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REVIEW

Luteinizing hormone receptor mutations and sex differentiation

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Mutations in the luteinizing hormone (LH) receptor gene have been found in patients with abnormalities in their sexual differentiation. In this paper we review results obtained in the studies of these LH receptor mutations. Activating and inactivating mutations are discussed with respect to the mechanism of action of LH/human chorionic gonadotrophin but also in light of their impact of the present knowledge of the physiology of sex differentiation and gonadal function.

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In the process of sex differentiation two major switches can be distinguished. First, expression of the SRY (sex-determining region Y) gene on the Y chromosome is responsible for induction of testis formation from the indifferent gonads (1, 2), a process that also requires other autosomal or X chromosomal genes. Subsequently, the differentiating testicular cells establish hormonal control of further development of the urogenital tract and sinus. The Sertoli cells of the fetal testis secrete anti-Müllerian hormone (AMH), a member of the transforming growth factor-β (TGF-β)/activin family of growth and differentiation factors (3). Anti-Müllerian hormone binds to an AMH receptor that is present in the mesenchymal cells surrounding the female anlagen of the urogenital tract, the Müllerian ducts (4, 5). The action of AMH results in regression in the Müllerian ducts in the male. Development of male internal and external genitalia is dependent on the Leydig cell product testosterone, which in some target tissues is converted to dihydrotestosterone (6). The Wolffian ducts, the anlagen of the male urogenital tract, develop into the epididymides, vasa deferentia and seminal vesicles. The other male internal and external genitalia are also formed under the influence of androgens: the prostate, penis and scrotum.

The obligatory role of androgens in sex differentiation is exemplified by the female external phenotype of 46,XY individuals that are insensitive to androgens because of mutations in their androgens receptor gene (7).

Leydig cells

Androgens are produced by a single cell type in the testis, the Leydig cells (for reviews see Refs. 8 and 9). Leydig cells develop from mesonephric cells that migrate into the gonads during the period of formation of the bipotential gonads (10). Human fetal Leydig cells initially differentiate and proliferate under the influence of hCG (11). Subsequently, after the establishment of the pituitary–gonadal axis, LH comes into play. Fetal Leydig cell activity is responsible for virilization of the male internal and external genitalia. Postnatally and in childhood, LH levels are low. During puberty rising LH levels again stimulate Leydig cell to start producing androgens, which results in the establishment of the adult male secondary sexual characteristics.

Both hCG and LH signal through the LH receptor. The LH receptor belongs to the class of GTP binding protein (G protein)-coupled receptor that display specific features such as a domain of seven transmembrane helices (12, 13). The LH receptor together with the receptors for TSH and FSH are characterized by an additional large extracellular domain that contains 14 leucine-rich repeats and is responsible for ligand binding (14–17). The subgroup of G protein-coupled receptors transduces the signal of the hormone mainly to adenylyl cyclase through the stimulatory G protein G_s. In addition, TSH and LH have been shown to activate the phosphatidylinositide pathway, indicating involvement of G_q (15, 18). Either LH- or hCG-stimulated adenylyl cyclase results in an increase of the cellular cAMP level, activation of cAMP-dependent protein kinase and phosphorylation of several proteins. Recently, an interesting substrate for phosphorylation was detected in steroidogenic cell types. The protein, steroidogenic acute regulatory protein (StAR), is a mitochondrial protein with a short half-life (19) that is involved in the transport of cholesterol through the inner mitochondrial membrane. Mutations in STAR

that inactivate the protein also cause congenital lipoid adrenal hyperplasia (20), which indicates the importance of STAR in steroidogenesis. The STAR-activated transport of cholesterol results in increased availability of the lipid to the Leydig cell mitochondrial cytochrome P-450 cholesterol side-chain cleavage enzyme that converts cholesterol into pregnenolone. Pregnenolone subsequently diffuses out of the mitochondria to the smooth endoplasmic reticulum where it is converted to testosterone.

Luteinizing hormone receptor expression studies have been mainly performed in the rat and mouse. Leydig cell expression of LH receptor mRNA can be found as early as at 15 days post coitum in the rat (21). However, the alternatively spliced mRNA species expressed in these early Leydig cells do not encode the complete receptor protein molecule, and produce truncated LH receptors that are inactive because they cannot couple to G_s. Messenger RNA molecules that encode a functional full-length LH receptor are expressed approximately 1 day later (21); in the ovary this transition takes more than 2 weeks (22). The physiological significance of these alternative splicing products is unclear, although it has been suggested that the alternative splicing of gene products is a by-product of an alternative way of regulation of LH receptor gene expression (21, 22). Fetal testicular LH receptor mRNA expression has not been documented in humans. However, binding of hCG to fetal testicular tissue and the correlated hCG and testosterone levels during fetal life suggest that functional LH receptors are expressed in Leydig cells at an early fetal age of 9 weeks (11, 23, 24).

Disorders of sex differentiation

Many disorders of male sex differentiation have been described, all of which are due to either insufficient availability of testosterone (androgens) or the lack of response of the target tissues to this hormone. Mutations in the androgen receptor gene lead to androgen insensitivity of target tissues, which causes partial or complete male pseudohermaphroditism (25). Before puberty a similar external phenotype is initially found in cases with deficiency of the enzyme 5α-reductase type 2 that converts testosterone to the more active metabolite dihydrotestosterone (6), although at puberty the testes start to secrete high amounts of testosterone, which sometimes results in extensive virilization. Also, changes in the activity of steroidogenic enzymes in the testosterone synthesis pathway, such as C17-20-lyase/17α-hydroxylase or 3β-hydroxysteroid dehydrogenase, affect male sex differentiation. Because these enzyme dysfunctions also manifest themselves in the adrenal gland, they also cause adrenal hyperplasia (26).

In two familial disorders—familial male-limited precocious puberty and Leydig cell hypoplasia—Leydig cell function is altered without changes in steroidogenesis in other organs such as the adrenal gland. In both syndromes, LH receptor function has been shown to be modified. In the following sections, mutational changes in the LH receptor in these two syndromes and their consequences for Leydig cell function will be dicussed.

Familial male-limited precocious puberty

Familial male-limited precocious puberty (FMPP), also named testotoxicosis, has been described initially in 1981 (27). This familial form of precocious puberty is characterized by an excessive secretion of testosterone by Leydig cells in the absence of elevated levels of pituitary gonadotrophins (28, 29). It is different from true (central) precocious puberty, which involves premature activation of the hypothalamic GnRH pulse generator (30). In FMPP, excessive androgen production occurs probably also in the fetus, because enlargement of the external genitalia has been noted at birth in some cases (31). A circulating testis-stimulating factor that activates only human Leydig cells, but not rodent Leydig cells, has been suggested to be the cause of FMPP (32), but this report was not confirmed. The increased testosterone production might also be the result of an inherent change in the Leydig cells themselves, and therefore the LH receptor gene of these patients was investigated.

Mutations in the LH receptor could change the behaviour of the receptor in such a manner that it would signal continuously through G_s to the adenylyl cyclase system, activating the Leydig cells without the ligand being present. In their investigations of the α_1-adrenergic receptor, Lefkowitz and co-workers (33) had shown that changing an alanine residue in the third intracellular loop of this receptor to any other amino acid residue resulted in different levels of constitutive activation of the phosphatidylinositide pathway without ligand being present. Furthermore in mice that dominantly express a coat-colour trait, an MSH receptor gene that consititutively activates adenylyl cyclase was described (34).

Familial diseases as a result of such constitutively activated G protein–coupled receptors had not been described at that time, but on the basis of these reports, DNA analysis of the LH receptor gene was undertaken with emphasis on the third intracellular loop and the sixth transmembrane segment. Two mutations were reported, methionine at position 571 to isoleucine (35) and aspartate at position 578 to glycine (35, 36). When transfected into COS or HEK293 cell lines, the cells that expressed the mutant receptor species indeed showed an increased basal cAMP level when compared to cells expressing the wild-type receptor (Fig. 1).

When hCG was added to the transfected cells, a dose–response relationship was found with an ED_{50} which is very similar to the ED_{50} of the wild-type receptor. To

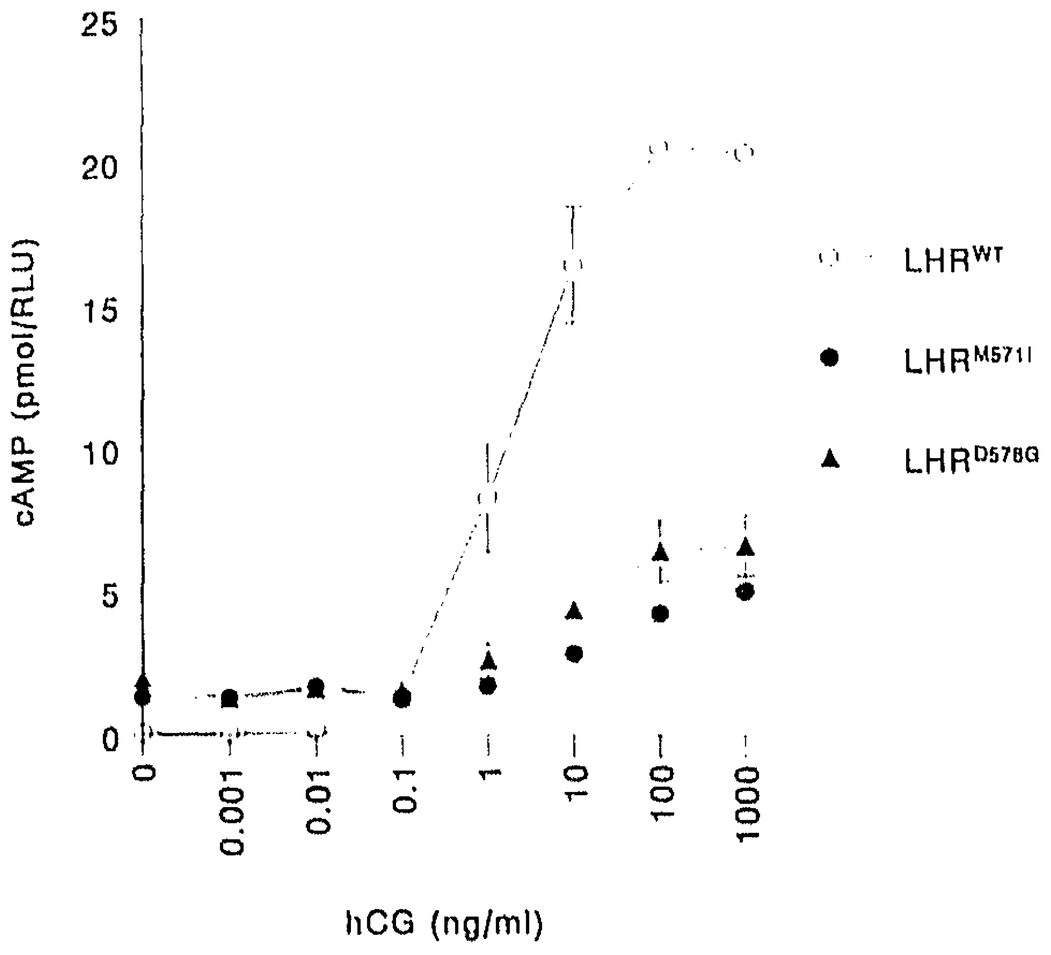

Fig. 1. Mutation in patients with familial male-limited precocious puberty causes constitutive LH receptor activity. Human chorionic gonadotrophin (hCG)-dependent cAMP production in cells expressing the wild-type or mutant (methionine[571] to isoleucine or aspartate[578] to glycine) LH receptor. Mutant receptors show an increased basal cAMP production but a decreased maximal response to hCG. (Figure taken from Ref. 54.)

date, eleven different LH receptor mutations in FMPP have been described (Fig. 2). Most mutations are found in the sixth transmembrane segment and flanking third intracellular loop. However, two mutations (methionine[398] to threonine and isoleucine[542] to leucine) were found in other transmembrane segments, the second and fifth, respectively. These findings indicate that the sixth transmembrane segment and the third intracellular loop are major determinants of G protein coupling in the LH receptor, but that the second and fifth transmembrane segments are also involved. Data obtained with other G protein-coupled receptors implicate similar receptor regions in G protein coupling (see Refs. 37 and 38). Careful examination of the amino acid changes found in FMPP patients reveals that although some alterations involve a major change of amino acid type, such as aspartate[578] to glycine or tyrosine, or cysteine[581] to arginine, other amino acid changes such as alanine[568] to valine or isoleucine [575] to leucine, do not. It seems that any change of several amino acid residues may result in a constitutively active receptor. The receptor molecule appears to be finely tuned, and can be thought to be activated similar to a spring or mouse-trap when either the hormone binds or an amino acid is changed. The amino acid residues that are changed in FMPP patients are obvious candidates in future investigations of the structure–function relationship of the LH receptor during activation by LH or hCG.

It would appear that the LH receptor mutations found in FMPP patients are indeed the cause of their precocious puberty, but it is difficult to obtain formal proof. One of the experiments that could provide such proof would be a transgenic mouse with a targeted activating LH receptor gene mutation. In another approach, we have chosen to use a mouse Leydig cell line, MA-10 cells (39), to test the mutations that were found in FMPP patients. The MA-10 cells were transfected with wild-type and mutated LH receptor cDNA constructs, and basal and maximally hCG-stimulated pregnenolone production was determined. Because MA-10 cells carry the mouse wild-type LH receptor, the transfected cells partially mimic the situation in Leydig cells of an FMPP patient, where also both wild-type and mutated alleles are expressed. Figure 3 shows that mutated LH receptors have a

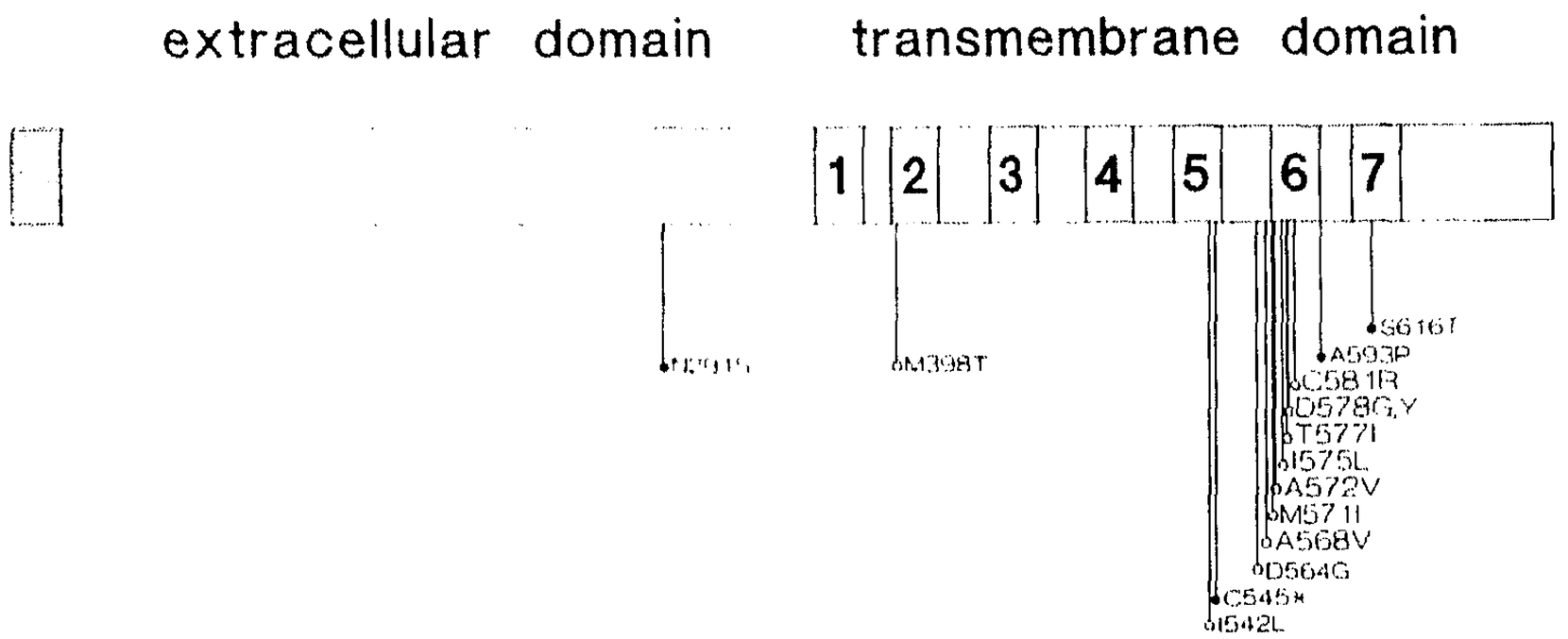

Fig. 2. Overview of all LH receptor mutations known to date. The horizontal bar indicates the LH receptor protein with its domains. The striped segment at the N-terminus indicates the signal sequence, and the numbered speckled areas indicate the seven different transmembrane α-helices that constitute the transmembrane domain. Mutations in patients with familial male-limited precocious puberty are indicated by the open circle at the end of the line, and Leydig cell hypoplasia mutations by a closed circle. The numbering of the mutations is according to Minegishi et al. (16). The mutations are taken from the following references: N291S, Refs. 50; M398T, Refs. 54 and 55; I542L, Ref. 55; C545X, Ref. 56; D564G, Ref. 55; A568V, Ref. 57; M571I, Refs. 35 and 54; A572V, Ref. 58; I575L, Ref. 59; T577I, Ref. 60; D578G, Refs. 35, 36 and 54; D578Y, Ref. 55; C581R, Ref. 55; A593P, Ref. 48; S616Y, Ref. 50.

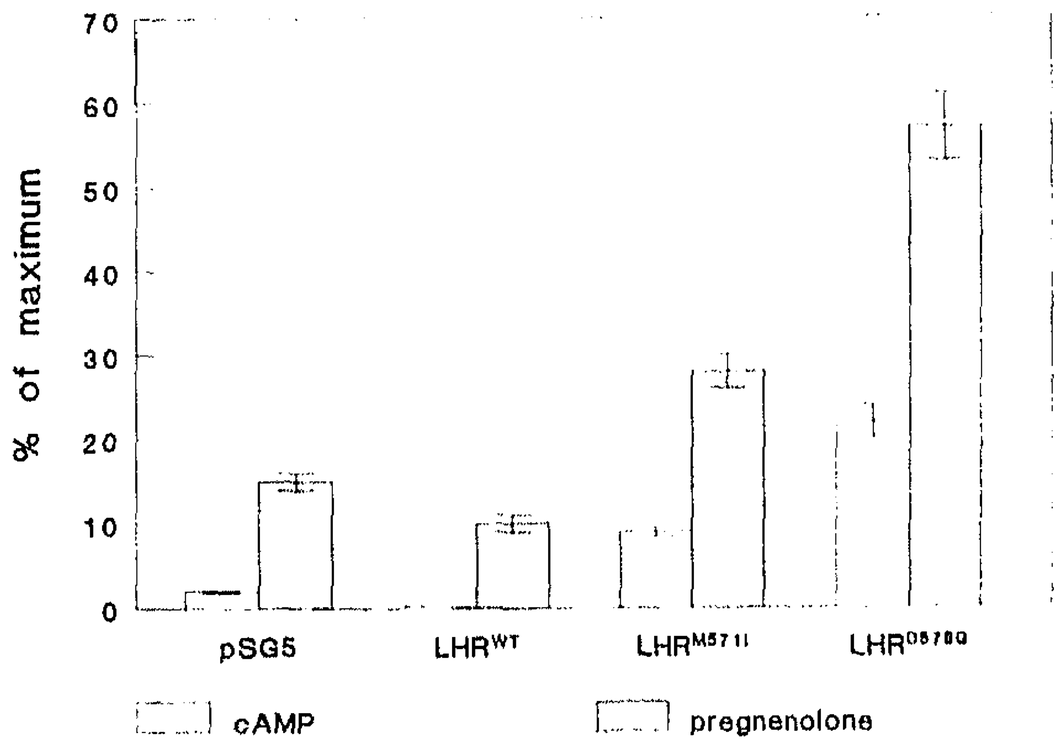

Fig. 3. Effect of mutant familial male-limited precocious puberty LH receptor expression on basal cAMP and pregnenolone production by mouse tumour Leydig cells (MA10). Cells transfected with wild-type and mutant LH receptors were incubated in the absence or presence of a maximally stimulating dose of hCG. Basal cAMP or pregnenolone production is expressed as a percentage of the maximal response to hCG. Both cAMP and pregnenolone productions are increased in cells transfected with mutant receptor, in the absence of hCG or LH.

dominant phenotype; cells transfected with a constitutively active LH receptor show a higher cAMP level and, more importantly for the FMPP phenotype, also a higher steroid production. These results demonstrate that in Leydig cells of FMPP patients the increased basal cAMP level in the absence of hormone will indeed result in increased testosterone production, and subsequently in precocious puberty.

Leydig cell hypoplasia

The clinical mirror-image of FMPP is the Leydig cell hypoplasia syndrome (LCH). Patients can present with a relatively mild undervirilization of the external genitalia, or with a more severe form with complete male pseudohermaphroditism (40–47). The testes of the patients show low numbers of Leydig cells in the mildly affected patients, to a complete absence of adult Leydig cells in the pseudo-hermaphrodites. The syndrome of Leydig cell hypoplasia is an autosomal recessive condition. In some studies testicular LH binding was absent, which could either be the cause or the consequence of the absence of Leydig cells (47). In analogy with the studies on FMPP, the LH receptor gene in LCH patients was investigated (48, 49). In contrast with the LH receptor gene in FMPP patients, it can be expected that DNA changes the result in a loss of function of the LH receptor gene will be found anywhere in the gene and may range from amino acid changes, to shifted reading frames or deletions of part of the gene. Two groups have reported on LH receptor gene mutations in LCH patients. In one of these papers, two 46,XY sisters born from consanguineous parents were found to be homozygous for a missense mutation that resulted in the change of alanine at position 593, located just outside the sixth transmembrane segment, into a proline residue (Figure 4)(48). When expressed in transfected cells, this mutated LH receptor was found to bind hCG with a normal K_d, although the receptor was expressed at a much lower level than the wild-type receptor. However, the proline residue changed the LH receptor in such a way that, in spite of the high-affinity hCG binding, no couping to adenylyl cyclase could be demonstrated in the absence or presence of hCG (Fig. 4). In another patient the situation was more complex (49). One allele of the LH receptor gene was found to contain a nonsense mutation resulting in the introduction of a stop codon at position 545 in the fifth transmembrane segment of the transmembrane domain. Similar to the alanine^{593} to proline mutation, in vitro expression of this truncated receptor showed diminished binding with the same affinity compared to the wild-type LH receptor, and no coupling to adenylyl cyclase. The other LH receptor allele did not show the same mutation, suggesting that the patients were compound heterozygotes. The identity of the mutation in this LH receptor allele has not been established yet (49). Two additional mutations (asparagine^{291} to serine and serine ^{616} to tyrosine) have been published recently in an abstract (50). The mutation at position 291 lies in the extracellular domain, and this receptor molecule may be expected to have lost ligand binding. This mutation has not been tested in vitro, however. The serine^{616} to tyrosine mutation showed similar behaviour in vitro to the LH receptors carrying an alanine^{593} to proline mutation or a stop codon at position 545.

The mutations described above were found in severe cases of LCH. In patients that are affected more mildly, amino acid changes may be found that do not abolish receptor function completely, but rather may result in decreased activity such as diminished LH binding affinity or less-efficient G protein coupling. Milder mutations may allow correlations between the properties of the amino acid residues that have changed and the severity of the LCH syndrome, and hopefully will give directions toward the further functional characterization of the receptor subdomains, such as the domains involved in the regulation of receptor transport from the endoplasmic reticulum to the plasma membrane or glycosylation of the extracellular ligand binding domain.

Concluding remarks

Although identification of LH receptor gene mutations has just started, a distinct pattern of mutations appears to evolve. Fourteen mutations that have been published are located in the transmembrane domain of the LH receptor; only one mutation (asparagine^{291} to serine) changes the extracellular LH binding domain, although the effect of this mutation on LH receptor function was

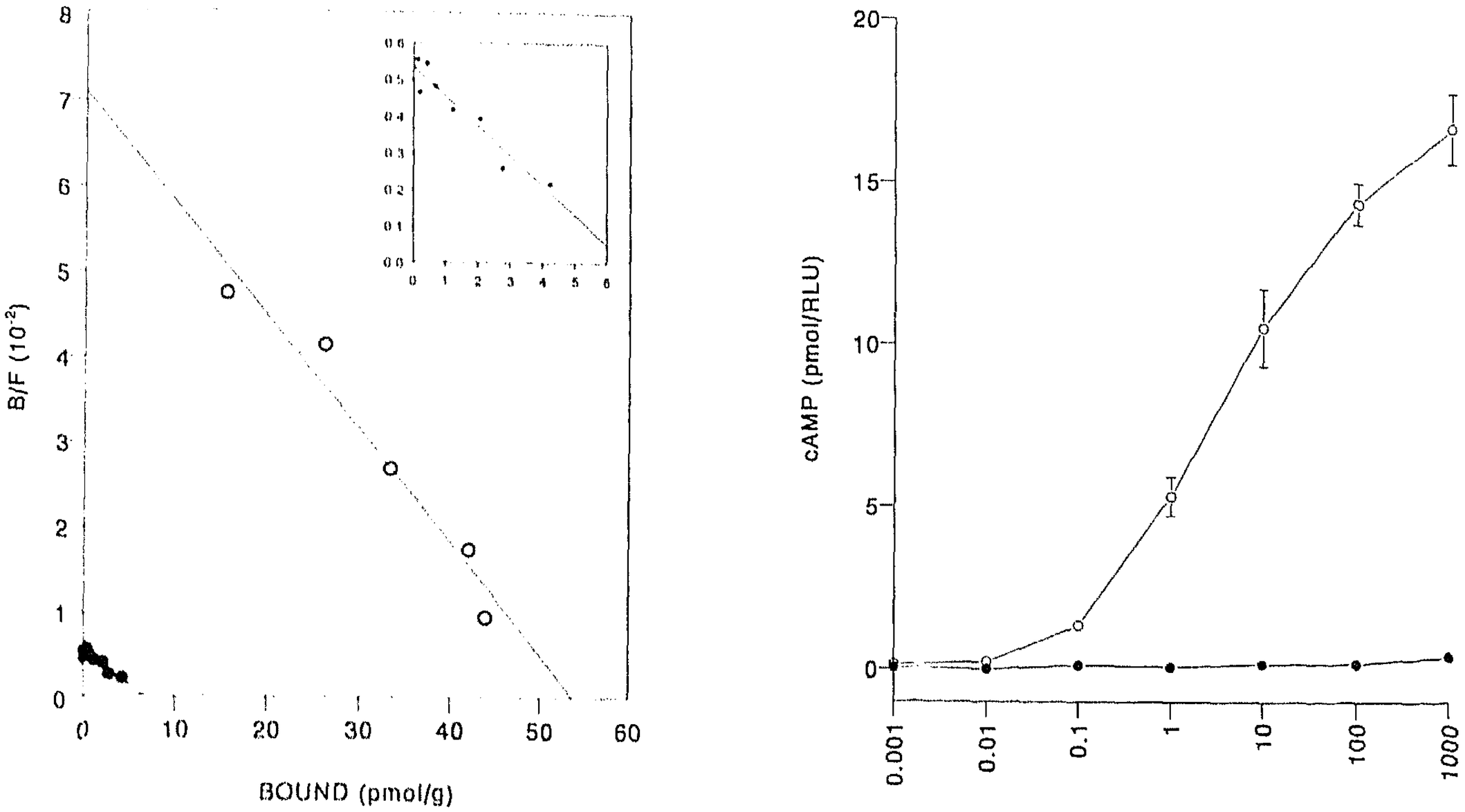

Fig. 4. In vitro studies of mutant Leydig cell hypoplasia and wild-type LH receptor gene constructs. Left: Scatchard plot of the mutant (alanine^{593} to proline) LH receptor (closed circles; enlarged in inset) shows reduced maximal hormone binding but normal affinity, compared to the wild-type receptor (open circles). *Right:* hCG-dependent cAMP production in wild-type receptor (open circles) and mutant receptor (closed circles) expressing HEK293 cells. The alanine^{593} to proline mutation (Leydig cell hypoplasia) completely abolishes hCG-dependent cAMP production. (Figure taken from Ref. 48 with permission of *Nature Genetics*.)

not tested in vitro. However, this overrepresentation of the transmembrane domain should not be emphasized too much because this region was the domain that was first subjected to DNA analysis. Recently, the intron–exon structure of the other exons of the large human LH receptor gene has been determined, allowing the study of the other exons of the LH receptor gene (51). Most LH receptor mutations found in FMPP patients are located in the third intracellular loop or in the sixth transmembrane segment (Fig. 2). This finding underscores the importance of this receptor region in G protein coupling, as was already suggested from studies using site-directed mutagenesis (see Ref. 52). However, it is of interest that the two mutations that were identified in the LCH patients both lie in the last exon that encodes the transmembrane domain. It remains to be established by the identification of other LCH LH receptor gene mutations whether a selective pressure exists against mutations in the other exons that encode the extracellular LH/hCG binding domain.

Now that the molecular basis of the syndromes of FMPP and LCH has been established, conclusions can be drawn about the relative importance of LH/hCG during sexual differentiation of the male and the role of androgens in spermatogenesis. In the LCH patients with the alanine^{593} to proline mutation the absence of LH/hCG stimulation of Leydig cells did not lead to a complete absence of androgens. The presence of the androgen-dependent epididymides and vasa deferentia associated with the testes of these LCH patients indicates that there is autonomous (LH/hCG-independent) androgen production by Leydig cells during fetal male sex differentiation. Probably this autonomous androgen production is relatively low, and only effective at a small distance from the developing testis, as indicated by the absence of other androgen–dependent organs such as the prostate or seminal vesicles. Epididymides and vasa deferentia are not found in patients that suffer from complete androgen insensitivity and do not have a functional androgen receptor gene (25). Recently, differentiation of epididymides and vasa deferentia was described in a mouse line without a functional glycoprotein α-subunit gene (53). Because mice do not have a hCG homologue, this androgen-induced diferentiation must have occurred independently of LH.

The high androgen production in FMPP patients that causes the very early precocious puberty does not seem to have adverse effects in adult life. Patients with FMPP

have the short stature associated with precocious puberty but abnormalities such as Leydig cell neoplasia or infertility have not been reported, with the exception of one individual in a large pedigree who was reported to be infertile (28). Postpubertal LH-stimulated androgen levels are much higher than the androgen productions in the FMPP boys, and may explain the absence of effects of the activating LH receptor mutations in adult life.

The female carriers of an FMPP LH receptor gene mutation, which in men leads to precocious puberty, seem to be without symptoms. In the light of the precise regulation of the menstrual cycle in women, this seems puzzling. Although no studies of female carriers of FMMP LH receptor mutations have been reported, expression of constitutively active LH receptors in preovulatory follicles might lead to premature ovulations. However, there is no indication of impaired fertility in the female carriers. Possibly, at the time of ovulation the follicular LH receptors have to be stimulated to a high degree to induce ovulation. As can be seen readily from the hCG dose–response curves depicted in Fig. 1, the basal cAMP level is increased in cells expressing a constitutively active receptor, but an increase of the hormone concentrations leads to further enhancement of cAMP production, thus allowing granulosa cells of female FMPP carriers to respond normally to the ovulatory LH peak.

Inactivating LH receptor mutations has no effect on female sex characteristics because the LH receptor is not expressed in ovaries before birth (21). However, a 46, XX sister in the family described by Kremer et al. (48) was found to be homozygous for the same inactivating LH receptor gene mutation, and presents with primary amenorrhoea, but has normally developed sex characteristics (Dr S Toledo, pers. comm.) (48). This is consistent with the requirement for an active LH signal transduction system for ovulation to occur. In this respect it is of interest to note that recently an inactivating mutation in the FSH receptor gene has been found in women with ovarian dysgenesis. In these women, the inability of the ovaries to respond to FSH results in the absence of follicle growth, low gonadal hormone production and variably developed secondary sex characteristics in the presence of high levels of FSH and LH. It is tempting to speculate that other patients with female infertility may also be carriers of more or less severe LH or FSH receptor gene mutations. A combination of two mutations with a mild phenotype (two alleles) or of one mutation with a severe phenotype (one allele) could lead to a decreased LH or FSH receptor response and disturbance of the precise timing that is necessary for ovulation of the preovulatory follicle.

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