusually early clinical expression. In addition, while the mutations Cys-581 → Arg and Ile-542 → Leu both had elevated levels of basal cAMP, they did not further increase cAMP production in response to hCG. These are then two constitutively activating mutations of the hLHR that appear ligand-unresponsive. Although reproductive potential is not impaired by these mutations, the clinical significance of this finding will require additional study. We speculate that alterations of Ile-542 and Cys-581 may disrupt the proposed low-affinity ligand-binding domain within the membrane and thereby impede ligand-binding and/or signal transduction.

(*v*) The region in which activating hLHR mutations have been found is no longer constrained between residues 571 and 578 in the sixth transmembrane helix. We have described four additional activating mutations that encompass the fifth transmembrane helix, the intervening third cytoplasmic loop, and further down the sixth transmembrane helix. The region containing the known activating mutations now includes residues 542–581. Thus, there is a much wider region containing at least 120 bp within exon 11 that contain hotspots for receptor activation.

(*vi*) All seven known constitutively activating mutations of the hLHR gene in patients with FMPP resulted from a single-base-pair substitution in exon 11. Neither insertions, microdeletions, chain terminations, nor translocations have been found in exon 11, nor have mutations in other regions of the hLHR been identified as causes of FMPP.

Of the seven mutations observed in FMPP, three substitute a neutral or nonpolar residue for the acidic residue aspartate, two substitute the nonpolar residues isoleucine and methionine with another nonpolar residue (leucine or isoleucine) of similar size, one substitutes a nonpolar residue (isoleucine) for a polar residue (threonine), and one substitutes the basic residue arginine for cysteine. Five mutations (Ile-542 → Leu, Asp-564 → Gly, Thr-577 → Ile, Asp-578 → Gly, Cys-581 → Arg) either create or delete recognition sites for restriction endonucleases, which facilitates rapid screening for these defects. The residues Ile-542, Asp-564, Asp-578, and Cys-581 are conserved in the glycoprotein hormone receptors. Ile-542 and Cys-581 are also found in the family of human opsins and rhodopsin, whereas Cys-581 is found in the human thromboxane $A_2$ receptor, α- and β-adrenergic, dopamine, and serotonin receptors (20), suggesting that this is a highly conserved residue with an important role in receptor function.

Implicit in the activation of G protein-coupled receptors is a release of conformational constraints following ligand binding (21, 22). Five human diseases are known to be caused by constitutive activation of G protein-coupled receptors: retinitis pigmentosa due to mutant rhodopsin (23), hyperfunctioning thyroid adenomas and autosomal dominant toxic thyroid

Table 1. Location of known mutations of the hLHR in FMPP

| Position in cDNA | Nucleotide change | Amino acid change | Location | Affected families, no. (ref.) |
|---|---|---|---|---|
| 1624 | A → C | Ile-542 → Leu | TM helix V | 4 |
| 1691 | A → G | Asp-564 → Gly | 3rd loop | 1 (19) |
| 1713 | G → A | Met-571 → Ile | TM helix VI | 2 (5, 6) |
| 1730 | C → T | Thr-577 → Ile | TM helix VI | 1 (6) |
| 1732 | G → T | Asp-578 → Tyr | TM helix VI | 2 |
| 1733 | A → G | Asp-578 → Gly | TM helix VI | 38 (4, 5, 7, 8) |
| 1741 | T → C | Cys-581 → Arg | TM helix VI | 1 |

TM, transmembrane.

hyperplasia due to mutations in the thyroid-stimulating hormone receptor gene (24, 25), autosomal dominant hypocalcemia due to a mutation in the calcium-sensing receptor gene (26), and FMPP due to mutations in the hLHR gene (4–8). Rhodopsin is maintained in an inactive state by a salt bridge between Glu-113 and Lys-296 in the third and sixth transmembrane helices, respectively (27, 28). Mutations in either residue abolish this salt bridge and cause constitutive activation of rhodopsin. The Asp-578 → Tyr and Asp-564 → Gly mutations of the hLHR, like the Asp-578 → Gly mutation, substitute a neutral for an acidic residue and thus might disrupt either electrostatic or hydrogen bond interactions with residues in adjacent helices, analogous to the activating mutations in rhodopsin. The Ile-542 → Leu, Thr-577 → Ile, and Met-571 → Ile mutations are conservative, substituting one nonpolar residue with another. However, because of change in size or bulkiness of side chains, these substitutions may also modify the relative positions of helices 5 and 6 and the position or accessibility of the third cytoplasmic loop, allowing the hLHR to assume a partially active conformation. Substitution of Cys-581 by arginine introduces a positively charged residue into helix 6, which is likely to disrupt electrostatic or hydrogen bond interactions with adjacent helices.

In summary (see Table 1), we have identified four additional point mutations within transmembrane helices 5 and 6 and the intervening third cytoplasmic loop of the hLHR that are responsible for constitutive activation of testis Leydig cells, leading to FMPP. The mutations Asp-578 → Gly and Ile-542 → Leu account for 90% of the inherited forms of FMPP. Three of our additional mutations were found in sporadic cases of FMPP; therefore, Asp-578 → Gly can no longer be presumed to account for all sporadic cases. Two other patterns of activating hLHR mutations have been identified: one where a genotype leading to higher basal cAMP formation appears to correlate with the phenotype of earlier clinical expression and one where the constitutively activated mutant receptors are ligand-unresponsive. Knowledge of these additional mutations will facilitate genetic counseling and diagnosis, as well as provide the basis to study the three-dimensional conformation of the receptor domains involved in ligand-binding and G protein activation.

We thank Drs. B. Albertson, S. LaFranchi, G. August, R. Banks, L. Deeb, M. Donaldson, M. Jenner, D. Jelly, D. Hadden, L. Low, K. Parker, B. Pfeiffer, P. Clark, W. Clarke, A. Rogol, B. Bercu, F. Diamond, E. Jorgensen, D. Shulman, A. Root, B. Wilson, and D. Zimmerman for providing samples from their patients and Janet Jones, R.N., for dedicated assistance. W.-Y.C. was supported in part by National Institutes of Health Grant HD 32644.

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