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Male pseudohermaphroditism due to a homozygous missense mutation of the luteinizing hormone receptor gene

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Leydig cell hypoplasia is a rare autosomal recessive condition that interferes with normal development of male external genitalia in 46,XY individuals. We have studied two Leydig cell hypoplasia patients (siblings born to consanguineous parents), and found them to be homozygous for a missense mutation (Ala593Pro) in the sixth transmembrane domain of the luteinizing hormone (LH) receptor gene. *In vitro* expression studies showed that this mutated receptor binds human choriogonadotropin with a normal K_D, but the ligand binding does not result in increased production of cAMP. We conclude that a homozygous LH receptor gene mutation underlies the syndrome of autosomal recessive congenital Leydig cell hypoplasia in this family. These results have implications for the understanding of the development of the male genitalia.

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The process that leads to the development of male sex characteristics is generally thought to consist of three sequential steps[1]. The first is the establishment of male genetic sex by the XY chromosome pattern at conception. The second involves the induction of male gonadal sex, that is testis formation, through the action of SRY (sex determining region of the Y chromosome). The third step consists of the development of internal and external male genitalia, a process which is basically controlled by two factors, testosterone and anti-Müllerian hormone (AMH), both produced by the fetal testis. The Sertoli cell product, AMH, prevents the formation of the müllerian duct derivatives (fallopian tubes, uterus and the upper part of the vagina), whereas the development of the male internal and external genitalia is dependent on the Leydig cell product, testosterone, which is converted to dihydrotestosterone in certain target tissues. Various genetic defects in this pathway have been reported, supporting the concept of sequential determination of male genetic, gonadal and phenotypic sex[2].

An important step in this differentiation cascade is the development of the Leydig cells. This apparently begins independent of stimulation by gonadotropins at approximately the same time that the formation of the testicular cords occurs[3], although the process still occurs in cases where testicular cords do not develop[4]. Leydig cell differentiation is crucial for sufficient androgen production by the testis. Absence of Leydig cells or insufficient Leydig cell differentiation can occur as an autosomal recessive condition[5–13]. The phenotype of inherited Leydig cell hypoplasia ranges from extreme forms presenting as 46,XY females to milder forms in which males present with hypergonadotropic hypogonadism and a micropenis[9,10]. Testicular luteinizing hormone (LH) binding was decreased or absent in some studies, which could be either the cause or the consequence of hypoplasia of Leydig cells[5,6]. Here, we report studies of the LH receptor gene in siblings born to consanguineous parents who have male pseudohermaphroditism due to Leydig cell hypoplasia.

Clinical details

Our study examined two 46,XY siblings who presented with female external genitalia, primary amenorrhea, and lack of breast development. Their parents are first cousins,

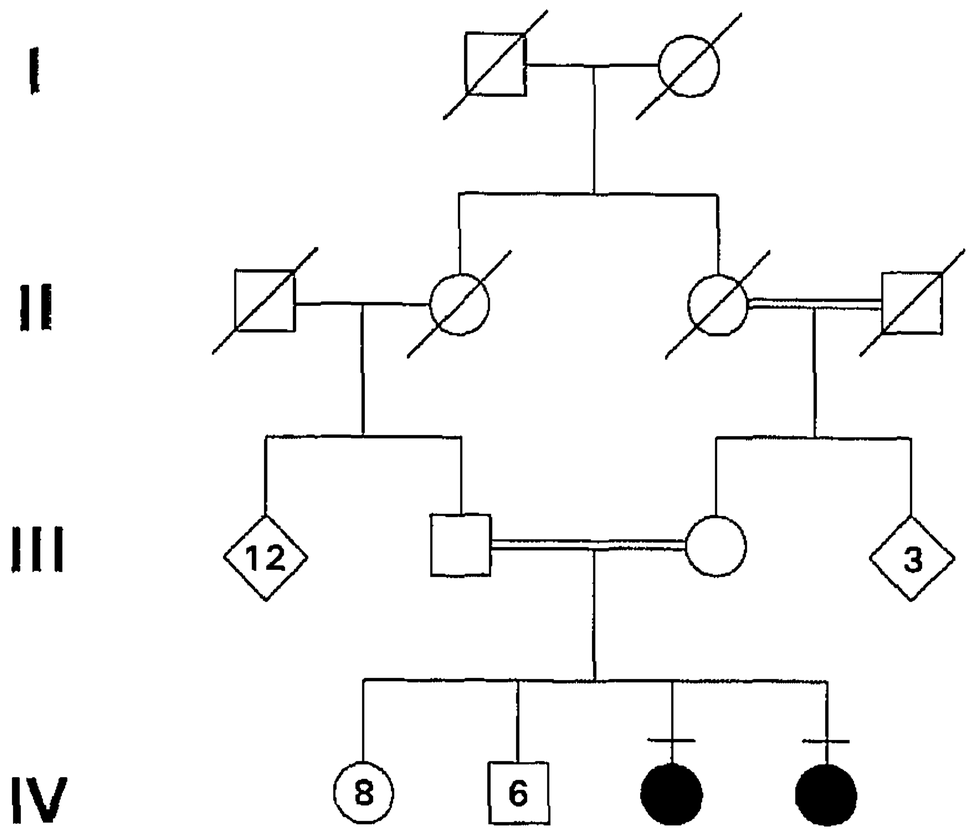

Fig. 1 Pedigree of two siblings with male pseudohermaphroditism and Leydig cell hypoplasia.

and there are 14 additional siblings (Fig. 1). Both cases had a short blind ending vagina, without uterus or fallopian tubes. Serum levels of testosterone and testosterone precursors were abnormally low, and did not respond to stimulation by hCG. Basal levels of LH were markedly increased, but follicle-stimulating hormone (FSH) was within the normal range. The gonads were removed, and upon histological examination found to be testes with normal Sertoli cells, but no mature Leydig cells (Fig. 2).

Homozygous mutation in the LH receptor gene

The LH receptor gene of the two patients with male pseudohermaphroditism was studied for the presence of mutations by single strand conformational polymorphism (SSCP) analysis. We began by studying exon 11 which contains the transmembrane and cytoplasmic domains, with two sets of primers (see Methodology). Amplification with primer set 1 produced a fragment showing abnormal migration. This fragment comprises the third extracellular loop and the seventh transmembrane segment, as well as parts of the sixth transmembrane segment and the intracellular tail.

This part of the LH receptor gene was then sequenced in the two affected siblings and in two unaffected controls with primers 1a and 1b as sequencing primers. A GCC to CCC transversion was detected at position 1787 in the patients (Fig. 3*a*), producing an Ala593Pro substitution in the sixth transmembrane domain (Fig. 3*b*). The normal sequence could not be detected, indicating homozygosity of the mutated allele. The presence of two copies of the LH receptor gene was confirmed by dosage analysis (data not shown).

The mutation inactivates signal transduction

The Ala593Pro mutation was constructed *in vitro*, and the resulting mutant LH receptor cDNA transiently expressed in human embryonic kidney 293 cells. The high affinity (mean $K_D = 0.8 \pm 0.15$ nM; n=2) binding of [^{125}I]-labelled choriogonadotropin (hCG) to partially purified membrane preparations was no different from binding to membrane preparations from cells transfected with the wild type LH receptor cDNA (mean $K_D = 0.5 \pm 0.4$ nM; n=2) (Fig. 4). The difference in the values of B_{max} of the different membrane preparations is partly due to experimental variation in transfection efficiency (five-fold less for the mutant LH receptor construct), and partly to reduced expression of the mutant receptor at the cell membrane. The number of mutant or wild-type receptors expressed per transfected cell varied between 1,000 and 3,000, which is similar to the figure found *in vivo*[14]. In contrast, when hCG-induced cAMP production was determined, no hormonal effect was detected for the mutated LH receptor, even at very high (1000 ng/ml) hCG concentrations (Fig. 4). These results indicate that the missense Ala593Pro mutation completely abolishes signal transduction, probably at the level of coupling to the

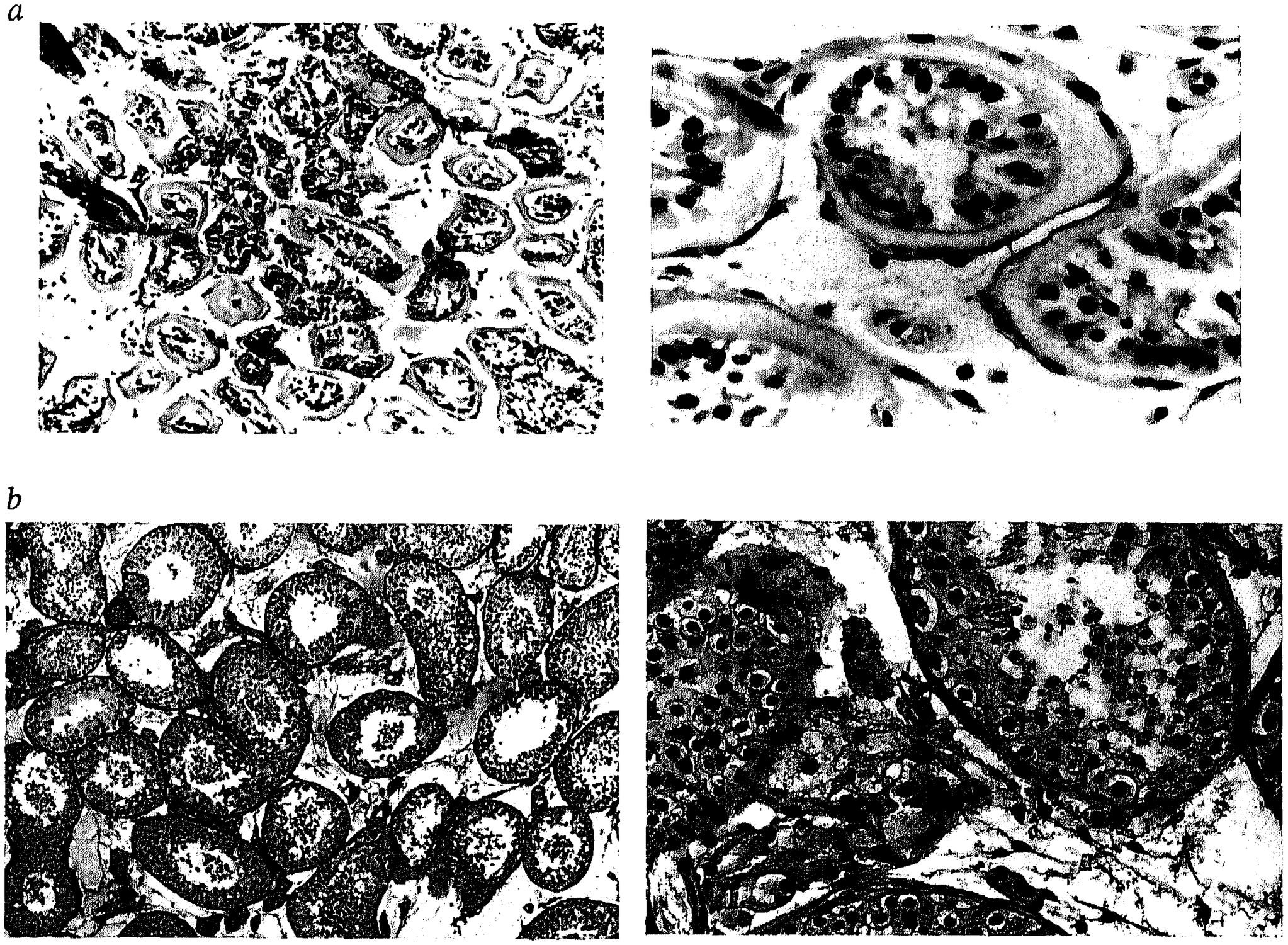

Fig. 2 *a*, Light photomicrographs of seminiferous tubules and interstitium of one of the patients showing hyalinization of the basal membrane and normal peritubular myoid cells. In the interstitium few Leydig cells, with immature characteristics, are found. The seminiferous epithelium contains Sertoli cells, and almost no germ cells. *b*, light photomicrographs of seminiferous tubules and interstitium of control testis with fully developed interstitium and spermatogenic epithelium. (Left, 100X; right, 400X. H/E staining).

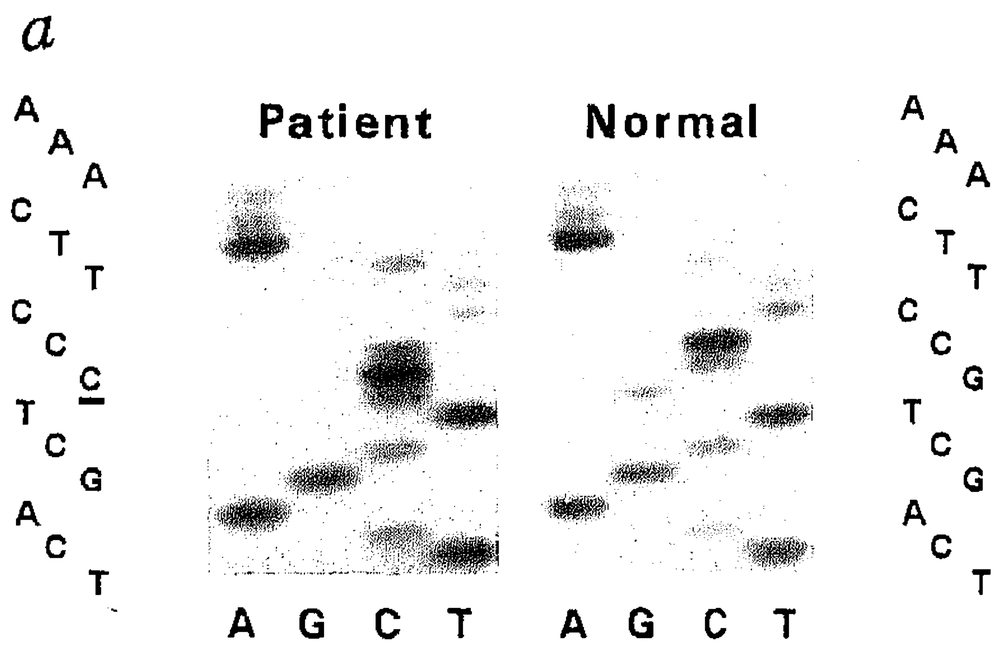

Fig. 3 LH receptor mutation in patients with pseudohermaphroditism. *a*, DNA Sequence of case 1. A G to C homozygous transversion is found at nucleotide 1787, compared to the normal control. *b*, Localization of the Ala593Pro mutation in the sixth transmembrane domain of the LH receptor in the two siblings with male pseudohermaphroditism. The localization of two missense mutations in families with male-limited precocious puberty[15,16] are shown for comparison. The right hand panel shows the overall structure of the LH receptor with seven transmembrane segments, separating three intracellular and three extracellular loops.

stimulatory GTP-binding protein G_s, although changes in post-synthesis receptor transport to the plasma membrane cannot be excluded. Ligand binding is less likely to be affected because this function resides in the N-terminal half of the LH receptor molecule[15].

Discussion

A homozygous missense mutation in the sixth transmembrane domain (Ala593Pro) of the LH receptor gene underlies the syndrome of male pseudohermaphroditism due to Leydig cell hypoplasia in our family. When the mutant LH receptor gene was transiently expressed in HEK293 cells, we observed hormone binding to the receptor albeit at lower maximal capacity. No increase in cAMP occured on stimulation with hCG, even at very high hCG concentrations. Therefore, this mutation precludes the normal increase in cAMP in response to LH/hCG, which renders the mutant receptor non-functional.

The finding of an LH receptor gene defect as a cause of inherited Leydig cell hypoplasia is consistent with previous studies of other patients which have indicated reduced LH binding capacity of testicular tissue[5,6] and absence of a testosterone response to LH[5–13]. It is likely that a wide array of mutations of the LH receptor will be found in other families with autosomal recessive forms of male pseudohermaphroditism. Such mutations could conceivably affect LH binding, G protein activation, post-translational modification or post-synthesis transport, or a combination of these processes. In addition, large LH receptor gene deletions without any LH receptor mRNA and protein expression cannot be excluded.

It is likely that milder forms of LH receptor defects exist, in which sufficient residual activity is present to allow partial masculinization of external genitalia. In fact, patients have already been described in whom Leydig cell hypoplasia was associated with hypergonadotropic hypogonadism, and micropenis, but not hypospadias[9,10].

The Ala593Pro mutation abolishes LH receptor function *in vitro* (Fig. 4). This is very different from the effect of missense mutations of the LH receptor gene that have been found in patients with autosomal dominant LH independent male precocious puberty[16,17] (Fig 3*b*; unpublished observations), although they also are located near the sixth transmembrane segment. These latter mutations — Met571Ile and Asp578Gly — act in a dominant fashion by constitutively activating cAMP production[16] (R.K.), and appear to be subtle: They might result in a conformational change that facilitates ligand-independent coupling to the G-protein. The Ala593Pro mutation, by contrast, might introduce a more drastic conformational change in the receptor caused by the constrained angle conferred by the proline residue. Such mutations are more likely to render the receptor inactive.

Many studies indicate that undifferentiated Leydig cells require LH for proliferation and differentiation. In humans, fetal Leydig cell number and testosterone production correlate with plasma hCG levels[18–20]. This is consistent with experimental studies in rats that have shown that full-length LH receptor mRNA is expressed in testis from day 16.5 of gestation onwards, suggesting an active role for LH/hCG-dependent testosterone production in the determination of male external genitalia[21]. After specific removal of differentiated Leydig cells from adult rat testes, immature Leydig cells reappear more quickly if LH is present[22]. Furthermore, LH can induce morphological differentiation of immature to mature Leydig cells in neonatal rats[23], and this differentiation is parallelled by an increase in the activity of the enzymes that are responsible for testicular steroidogenesis[24].

The initiation of androgen synthesis by Leydig cells early in fetal life may be independent of LH or hCG as indicated by data from rats and rabbits[3,25]. Our findings may support this notion, because testes with associated epididymis and vas deferens were found in both patients. These wolffian duct-derived structures can only have been formed in the presence of Leydig cell androgens at some point in fetal development. Our findings further demonstrate that at a later fetal stage the absence of a functional LH receptor interferes with Leydig cell proliferation and maturation. In patients with Leydig

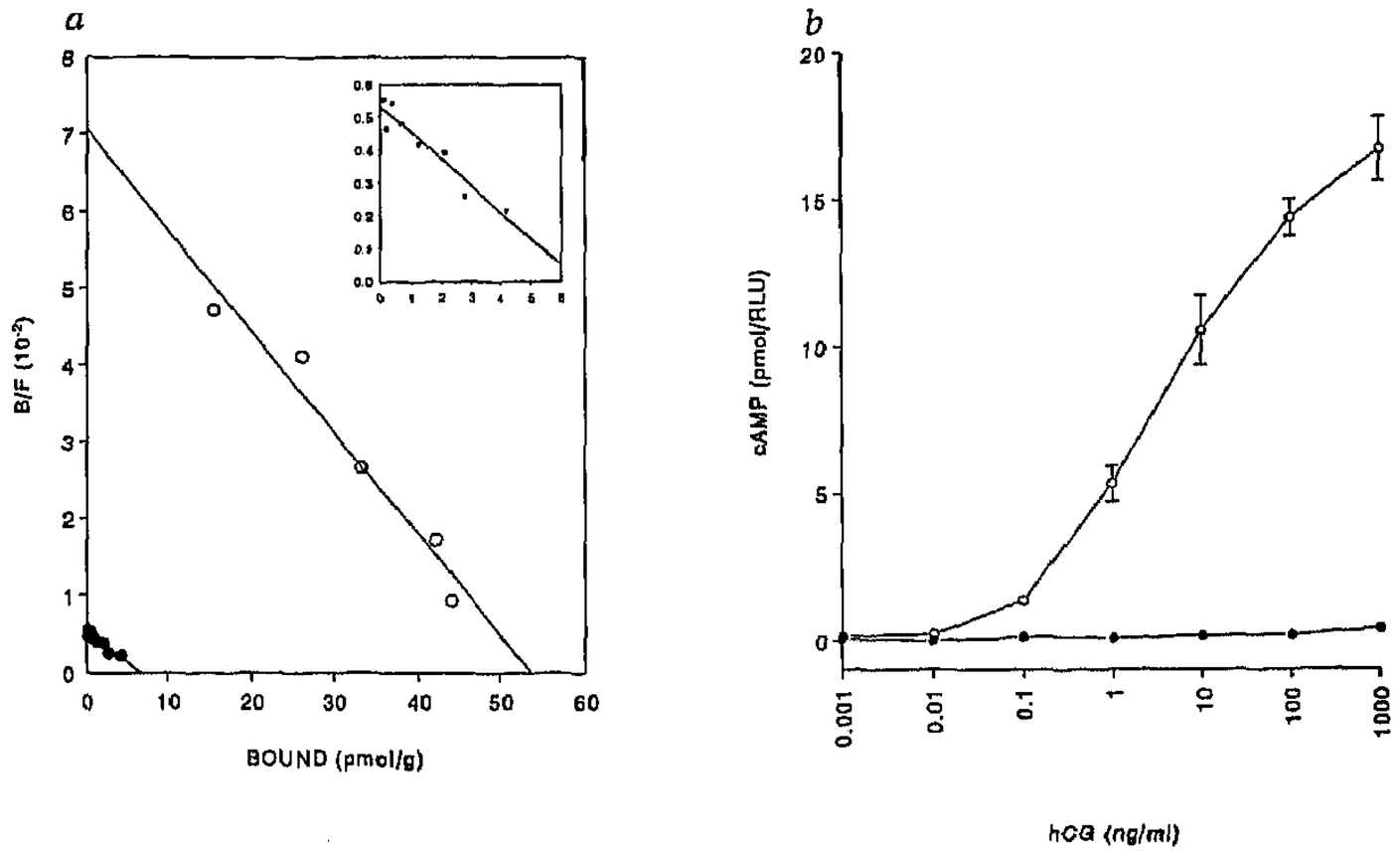

Fig. 4 *In vitro* studies of mutant and wild-type LH receptor gene constructs. *a*, Scatchard plot of the mutant receptor (filled circles; enlarged in inset) shows reduced maximal hormone binding but normal affinity, compared to the wild-type receptor (open circles). *b*, Right-hand panel: hCG-dependent cAMP production in wild-type receptor (open circles) and mutant receptor (closed circles) expressing HEK293 cells. The Ala593Pro mutation (LCH) completely abolishes hCG dependent cAMP production.

cell hypoplasia, the testes contain normal Sertoli cells, with no mature Leydig cells. Sometimes, small clusters of immature Leydig cells are present[7]. The histological findings in Leydig cell hypoplasia can now be interpreted as reflecting lack of stimulation through the LH receptor.

No abnormal female sex characteristics have been noted in sisters of patients with this form of male pseudohermaphroditism[12], which is consistent with experimental results that indicate absence of a functional ovarian LH receptor until after birth[21]. However, LH receptor function is required for a normal female menstrual cycle. Hence, the presence of primary amenorrhea in a 46,XX sister of the patients described above should be of particular interest. (As this patient lives approximately 4,000 kilometres from our centre in rural Brazil, we have not yet been able to contact the patient and obtain a blood sample.) Further studies should reveal if she is indeed homozygous for the same LH receptor missense mutation. Primary amenorrhea with ovarian resistance to luteinizing hormone might conceivably be the female counterpart to the pattern of male pseudohermaphroditism studied here. Amenorrhea has been noted in another sister of a patient with pseudohermaphroditism due to Leydig cell hypoplasia[13].

In conclusion, the finding of an LH receptor defect in male pseudohermaphroditism with testicular Leydig cell hypoplasia provides a biological basis for this disorder. Our data support a crucial role for the LH receptor in Leydig cell differentiation, and hence in the regulation of embryonal production of testosterone by the male gonad.

Methodology

Patients. The patients are two siblings born to consanguineous first cousin parents. The patients originate from a small village in rural north/northeast Brazil (Piaui State). They were referred at ages 37 and 42, because of primary amenorrhea and lack of breast development. Both cases had a eunuchoid habitus, absence of breast tissue, and sparse axillary hair. The external genitalia were female with distinct urethral and vaginal orifices, and triangle-shaped pubic hair. Pelvic ultrasound examination showed no internal genitalia, except for the testes which were located in the inguinal region. A genitogram demonstrated only the vertical component of the urethra and a short blind ending vaginal pouch. The karyotype was 46,XY. Baseline testosterone levels were low (0.69 and 0.53 nmol l^{-1} respectively; normal 8.3–35.8 nmol l^{-1}). Testosterone precursors such as DHEA, 17α-hydroxyprogesterone, progesterone, androstenedione, and estradiol were low compared to normal males. LH levels were elevated to 23.3 and 19.0 IU l^{-1}; normal 1-8.4 IU l^{-1}). FSH and inhibin levels were normal. A stimulation test with hCG (6000 IU i.m.) elicited no increase in testosterone levels. The LHRH test (100 μg IV) elicited a LH hyperresponse, and normal FSH increase. An acute adrenal cortex stimulation test using 1-24 synthetic ACTH (Cortrosyn, Organon, Oss, the Netherlands) did not reveal defects in enzymatic steps leading to adrenal androgen synthesis. The patients underwent bilateral gonadectomy. Testes were removed with normal vasa deferens and epididymides. Histopathological examination revealed seminiferous tubules with thickened membranes, few germ cells and a normal number of Sertoli cells. The interstitial space contained vascular conjunctive stroma and fibroblasts with absence of Leydig cells.

Mutation analysis. Genomic DNA was isolated from peripheral blood as described[26]. Because other family members live approximately 4,000 kilometres from Sao Paulo, only the index cases were studied.

DNA fragments were amplified with PCR and analysed by SSCP analysis[17] with the following adaptations for primer set 1 (see below): 1 mM $MgCl_2$ in the PCR and an annealing temperature of 58 °C. Two different sets of primers were used in the PCR: (1a) 5'–CGATTTCACCTGCATGGC–3' (nt 1731–1748), (1b) 5'–CCCGACGTTTACAGCAGCC–3' (nt 1924–1942); (2a) 5'–TATCCCATCAATTCTTGTGCC–3' (nt 1834–1854), (2b) 5'–GGATTGAGAAGGCTTATTTGATCC–3' (nt 2005–2028). Nucleotide numbers are according to Minegishi *et al.*[27]

For sequencing, the DNA fragments were separated on a 1.5% LMP agarose gel BRL Life Technologies and purified with the gelase system (Epicentre Technologies) according to the "fast protocol" given by the manufacturer. About 50 fmol of the purified DNA fragments and the 1a and 1b primers were used for sequencing with the cycle sequencing kit of BRL (BRL Life technologies).

***In vitro* expression.** The wild type human LH receptor expression plasmid (pSG5-hLHR) was constructed by cloning the human LH receptor cDNA (nucleotides –3 to 2374 relative to the translation start site) into the *Eco*RI site of pSG5, an expression vector that contains the SV40 large T early promoter, intron II of the rabbit β-globin gene, and an SV40 polyadenylation signal[24]. The protein encoded by this cDNA differs from the published human LH receptor[27] by the insertion of two amino acids (Leu and Gln) at positions 20 and 21 (E.M., unpublished observations). The Ala593Pro mutation was constructed in pSG5-hLHR by site-directed mutagenesis using PCR[29], yielding pSG5-hLHR4. Mutagenesis was confirmed by sequence analysis, and revealed an additional nucleotide change (C to A) at position 1983 in the pSG5-hLHR4 vector. This change does not alter the amino acid encoded by this codon. Human embryonic kidney 293 (HEK293) cells were transiently transfected as described before[30]. 48 h after transfection, [^{125}I]-hCG (Dupont) binding to partially purified membranes was determined according to Ketelslegers & Catt[31]. For the estimation of hCG-dependent cAMP production, HEK293 cells were co-transfected in 12 well tissue culture plates (Costar, Cambridge, MA, USA) with a luciferase expression plasmid pRSVluc[32] and pSG5, pSG5-hLHR or pSG5-hLHR4. 48 h after transfection cells were incubated for 2 h with culture medium containing 0.1% BSA and 0.2 mM isobutyl-methylxanthine (Sigma) and different concentrations of hCG (urinary

hCG; Organon International, Oss, The Netherlands). Medium was collected and cAMP production determined as described before[33,34]. The remaining cells were lysed, and luciferase activity was determined[35]. The final cAMP production in the transfected cells was determined by subtraction by the basal cAMP production in pSG5-transfected cells, and subsequent correction for transfection efficiency using the luciferase activities of the same wells.

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