A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty

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FAMILIAL male precocious puberty (FMPP) is a gonadotropin-independent disorder that is inherited in an autosomal dominant, male-limited pattern[1–5]. Affected males generally exhibit signs of puberty by age 4. Testosterone production and Leydig cell hyperplasia occur in the context of prepubertal levels of luteinizing hormone (LH)[3–5]. The LH receptor is a member of the family of G-protein-coupled receptors[6,7], and we hypothesized that FMPP might be due to a mutant receptor that is activated in the presence of little or no agonist[8–12]. A single A → G base change that results in substitution of glycine for aspartate at position 578 in the sixth transmembrane helix of the LH receptor was found in affected individuals from eight different families. Linkage of the mutation to FMPP was supported by restriction-digest analysis. COS-7 cells expressing the mutant LH receptor exhibited markedly increased cyclic AMP production in the absence of agonist, suggesting that autonomous Leydig cell activity in FMPP is caused by a constitutively activated LH receptor.

Genomic DNA was isolated from affected males from eight different FMPP families, and polymerase chain reaction (PCR) was used to amplify a fragment of LH receptor (LHR) DNA encoding amino-acid residues 441 to 594 (which includes most of transmembrane helices 3 to 6, the second extracellular loop, and the second and third intracellular loops)[6,7]. PCR products were screened for heterozygous mutations using temperature-gradient gel electrophoresis (TGGE)[13]. PCR product from normal individuals migrated as a single band in a gradient of increasing temperature, but PCR products from all patients with FMPP migrated as multiple bands (Fig. 1*a*). The abnormal pattern of bands was identical for all patients.

PCR product from patient V-4 was subcloned and sequenced, revealing a heterozygous A → G transition at nucleotide 1,733 in codon 578 (Fig. 1*b*). This result was confirmed by direct sequencing of PCR products from patients from two other families. The mutation (GAT to GGT) encodes a substitution of glycine for an aspartate in transmembrane helix 6 (Fig. 1*c*) and creates a recognition site for the restriction endonuclease *Msp*I. Restriction digests were positive for the Asp 578 → Gly mutation in all patients who had abnormal TGGE patterns, and analysis of PCR products from three generations of one well characterized FMPP kindred indicates linkage of the LHR mutation to FMPP (Fig. 2).

Although the eight FMPP families are not known to be related, six of them originate in the same geographical region, and the surname of the kindred shown in Fig. 2 appears in the line of descent of two other families. We suspect that there is a common ancestral origin for the Asp 578 → Gly mutation, but that different mutations may be found in other families with FMPP.

To assess the functional effect of the Asp 578 → Gly mutation directly, wild-type and mutated human LHR were transiently expressed in COS-7 cells and cAMP accumulation was measured (Fig. 3). Cells transfected with wild-type LHR DNA had the same basal cAMP production as cells transfected with vector DNA. Human chorionic gonadotropin (hCG) produced a concentration-dependent increase in cAMP production in cells expressing the wild-type LHR, with an EC_{50} (50% effective concentration) of 4 ng ml^{-1} and mean maximal stimulation of 11.5-fold (range 2.9- to 18.7-fold; $n=5$).

In contrast to the wild-type receptor, the mutant LHR produced a 4.5-fold increase in basal cAMP production in COS-7 cells (range 1.8- to 8.3-fold; $n=5$), indicating that it was constitutively active. There was a significant increase in basal cAMP production even when cells were transfected with 25-fold less mutant DNA (data not shown). The mutant receptor was capable of responding to increasing concentrations of hCG, with an EC_{50} similar to that of the wild-type receptor. Maximal hCG-stimulated cAMP production in mutant-transfected cells was variable, but did not differ significantly from that in wild-type-transfected cells (range 75 to 135% of wild-type maximum). Agonist-independent stimulation of cAMP production by mutant receptors represented 42% (range 32 to 62%; $n=5$) of the maximal stimulation produced by hCG.

A dominant mutation that leads to constitutive activation of the LHR-mediated cAMP signalling pathway can explain the pathophysiology of FMPP, including its male-limited inheritance pattern. LHR-mediated effects, including testosterone production, involve increased production of cellular cAMP[14]. The normal onset of puberty in boys is attributed to a rise in circulating LH levels. Concentrations of LH found in the serum of pubertal boys[15] produce less than half-maximal activation of normal, recombinant human LHR[7]. We postulate that intracellular cAMP accumulation triggered by the unoccupied mutant receptor is sufficient to cause Leydig cell hyperfunction and hyperplasia. The age of pubertal onset in FMPP may depend on the extent to which the mutant allele is expressed as protein and on the relative expression of other genes critical for Leydig cell maturation. Because LH is sufficient to trigger steroidogenesis in Leydig cells but both LH and FSH are required to activate ovarian steroidogenesis, inappropriate activation of LHR alone would not be expected to cause precocious puberty in females. For example, hCG-secreting germ-cell tumours cause sexual precocity in males, but not females[16]. The observation that plasma testosterone in boys with FMPP increases normally in response to high doses of exogenous hCG[4,5] is consistent with the ability of the mutant LHR in COS cells to respond to high concentrations of hCG with the same maximal response as wild-type LHR (Fig. 3).

Other constitutively active G-protein-coupled receptors have been produced by mutating residues in the C-terminal portion of the third intracellular loop, adjacent to helix 6 (refs 8–12). A model of the arrangement of the transmembrane α-helices in G-protein-coupled receptors[17] places Asp 578 in the middle of helix 6, oriented towards the internal cleft. The aspartic acid at position 578 is conserved in all glycoprotein hormone receptors, but is not found in any other G-protein-coupled receptor[18]. The corresponding residue in cone opsins (Phe or Tyr at residue 277) lies near the retinal chromophore and is critical in determining the spectral absorption properties that are the basis of colour vision[19]. A phenylalanine residue occupies the equivalent position in all cationic neurotransmitter receptors, and is postulated to participate in a network of interhelical, aromatic interactions which help form the agonist-binding pocket and may be involved in propagating a conformational signal to the intracellular loops[18].

We speculate that in the inactive receptor state, Asp 578 is engaged in electrostatic or hydrogen-bond interaction with one

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or more residues in an adjacent helix, thus constraining the conformation of helix 6. When agonist binds to LHR these bonds are disrupted and a conformational change ensues. Substitution of the polar aspartate residue with glycine, a residue incapable of forming electrostatic or hydrogen bonds, would allow LHR to assume a partially active conformation in the absence of agonist. The substitution appears to have a negligible effect on agonist affinity, which is determined primarily by binding to the large N-terminal extracellular domain[20], or on the ability of the receptor to be maximally activated by agonist.

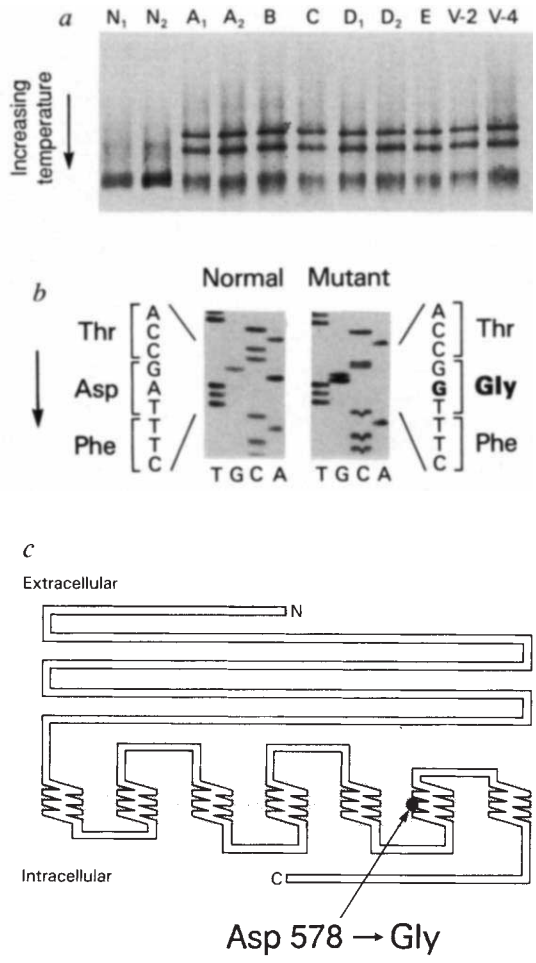

FIG. 1 *a*, Temperature-gradient gel electrophoresis (TGGE) of PCR-amplified DNA fragments encoding amino-acid residues 441 to 594 of the human LHR from 2 normal males (N_1 and N_2) and from 9 FMPP patients from 6 different families. TGGE identifies DNA duplexes containing base-pair mismatches on the basis of their altered melting properties in comparison to wild-type DNA duplexes[13,28]. The abnormal pattern of bands produced by each patient sample consists of the wild-type homoduplex band, two DNA heteroduplexes that melt at a lower temperature than the wild-type band, and a poorly focused mutant homoduplex band that melts at a slightly higher temperature than the wild-type band. This suggested that the DNA fragments from FMPP patients contained a heterozygous substitution of a G·C for an A·T base pair. DNA samples from patients from 2 other families produced the same abnormal pattern of bands (data not shown). *b*, Subcloned PCR product from subject V-4, showing normal (left) and mutant (right) sequences. Four clones had wild-type LHR sequence, and three clones showed an A-to-G transition at nucleotide 1,733. The heterozygous mutation encodes substitution of Asp 578 with Gly. The base change causes a compression of the 2 adjacent cytosines in the sequencing ladder. *c*, Schematic representation of the membrane topology of the human LHR, indicating the position of Asp 578 in the middle of the sixth transmembrane helix.

METHODS. The study was approved by the Clinical Research Committee of the NICHD and informed consent was obtained for all participants. Subject A_1 was patient 8 in ref. 29 and is the third cousin of subject A_2; subject D_1 is the son of subject D_2; subject E was patient 2 in other studies[29,30] and is the son of patient C.C. in ref. 2; subjects V-2 and V-4 are cousins (see Fig. 2 pedigree); subject V-4 was patient 1 in ref. 30 and patient 5 in ref. 29. PCR primers were selected based on the predicted melting behaviour of GC-clamped LHR DNA fragments (EZMELT, The Telluride Group, Newton, Massachusetts; and MELT87, L. Lerman, MIT). A GC clamp facilitates TGGE detection of single base substitutions within a DNA fragment[28]. Primers 5′CGCCCGCCGCGCCCCGCGCCCGCCCCGCCGCCCCCGCCCCCACTGCTGGCTTTTTCACTGTATT3′ and 5′TGAAGGCAGCTGAGATGGCAAAAA3′ generate a 504-bp DNA fragment consisting of a 40-bp GC clamp (underlined) and nucleotides 1,320 to 1,783 of the LHR sequence[6,7]. Each PCR reaction mixture (100 µl) contained 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.001% gelatin, 25 pmol each primer, 2.0 mM $MgCl_2$, 200 µM each nucleoside triphosphate, 2.5 U *Taq* polymerase (Perkin Elmer–Cetus), and 5 µl (~1 µg) of crude genomic DNA prepared from fresh or frozen blood samples. PCR conditions were: 5 min at 95 °C, 30 cycles of 1 min at 57 °C, 30 s at 72 °C and 1 min at 95 °C, and a final extension of 3 min at 72 °C. A commercial apparatus (Diagen) was used for TGGE. Gels were 3% polyacrylamide (50:1 acrylamide:bisacrylamide), 8 M urea, 1 mM EDTA, 2% glycerol, 20 mM MOPS-NaOH, pH 8.0. PCR fragments were denatured (at 95 °C for 5 min) and reannealed (50 °C, 15 min) in loading buffer (20 mM MOPS-NaOH, pH 8.0, 5 mM EDTA, 0.1% bromophenol blue, 2% glycerol). Each lane had 1 µl of PCR reaction loaded and samples were electrophoresed in 20 mM MOPS-NaOH, 1 mM EDTA, pH 8.0, at 280 V for 75 min over a linear temperature gradient of 35 to 55 °C. DNA was visualized using silver staining. PCR product generated from subject V-4 genomic DNA was blunt-end-ligated into the *Sma*I site of pBluescript (Stratagene); plasmid subclones were sequenced by standard methods.

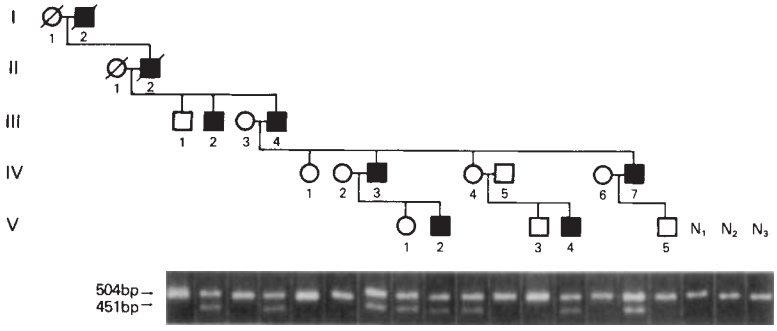

FIG. 2 *Msp*I restriction enzyme analysis of DNA from members of a family with FMPP and 3 unrelated normal males (N_1, N_2, N_3). Affected males are shown as solid squares. Subjects I-2 and II-2 were classified as affected on the basis of short stature. An A-to-G mutation at nucleotide 1,733 of the LHR gene creates the recognition site for *Msp*I (C/CGG). Normal PCR product (504 bp) will not be cut by *Msp*I, but DNA containing the mutant sequence is digested into two fragments of 451 and 53 bp. DNA from subjects who are heterozygous for the mutation will yield both uncut and digested fragments. The 53-bp fragment was run off the bottom of the gel in this experiment. The proband (V-4), his cousin (V-2), all other affected males in the pedigree (III-2, III-4, IV-3, IV-7), and an obligate carrier female (IV-4) showed the heterozygous digest pattern, whereas unaffected males (III-1, IV-5, V-3) and unrelated normal males (N_1, N_2, N_3) did not, suggesting linkage of the LHR mutation to FMPP. Restriction analysis indicates that the DNA of female IV-1 is normal, but that her niece (V-1) carries the Asp 578 → Gly mutation. Amniocyte DNA from a male fetus (V-5) at risk for inheriting FMPP was normal. DNA from affected individuals in the 7 other FMPP families showed the heterozygous digest pattern, but 40 additional control DNA samples did not (data not shown).

METHODS. PCR products (Fig. 1 legend) were digested for 16 h with 40 units of *Msp*I (New England Biolabs), separated on a 6% polyacrylamide gel (Novex), and visualized with ethidium bromide.

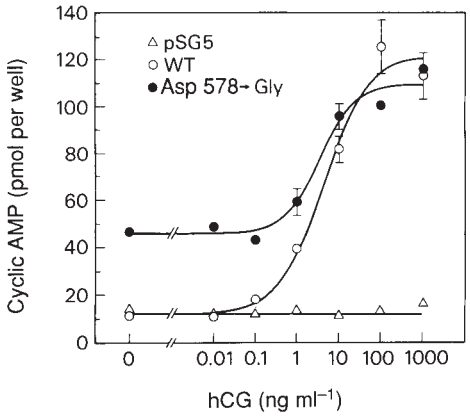

FIG. 3 Basal and human chorionic gonadotropin (hCG)-stimulated cAMP accumulation in COS-7 cells transfected 48 h earlier with pSG5 vector alone (pSG5) wild-type LHR DNA (WT), or mutant LHR DNA (Asp 578 → Gly). Data points are mean ± s.e. from 3 (hCG) or 6 (basal) replicate wells from a single experiment. If not shown, s.e. is smaller than the symbol. Results were similar in 4 additional experiments.
METHODS. Methods for mutagenesis, transfection and cAMP assay have been described[12]. Human LHR cDNA was inserted into a M13mp18 vector and oligonucleotide-mediated site-directed mutagenesis was used to generate clones encoding the Asp 578→Gly mutation (T7GEN kit, US Biochemical). *Hae*III was used instead of *Msp*I for parental strand digestion. Wild-type and mutant clones were inserted into the *Eco*RI site of the pSG5 expression vector (Stratagene). Mutagenesis was confirmed by DNA sequencing of the final construct and plasmid DNA was purified by CsCl gradient ultracentrifugation. COS-7 cells ($\sim 10^7$ cells) were transfected by electroporation (Bio-Rad) with 25 μg plasmid DNA, suspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, and transferred to 24-well plates ($\sim 10^5$ cells per well). Equivalent transfection efficiency was confirmed by co-transfecting pSVGH and measuring growth hormone concentration in the medium. Forty-eight hours after transfection, cells were washed and then incubated for 1 h at 37 °C with 0.2 ml Hanks' balanced salt solution containing 0.5% bovine serum albumin, 20 mM HEPES, pH 7.4, 10 mM LiCl and 0.5 mM 3-isobutyl-1-methylxanthine alone or with 0.01 to 1,000 ng ml^{-1} hCG (CR-127, 14,900 IU mg^{-1}; National Hormone and Pituitary Program). Perchloric acid was added to each well, samples were centrifuged, and total cAMP in aliquots of neutralized supernatant was determined by ^{125}I radioimmunoassay (DuPont). Membrane protein and growth hormone concentration per well varied by less than 10% between wild-type and mutant transfections.

Constitutively activating point mutations have been described in other classes of transmembrane receptors[21,22]. Inactivating mutations of G-protein-coupled receptors can serve as a mechanism of human disease[23,24], and the identification of amino-acid substitutions that cause constitutive activation of adrenergic receptors *in vitro* led to the prediction that such mutations might also be pathogenic[8,9]. Activating mutations of the melanocyte-stimulating hormone receptor have been found in mice with dominantly inherited hyperpigmentation[25], and activating mutations of rhodopsin have been described in a severe form of retinitis pigmentosa[26] and in congenital stationary night-blindness[27]. FMPP provides the first example of an inherited human disease that is due to a constitutively activating mutation in a G-protein-coupled hormone receptor. □

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Prevention of lung reperfusion injury in rabbits by a monoclonal antibody against interleukin-8

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RE-ESTABLISHING blood flow to ischaemic tissues causes greater injury than that induced during the ischaemic period[1,2]. This type of tissue injury, reperfusion injury, is involved in frostbite, multiple organ failure after hypovolaemia and in myocardial infarction[1]. Depletion of neutrophils alleviates reperfusion injury, implying a causal role of neutrophil infiltration[3,4]. Among members of the recently discovered family of chemotactic cytokines (chemokines)[5–8], interleukin-8 (IL-8)[5,9–13] is a major neutrophil chemotactic and activating factor produced by various types of human cells. We investigated its pathophysiological role in a rabbit model of a lung reperfusion injury. Reperfusion of ischaemic lung caused neutrophil infiltration and destruction of pulmonary structure, as well as local production of IL-8. Furthermore, the administration of a neutralizing monoclonal antibody against IL-8 prevented neutrophil infiltration and tissue injury, proving a causal role of locally produced IL-8 in this model.

In acute inflammation, neutrophils infiltrate down a concentration gradient of chemotactic factor(s) after adherence to endothelial cells through adhesion molecules[14]. Antibodies against those adhesion molecules inhibited the reperfusion injury[15–17], suggesting their essential role in the injury. Leukotriene B_4 (LTB_4) was produced in reperfusion injuries[18–21] but the effects of its specific antagonists on the injury were

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