A Homozygous Mutation in the Luteinizing Hormone Receptor Causes Partial Leydig Cell Hypoplasia: Correlation between Receptor Activity and Phenotype

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Leydig cell hypoplasia (LCH) is characterized by a decreased response of the Leydig cells to LH. As a result, patients with this syndrome display aberrant male development ranging from complete pseudohermaphroditism to males with micropenis but with otherwise normal sex characteristics. We have evaluated three brothers with a mild form of LCH. Analysis of their LH receptor (LHR) gene revealed a homozygous missense mutation resulting in a substitution of a lysine residue for a isoleucine residue at position 625 of the receptor. *In vitro* analysis of this mutant LHR, LHR(I625K), in HEK293 cells indicated that the signaling efficiency was significantly impaired, which explains the partial phenotype. We have compared this mutant LHR to two other mutant LHRs, LHR(A593P) and LHR(S616Y), identified in a complete and partial LCH patient, respectively. Although the ligand-binding affinity for all three mutant receptors was normal, the hormonal response of LHR(A593P) was completely absent and that of LHR(S616Y) and LHR(I625K) was severely impaired. Low cell surface expression explained the reduced response of LHR(S616Y), while for LHR(I625K) this diminished response was due to a combination of low cell surface expression and decreased coupling efficiency. For LHR(A593P), the absence of a reduced response resulted from both poor cell surface expression and a complete deficiency in coupling. Our experiments further show a clear correlation between the severity of the clinical phenotype of patients and overall receptor signal capacity, which is a combination of cell surface expression and coupling efficiency. (Molecular Endocrinology 12: 775–784, 1998)

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INTRODUCTION

Male sex differentiation begins when the sex-determining factor (SRY) is expressed from the Y chromosome, which causes the indifferent gonads to develop into testes (1). In the testis, testicular cords are formed containing Sertoli cells and germ cells, while mesenchymal cells migrate into the interstitial space between the cords, giving rise to fetal Leydig cells (2). The testicular Sertoli cells and Leydig cells produce, respectively, anti-Müllerian hormone and testosterone, two hormones that are essential for correct differentiation of both primary and secondary sex characteristics (for review see Refs. 3–5). Anti-Müllerian hormone triggers the regression of the müllerian duct, the anlagen of the female urogenital tract, while testosterone,

in some target tissues after its reduction to dihydrotestosterone, stimulates differentiation and growth of the epididymides, vasa deferentia, the prostate, seminal vesicles, and other parts of the male urogenital tract including the formation of the penis. Before birth, proliferation and differentiation of Leydig cells and their production of androgens are dependent on the placental hormone, human CG (hCG). Prenatal disturbance of male sex differentiation leads to a variety of phenotypes ranging from males with micropenis, individuals with ambiguous genitalia and hypospadias, to complete male pseudohermaphrodites (5). These phenotypes are either due to insensitivity of the target cells to androgens (5, 6) or to impaired testicular androgen production as a result of a steroidogenic enzyme defect [*e.g.* 17β-hydroxysteroid dehydrogenase type II (7)] or reduced sensitivity of the Leydig cels to LH [Leydig cell hypoplasia (LCH) (8, 9)]. Two types of LCH have been described (10). LCH type I is the severe form of LCH identified in 46 XY individuals displaying a predominantly female phenotype, which is caused by mutations in the LH receptor (LHR) that completely disrupt LH signaling (11–13). Milder forms of LCH (type II), initially described in 1985 by Toledo *et al.* (14), are also caused by a mutation in the LHR gene, but this defect disrupts LHR signaling less severely, and patients are characterized by hypospadias or micropenis (13, 15, 16).

In the present paper we report the identification and characterization of a homozygous mutation in the LHR in three brothers with LCH type II. This mutation partially inactivates LH signaling, which explains the phenotype. In addition, we have compared this mutation to other missense mutations previously identified in patients with LCH. Our data show that a clear correlation exists between receptor activity and the resulting phenotype.

RESULTS

Clinical Details

Three brothers with normal karyotype were referred at the age of 28, 35, and 51 with micropenis, absence of pubertal signs, and infertility. LH and FSH levels were elevated but responded normally to GnRH. Basal testosterone and androstenedione levels, however, were low and responded poorly to hCG (Table 1; data not shown). Some variation in their basal and hCG-stimulated testosterone levels was observed among the brothers. However, this variation is probably the result of interindividual variation that is also observed in the normal population (Table 1) (17). Histological analysis of a testis biopsy of one of the patients showed seminiferous tubules with clearly thickened basal lamina and an interstitium that lacked mature Leydig cells (Fig. 1A). Spermatogenesis was present but did not extend beyond the elongated spermatid stage (Fig. 1B). Taken together these observations indicate that these patients have a mild form of LCH (LCH type II).

Table 1. Basal and hCG-Induced Testosterone Levels of Three Brothers with Mild Leydig Cell Hypoplasia

hCG test (10000 IU im)	Case I	Case II	Case III	Normal
Testosterone (basal)[a]	2.28	3.26	2.99	10–33[b]
Testosterone (after hCG)[a]	4.17	20.4	10.9	36–60[b]

[a]Testosterone in nanomoles per liter.
[b]Basal and hCG-induced levels in normal men (17).

DNA Analysis of the Patients

Single-strand conformation polymorphism (SSCP) analysis of the LHR gene in two of the affected brothers was first performed on exon 11 because in the transmembrane domain (TMD) of the receptor most of the subtle mutations have been identified. An aberrant migration pattern was found in one of the PCR fragments from both affected brothers while normal individuals showed only control bands (Fig. 2A). Sequencing of the PCR fragment revealed a T-to-A transversion at position 1874 of the cDNA (18), changing codon 625 from ATA for isoleucine to AAA for lysine (Fig. 2B). Isoleucine 625 is located in the seventh transmembrane segment of the receptor near the cytoplasmic tail. Both tested brothers were homozygous for the DNA change, and the mutation was not identified in any of the other samples that were analyzed ($n = 25$). Therefore, we conclude that the missense mutation segregates with the disease, although genomic DNA of the other family members could not be analyzed.

I625K Mutation Partially Inactivates Signal Transduction

The I625K mutation was introduced in a wild-type human LHR (hLHR) cDNA expression vector to produce pLHR(I625K). To determine the effect of the mutation on LH signal transduction, HEK293 cells were cotransfected with a luciferase reporter construct containing a cAMP-responsive promoter (CRE_6-Lux) (19) and the wild-type expression vector (pLHR(WT)) or pLHR(I625K). We used a CRE-driven luciferase reporter because this response is more sensitive and more conveniently measured than the regularly used cAMP response (not shown). Transiently transfected cells expressing the wild-type LHR responded to hCG with a 32-fold increase of luciferase activity whereas cells transfected with LHR(I625K) showed only a 18-fold increase (Fig. 3A). Furthermore, the EC_{50} of this response in the LHR(I625K) shifted more than a 1 order of magnitude to the right compared with the EC_{50} obtained with the wild-type LHR (Table 2). The total number of binding sites and the affinity for hCG of LHR(I625K) were not different from those of the wild-type receptor (Table 2).

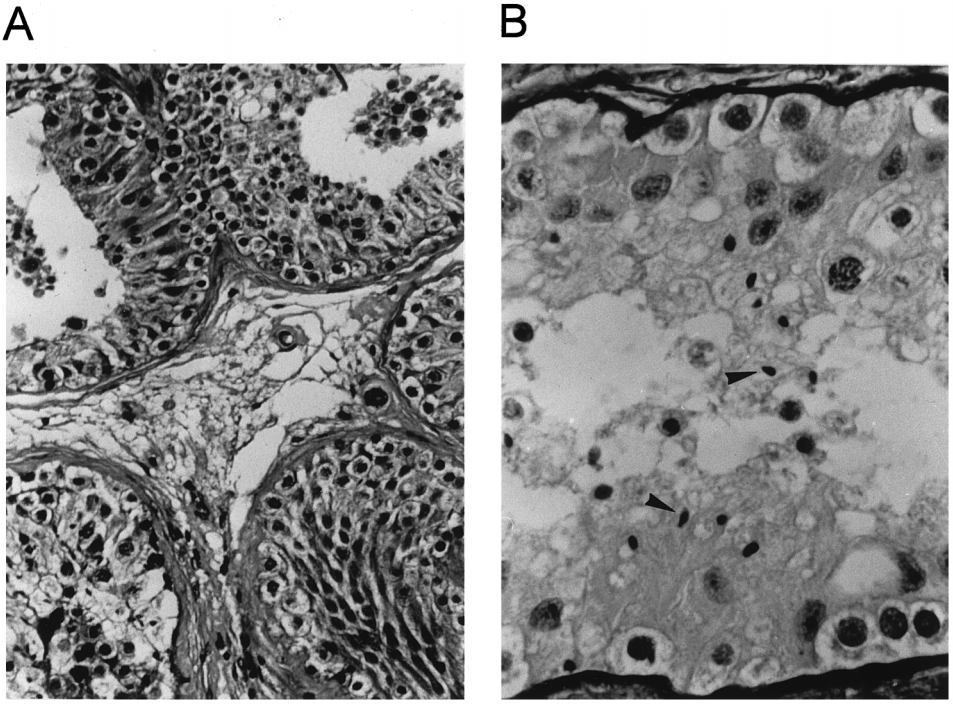

Fig. 1. Testis Histology of Testis of a Patient with Mild LCH

A, Testis biopsy taken from one of the patients. It shows seminiferous tubules with thickened basal lamina and spermatogenesis arrested in elongated spermatid stage. In the interstitium, mature Leydig cells are absent. Magnification 160×. B, Higher magnification (400×) of one tubule with elongated spermatids (see *arrow*). Sections were stained with hematoxylin/eosin.

Correlation of Receptor Activity and Phenotype

To date, three different missense mutations in the LHR gene, A593P, S616Y, and, in the present report, I625K, have been identified in patients with LCH (11, 13, 15). The extent of the syndrome probably depends on the residual level of androgen production by the Leydig cells, which in turn might be a function of the severity of the effect of the mutation on LH signaling. Therefore, we compared the characteristics of the different mutant LHRs in HEK293 cells (Fig. 3B and Table 2). The three mutant receptors bound hCG with similar affinity as the wild-type receptor (Table 2). The hCG-induced cAMP regulatory element (CRE) activity of all three mutant receptors was impaired (Fig. 3B). As previously shown, LHR(A593P) did not respond to hCG at all, while the maximal response of LHR(S616Y) and LHR(I625K) was reduced to 50% and 65%, respectively (Table 2). In addition, the EC_{50} of CRE induction by hCG of both mutant receptors shifted to the right by a factor of 20. The results obtained with LHR(S616Y) confirm those of Laue *et al.* (15); however, they contradict the results of Latronico *et al.* (13), who were unable to show signaling of LHR(S616Y). A possible explanation for this discrepancy may be the poor sensitivity of the cAMP production assay. The CRE luciferase assay used here is much more sensitive, and in this case appears to be clearly superior to a cAMP assay.

A reduced hormonal response of the mutant LHRs could be caused by a reduced expression of the LHR. Therefore, the amount of LHR receptor protein on a Western blot (Fig. 4) and the total number of binding sites were determined (Table 2). To visualize the LHR molecules on Western blot, the LHR cDNAs were provided with an hemagglutinin (HA) epitope tag at the 3′-end of the open reading frame and overexpressed in COS-1 cells. The HA tag had no effect on the number of binding sites or on the hCG-induced CRE response (data not shown). In COS-1 cells transfected with the wild-type LHR cDNA, two major LHR-specific bands of approximately 65 and 220 kDa were observed (indicated with *arrows*). Furthermore a minor band of approximately 300 kDa was present. The two largest bands are probably multimers or aggregates but they could also represent glycosylated forms of the receptor. The two additional bands of approximately 55 and 70 kDa are the result of nonspecific binding of the HA antibody, as they also occur in cells transfected with the empty expression vector (Fig. 4, *right lane*). In cells transfected with the different mutant LHR cDNAs, a similar profile was observed but the intensity of the LHR-specific bands was less, indicating that the amount of LHR protein is much less. The total number of binding sites (B_{max}) was also determined as a measure of the amount of receptor that has been inserted properly into the plasma membrane. The wild-type LHR and LHR(I625K) had the same

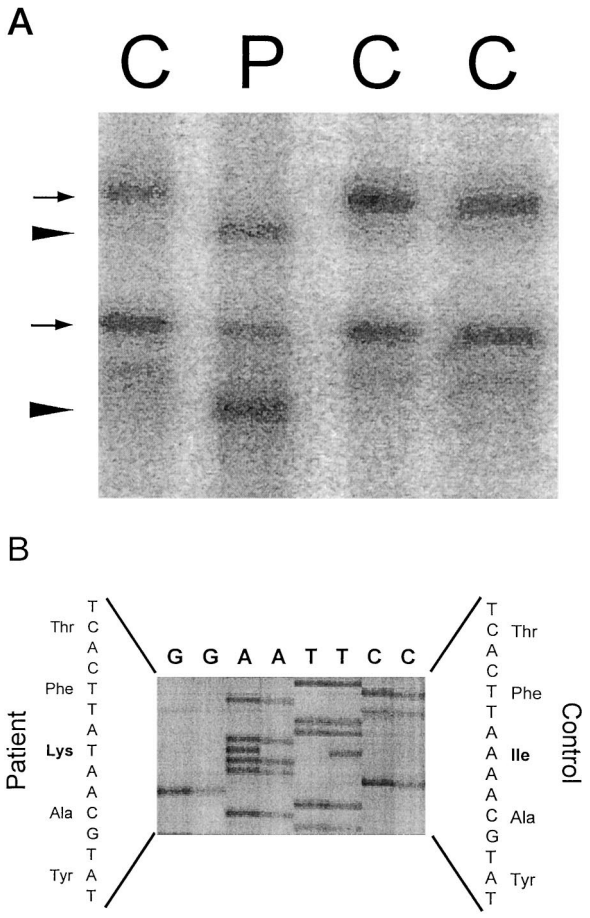

Fig. 2. Homozygous Missense Mutation in LHR Gene

A, SSCP in the coding sequence in patient with mild LCH (patient). For comparison, SSCP patterns of three normal individuals (contol) are shown. Normal and aberrant SSCP patterns are indicated by *arrows* and *arrowheads*, respectively. B, Genomic sequence of the LHR gene (from position 1867 to 1882) of patient with mild LCH (left) and normal individual (right). A homozygous T-to-A conversion was identified at position 1874 of the LHR gene in the patient.

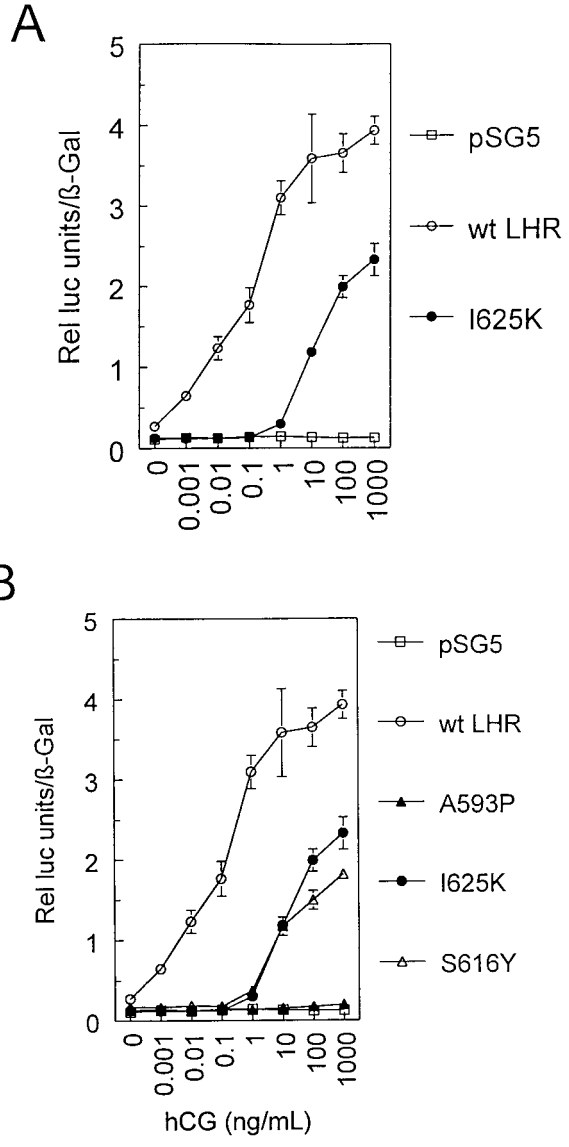

Fig. 3. hCG-Induced CRE Activation by Wild-Type and Mutant LHR

HEK 293 cells were transfected with the expression vector pSG5 containing the wild-type or indicated mutant hLHR cDNA in combination with the cAMP responsive construct, $pCRE_6Lux$. The expression vector pRSV-lacZ was included in the transfection assay as a control for transfection efficiency. Basal and hCG-induced luciferase activity divided by β-galactosidase activity determined in the same cell lysate was plotted as means $\pm$ SEM (n = 4) against the dose of hCG. The results of one representative experiment out of three is shown.

number of binding sites, but the B_{max} values of LHR(A593P) and LHR(S616Y) were reduced to 0.5% and 14%, respectively (Table 2). These results indicate that the processing and/or the transport of the latter two mutant receptors to the cell surface was impaired. In conclusion, the expression studies show mutant LHR molecules do not behave as wild-type molecules with respect to stability and/or trafficking to the cell surface. We, therefore, decided to measure the receptor activity of those receptor molecules that present at the cell surface because these are responsible for signal transduction. Thus, basal and hCG-induced CRE activity per cell surface binding-site of the different mutants was determined and was compared with activity of the wild-type receptor expressed at different levels. Thus, a reference curve for the wild type LHR was constructed by transfecting different amounts (0.1, 1, and 10 μg) of expression vector in HEK293 cells. In these cells, basal and hCG-induced CRE luciferase activity as well as the number of cell surface hCG-binding sites was determined (Fig. 5A). Subsequently, 10 μg of the various mutant LHR expression vectors were transfected into HEK293 cells, and the same parameters were determined and compared with the wild-type LHR reference curve (Fig. 5B). In wild-type LHR-expressing cells, both basal and hCG-

Table 2. Comparison of Different Mutant LHRs Identified in Patients with LCH

Mutation	Wild Type	I625K	S616Y	A593P
Binding				
K_d (nM)	2.4	2.5	1.4	0.6
B_{max} [pmol/mg protein]	2.5	1.7	0.36	0.01
CRE response				
EC_{50} (ng/ml)	0.5	10	10	ND[b]
Basal[1]	1.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.2	1.0 ± 0.1
Max.[1]	30 ± 1.4	18 ± 1.6	14 ± 0.6	1.5 ± 0.2

[a] Relative luciferase units (means ± SEM).
[b] ND, not detectable.

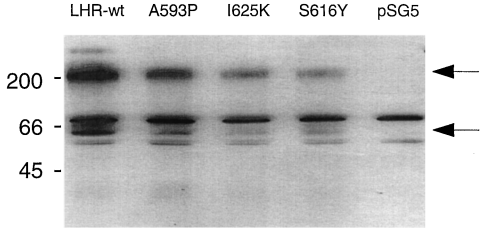

Fig. 4. Western Blot of Mutant LHRs Expressed in COS-1 Cells

COS-1 cells were transfected with the empty expression vector pSG5 or with pSG5 containing the indicated wild-type or mutant HA-tagged hLHR cDNA. Three days after transfection, the cells were harvested and subjected to 10% SDS-PAGE followed by Western blotting. Specific bands were visualized using immunostaining with a HA tag-specific monoclonal antiserum. The *arrows* indicated the two most predominant bands that are specific for the wild-type LHR. On the *left* the migration and the molecular weights of three standard proteins are indicated.

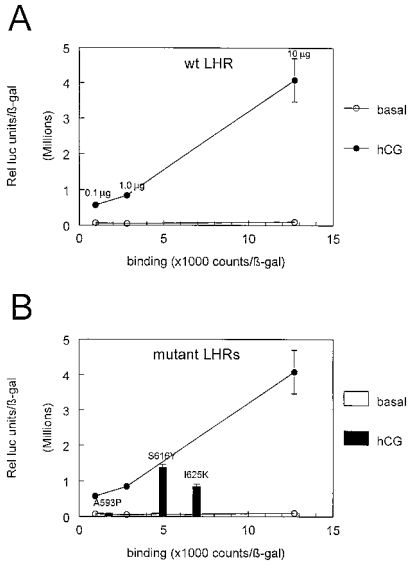

Fig. 5. Comparison of LHR Signaling Capacity per Cell Surface Binding Site

HEK 293 cells were cotransfected with the cAMP responsive reporter gene, $pCRE_6Lux$ in combination with either different amounts (10, 1, 0.1 μg) of wild-type LHR cDNA expression vector (A) or with equal amounts (10 μg) of expression vectors containing the wild-type hLHR cDNA or mutant cDNA A593P, S616Y, or I625K (B). Basal and hCG-induced luciferase activity (n = 4) determined in cell lysate is plotted as means ± SEM against the number of binding sites (n = 2). The results of one representative experiment out of two is shown.

induced CRE activity increased proportionally with the number of cell surface binding sites. Cell surface expression of all mutant receptor was reduced compared with the wild-type receptor when 10 μg expression vector DNA were also used in the transfection. In addition, LHR(A593P) completely lacked hormone-dependent signaling activity. In contrast, LHR(S616Y), which is also poorly expressed at the cell surface, displayed hardly reduced signaling activity compared with the wild-type receptor at similar receptor densities. The expression of LHR(I625K) was also reduced although to a lesser extent than LHR(S616Y). In addition, however, this mutant receptor displayed a reduced signaling capacity.

DISCUSSION

The characteristics of LCH II that are found in the family investigated in the present paper are caused by a homozygous T^{1874} to A mutation in the LHR gene. This base change results in a substitution of a neutral hydrophobic isoleucine residue by a positively charged lysine residue at position 625 of the LHR. This residue is located at the border between the seventh transmembrane segment and the cytoplasmic tail of the receptor. The substitution severely impairs hCG-induced receptor activation, without interfering with the total number of binding sites and the affinity for the ligand. The reduced hCG response, however, does not completely preclude all Leydig cell responses. An hCG challenge elicited a slight plasma testosterone response, which probably explains why spermatogenesis in these patients progressed up to late stages of spermatid differentiation. Spermiation, the last complicated step of sperm release involving major recon-

struction of the spermatogenic epithelium, did not occur, which is in accordance with the dependence of this step on sufficient androgens (20). In patients with severe LCH who display very low levels of androgens, spermatogenesis does not occur at all (11), indicating that the first stages of spermatogenesis may also be androgen-dependent, although an additional effect of cryptorchidism in complete LCH patients cannot be excluded.

The only other reported point mutation causing LCH type II, S616Y, is located in the same receptor domain, approximately two α-helical turns toward the extracellular side in TMD 7 (Fig. 5) (13, 15). Two patients with this mutation have been identified independently. One compound heterozygote patient carrying the S616Y mutation in combination with a completely inactive LHR gene (deletion of exon 8) had a small penis and severe hypospadias, while the other patient, homozygous for S616Y, had a phenotype similar to the patients described in the present paper, micropenis but no other indications of aberrant male sex differentiation. Both patients with the S616Y mutations were too young to be informative about the effect of their LHR mutation on spermatogenesis.

The extent of the phenotype of patients correlates well with the effect of the mutation on both receptor expression and responsiveness to hCG. Homozygous presence of two LHR(A593P) allelles, giving rise to a receptor that is poorly expressed and deficient in signaling, is associated with complete pseudohermaphroditism. Similar phenotypes are observed in patients having a premature stop codon in both alleles; severe truncation of the LHR also results in complete disruption of signal transduction (12, 13). Homozygocity for a S616Y or a I625K mutation both cause a milder identical phenotype (micropenis). The overall receptor activity of these two mutant receptors is equally reduced, albeit this reduction results from different mechanisms. LHR(S616Y) is poorly expressed but responds normally to hCG while LHR(I625K) is both poorly expressed and impaired in responding to hCG. In addition, both mutant receptors require a higher dose of hCG to respond compared with the wild-type receptor. When the LHR(S616Y) allele is combined with a completely inactive LHR allele (15), the phenotype of the compound heterozygous patient is intermediate between the mild and the complete form of LCH. (micropenis with severe hypospadia). These correlations of patient phenotype with receptor behavior *in vitro* suggest that there may be no clear distinction between complete and partial feminization of external genitalia due to LH insensitivity (LCH type I and type II) as proposed by Toledo *et al.* (14). Rather, a continuous range of phenotypes from complete pseudohermaphrodism to patients that are only mildly affected is observed depending on the different effects of mutations on LHR signal transduction and on specific combinations of abnormal alleles. During the preparation of this manuscript, another mutation causing partial LCH was described (16). This patient displayed sexual ambiguity at birth due to a homozygous mutation (C131R) in the extracellular domain of the receptor. The signaling of the receptor was detectable but severely impaired, which is in line with the observed phenotype.

Remarkably, LHR(A593P), LHR(S616Y) and LHR(I625K) display normal binding affinity while their EC_{50} is severly affected. Thus, only the biological response of these receptors is impaired. Similar discrepancies have been observed in the TSH receptor (21), which underscores the separate position of the glycoprotein hormone receptors within the G protein-coupled receptor (GPCR) superfamily and supports the hypothesis that these receptors contain two independent domains: a ligand-binding domain and a TMD that transduces the signal into the cell. Only the function of this latter domain is affected in the three mutant receptors.

The different characteristics of the tested mutants also provide clues as to the importance of subdomains of the receptor molecule. Both the S616Y and I625K mutations are located on the cytoplasmic side of TMD 7, close to a conserved region in the superfamily of GPCRs, the NPXXY motif (22). *In vitro* studies of other GPCRs, including the glycoprotein hormone receptors, have indicated that the NPXXY motif is important for ligand-induced receptor activation (23–25) and receptor sequestration (26). Indeed, I625K and S616Y may affect the function of the NPXXY motif and in this way decrease hCG-induced receptor activation.

Based on homology studies of a number of GPCRs, Baldwin (22, 27) has suggested a model of the most probable orientation of the seven-transmembrane α-helices in the membrane (Fig. 6). In this model, which appears to agree well with both our own preliminary model (F. Fanelli, personal communication) and a recently published LHR model (28), the hydrophobic isoleucine 625 points toward the hydrophobic phospholipid membrane. Introduction of a positive charge by the exchange of a lysine residue at this position may cause the α-helix of TMD 7 to rotate and move residue 625 toward the hydrophilic core of the TMD (see *arrow* in Fig. 6). Alternatively, TMD 7 may be shifted toward the cytoplasm. Movement of parts of TMD 7 could be facilitated by the presence of two helix-breaking proline residues. Furthermore, a turn of TMD 6 has been observed in a constitutively active mutant β_2-adrenergic receptor (29), indicating that such a turn can affect signal transduction. A slight rotation of TMD 7 changes the position of the conserved NPXXY motif in relation to the rest of the receptor and in that manner may reduce the response to hCG. However, the change must be small because the mutation does not severely affect proper folding. In the same model, serine 616 points toward TMD 1 and 2 in the hydrophilic pocket between the transmembrane helices of the receptor (F. Fanelli, personal communication). This serine is located exactly one helical turn N-terminal of the asparagine in the NPXXY motif and is very well conserved in the GPCR family, which indicates that the size of this residue and its ability to form

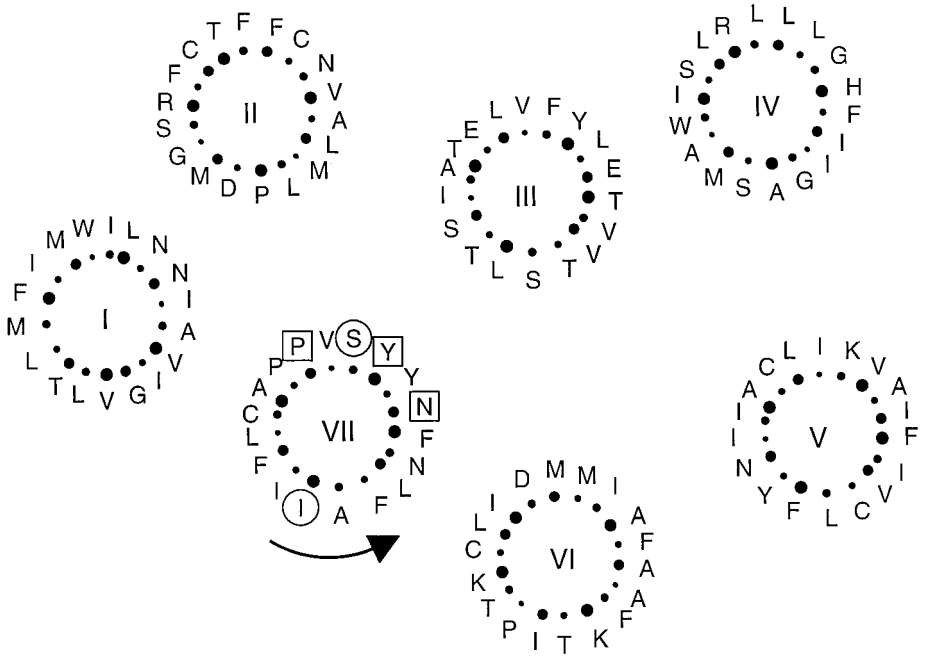

Fig. 6. Model of the TMD of the hLHR

Projection of the cytoplasmic part of the TMD of the LHR according to the model proposed by Baldwin (22, 27). The view is from the intracellular side toward the outside. The size of the dots indicate the distance of the amino acid to the cytoplasm (a *large dot* means that the amino acid is close to the cytoplasm). The amino acids of the NPXXY motif are indicated in *squares*; the two amino acids mutated in patients with partial LCH are indicated in *circles*. The amino acid mutated in the patient with complete LCH (A593P) is located at the border between TMD 6 and extracellular loop 3 and is therefore absent in the projection.

hydrogen bonds may be important. According to molecular modeling (F. Fanelli, personal communication) a tyrosine residue at this position may fit in the receptor pocket, although its side chain points toward TMD 3 instead of TMD 1 and 2. However, this fit must be poor because receptor folding is disturbed as indicated by the reduced cell surface expression. The intrinsic receptor activity is, however, unaffected, which indicates that the bulky tyrosine side chain does not interfere with receptor activation, once it is in place.

In conclusion, partial LCH in the present family is due to a homozygous missense mutation (I625K) in TMD 7 of the LHR. This mutation causes severe impairment in hormone-dependent receptor signaling. Detailed analysis of three missense mutations that result in LCH revealed a clear inverse relationship between residual receptor activity and severity of the clinical phenotype.

MATERIALS AND METHODS

Patients

The patients studied here have a mild form of LCH that was designated LCH type II (14). In short, three brothers, born to consanguineous parents were referred at the ages of 28, 31, and 51, because of infertility due to azoospermia. The patients, all with a 46 XY karyotype, had male external genitalia with adult-sized testis, but an undervirilized penis (micropenis). Baseline levels of testosterone were low and a single hCG injection (10,000 IU Pregnyl; Organon International, Oss, The Netherlands) elicited a slight but significant increase of serum testosterone levels (Table 1). Levels of intermediates of the testosterone biosynthetic route were not elevated, indicating absence of enzyme defects (not shown). An acute adrenal cortex stimulation using 250 μg Cortrosyn (Organon International) induced a normal elevation of corticosteroids, showing that adrenal steroid production was normal (data not shown). LH and FSH levels were elevated, but the pituitary responded normally to GnRH (100 μg iv; Ayerst Laboratories, Rouse Point, IL) (data not shown). LH bioactivity of one of these patients was tested and was found to be normal. The two younger brothers were treated with testosterone enanthate (250 mg/3 weeks; Organon International). After 2 yr of treatment, both patients showed sufficient virilization but penis size remained inadequate. In only one of the patients did treatment result in a significant increase in sperm count (from azoospermia to 3×10^6/ml) and fertility. All procedures were carried out in the course of normal patient care after appropriate informed consent had been obtained.

SSCP and Sequence Analysis

Genomic DNA was extracted from peripheral blood (30) of two of the affected brothers. Six overlapping fragments of exon 11 of the LHR gene were amplified by PCR and analyzed by SSCP as described previously (Ref. 31 and H. Kremer *et al*., submitted). For sequencing, PCR fragments were treated with alkaline phosphatase and exonuclease I and sequenced using the USB sequencing kit for PCR fragments (US Biochemical Corporation, Cleveland, OH).

Construction of Mutant hLHR Expression Vectors

Wild-type hLHR cDNA was introduced in the expression vector pSG5 (33), resulting in pLHR(WT) (34). Mutations were introduced into this construct using standard PCR mutagenesis (34, 35) with the primers (Pharmacia, Uppsala, Sweden) described below. The nucleotides that differ from the wild-type hLHR cDNA are indicated in bold.

LHR1512FOR: 5′-GTC GGT GTC AGC AAT TAC-3′;
LHR2182REV: 5′-GTT AAA ATT ACT GGT ACA GG-3′;
LHR616SYFOR: 5′-CCC ATC AAT T**A**T TG**C** GC**A** AAT CCA TTT-3′;
LHR616SYREV: 5′-AAA TGG ATT TGC GCA ATA ATT GAT GGG-3′;
LHR625IKFOR: 5′-G TAT GCA A**A**A TTC ACT AAG-3′;
LHR625IKREV: 5′-CTT AGT GAA T**T**T TGC ATA C-3′.

For constructing pLHR(I625K), primer sets LHR1512FOR/LHR625IKREV and LHR625IKFOR/LHR2181REV were used separately to perform the first PCR amplification. After mixing of the fragments, the final mutant fragment was obtained by PCR using the primer set LHR1512FOR and LHR2181REV. To construct the mutant hLHR expression vector, a *Bst*XI-*Hpa*I fragment of the reamplified fragment (669 bp) was used to replace the wild-type sequence in pLHR(WT), resulting in pLHR(I625K). For the construction of pLHR(S616Y), a similar strategy was used using the LHR616 primer set. Both constructs were checked by DNA sequencing. The construct pLHR(A593P) was described previously (11).

Transfection of COS-1 and HEK293 Cells

COS-1 and HEK293 cells were maintained in culture medium (DMEM/Ham's F12 (1:1 vol/vol) (GIBCO BRL, Gaithersburg, MD), 2×10^5 IU/liter penicillin (Brocades Pharma, Leiderdorp, The Netherlands) and 0.2 g/liter streptomycin (Radium Farma, Milan, Italy) and 5% and 10% FCS (SEBAK, Aidenbach, Germany), respectively, and were incubated in a humidified incubator at 37 C and 5% CO_2. Before transfection the cells were seeded at 15% confluence in 75-cm^2 flasks (Nunc, Roskilde, Denmark) and transfected the next day with 1 ml precipitate containing 20 μg DNA (36).

cAMP Reporter Activity Measurements

For measuring the hormonal response of the different mutants, HEK293 cells were cotransfected with pCRE$_6$Lux (19), pRSVlacZ (37) and pSG5, pLHR(WT), pLHR(A593P), pLHR(S616Y) or pLHR(I625K) (10 μg expression construct, 1 μg pRSVlacZ, 2 μg pCRE$_6$Lux, and 7 μg carrier DNA per ml precipitate). Three days after transfection the hCG-dependent CRE response was determined in 24-well tissue culture plates (Costar, Cambridge, MA) by incubating the cells for 4 h in culture medium containing 0.1% BSA with increasing concentrations of hCG (0.001 to 1000 ng/ml; urinary hCG; Organon International). Subsequently, the cells were lysed and luciferase activity was measured (38). β-Galactosidase activity of the lysates was determined to correct for transfection efficiency (37).

Scatchard Analysis

To determine the binding affinity (K_d) and total receptor number (B_{max}), HEK293 cells were transfected with pSG5, pLHR(WT), pLHR(A593P), pLHR(S616Y), or pLHR(I625K) (10 μg expression construct and 10 μg carrier DNA per ml precipitate). Three days after transfection, Scatchard analysis using chloramine T ^{125}I-labeled hCG (39) was performed on purified membranes according to Ketelslegers and Catt (40).

cAMP Reporter Activity per Cell Surface Receptor Number

For determining the receptor activity per receptor number expressed at the cell surface, HEK293 cells were transfected with pCRE$_6$Lux, pRSVlacZ and pSG5, pLHR(WT), pLHR(A593P), pLHR(S616Y), or pLHR(I625K). Three days after transfection a part of the transfected cells was used to measure the basal and maximal hCG (1000 ng/ml) CRE response as described above while the rest of the cells were used to measure LHR cell surface expression. Cell surface expression was determined as described previously (41, 42). Briefly, transfected cells were harvested and resuspended in binding buffer (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1% BSA, 5 mM sodium azide, 200 mM sucrose) containing ^{125}I-labeled hCG (1.5×10^6 cpm) in the presence or absence of a 1,000 fold excess of unlabeled hCG in a volume of 0.2 ml. The sodium azide was added to prevent internalization. After incubation for 1 h at 37 C, the cells were washed twice with excess of binding buffer, after which the binding as measured by the amount of radioactive hCG bound to the cells was counted in a γ-counter.

SDS-PAGE

To determine LHR expression on Western blots, LHR cDNAs were extended with an HA immuno tag (YPYDVPDYAS) at the 3′-end. This HA tag did not affect the number of binding sites or hormone-dependent signaling (not shown). COS-1 cells were transfected with pSG5, pLHR(WT)HA, pLHR(A593P)HA, pLHR(S616Y)HA, or pLHR(I625K)HA (10 μg expression plasmid and 10 μg carrier DNA per ml precipitate). Three days after transfection, the cells were washed twice with PBS and harvested in 1 ml PBS. After the protein concentration was determined (Bradford), equal amounts of protein (3.5 μg) were separated on 10% SDS/PAGE (43) and subsequently blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany) using the Mini-Protean II gel electrophoresis and electro-blotting apparatus (Bio-Rad, Hercules, CA). The tagged LHR proteins were visualized using the Renaissance Western blot chemiluminescence detection kit (DuPont/NEN, Du Pont de Nemours GmbH, Dreieich, Germany) using as primary antibody a 1:500 dilution of the HA-specific monoclonal antiserum 12C5.

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