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Characterization of heterogeneous mutations causing constitutive activation 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. A heterozygous mutation encoding substitution of Asp^{578} with Gly in transmembrane helix 6 of the G protein-coupled receptor for luteinizing hormone (LHR) has been found in affected males from nine American FMPP families. Cells expressing the mutant LHR exhibit markedly increased cyclic adenosine monophosphate (cAMP) production in the absence of agonist, suggesting that autonomous Leydig cell activity in FMPP is caused by a constitutively activated LHR. We have now analyzed genomic DNA from affected males from six additional FMPP families. PCR was used to amplify a fragment of the LHR gene encoding amino acid residues 441–594. None of the six new samples contained the Asp^{578}→Gly mutation, as indicated by absence of digestion with *MspI*. PCR products were then screened for heterozygous mutations using temperature-gradient gel electrophoresis. DNA fragments from two of the patients migrated abnormally. Direct sequencing of PCR product from one affected German male revealed a heterozygous mutation (ATG→ATA) encoding Met^{571}→Ile at the cytoplasmic end of helix 6, the same mutation that has been reported in another European FMPP kindred. Affected males in the second family had a novel Thr^{577}→Ile mutation (ACC→ATC). Mutations in different portions of the LHR or in a different gene may be responsible for disease in the other FMPP kindreds. Agonist binding and functional coupling of the mutant receptors to the cAMP and inositol phosphate pathways were studied by transiently expressing them in COS-7 cells. Agonist affinity was unaffected by the mutations. Like the Asp^{578}→Gly mutant receptor, the two newly identified mutant receptors triggered agonist-independent production of cAMP, but not of inositol phosphates, suggesting that autonomous testosterone production in FMPP can be explained by constitutive activation of the cAMP pathway alone.

INTRODUCTION

Familial male precocious puberty (FMPP, familial testotoxicosis, MIM #176410), is inherited in an autosomal dominant, male-limited pattern (1–3). Onset of puberty generally occurs by the age of 4 years. Testosterone production and Leydig cell hyperplasia occur autonomously, in the context of prepubertal levels of luteinizing hormone (LH).

The LH receptor (LHR) is a member of the family of G protein-coupled receptors and its structure is predicted to consist of a large extracellular domain connected to a bundle of seven α-helices that span the membrane (4). Puberty is normally initiated when LH produced by the pituitary binds to LH receptors in the testicular Leydig cells (2). Small increases in cellular cyclic adenosine monophosphate (cAMP) levels trigger production of testosterone (4,5).

It was suggested that FMPP could be due to a dominant mutation in the LHR gene that produced a receptor that could undergo spontaneous activation in the absence of agonist, and we and others recently described a heterozygous A to G mutation encoding substitution of Asp^{578} with Gly (D578G) in the sixth transmembrane helix of the LHR in nine different

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FMPP families (6,7). This mutation creates a recognition site for the endonuclease *Msp*I, and restriction digest analysis revealed linkage of this mutation to FMPP. COS-7 cells expressing D578G mutant LH receptors exhibit increased cAMP production in the absence of agonist, supporting the hypothesis that autonomous Leydig cell activity in FMPP is caused by a constitutively activated receptor (6).

A heterozygous mutation of Met^{571} to Ile has been identified in affected males from one other FMPP kindred, but the behavior of this mutant LHR was not characterized (7). Analysis of the same region of the LHR gene from one other male with FMPP and from two males with sporadic gonadotropin-independent precocious puberty was normal (7).

Seven of the nine FMPP families with the D578G mutation (6,7) originated in the southeastern United States, suggesting that they might have shared a common ancestor. To determine how many different mutations might be responsible for FMPP, we analyzed genomic DNA from affected males from six new FMPP families: two from Germany, three from France, and one family from the western United States, with mixed Caucasian–Native American ancestry. Functional properties and agonist binding of the mutant receptors were studied after transient expression in COS-7 cells. A preliminary account of this work has been published in abstract form (8,9).

RESULTS

*Msp*I digestion

Genomic DNA was isolated from affected males from the six new kindreds. PCR was used to amplify a fragment of the LHR gene encoding amino acid residues 441 to 594 and the PCR products were incubated with *Msp*I (Fig. 1). The PCR product from a normal individual, seen on the far left, is not cut by *Msp*I, but DNA from an affected male known to have the D578G mutation (6) yields both uncut and digested fragments. *Msp*I digests were negative in all six new patients, indicating that none of them had the D578G mutation.

Temperature-gradient gel electrophoresis

The same PCR products were then screened for the presence of other heterozygous mutations using temperature-gradient gel electrophoresis (Fig. 2). This method rapidly identifies mutation-containing DNA duplexes on the basis of their altered melting properties (10). DNA from a normal control migrates as a single major band, but DNA from a patient known to have one allele encoding the D578G mutation produces an abnormal pattern of bands. DNA fragments from patients 1, 2, 3, and 6 migrated normally, suggesting that they do not have a mutation in this region of the LHR. DNA from patients 4 and 5 produced distinct, abnormal patterns that differed from that produced by the D578G mutation.

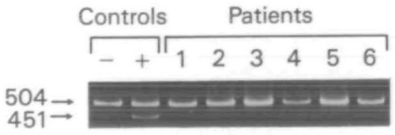

Figure 1. *Msp*I restriction enzyme analysis of PCR products (504 bp) generated from genomic DNA from a normal male (– control), an FMPP patient known to have one allele encoding the D578G mutation (+ control), and six new FMPP patients. *Msp*I digests were negative in all six patients, indicating that none had the D578G mutation.

DNA sequencing

Direct sequencing of PCR product from patient 4, who has mixed Caucasian–Native American ancestry, revealed a novel mutation of the codon directly adjacent to that for Asp^{578} (Fig. 3A). A heterozygous C to T change encoded replacement of Thr^{577} with Ile (T577I). Patient 5, who is German, had a heterozygous G to A mutation encoding substitution of Met^{571} at the cytoplasmic end of helix 6 with an Ile residue (M571I) (Fig. 3B). This is the same mutation that has been linked to FMPP in another European kindred (7).

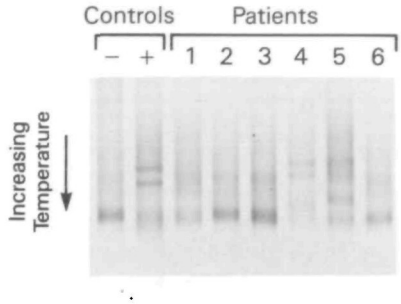

Figure 2. Temperature-gradient gel electrophoresis of the same PCR products analyzed in Fig. 1. DNA from a normal male (– control) migrates as a single major band, but DNA from an FMPP patient known to have one allele encoding the D578G mutation (+ control) produces an abnormal pattern of bands. DNA fragments from FMPP patients 1, 2, 3, and 6 migrated normally, suggesting that they do not have a mutation in this region of the LHR gene. DNA from patients 4 and 5 produced distinct, abnormal heteroduplex patterns that differed from that produced by the D578G mutation.

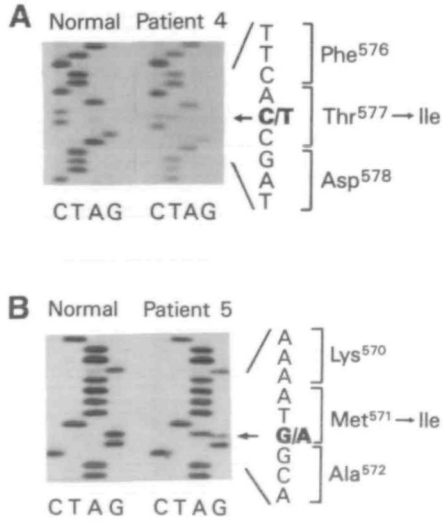

Figure 3. Sequence analysis of the LHR gene in two FMPP patients. PCR products that migrated abnormally in temperature-gradient gel electrophoresis were directly sequenced as described in Methods. (**A**) Patient 4 is heterozygous for a C to T mutation of nucleotide 1730. This mutation encodes substitution of Thr^{577} with Ile. (**B**) Patient 5 is heterozygous for a G to A mutation of nucleotide 1713. This mutation encodes substitution of Met^{571} with Ile.

*Cla*I digestion

The C to T mutation in codon 577 creates a recognition site for the restriction endonuclease *Cla*I (AT/CGAT). PCR products from unrelated normal individuals and patient 4's mother were not cut by *Cla*I, but DNA from patient 4, his affected brother and his affected father all yielded both uncut and digested fragments (Fig. 4). Because the paternal grandfather is reportedly unaffected, the T577I mutant allele must either be derived from the paternal grandmother, who is Native American, or represents a new mutation in the father. DNA samples from the grandparents were not available for analysis.

COS-7 expression studies

Initial characterization of the D578G mutant LHR included only measurement of cAMP accumulation (6), but normal LHR is also capable of stimulating hydrolysis of phosphoinositides (PI) by phospholipase C (11,12). To fully assess the functional consequences of the D578G mutation and the two newly identified mutations, wild-type (WT) and mutated human LH receptors were transiently expressed in COS-7 cells and cAMP production, PI hydrolysis and radiolabeled agonist binding were measured in intact cells.

Cells transfected with WT receptor had the same low basal cAMP accumulation as cells transfected with vector DNA alone (6), and the receptor agonist human chorionic gonadotropin (hCG) produced a concentration-dependent increase in cAMP production in cells expressing the WT receptor, with an EC_{50} of 4 ng/ml and a maximal stimulation of 14-fold over basal (Fig. 5A).

Unlike the WT receptor, the M571I and T577I mutant LHR both produced a 5-fold increase in cellular cAMP production when assayed in the absence of agonist, the same effect obtained with the D578G receptor (Fig. 5A). Mutant receptors were also capable of responding to increasing concentrations of hCG with additional stimulation of cAMP production. Maximal hCG-stimulated cAMP production in mutant-transfected cells was the same (M571I, T577I) or slightly lower (D578G) than that in WT-transfected cells (Fig. 5A).

Cells transfected with the WT LHR also exhibited increased production of inositol phosphates in response to hCG (Fig. 5B), but at least 10 ng/ml hCG was required to produce a response. The maximum concentration of hCG that was tested (1000 ng/ml) produced a 5-fold stimulation of basal inositol phosphate levels. Unlike the findings with cAMP production, none of the mutant LHR elicited a marked increase in inositol phosphate production in the absence of hCG, although the M571I LHR consistently produced a small, significant stimulation of basal inositol phosphate levels (1.33 ± 0.05 fold, $n = 7$, $P<0.01$).

Binding experiments performed in parallel with the functional studies demonstrated that none of the amino acid substitutions affected affinity for hCG (Table 1). The concentations of the three mutant receptors expressed at the COS cell surface (B_{max}) averaged only 54–66% of the WT receptor concentration.

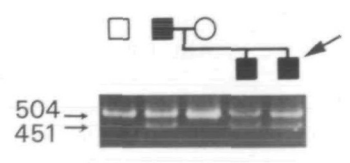

Figure 4. *Cla*I restriction enzyme analysis of PCR products from a normal male (open square), and the family of patient 4 (affected males are shown as solid squares). Only DNA that contains the T577I mutant sequence will be digested by *Cla*I. DNA from patient 4 (arrow), his affected brother, and his affected father yielded both uncut (504 bp) and digested (451 bp) fragments, but the DNA from patient 4's mother was not digested.

DISCUSSION

It has recently been shown that naturally occurring mutations in G protein-coupled receptor genes can serve as a mechanism of human disease, either by causing loss of receptor function, or by promoting constitutive receptor activation (reviewed in

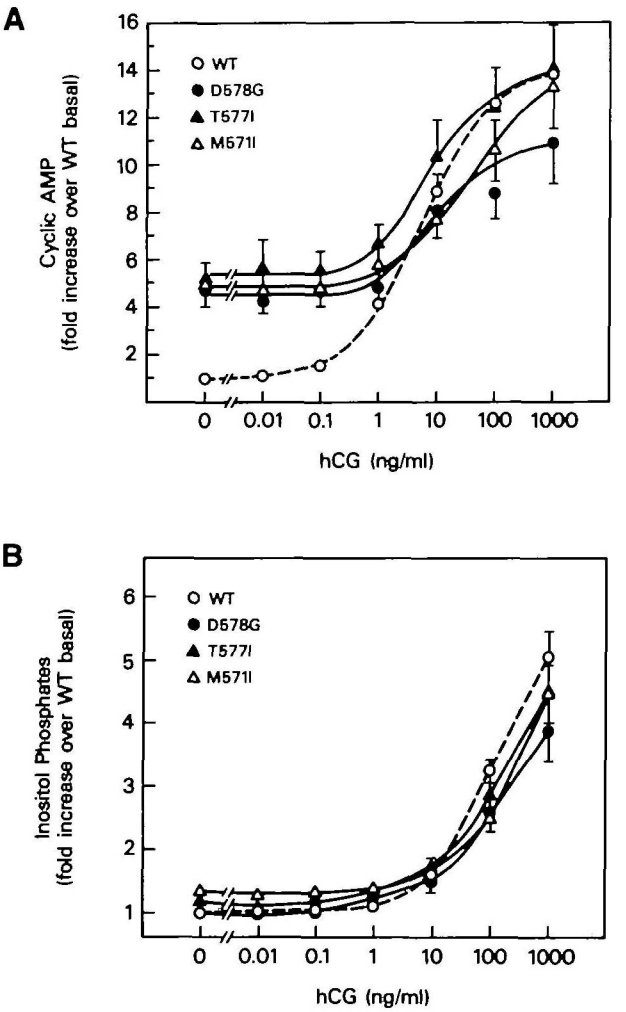

Figure 5. Basal and hCG-stimulated accumulation of cAMP (**A**) and inositol phosphates (**B**) in COS-7 cells transfected 48 h earlier with wild-type LHR DNA (WT), or LHR DNA encoding the D578G, T577I, or M571I mutations. Data points are mean ± SE from 7 to 16 experiments, expressed as fold-increase over basal accumulation in WT-transfected cells. For some points, SE is smaller than the symbol. Basal cAMP accumulation in WT-transfected cells was the same as that in cells transfected with vector alone, and basal inositol phosphate accumulation was slightly lower than that in vector-transfected cells (data not shown).

Table 1. ^{125}I-hCG binding in COS-7 cells transfected with wild-type (WT) and mutant LH receptors

	WT	D578G	T577I	M571I
K_d, nM	3.5	2.2	2.9	4.3
(range)	(0.8–5.3)	(1.0–4.5)	(1.7–4.6)	(2.1–12.0)
B_{max},% WT	100[a]	66 ± 13	62 ± 6	54 ± 12
# experiments	10	5	6	5

[a]WT B_{max} averaged 157 ± 20 fmol/well

reference 13). In addition to the discovery of activating LHR mutations in FMPP, activating mutations of rhodopsin have been described in certain forms of retinitis pigmentosa and congenital night blindness (14–16), and several different activating mutations of the TSH receptor (TSHR) have been found in sporadic (17,18) and inherited (19) forms of hyperthyroidism.

The observation that seven of the first nine FMPP families with the LHR D578G mutation (6,7) originated in the southeastern United States initially raised the possibility of a founder effect for this mutation. Recent reports describing the identical D578G mutation as the cause of FMPP in many more apparently unrelated families (20), and as the cause of several sporadic cases of gonadotropin-independent precocious puberty in boys (21,22), suggest that A to G mutation of nucleotide 1733 in the LHR gene is in fact a recurrent event.

Because of the known importance of the second and third intracellular receptor loops in coupling to G proteins (23–25), we and others focused our initial search for activating mutations on the part of the LHR gene that encoded those loops and their flanking transmembrane helices (6,7,20). Our finding that heterogeneous LHR mutations in this region are associated with some but not all cases of FMPP is consistent with the results from others (7,20).

The T577I and M571I mutant receptors, like the original D578G receptor, cause constitutive activation of the cAMP pathway in COS-7 cells. The ability of each of the three receptors to produce agonist-independent stimulation of cAMP accumulation is similar, as is their ability to mediate additional, agonist-dependent stimulation (Fig. 5A).

Cells transfected with the WT LHR not only produce cAMP in response to hCG, but also exhibit increased production of inositol phosphates in response to high concentrations of the agonist (Fig. 5B). These results confirm other studies showing that murine (11) and human (12) LHR can couple to dual signaling pathways in transfected cells, albeit with different efficiency. As with other members of the glycoprotein hormone receptor group, such as the TSHR, coupling to the cAMP and PI hydrolysis pathways is likely to be mediated by members of two different G protein families, G_s and $G_{q/11}$, respectively (26). Because such high concentrations of agonist are required to produce activation, the physiological significance of LHR coupling to the PI pathway in the testes is questionable (4). In contrast to the results with cAMP (Fig. 5A), the mutant LH receptors cause little or no constitutive activation of the PI pathway (Fig. 5B). This behavior is comparable with that of the constitutively active mutant TSHR identified in some patients with hyperthyroidism (17,19), and suggests that in both cases autonomous endocrine activity may be related to stimulation of the cAMP pathway alone. The ability of the mutations described here and elsewhere to selectively promote (17,19,27) or inhibit (27–31) coupling to one of two parallel signaling pathways may provide insight into the mechanism by which a single receptor differentially activates multiple G protein-linked effector systems.

None of the helix 6 mutations causes a significant change in agonist affinity, and the concentration of receptors at the cell surface measured by agonist binding is actually decreased compared with the WT receptor (Table 1), ruling out the possibility that increased basal cAMP accumulation by the mutants was due to increased receptor number. The mutant receptors may be less efficiently incorporated into the cell membrane or be less stable than the WT receptor. Some activating mutations of catecholamine receptors have been shown to be associated with increased affinity for agonist (25). The lack of change in affinity for hCG is consistent with the fact that, in the case of glycoprotein hormone receptors, sequences in the large extracellular domain are the major determinant of agonist binding affinity (4).

The aspartate, methionine, and threonine residues found to be mutated in FMPP patients are conserved in all mammalian glycoprotein hormone receptors (4,32,33), and in partially homologous G protein-coupled receptors cloned from invertebrates (34,35). Although the three-dimensional structure of G protein-coupled receptors is not known, models of the possible orientation of residues in the transmembrane bundle have been constructed (33,36,37). According to one model (36,37), Met^{571} is predicted to lie two helical turns away from, and directly below Asp^{578}, with both residues pointed toward the internal cleft of the receptor, and Thr^{577} would be oriented toward helix 7 and the surrounding membrane lipid.

The localization and characterization of mutations that mimic agonist occupancy may provide insight into the normal mechanism of receptor activation (25). We speculate that in the inactive receptor state the conformation of LHR helix 6 is constrained. When LH or hCG binds to the receptor, this constraint is broken, and a conformational change ensues. Substitution of certain residues in helix 6 may cause structural changes that weaken the constraint and therefore partially mimic agonist occupancy. The activating effect of D578G may be due to the loss of a stabilizing interhelical bond (6,33). For example, disruption of a critical interhelical electrostatic interaction appears to explain constitutive activation of certain mutant rhodopsin molecules (14–16). The mechanisms by which substitutions at positions 571 and 577 in the LHR cause spontaneous activation remain unclear, but it is possible that the side chain of Thr^{577} normally provides helix stabilization by participating in an intrahelical hydrogen-bond (38). The potential importance of this particular region of helix 6 in maintaining the inactive conformation of glycoprotein hormone receptors is underscored by the recent discovery that some hyperfunctioning thyroid adenomas contain somatic point mutations of human TSHR residues equivalent to LHR Asp^{578}, Thr^{577}, and Phe^{576} (i.e. TSHR D633Y, D633E, T632I, and F631C) (18).

In conclusion, we have demonstrated that at least three different activating LHR gene mutations are associated with FMPP. The location of the mutations is consistent with other data showing that residues at the base of receptor helix 6 and in the adjacent C-terminal portion of the third intracellular loop play a critical role in receptor-G protein coupling (17,18,23–25,27,28). The three mutant LH receptors behave very similarly in expression studies, and there is, as yet, no evidence that different mutations are associated with discernible FMPP

phenotypes. In addition to the helix 6 mutations discussed here, naturally occurring mutations in helix 2 of the melanocyte stimulating hormone receptor (39), and in helices 3 and 7 of the TSHR (19), are capable of triggering agonist-independent activation of the cAMP pathway. Mutations in these or other portions of the LHR (or possibly mutations in a different gene) may be found in the four FMPP patients who had normal temperature-gradient gel electrophoresis results and in other FMPP patients who have been shown to lack a mutation in helix 6 (7,20).

MATERIALS AND METHODS

Patients

All patients presented with early pubertal development, excessive growth velocity, advanced bone age, mature levels of testosterone, and a history of precocious puberty in at least one other male family member. Patients 1–5 had documented prepubertal gonadotropin levels, but patient 6 was already in advanced puberty when first evaluated (9). Patients 1–3 were from France, and have previously been reported as subjects 5, 1, and 2, respectively, in table II of reference 40. Patient 4 presented at age 3½ with an 8 month history of increasing penile size and a 4 month history of pubic hair growth, rapid height increase, recurrent priapism, and aggressive behavior. Evaluation revealed: (a) no café au lait spots; (b) prepubertal serum gonadotropin concentrations, with no rise in serum LH bioactivity following gonadorelin administration; (c) serum testosterone concentrations 190–236 ng/dL; (d) normal adrenal androgen response to cosyntropin; (e) skeletal maturity 7–8 years; and (f) normal cranial computerized tomography scan. Treatment with ketoconazole was successful. Patient 5 is the father of the patient first described by Rabl *et al.* (41), and is of Bavarian ancestry. There was no evidence for phenotypic heterogeneity among the different families.

Mutation detection

Methods used for PCR and TGGE have been described (6). Primers LH1 (5′-CGCCCGCCGCGCCCCGCGCCCGCCCCGCCGCCCCCGCCCCCACTG-CTGGCTTTTTCACTGTATT-3′) and LH2 (5′-TGAAGGCAGCTGAG-ATGGCAAAAA-3′) generate a 504 bp DNA fragment consisting of a 40 bp GC-clamp (underlined) and nucleotides 1320–1783 of the human LHR sequence (32). Each PCR reaction mixture (100 µl) contained 10 mM Tris−HCl, pH 8.3, 50 mM KCl, 0.001% gelatin, 25 pmol of each primer, 2.0 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 2.5 U *Taq* polymerase (Perkin-Elmer Cetus), and 5 µl (about 1 µg) of crude genomic DNA prepared from fresh or frozen blood samples. PCR conditions were 5 min at 95°C, 35 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.

TGGE was performed using a commercial apparatus (Diagen). 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 (98°C, 5 min) and reannealed (50°C, 15 min) in the presence of 10 mM EDTA, then diluted in loading buffer (20 mM MOPS-NaOH, pH 8.0, 5 mM EDTA, 0.1% bromophenol blue, 2% glycerol). Two µl of PCR reaction were loaded per lane and samples were electrophoresed in 20 mM MOPS-NaOH, 1 mM EDTA, pH 8.0, at 280 V for 90 min over a linear temperature gradient of 35–55°C. DNA was visualized with silver staining.

DNA sequencing

Single-stranded DNA was generated from PCR products using the primer LH1, and purified templates were then sequenced directly (Sequenase, US Biochemical) with the primer LH2.

Restriction enzyme digests

PCR products were digested at 37°C with 40 U of *Msp*I (New England Biolabs) or 10 U of *Cla*I (Boehringer Mannheim) and separated on a 6% acrylamide gel (Novex). In both cases, the enzymes cut after nucleotide 1730 of the coding sequence when a mutation was present, producing fragments of 451 and 53 bp. The 53 bp fragment was routinely run off the bottom of the gel. DNA was visualized with ethidium bromide and UV light.

Mutagenesis and expression studies

Methods used for mutagenesis, transfection, binding, cAMP, and inositol phosphate assays have been described (6,27). Human LHR cDNA was inserted into a M13mp18 vector and oligonucleotide-mediated, site-directed mutagenesis was used to generate clones encoding the desired mutation (T7GEN kit, US Biochemical). WT 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 (~10^7 cells) were transfected by electroporation (Bio-Rad) with 25 µg plasmid DNA. Cells intended for binding assays were suspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, and transferred to 6-well plates (~5×10^5 cells/well). Cells for cAMP and inositol phosphate assays were suspended in inositol-free medium supplemented with 10% fetal calf serum and 2.5 µCi/ml myo-[2-^{3}H]inositol (DuPont) and were transferred to 24-well plates (~10^5 cells/well). Forty-eight hours after transfection cells were washed with assay buffer (Hanks' balanced salt solution, 0.5% crystalline bovine serum albumin, 20 mM Hepes-NaOH, pH 7.4). ^{125}I-hCG binding was measured by incubating cells for 8 h at 4°C in 1 ml assay buffer containing approximately 300 000 c.p.m. ^{125}I-hCG (CR-127, 14 900 IU/mg, National Hormone and Pituitary Program; labeled to about 40 µCi/µg by Hazelton Washington) and 0–10^{-7} M unlabeled hCG. The program LIGAND (42) was used to analyze binding data. Data were best fit with a one-site model. Cyclic AMP and inositol phosphate production were measured concurrently by incubating cells for 1 h at 37°C in 0.2 ml assay buffer containing 10 mM LiCl, 0.5 mM IBMX and 0–1000 ng/ml hCG. 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). Total inositol phosphates were measured using Dowex AG1-X8 anion exchange column chromatography. COS-7 cells transfected with pSG5 vector alone were not stimulated by hCG and did not exhibit specific ^{125}I-hCG binding. K_d values are expressed as the geometric mean and range, and other data are shown as mean ± standard error.

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