

## 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<sup>1-5</sup>. 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)<sup>3-5</sup>. The LH receptor is a member of the family of G-protein-coupled receptors<sup>6,7</sup>, and we hypothesized that FMPP might be due to a mutant receptor that is activated in the presence of little or no agonist<sup>8-12</sup>. 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)<sup>6,7</sup>. PCR products were screened for heterozygous mutations using temperature-gradient gel electrophoresis (TGGE)<sup>13</sup>. 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. 1a). 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. 1b). 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. 1c) and creates a recognition site for the restriction endonuclease *MspI*. 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<sub>50</sub> (50% effective concentration) of 4 ng ml<sup>-1</sup> 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<sub>50</sub> 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<sup>14</sup>. 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<sup>15</sup> produce less than half-maximal activation of normal, recombinant human LHR<sup>7</sup>. 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<sup>16</sup>. The observation that plasma testosterone in boys with FMPP increases normally in response to high doses of exogenous hCG<sup>4,5</sup> 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  $\alpha$ -helices in G-protein-coupled receptors<sup>17</sup> 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<sup>18</sup>. 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<sup>19</sup>. 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<sup>18</sup>.

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

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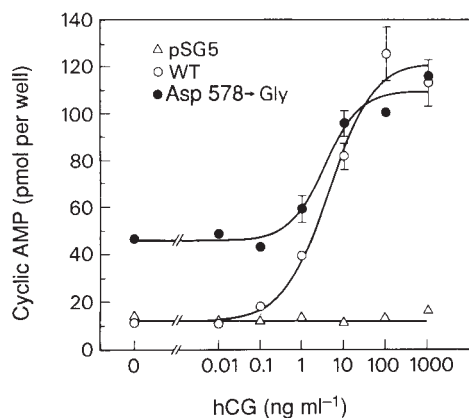


FIG. 3 Basal and human chorionic gonadotropin (hCG)-stimulated cAMP accumulation in COS-7 cells transfected 48 h earlier with pSG5 vector alone (pSG5) wild-type LHR DNA (WT), or mutant LHR DNA (Asp 578 → Gly). Data points are mean  $\pm$  s.e. from 3 (hCG) or 6 (basal) replicate wells from a single experiment. 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<sup>12</sup>. 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  $\mu$ g 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<sup>-1</sup> hCG (CR-127, 14,900 IU mg<sup>-1</sup>; 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 <sup>125</sup>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<sup>21,22</sup>. Inactivating mutations of G-protein-coupled receptors can serve as a mechanism of human disease<sup>23,24</sup>, 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<sup>8,9</sup>. Activating mutations of the melanocyte-stimulating hormone receptor have been found in mice with dominantly inherited hyperpigmentation<sup>25</sup>, and activating mutations of rhodopsin have been described in a severe form of retinitis pigmentosa<sup>26</sup> and in congenital stationary night-blindness<sup>27</sup>. 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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- Stone, R. K. *Am. J. med. Sci.* **24**, 561–564 (1852).
- Walker, S. H. *J. Pediatr.* **41**, 251–257 (1952).
- Schedewie, H. K. *et al. J. clin. Endocr. Metab.* **57**, 271–278 (1981).
- Rosenthal, S. M., Grumbach, M. M. & Kaplan, S. L. *J. clin. Endocr. Metab.* **57**, 571–578 (1983).
- Holland, F. J. *Endocr. Metab. Clin. North Am.* **20**, 191–210 (1991).
- Minegishi, T. *et al. Biochem. biophys. Res. Commun.* **172**, 1049–1054 (1990).
- Jia, X.-C. *et al. Molec. Endocr.* **5**, 759–768 (1991).
- Allen, L. F., Lefkowitz, R. J., Caron, M. G. & Cotecchia, S. *Proc. natn. Acad. Sci. U.S.A.* **88**, 11354–11358 (1991).
- Kjelsberg, M. A., Cotecchia, S., Ostrowski, J., Caron, M. G. & Lefkowitz, R. J. *J. biol. Chem.* **267**, 1430–1433 (1992).

- Samama, P., Cotecchia, S., Costa, T. & Lefkowitz, R. J. *J. biol. Chem.* **268**, 4625–4636 (1993).
- Ren, Q., Kurose, H., Lefkowitz, R. J. & Cotecchia, S. *J. biol. Chem.* **268**, 16483–16487 (1993).
- Kosugi, S., Okajima, F., Ban, T., Hidaka, A., Shenker, A. & Kohn, L. D. *Molec. Endocr.* **7**, 1009–1020 (1993).
- Riesner, D. *et al. Electrophoresis* **10**, 377–389 (1989).
- Hunzicker-Dunn, M. & Birnbaumer, L. in *Luteinizing Hormone Action and Receptors* (ed. Ascoli, M.) 57–134 (CRC, Boca Raton, 1985).
- Oenter, K. E., Uriarte, M., Rose, S. R., Barnes, K. M. & Cutler, G. B. Jr *J. clin. Endocr. Metab.* **71**, 1251–1258 (1990).
- Sklar, C. A., Conte, F. A., Kaplan, S. L. & Grumbach, M. M. *J. clin. Endocr. Metab.* **53**, 656–660 (1981).
- Baldwin, J. M. *EMBO J.* **12**, 1693–1703 (1993).
- Trumpp-Kallmeyer, S., Hoflack, J., Bruinvels, A. & Hibert, M. J. *Med. Chem.* **35**, 3448–3462 (1992).
- Neitz, M., Neitz, J. & Jacobs, G. J. *Science* **252**, 971–974 (1991).
- Tsai-Morris, C. H., Buczko, E., Wang, W. & Dufau, M. L. *J. biol. Chem.* **265**, 19385–19388 (1990).
- Bargmann, C. I., Hung, M.-C. & Weinberg, R. A. *Cell* **45**, 649–657 (1986).
- Yaghai, R. & Hazelbauer, G. L. *Proc. natn. Acad. Sci. U.S.A.* **89**, 7890–7894 (1992).
- Dryja, T. P. *et al. New Engl. J. Med.* **323**, 1302–1307 (1990).
- Rosenthal, W. *et al. Nature* **359**, 233–235 (1992).
- Robbins, L. S. *et al. Cell* **72**, 827–834 (1993).
- Robinson, P. R., Cohen, G. B., Zhukovsky, E. A. & Oprian, D. D. *Neuron* **9**, 719–725 (1992).
- Dryja, T. P., Berson, E. L., Rao, V. R. & Oprian, D. D. *Nature Genet.* **4**, 280–283 (1993).
- Myers, R. M., Sheffield, V. C. & Cox, D. R. in *PCR Technology: Principles and Applications for DNA Amplification* (ed. Erlich, H. A.) 71–88 (Stockton, New York, 1989).
- Laue, L., Jones, J., Barnes, K. M. & Cutler, G. B. Jr *J. clin. Endocr. Metab.* **76**, 151–155 (1993).
- Laue, L. O. H. *et al. New Engl. J. Med.* **320**, 496–502 (1989).

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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<sup>1,2</sup>. This type of tissue injury, reperfusion injury, is involved in frostbite, multiple organ failure after hypovolaemia and in myocardial infarction<sup>1</sup>. Depletion of neutrophils alleviates reperfusion injury, implying a causal role of neutrophil infiltration<sup>3,4</sup>. Among members of the recently discovered family of chemotactic cytokines (chemokines)<sup>5–8</sup>, interleukin-8 (IL-8)<sup>5,9–13</sup> 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<sup>14</sup>. Antibodies against those adhesion molecules inhibited the reperfusion injury<sup>15–17</sup>, suggesting their essential role in the injury. Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) was produced in reperfusion injuries<sup>18–21</sup> but the effects of its specific antagonists on the injury were

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