CLINICAL STUDY

**Homzygous mutation within the conserved Ala-Phe-Asn-Glu-Thr motif of exon 7 of the LH receptor causes male pseudohermaphroditism**

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**Abstract**

**Background:** Human chorionic gonadotropin/luteinizing hormone (hCG/LH) function in the male is mediated by the LH receptor (LHR) and is crucial for the normal development of internal and external genitalia. We report a 46, XY patient who presented at the age of 16 with a female phenotype and delayed puberty. Gonads were located bilaterally in the inguinal canal, removed surgically and showed hypoplastic Leydig cells. Immunostaining for the LHR revealed that some Leydig cell progenitors were positive, while others were negative, reflecting different developmental stages of Leydig cell maturation.

**Methods and Results:** Molecular analysis of the LHR was performed on DNA extracted from blood samples of the patient, her parents and sister. The 11 exons of the LHR gene were amplified by PCR and subjected to further single stranded conformation polymorphism (SSCP) analysis. Aberrant migration patterns were observed in exon 7. Upon sequencing, a homozygous T to G transversion was identified, resulting in a F194V substitution located in the extracellular domain. The parents and sister were heterozygous carriers of this mutation. Functional studies in transiently transfected COS-7 cells with the F194V LHR mutation showed the lack of cAMP production upon hCG stimulation, indicating complete inactivation of the receptor due to impaired trafficking of the receptor to the membrane. The mutation is located within a stretch of five amino acids Ala (A)-Phe (F)-Asn (N)-Gly (G)-Thr (T), highly conserved in glycoprotein hormone receptors. For the follicle-stimulating hormone (FSH) receptor (FSHR) loss-of-function mutations have been allocated to this region, a homozygous A189V mutation resulting in a resistant ovary syndrome and impaired spermatogenesis and a heterozygous N191I mutation with no apparent phenotype. Further mutational and functional analysis of the AFN region in the LHR and FSHR revealed that the integrity of this amino acid sequence is crucial for receptor function.

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**Introduction**

Male sexual development in primates and humans comprises initial sex determination, followed by sexual differentiation into the male phenotype. While determination is mainly driven by SR Y, the testis determining gene located on the Y chromosome, which induces the bipotential gonad to develop into a testis, differentiation is exclusively regulated by hormones. Male phenotypic development is under the control of anti-Müllerian hormone, causing Müllerian duct regression, and testosterone, essential for transformation and virilization of external genitalia (1). Fetal Leydig cell differentiation and testosterone production is stimulated by human chorionic gonadotropin (hCG) produced by the trophoblast and mediated by the hCG/luteinizing hormone (LH) receptor. After birth LH takes over the role of hCG to maintain testosterone production (2).

Aberrant hormone binding or signal transduction of hCG/LH through the LH receptor (LHR) directly affects androgen production, leading to disturbances of fetal as well as pubertal male development (3). The clinical
phenotypes resulting from altered hCG/LH–LHR interaction in genetically male subjects range from male pseudohermaphroditism, characterized by female external phenotype with a blind ending vagina, lack of breast development and primary amenorrhea, to incomplete virilization of the external genitalia with micropenis and/or hypospadias (1–3). In severe cases the responsiveness of Leydig cells to hCG is abolished, resulting in Leydig cell hypoplasia (LCH). Two types of LCH have been described (1). Type I LCH is the most severe form, resulting in a female phenotype and is caused by inactivating mutations in the LHR which completely prevent hCG/LH signal transduction and thus testosterone production. Patients with type II LCH are characterized by milder signs of androgen deficiency and are generally hypogonadal. This milder form is derived from mutations of the LHR, which only partially inactivate signal transduction and retain some responsiveness to hCG/LH (4).

Numerous activating mutations have been described for the LHR (5) but to date only a few inactivating mutations are known (2, 6–8). One possible explanation for this phenomenon could be that activating mutations are much more compatible with reproduction, e.g. females do not manifest any abnormal phenotype and males display signs of precocious puberty not affecting fertility, whereas inactivating mutations, besides their autosomal recessive inheritance, lead to infertility and will not be forwarded to the next generation. Mutations of the closely related follicle-stimulating hormone (FSH) receptor (FSHR) occur even less frequently: only one activating mutation has been described up to now, and only very few inactivating mutations of the FSHR causing gonadal dysgenesis, secondary amenorrhea, or impaired spermatogenesis have been identified (2, 9, 10). While activating mutations are located almost exclusively in the transmembrane domain, inactivating mutations or deletions are spread throughout N-terminus, C-terminus and transmembrane domains of the receptor. Overall, no putative hot-spot for inactivating mutations has been discernible for the gonadotropin receptors.

In the present report, we describe the case of a patient with male pseudohermaphroditism caused by a homozygous inactivating F194V mutation of the LHR located in a highly conserved stretch of five amino acids of exon 7. Within this amino acid motif, inactivating mutations have been described for the closely related FSHR, underlining the crucial function of this motif for receptor functioning.

**Subject and methods**

### Case report

A 16-year-old girl was referred to the Department of Internal Medicine and Endocrinology, University of Tübingen, Germany, because of delayed puberty, since her sister, younger by 1 year and 7 months experienced menarche at the age of 14 years. Both parents were immigrants from the same rural village of 800 inhabitants in the northern part of Greece. Close consanguinity is excluded, but not a distant relationship.

The phenotypic female of 55 kg was 171 cm tall with an upper/lower segment ratio of 0.84. Breast development was infan tile (Tanner stage I). Some sporadic pigmented terminal hairs were present in the pubic region (stage II), not so in the axillae. The external genitalia showed infantile labia majora et minora and a vaginal orifice (urogenital sinus) of 1 cm depth. There were no signs of virilization whatsoever. No dysplastic stigmata were detected; no goiter (sonotomographic volume 5 ml) and no clinical signs of overt hypothyroidism. Pigmentation was normal and homogeneous. In the inguinal canal tender testicle-like structures were palpable on both sides and measured $25 \times 10 \times 9$ mm by sonography. No other structures of internal genitalia, such as derivatives of the Wolffian and Müllerian ducts, were discernible by ultrasound or magnetic resonance imaging (MRI).

Radiography of the left hand and wrist showed a bone age of 13 years by the Tanner method of maturat ion indicators. Magnetic resonance imaging of the brain was normal, especially with respect to the anterior and posterior pituitary, the stalk, pineal, optic chiasm, hypothalamic structures, and the septum pelliculum. A normal male karyotype (46, XY) was detected. DNA sequencing revealed no indication of gene defects of the androgen receptor and the 5-alpha reductase 2 enzyme. Adrenocorticotropic (ACTH), cortisol, androstandione, dehydroepiandrosterone (DHEA) sulfate,

### Table 1 Endocrine parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>A</th>
<th>B</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT₄ (ng/dl)</td>
<td>1.3</td>
<td>1.2</td>
<td>0.7–1.5</td>
</tr>
<tr>
<td>TT₃ (ng/ml)</td>
<td>1.81</td>
<td>2.30</td>
<td>0.8–2.2</td>
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<tr>
<td>TBG (mg/l)</td>
<td>19</td>
<td>23</td>
<td>12–30</td>
</tr>
<tr>
<td>TSH (0') (mIU/l)</td>
<td>1.7</td>
<td>1.3</td>
<td>0.3–2.5</td>
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<tr>
<td>TSH (30') (mIU/l)</td>
<td>11.9</td>
<td>10.9</td>
<td>5.0–22.5</td>
</tr>
<tr>
<td>PRL (0') (ng/ml)</td>
<td>22</td>
<td>34</td>
<td>5–20</td>
</tr>
<tr>
<td>PRL (30') (ng/ml)</td>
<td>91</td>
<td>115</td>
<td>10–120</td>
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<td>GH (ng/ml)</td>
<td>0.6</td>
<td>10.4</td>
<td>0.5–10.0</td>
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<td>IGF-I (ng/ml)</td>
<td>313</td>
<td>171</td>
<td>90–500</td>
</tr>
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<td>ACTH (pg/ml)</td>
<td>27.7</td>
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<td>10–50</td>
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<tr>
<td>Cortisol (µg/8)</td>
<td>18.9</td>
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<td>6.0–22.0</td>
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<td>Transcortin (mg/l)</td>
<td>5.2</td>
<td>9.2</td>
<td>3.0–8.5</td>
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<td>Aldosterone (pg/ml)</td>
<td>108</td>
<td>—</td>
<td>40–310</td>
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<tr>
<td>Renin activity (ng AngI/ml/h)</td>
<td>2.03</td>
<td>—</td>
<td>0.15–2.12</td>
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<tr>
<td>Potassium (K⁺) (mmol/l)</td>
<td>4.3</td>
<td>3.9</td>
<td>3.5–4.6</td>
</tr>
</tbody>
</table>

*30 min after l.v. stimulation by 400 µg TRH.

FT₄, free thyroxine; TT₃, total triiodothyronine; TBG, thyroid-binding globulin; PRL, prolactin; GH, growth hormone; IGF-I, insulin-like growth factor I; AngI, angiotensinogen type 1.
aldosterone, and renin activity were normal (Tables 1 and 2). Stimulation by hCG i.m. did not result in an increase in gonadal steroids after 72 h (Table 3). Furthermore, plasma concentrations of testosterone and estradiol were minimal, LH was increased and FSH was normal (Table 2).

Because of the high risk of neoplastic growth in dysontogenetic gonads, both inguinal gonads were removed by herniotomy. Macroscopically, they were normally differentiated small testicles with regular appendages and thready vasa deferentia. After gonadectomy the patient underwent estrogen replacement therapy, first estradiol valerate 1 mg per day for 6 months, then 2 mg for another 6 months, and afterwards 20 mg ethinyl estradiol daily. Meanwhile, 22 months after gonadectomy, she developed breasts (Tanner stage III-IV) and pubic hair (stage IV). Her height increased to 178 cm. Thyrotropin (TSH) levels rose to 2.7 mIU/l before and 22.9 mIU/l after thyrotropin releasing hormone (TRH) stimulation, indicating borderline primary hypothyroidism without detectable thyroid-specific autoantibodies. Therefore, the patient is treated intercurrently with 75 mg thyroxine daily with subsequent normalization of TSH to 1.3 mIU/l before and 10.9 mIU/l 30 min after TRH stimulation (Table 1, column B). The slight hyperprolactinemia is monitored and considered functional, since a second MRI showed neither a pituitary adenoma nor any hypothalamic abnormality.

Table 2 Time course of gonadal endocrine parameters before and after gonadectomy. Gonadectomy was performed in April 1998 (4/98).

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>LH (IU/l)</td>
<td>18/46*</td>
<td>17/43*</td>
<td>17/46*</td>
<td>29/153*</td>
<td>32/240*</td>
<td>37/322</td>
<td>1.5–10.0</td>
<td>1.5–10.0</td>
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<tr>
<td>FSH (IU/l)</td>
<td>12/16*</td>
<td>11/15*</td>
<td>65/86*</td>
<td>95/205*</td>
<td>86/170*</td>
<td>39/111</td>
<td>2.0–25.0</td>
<td>2.0–25.0</td>
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<tr>
<td>Estradiol (pg/ml)</td>
<td>9</td>
<td>8</td>
<td>6</td>
<td>39(1)</td>
<td>67(1)</td>
<td>23(2)</td>
<td>&lt;50</td>
<td>20–60</td>
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<td>Testosterone (ng/ml)</td>
<td>0.17</td>
<td>0.22</td>
<td>0.13</td>
<td>0.13</td>
<td>0.18</td>
<td>3.0–8.0</td>
<td>&lt;0.60</td>
<td></td>
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<tr>
<td>Androstenedione (ng/ml)</td>
<td>1.34</td>
<td>1.44</td>
<td>1.18</td>
<td>0.91</td>
<td>0.91</td>
<td>1.32</td>
<td>1.20–2.50</td>
<td>1.20–2.50</td>
</tr>
<tr>
<td>DHEA sulfate (ng/ml)</td>
<td>1930</td>
<td>1940</td>
<td>1690</td>
<td>1820</td>
<td>1350</td>
<td>1440</td>
<td>500–2500</td>
<td>500–2500</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>66</td>
<td>74</td>
<td>58</td>
<td>92</td>
<td>71</td>
<td>101</td>
<td>15–50</td>
<td>30–90</td>
</tr>
</tbody>
</table>

* Before and 30 min after i.v. stimulation by 100 µg LH-RH.
(1), Replacement by estradiol valerate 1 mg and 2 mg daily; (2), replacement by ethinyl estradiol 20 µg daily.
SHBG, sex hormone binding globulin.

Table 3 hCG stimulation test with 5000 IU i.m.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0h (basal)</th>
<th>48 h</th>
<th>72 h</th>
<th>Normal range</th>
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<tbody>
<tr>
<td>Estradiol (pg/ml)</td>
<td>7</td>
<td>9</td>
<td>7</td>
<td>1.5–2.5 x*</td>
</tr>
<tr>
<td>Testosterone (ng/ml)</td>
<td>0.27</td>
<td>0.27</td>
<td>0.25</td>
<td>1.5–2.5 x*</td>
</tr>
<tr>
<td>Androstenedione (ng/ml)</td>
<td>1.54</td>
<td>1.66</td>
<td>1.71</td>
<td>—</td>
</tr>
<tr>
<td>DHEA sulfate (ng/ml)</td>
<td>1520</td>
<td>1400</td>
<td>1260</td>
<td>—</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>88</td>
<td>79</td>
<td>84</td>
<td>see Table 2</td>
</tr>
</tbody>
</table>

* Normal response is an increase by 1.5 to 2.5 of the basal value at time 0.
SHBG, sex hormone binding globulin.

DNA isolation and PCR analysis

DNA was isolated from blood samples obtained from the patient as well as from her sister, father and mother. In addition, blood samples from a male volunteer with normal hormonal levels and normal sperm counts according to the WHO criteria were taken. Genomic DNA was purified using the Nucleon Kit (Herolab, Braunschweig, Germany). Exons 1–11 of the LHR gene were amplified using the primers and cycling conditions described by Atger et al. (11). Each PCR sample (25 µl) contained 10 nmol/l Tris – HCl (pH 8.3), 50 nmol/l KCl, 0.01% gelatin, 2 nmol/l MgCl2, 0.2 mmol/l deoxy-NTPs, 2 U Taq polymerase (Promega Corp., Heidelberg, Germany), 20 pmol/l primer, and 200 ng DNA. The amplified products were subjected to 1–2% agarose gel electrophoresis for further analysis.

Single stranded conformation polymorphism (SSCP) and in vitro mutagenesis

The entire LHR gene consisting of 11 exons was screened for mutations either by direct sequencing (exon 11) or by SSCP gel electrophoresis. SSCP and sequence analysis were performed as described previously (12).

The human LH and FSH receptors cloned into the EcoRI restriction site of pSG5 (Stratagene, Heidelberg, Germany) were mutagenized by oligonucleotide-mediated site-directed mutagenesis using the Transformer site-directed mutagenesis kit (Clontech, ITC Biotechnology, Heidelberg, Germany). The selection primer (5'-GAAGTACAAAGTCATGTATTCA-3') converted the unique NdeI restriction site of pSG5 into NcoI. To introduce the different mutations (see Fig. 4) the following primers were used:

1. LHR-A193V 5'-GAAGTACAAAGTCATGTATTCA-ATGGGACGAC-3',
2. FSHR-A189V 5'-GAATACAAACTGTGTATTC-ATGGGACGAC-3'.

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3. LHR-F194V 5'-GTACAAAGTCATGCAATGG- GGACGG-3';
4. FSHR-F190V 5'-TACACACTGTGCAATGG- AAACCCAC-3';
5. LHR-N195I 5'-GAATGAAGTACAAAGTCATGCAATGGGACGAC-3';
6. LHR-N191I 5'- GAATTACAAAAGGAAATGGGACGAC-ATTGGAACCCACTA-3'.

The mutations were confirmed by direct PCR sequencing. Plasmids were isolated and purified by anion exchange columns (Qiagen, Hilden, Germany).

**Transfection of COS-7 cells and cAMP assays**

COS-7 cells were transiently transfected as described previously (13). Briefly, 5 μg/well of plasmid DNA was transfected using a calcium-coprecipitation method. Cells were prelabeled with [3H]adenine (25–50 Ci/mmol, Dupont-NEN, Brussels, Belgium) and cAMP assays were performed 3 days after transfection. To measure cAMP accumulation, cells were stimulated for 1 h with 100 nmol/l hCG (from urine of pregnant women, 3000 U/mg, Sigma Chemical Co., St Louis, MO, USA) for wild-type (wt)-LHR and LHR mutants or 100 μM/ml porcine (p) FSH (Sigma) for wt-FSHR and FSHR mutants after washing the cells with serum-free DMEM containing 1 mmol/l 3-isobutyl-1-methylxanthine (Sigma). Termination of cAMP accumulation was performed by adding 1 ml 5% trichloric acid, and the cAMP content of cell extracts was determined by cation exchange chromatography as described by Schöneberg et al. (13). For concentration response curves, cells were stimulated with different hormone concentrations. To account for experimental variability, wt-receptor cDNA was included as an internal control in each transfection assay and functional data were expressed as x-fold increase of basal levels.

**125I-hCG displacement studies**

Binding experiments were performed as described by Schulz et al. (14). In brief, COS-7 cells were transiently transfected with either the wild-type LHR in the pSG5 vector or FSHR F194V. Cells were harvested three days after transfection and membrane homogenates were used for displacement studies. Incubations were carried out for 90 min at room temperature in a 0.25-ml volume. Each sample contained 0.1 nmol/l 125I-hCG (4712 Ci/mmol, Dupont-NEN) and membranes of 2 × 10^5 cells. Concentrations of non-labeled hCG ran from 10^-7 to 10^-11 mol/l. Non-specific binding was defined as binding in the presence of 10^-7 mol/l hCG.

Cell-surface ELISA

Assessment of LHR cell surface expression was principally performed as described previously (15) To study cell surface expression of the LHR, an HA epitope tag was engineered in the N-terminus of the receptor between Arg^20^ and Glu^27^ to construct HA-LHR. An N-terminally tagged V2 vasopressin receptor, kindly provided by Torsten Schöneberg, Berlin, served as a control. COS-7 cells plated in 24-well-plates at 5 × 10^4 cells per well were transfected with either the wild-type HA-LHR in pSG5 or HA-LHR F194V. Three days after transfection cells were fixed (4% formaldehyde in phosphate-buffered saline), and unspecific binding sites were blocked with DMEM/10% FCS (1 h, 37°C). Subsequently, cells were incubated at 37°C for 2 h with a peroxidase-labeled anti-HA-antibody (Roche Molecular Biochemicals). After removal of excess unbound antibody, H_2O_2 and o-phenylenediamine were added to serve as substrate and chromogen respectively. After 15 min the enzyme reaction was stopped by 1 mol/l H_2SO_4/0.05 mol/l Na_2SO_3 and color development was measured at 450 nm using an ELISA reader (Bio-Tek Instruments).

**Immunohistochemistry**

After fixation in Bouin’s solution the removed gonads were embedded in paraffin. The LH receptor monoclonal antibody P1B4 was a gift from Dr J Wimalasena (Department of Obstetrics and Gynecology, University of Tennessee, Knoxville, TN, USA). The antibody was raised against purified rat LH receptors, as described by Indrapichate et al. (16), and has been shown to bind to LH receptors in the human.

The immunostaining technique was performed on 5-μm paraffin sections on silan-coated glass slides. The sections used in this study were obtained from the patient under investigation and a control patient with normal testicular morphology. Sections were deparaffinized and endogenous peroxidase was blocked with 1% H_2O_2 in methanol for 30 min. The slides were subsequently washed in 0.01 mol/l Tris-buffered saline (TBS; pH 7.4), incubated with 0.1 mol/l glycine in TBS for 30 min and rinsed with TBS. The slides were blocked with 10% normal goat serum for 30 min and then incubated overnight at 4°C with the antibody against the LH receptor at a dilution of 1:5000 in TBS with 0.05% acetylated BSA (Aurion, Wageningen, The Netherlands). Following this incubation the slides were rinsed with TBS and then incubated for 60 min with a biotinylated goat-anti-mouse antibody (Vectastain Kit Elite, Vector Lab., Burlingame, CA, USA) diluted 1:200 in TBS containing 0.05% acetylated BSA. Slides were again washed in TBS and subsequently incubated for at least 60 min with the components avidin (A) and biotin (B) of the ABC staining kit (Vector Lab). Both components (A and B) were
diluted 1:2000 and prepared at least 15 min before addition to the sections. Slides were washed again in TBS and Tris–HCl (0.05 mol/l, pH 7.6), and bound antibody was visualized using a 0.6 mg/ml solution of 3,3'-diaminobenzidine tetrachloride (Sigma Chemical Co.) in Tris–HCl, to which 0.03% H₂O₂ was added. All slides were subsequently counterstained with Mayer's hematoxylin.

Pilot experiments had previously demonstrated that the dilution of the LHR antibody used in this study gave optimal staining results. Control experiments were carried out and representative sections are shown in Fig. 3B and D. In these control experiments the primary antibody was either omitted from the procedure (not shown), or normal mouse serum was employed instead of the first antibody. Non-specific background staining was not observed.

Results

Molecular analysis of the LHR gene

Analysis of the LHR gene from the patient by SSCP revealed an aberrant migration pattern of exon 7 compared with two controls (Fig. 1). Sequencing of the total coding region including the adjacent splice sites of exon 7 showed a homozygous mutation at nucleotide position 580, leading to a T to G transversion. The mutation was located in the extracellular domain of the LHR and caused an amino acid change from F194 to V. The observed nucleotide change eliminates a restriction site for BsmI, which was used further to screen the family members for the presence of this mutation. In the presence of the BsmI restriction site the DNA fragment (194 bp) can be cut, resulting in a 118 bp and a 76 bp fragment. As shown in Fig. 2, father, mother and sister are heterozygous carriers, whereas the proposita is homozygous for the T580G mutation.

Immunohistochemistry for the LHR in the testis of the patient

Sections of the gonads displayed testicular tubules with abundant Sertoli cells, some resting spermatogonia, and interstitially some sparse cells reminiscent of fibroblast-like Leydig cell precursors and hypoplastic fetal Leydig cells. Since LH is essential for the development of the adult-type Leydig cell population, no mature LHR positive adult-type Leydig cells were observed. Immunohistochemistry using a monoclonal antibody raised against purified LHR showed intense staining in the hypoplastic Leydig cells and fibroblast-like cells, while other cells with a fibroblast-like morphology seemed to lack LHR protein expression and were immuno-negative (Fig. 3).
Functional studies

The observed mutation is located in a stretch of five amino acids, consisting of Ala (A)-Phe (F)-Asn (N)-Gly (G)-Thr (T), completely conserved within the glycoprotein hormone receptors and among all mammalian species. Since naturally occurring mutations within this motif have already been described for the FSHR, we designed a mutational analysis approach based on known mutations to elucidate the importance of the A-F-N-G-T motif for LHR and FSHR function.

According to the previously described two mutations in the FSHR (8) and the identified mutation in the LHR (this manuscript), the following amino acid changes were introduced into the FSHR: A189 was replaced by V, F190 by V and N191 was replaced by I. In parallel, the LHR was mutated, whereby A193 was replaced by V, F194 by V and N195 was replaced by I (Fig. 4).

Transfection of COS-7 cells with the different receptor constructs and subsequent stimulation with hCG (100 nmol/l) and pFSH (100 mU/ml) resulted in a robust increase in cAMP production mediated by the wt-LHR and wt-FSHR respectively (Fig. 5). The mutation identified in our patient, F194V for the LHR and the F190V mutation in the FSHR dramatically decreased cAMP production (Fig. 6A). The previously described inactivating mutation of the FSHR A189V (2) displayed only a weak cAMP accumulation upon stimulation with pFSH. Similarly, the corresponding LHR mutation A193V led to a reduced maximum cAMP level as compared with the wt-LHR receptor when stimulated with hCG. Mutation of the putative glycosylation site N195I in the LHR and N191I in the FSHR resulted in a reduced cAMP production for both receptors, but was more obvious for the FSHR.

To characterize the mutant receptors in more detail, concentration response curves were obtained for receptor constructs which gave a maximum stimulation of more than twofold over basal levels. For LHR mutants (Fig. 5B) the EC50 value for N195I (0.13 nmol/l) was reduced by a factor of 7 as compared with the wt-LHR receptor (0.91 nmol/l) and A193V (0.98 nmol/l). As shown in Fig. 5D, no major differences...
in the EC$_{50}$ values for the FSHR constructs were observed (wt-FSHR: 1.36 mU/ml; N191I: 3.0 mU/ml).

The identified F194V mutation in the LHR was studied further by performing dose-dependency experiments in which the wt or the mutated LHR were stimulated with increasing concentrations of hCG. Stimulation with 10$^{-2}$ mol/l hCG could evoke a nearly 25-fold increase in cAMP production in the wt-LHR over the basal levels whereas stimulation of the mutated receptor did not result in cAMP values significantly different from basal (Fig. 6A). Thus, the mutation completely inactivates signal transduction of the LHR. In order to investigate whether this impaired signal transduction is due to an effect on the signal transduction machinery or on the hormone binding capacity of the receptor we performed displacement experiments and studied the receptor expression at the membrane.

Using membranes prepared from identical numbers of cells transiently transfected either with wt-LHR cDNA or LHR-F194V cDNA, we observed specific binding of $^{125}$I-hCG which could be displaced by increasing amounts of unlabeled hCG. The IC$_{50}$ values were 0.44 and 0.25 nmol/l for wt-LHR and LHR-F194V respectively. These observations show that the ligand-binding domain of the mutated LHR is correctly folded and binds hCG with an affinity comparable with that of the wt-LHR (Fig. 6B). Thus, impaired ligand-binding to the receptor cannot account for the severe functional defect of LHR-F194V.

Measuring the surface expression of the wt- and the mutated LHR by a cell surface ELISA, using the expression of the vasopression receptor as an internal control (100%), revealed values of 10.1±0.8% for the wt-LHR and 1.2±1.0% for the F194V LHR ($n=2$). The data indicate that the mutated receptor when compared with the wt-LHR is presumably not expressed at all at the cell surface and that it remains completely cytosolic or stacked to inner cell membranes like the endoplasmic reticulum.

**Discussion**

**Patient**

The described patient displayed the full phenotype of Leydig cell hypoplasia (LCH) type I, characterized by a blind-ending vagina, lack of breast development and XY karyotype. This severe form of LCH indicates the complete absence of hCG/LH signal transduction in Leydig cells, which is also evident by the extremely low testosterone and estradiol levels in the presence of elevated levels of LH and normal FSH (Table 2). Testosterone, androstendione, and estradiol could not be increased by hCG stimulation (Table 3). However, in patients with LCH type II, caused by partially inactivating mutations of the LHR, hCG stimulation results in a slight but consistent increase in testosterone serum levels (4). This residual activity of the LHR seems to enable some degree of androgenization, resulting in a variable phenotypic picture ranging from individuals with ambiguous genitalia to male hypogonadism (2, 4). The functional studies on the F194V mutation in our patient are in full accordance with the observed clinical phenotype of LCH type I (1). Stimulation of COS-7 cells transiently transfected with the mutated receptor cDNA construct with 100 nmol/l hCG could not evoke any increase in cAMP production, thus indicating complete inactivation of the LHR (Figs 5 and 6). Thus the homozygous F194V mutation is the cause of the observed male pseudohermaphroditism in this patient. Father, mother and sister of the proposita are heterozygous carriers of this mutation, but do not show any phenotypical signs of an impaired LH/LHR interaction (Fig. 2). It is generally believed that heterozygous mutations within the gonadotropin receptors do not display any specific phenotype, since the normal allele can compensate for the loss-of-function mutation of the corresponding allele. Up to now there are no gene dose effects or preferential expression of one of the alleles described which could lead to a decreased LHR expression causing milder forms of hypovirilization or subtle signs of female infertility. However, homozygous

![Figure 4 Schematic representation of the Ala(A)-Phe(F)-Asn(N)-Gly(G)-Thr(T) motif located within exon 7 of the LHR (upper line) and FSHR (bottom line). The amino acid changes being introduced by in vitro mutagenesis are indicated. Numbering is according to the location of Asn which is position 195 in the case of the LHR and 191 for the FSHR. Additionally G was inserted between F and N to mimic the amino acid sequence in a recently described LGR.](image-url)
inactivating mutations of the LHR in females (46, XX) usually in some sisters of LCH cases, result in subclinical estrogen deficiency. These cases show normal development of external and internal female genitalia, maturation of primary and secondary sexual characteristics, but primary amenorrhea with occasional irregular spotting, infertility by anovulation, cystic ovaries, and osteopenia (17). The increase in FSH observed in the patient soon after gonadectomy (Table 2) reflects the ablation of the non-steroidal inhibitory function of the Sertoli cells mediated by inhibin. The persistent doubling of basal LH serum levels shows incomplete suppression by pure estrogen treatment. The hyperprolactinemia is in accordance with similar observations in patients undergoing male-to-female transsexual conversion under estrogen treatment after orchidectomy (18).

Until recently the clinical phenotype of LCH type I was suspected to be exclusively due to genetic alterations in the LHR gene. However, in 1999 Zenteno et al. (19) described a family with characteristic clinical symptoms of LCH, but were unable to identify any mutation or deletion in the LHR gene of affected family members. This might indicate defects in other loci presumably involved in Leydig cell differentiation, thus making LCH a genetically heterogeneous condition. In addition to these findings, mutations of or deletions in the LHR do not always give rise to inactivated or activated receptors. We have recently identified a patient in whom a homozygous deletion of exon 10 of the LHR did not cause inactivation of the receptor, but led to a receptor isoform capable of discriminating between LH and hCG action and responding only to hCG but not to LH. This patient displayed regular male sexual differentiation (due to intrauterine hCG action) but failed to enter puberty (20).

Histological examination of the testis from our patient revealed that the seminiferous tubules consist of Sertoli cells and some spermatogonia, indicative of the complete absence of spermatogenesis. This spermatogenic failure is caused by the lack of testosterone action during embryonic development, also crucial for testicular descent and later, during adulthood, for stimulation of spermatogenesis (21). Immunohistochemical staining of the testis using a monoclonal LHR antibody showed distinct positive staining in some of the interstitial cells. Fetal Leydig cell differentiation is a multistep process, during which

Figure 5 Functional analysis of LHR and FSHR mutants. To characterize mutant LH and FSH receptors, COS-7 cells were transfected with the different constructs. cAMP accumulation assays were performed as described under Subject and methods. Basal (open bars) and agonist-induced cAMP levels (shaded bars) (A and C) are presented as means of three to five independent experiments, each carried out in triplicate. For concentration response curves (B and D) transfected cells were stimulated with increasing concentrations of hCG (for LHR constructs) or pFSH (for FSHR constructs). The data are presented as means (×fold over basal) of two to four independent experiments, each carried out in duplicate.
undifferentiated mesenchymal cells initially acquire the steroidogenic machinery by an unknown mechanism. With the expression of the LHR and the secretion of hCG, maturation of fetal-type Leydig cells starts, associated with increased steroidogenesis (22). The development of the adult-type Leydig cell population depends strongly on LH and functioning LHRs. Thus the resistance to LH action caused by a non-functional LHR prevents the development of mature and functioning Leydig cells and is reflected by the non-differentiated morphology and the sparse LHR immunostaining of the Leydig cell progenitors observed in the testis of the patient.

In rodents male sexual differentiation is also strictly dependent on testosterone; however, since CG is lacking in lower mammalian species, a testicular paracrine regulation of testosterone biosynthesis in Leydig cells is assumed to play a pivotal role. Thus elimination of the LHR in knock-out mice (LuRKO-mice) at birth yields phenotypically normal male mice with testes and genital structures indistinguishable from their wild-type littermates (23, 24). Postnatally, sexual maturation, e.g. spermatogenesis and accessory sex organ maturation is blocked by the lack of testosterone, which then is purely LH-dependent. In part, this mouse model resembles the pathogenesis of Leydig cell hypoplasia caused by inactivating LHR mutations.

The potential role of the AFNGT motif

Within the extracellular domain the glycoprotein hormone receptors, LHR, FSHR and TSHR, share some characteristic motifs. The most prominent ones are the leucine-rich repeats, consisting of approximately 24 amino acids, spread throughout the whole extracellular domain (10, 24, 25). They are believed to form a horseshoe-shaped extracellular domain essential for ligand binding. Recently, it has been shown that the leucine-rich repeats are also present in other G-protein-coupled receptors identified in human and other species such as the fruitfly and sea anemone (26). These receptors bear a variable number of leucine-rich repeats, but their ligands are still unknown. Phylogenetic studies revealed that there are three subclasses of leucine-rich repeats containing G-protein-coupled receptors present; the TSHR, FSHR and LHR belong to one subclass of these receptors (26). While leucine-rich repeats are a hallmark for this type of receptor other conserved elements within the extracellular domain are difficult to identify. One common element which can be allocated within all three glycoprotein hormone receptors is the AFNGT motif, a stretch of five amino acids, completely conserved in the TSHR, FSHR and LHR and among different species (10).

Interestingly, the AFNGT motif, in part, contains a putative glycosylation signal site (NXS/T), whereas no function can be allocated to the two other amino acids (A and F). For the FSHR two mutations in this motif have been described in patients (10). One changes A189 into V, thereby leading to an almost complete inactivation of the receptor. Clinically, this inactivating mutation has been characterized on the female side by an FSH-resistant ovary and on the male side by impaired spermatogenesis. Extensive functional studies on this mutation revealed that the FSHR A189V
mutation has normal binding affinity but significantly reduced plasma membrane expression and signal transduction. Using confocal microscopy most FSHR immunoreactivity in cells transfected with the mutated cDNA appeared to be sequestered within the cells indicating a possible function of the AFNGT motif in receptor folding (27–30). The second FSHR described mutation within this motif led to the transition from N191 to I. Since the patient in whom this mutation was identified was a heterozygous carrier, no phenotype was described. Functional studies with this mutated FSHR in transiently transfected COS-7 cells revealed a decrease in cAMP production when compared with the wt-FSHR upon FSH stimulation (9).

The AFNGT motif is also present in the two recently identified human leucine-rich-repeats containing G-protein-coupled receptors (LGR 4 and 5) (26); however, it is interrupted by the insertion of a G or T residue, giving rise to an AF(G/T)NGT motif.

In our experimental approach we characterized the consequences of mutations within the AFNGT motif on signal transduction. For this purpose we have introduced into the FSHR and the LHR cDNA all previously described mutations in patients for the AFNGT motif. The functional studies revealed that the F194V LHR mutation identified in our patient almost completely inactivates the LHR and also the equivalently mutated FSHR (Figs 5 and 6). These findings were confirmed by the dose-dependency experiments. Displacement experiments revealed that binding per se was not grossly affected, but trafficking of the receptor protein to the cell membrane was impaired. Thus trapping of the receptor within the cell compartments seems to be the major mechanism for the observed inactivation of the F194V LHR.

The A189V mutation, originally described for the FSHR, displayed the expected reduction in cAMP production and the same mutation in the LHR A193V also markedly decreased cAMP production; however, some residual activity can be observed. This is in agreement with a recent study in which the effects of the A189V of the FSHR were studied in more detail. The mutation leads to an intracellular sequestration of the FSH and A193V in the LHR leads to intracellular trapping of the receptor, indicative of an impaired receptor integration in the plasma membrane. This is in full agreement with our data on the FSHR N191I mutant, in which the stimulation by pFSH could evoke a significant increase in cAMP production.

While in the FSHR at least one site has to be glycosylated to maintain receptor function, the LHR seems to be more flexible with respect to the extent of glycosylation, and N-linked carbohydrates do not seem to be essential for receptor function per se. For example, mutation of N193 to Q in the rat FSHR severely affects receptor integration in the plasma membrane. In subsequent experiments the adjacent T195 was mutated to A, thus removing the glycosylation motif site; however, this mutation did not have any effect on receptor function. This implies that glycosylation at position N193 is not crucial for receptor function, although the kind of amino acid at this position might be of importance. In our case we have introduced isoleucine (I) at position 193, a mutation previously described in a patient. Isoleucine is hydrophobic, whereas Q is a more polar residue. These different biochemical characteristics of I and Q might explain why we could observe a clear increase in cAMP production in the mutated 193I LHR upon hCG stimulation.

Concerning the mechanisms by which mutations at glycosylation sites might influence receptor function, Segaloff and colleagues (31, 32) demonstrated that glycosylations per se are not necessarily required for hormone binding; however, they are crucially involved in folding of the nascent receptor protein into a conformation compatible with hormone binding. This applies more to the FSHR than to the LHR.

Collectively, these experiments demonstrate that the AFNGT motif is crucial for gonadotropin receptor function. Mutations in this region presumably influence receptor folding, which in turn affects receptor trafficking to the cell membrane. This assumption is also evident from studies showing that the A189V mutation in the FSH and A193V in the LHR leads to intracellular sequestering of the receptor, indicative of an impaired membrane integration (2, 29, 30).

Assuming that such a change affects receptor folding/trafficking, the LGR-type receptors might have a different three-dimensional structure of the extracellular domain compared with the LHR and FSHR, where changes in this motif affect receptor folding.

In the LHR and FSHR the overall frequency of mutations is low (2, 10, 28, 33); in the case of the FSHR only two other compound heterozygous mutations, aside from the mutations in the AFNGT motif of the FSHR, have been identified. In the LHR, mutations in the AFNGT motif have been associated with a variety of clinical conditions, including infertility, polycystic ovary syndrome, and decreased fertility.

Segaloff and colleagues (31) have demonstrated the role of the putative glycosylation motif (NXS/T) within the AFNGT amino acid stretch for LHR and FSHR expression and function. In the rat FSHR an N192A mutation does not lead to inactivation of the receptor; instead, the mutated receptor displays somewhat reduced human FSH binding which is caused by a lower number of receptors being expressed at the plasma membrane. This is in full agreement with our data on the FSHR N191I mutant, in which the stimulation by pFSH could evoke a significant increase in cAMP production.

Collectively, these experiments demonstrate that the AFNGT motif is crucial for gonadotropin receptor function. Mutations in this region presumably influence receptor folding, which in turn affects receptor trafficking to the cell membrane. This assumption is also evident from studies showing that the A189V mutation in the FSH and A193V in the LHR leads to intracellular sequestering of the receptor, indicative of an impaired membrane integration (2, 29, 30). Assuming that such a change affects receptor folding/trafficking, the LGR-type receptors might have a different three-dimensional structure of the extracellular domain compared with the LHR and FSHR, where changes in this motif affect receptor folding.
motif, have been reported and for the LHR approxi-
mately 15 mutations have been described so far. The
identification of an inactivating mutation in the
AFNGT motif in exon 7 of the LHR (this report),
together with the previously described mutations in
this region for the FSHR, points to the crucial function
of this motif and makes exon 7 a prime candidate to
start with when screening glycoprotein hormone
receptors for mutations.

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