

# Mutant Luteinizing Hormone Receptors in a Compound Heterozygous Patient with Complete Leydig Cell Hypoplasia: Abnormal Processing Causes Signaling Deficiency

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Over the past 5 yr several inactivating mutations in the LH receptor gene have been demonstrated to cause Leydig cell hypoplasia, a rare autosomal recessive form of male pseudohermaphroditism. Here, we report the identification of two new LH receptor mutations in a compound heterozygous case of complete Leydig hypoplasia and determine the cause of the signaling deficiency at a molecular level. On the paternal allele of the patient we identified in codon 343 a T to A transversion that changes a conserved cysteine in the hinge region of the receptor to serine (C343S); on the maternal allele a T to C transition causes another conserved cysteine at codon 543 in *trans*-membrane segment 5 to be altered to arginine (C543R). Both of these mutant receptors are completely devoid of hormone-induced cAMP reporter gene activation. Using Western blotting of expressed LH receptor protein with a hemagglutinin tag, we further show that despite complete

absence of total and cell surface hormone binding, protein levels of both mutant LH receptors are only moderately affected. The expression and study of enhanced green fluorescent protein-tagged receptors confirmed this view and further indicated that initial translocation to the endoplasmic reticulum of these mutant receptors is normal. After that, however, translocation is halted or misrouted, and as a result, neither mutant ever reaches the cell surface, and they cannot bind hormone. This lack of processing is also indicated by reduced presence of an 80-kDa protein, the only *N*-linked glycosylated protein in the LH receptor protein profile. Thus, complete lack of signaling by the identified mutant LH receptors is caused by insufficient processing from the endoplasmic reticulum to the cell surface and results in complete Leydig cell hypoplasia in this patient. (*J Clin Endocrinol Metab* 87: 2506–2513, 2002)

**A**MONG THE VARIOUS disorders of sex differentiation, male pseudohermaphroditism is defined as a defective masculinization of internal and/or external genitalia in 46,XY individuals. Male pseudohermaphroditism can be caused by gonadal dysgenesis or defects in T action, such as androgen insensitivity or 5 $\alpha$ -reductase deficiency (1). Another cause of male pseudohermaphroditism is defective T synthesis as a consequence of an enzymatic defect in the steroidogenic pathway or an anomaly in Leydig cell differentiation referred to as Leydig cell hypoplasia (LCH).

LCH is a rare form of male pseudohermaphroditism with an autosomal recessive pattern of inheritance (2–6). LCH patients present with a female phenotype, primary amenorrhea, absence of secondary sex differentiation at puberty, and elevated LH levels with abnormally low T levels. In the testes of these patients mature Leydig cells are absent, indicating a primary defect of Leydig cell development. In addition, milder forms of LCH have been

described in phenotypic males with micropenis and/or hypospadias (7–10).

The underlying gene defect in LCH was first identified by Kremer *et al.* (11). They observed a homozygous missense mutation in the LH receptor (LHR) gene in a patient with the severe form of LCH. The mutation resulted in an alanine to proline transition in the sixth *trans*-membrane segment of the LHR protein, which completely obliterated transduction of the signal of LH binding to an intracellular increase in cAMP. After this report, a number of different LHR gene mutations were identified in patients with LCH, including in males with hypospadias and micropenis (reviewed in Refs. 12–16). As may be expected, the inactivating LHR gene mutations are not only found in the *trans*-membrane domain, but are scattered throughout the protein. In addition, the mutations include not only missense mutations, but also nonsense mutations and small and large deletions and insertions, all leading to a completely or partially inactive receptor molecule.

In the present paper we report the identification of two novel LHR mutations that cause LCH in the described patient. In addition, using enhanced green fluorescent protein

Abbreviations: EGFP, Enhanced green fluorescent protein; ER, endoplasmic reticulum; HA, hemagglutinin; hLHR, human LH receptor; LCH, Leydig cell hypoplasia.

(EGFP)-tagged receptors we show that lack of signaling by the mutant receptors is caused by aberrant intracellular processing of the mutant LHR protein.

## Subjects and Methods

### Subject

The patient was born at term after uncomplicated pregnancy and delivery as the first child of unrelated parents of Caucasian origin. No family history was noted. She presented at the age of 12 yr with a female phenotype and inguinal hernias. Further examination revealed a 46,XY karyotype, cryptorchid testes in the inguinal canal, a blind vaginal pouch, and absence of Müllerian duct derivatives. At the age of 14 yr no breast development was observed. T levels were low (0.21 ng/ml) and did not respond to an hCG stimulation test (0.19 ng/ml). In contrast, LH levels were high (68 IU/liter) and increased to 150 IU/liter after a GnRH challenge. Histological analysis of the testes, which were removed at 14 yr of age, revealed seminiferous tubules surrounded by thick hyalinized walls and absent spermatogenesis. In the interstitial space few immature, but no mature, Leydig cells could be found. Informed consent for this study was obtained from the mother of the subject.

### Mutational analysis

Genomic DNA was extracted from peripheral blood obtained from the patient and her mother. Using PCR, two overlapping fragments of exon 11 of the LHR gene were amplified with two primer sets: primer set 1: LHR1, 5'-ggctgaggctattatggcttt-3' (in intron 10); and LHR2, 5'-tggggaagcaataactgacc-3'; and primer set 2: LHR3, 5'-aagatggcacacatcact-3'; and LHR4, 5'-ttctcaatcaaccctttatg-3'. The PCR products (720 and 810 bp, respectively) were verified using gel electrophoresis, purified, and subsequently manually sequenced using a commercial sequencing kit (Amersham Pharmacia Biotech, Saclay, France). The sequence samples were analyzed on standard denaturing acrylamide gels (17).

### Construction of the mutant LHR cDNA expression vectors

The coding region of the LHR, extended with an immunotag [hemagglutinin 1 (HA1)] at the C-terminus (18), was placed downstream of the simian virus 40 large T antigen promoter in the expression plasmid pSG5 (19, 20), resulting in pSG5-human (h) LHR. The HA1 tag does not affect expression and signal transduction of the wild-type LHR (18). pSG5-hLHR-EGFP was constructed by inserting in-frame the EGFP coding sequence immediately downstream of the last codon of the LHR open reading frame. Therefore, EGFP was excised from pEGFP-N1 (CLONTECH Laboratories, Inc., Palo Alto, CA) as an *Sma*I-*Hpa*I (nucleotides 658 to 1518) fragment and cloned in the unique *Hpa*I site of pSG5-hLHR. The C543R mutation was introduced in the expression vectors pSG5-hLHR and pSG5-hLHR-EGFP using a previously described approach (20), except that primers LHR543CRFOR (5'-gccttctcataattcgtgctgtctacatt-3') and LHR543CRREV (5'-aatgtagcaagcacgaattatgaagaaggc-3') were used in the first PCR amplification reaction. The mutant expression vector obtained was named pSG5-hLHRC543R. For the C343S substitution, a 1028-bp fragment was obtained in a two-step PCR amplification strategy using the flanking primers 181FOR (5'-ctccctgtcaaatgatcc-3') and 11.1REV (5'-attgcacatgagaaacagg-3'). The mutation was introduced in the first PCR step using primers LHR343CSFOR (5'-ttacccaagacacccgaagtgcctctgaa-3') and LHR343CSREV (5'-ttcaggagcacttcgggtgtcttgggtaa-3'). The amplified mutated DNA fragment was digested with *Hind*III and *Bsu*36 I (positions 202 and 1082 of the LHR open reading frame), and the resulting fragment was exchanged for the wild-type *Hind*III-*Bsu*36 I fragment in pSG5-hLHR and pSG5-hLHR-EGFP, resulting in pSG5-hLHRC343S and its EGFP extended analog. Primers were purchased from Eurogentec (Seraing, Belgium), and mutagenesis was verified by DNA sequence analysis of the exchanged fragments.

### Analysis of signal transduction and hCG binding

COS-1 cells were maintained as previously described (18). For estimation of hormone-dependent induction of cAMP and total and cell

surface binding, subconfluent COS-1 cells were transiently transfected (21) with 2  $\mu$ g of the cAMP-reporter plasmid pCRE<sub>6</sub>Lux (22); 1  $\mu$ g pRSVlacZ (23) as a control for transfection efficiency; 10  $\mu$ g pSG5, pSG5-hLHR, pSG5-hLHR-EGFP, pSG5-hLHRC343S, or pSG5-hLHRC543R; and 8  $\mu$ g carrier DNA/75 cm<sup>2</sup> culture flask. In the receptor cotransfection experiment, 5  $\mu$ g of each receptor expression plasmid were combined. Two days after transfection the cells were trypsinized and plated in 24-well tissue culture plates (Nunc, Roskilde, Denmark) for luciferase and  $\beta$ -galactosidase measurements and in a 75-cm<sup>2</sup> tissue culture flask (Nunc) for total and cell surface hCG binding. To determine the cAMP response, cells were incubated the next day in culture medium containing 0.1% BSA and the indicated concentration of hCG (Organon, Oss, The Netherlands). After 6 h the cells were lysed, and luciferase activity was determined (24). hCG binding to intact cells as well as to cell membranes was performed as described previously (18, 25).

### Western blotting

SDS-PAGE was performed essentially as described previously (18). Briefly, COS-1 cells were transfected with 10  $\mu$ g of the expression vector pSG5 containing the indicated HA1-tagged LHR cDNA. Three days after transfection, the cells were harvested, and equal amounts of protein (10  $\mu$ g) were separated using 10% SDS-PAGE. To remove N-linked glycosylated groups, samples were boiled and treated with N-glycosidase F (Roche Molecular Biochemicals). After Western transfer, LHR protein was visualized using an HA1-specific monoclonal antibody in combination with the Renaissance chemiluminescence detection kit (NEN Life Science Products, Du Pont de Nemours, Dreieich, Germany).

### Microscopic analysis of hLHR-EGFP in COS-1 cells

COS-1 cells were transfected by the calcium phosphate DNA precipitation method (see above) on 2  $\times$  2 cm<sup>2</sup> Lab-Tek chamber slides (Nunc, Naperville, IL) with 2  $\mu$ g pSG5-hLHR-EGFP, pSG5-hLHRC343S-EGFP, or pSG5-hLHRC543R-EGFP. Twelve hours after transfection, precipitate was removed and replaced by fresh medium with 0.1% (wt/vol) BSA. The living cells were observed directly on the chamber slide using an inverted fluorescence microscope (Diaphot 200, Nikon, Champigny-sur-Marne, France) with a fluorescein isothiocyanate filter. This microscope was coupled to a CCD camera (Night Owl, EGG-Berthold, Evry, France) to record cells. The cells were first observed after 24, 48, and 72 h of transfection.

## Results

### Sequence analysis of exon 11 of the LHR gene

Sequence analysis of two overlapping PCR fragments of exon 11 of the LHR gene derived from genomic DNA revealed two heterozygous missense mutations (Fig. 1). At codon 343 we identified a TGT to AGT change resulting in a change from cysteine to serine at the protein level (Fig. 1A). In addition, a TGT to CGT modification was found at codon 543 that changed the cysteine at this position to arginine (Fig. 1B). The mother of the proband was also heterozygous at codon 543 for same mutation, indicating that this mutant allele was maternal. Unfortunately, DNA from the father was not available. The patient did not inherit both mutations from the mother, as we observed a homozygous wild-type sequence at codon 343 in the maternal DNA sample (Fig. 1A). Thus, the missense mutation at codon 343 was either derived from the father or had occurred *de novo*.

### Biochemical characterization and functional analysis of mutant receptors

To determine the effects of the missense mutations on the LHR protein, wild-type LHR and the two mutant receptors were expressed in COS-1 cells and visualized using Western

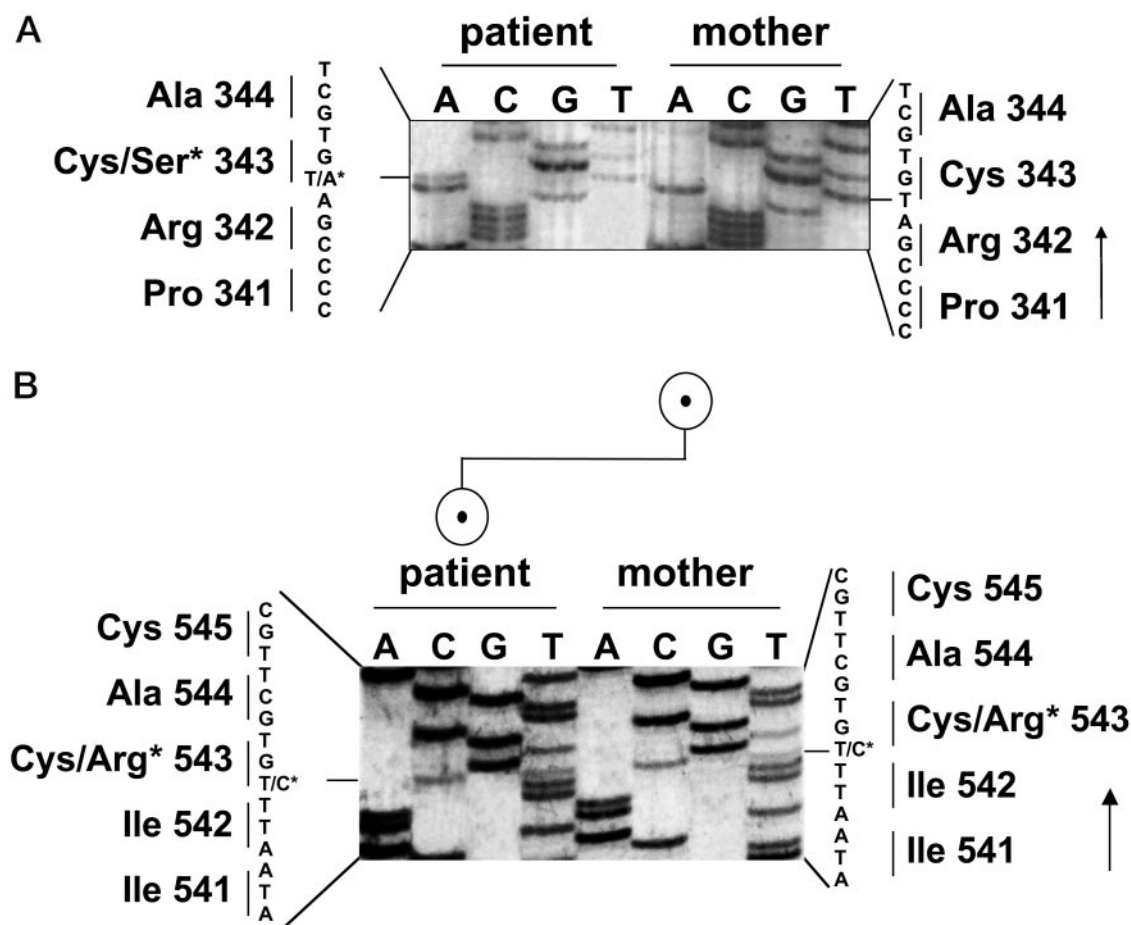


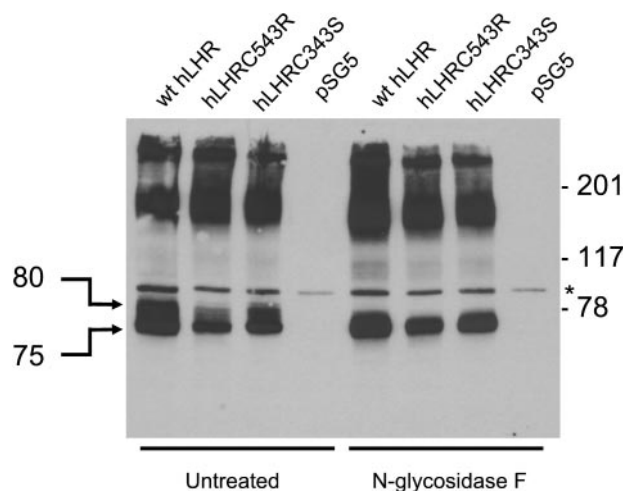
FIG. 1. Two heterozygous missense mutations were identified in LCH patient. Genomic sequence in the region of codon 343 (A) and codon 543 (B) of the LHR gene of the patient and her mother are shown. Mutations are indicated with an *asterisk*. The patient, but not her mother, is heterozygous at codon 343 for a T to A base change that results in an amino acid change from Cys to Ser; the patient and her mother are both heterozygous at codon 543 for a T to C nucleotide change that results in an amino acid change from Cys to Arg.

blotting (Fig. 2). In the control lane, containing proteins from COS-1 cells transfected with the empty expression vector pSG5, one protein band with molecular mass of 90 kDa was observed (Fig. 2, *asterisks*). As previously described, this band was due to nonspecific interaction of the HA1 antibody with an endogenous protein of COS-1 cells (18). In addition to this nonspecific signal, several bands of different intensities and molecular masses were observed in the COS-1 cells transfected with wild-type LHR. These bands probably represent different forms (glycosylated and/or multimerized) of the LHR protein. In addition to the two clearly visible bands with molecular masses of 75 and 80 kDa (indicated with *arrows*), multiple protein bands of different intensities of 150 kDa and larger were present. These latter bands may be the result of nonspecific association or incomplete solubilization of the hydrophobic LHR proteins. The appearance of the 80-kDa protein band was not sharp, suggesting that it may consist of multiple similarly sized proteins. In COS-1 cells transfected with the mutant LHR expression constructs, pSG5-hLHRC343S and pSG5-hLHRC543R, a similar pattern of protein bands was observed, with the distinct exception of the 80-kDa protein (Fig. 2), which is much more abundant in the wild-type LHR-expressing cells. In addition, the intensity of

all LHR-specific bands, especially the 75-kDa band, was reduced in COS-1 cells transfected with pSG5-hLHRC543R, suggesting that the expression of total LHR protein of this mutant receptor was slightly reduced. *N*-Glycosidase F treatment of the samples resulted in the complete disappearance of the heterogeneous 80-kDa band from both wild-type and mutant LHR-transfected cell extracts, indicating that this particular protein is *N*-glycosylated. None of the other bands showed a clear reduction in size after treatment, indicating that *N*-linked glycosyl groups are small, completely absent, or not accessible to the enzyme. The heterogeneous feature of the 80-kDa protein agrees with the fact that it is an *N*-linked glycosylated protein, because *N*-linked glycosylation is often heterogeneous (26).

Subsequently, we determined whether the mutant receptors after synthesis were properly transported to the cell surface and were able to display proper ligand binding. Cells transfected with pSG5-hLHR showed high affinity binding ( $K_d = 3.5$  nM) and a number of receptors equivalent to previous experiments (binding capacity, 25 fmol/mg protein) (18), whereas no binding was detectable to intact cells transfected with either the pSG5-hLHRC343S or pSG5-hLHRC543R expression vector (Table 1). These results indi-





**FIG. 2.** Expression of the mutant LHR cDNAs in COS-1 cells. COS-1 cells were transfected with the indicated HA-tagged expression vectors. Three days after incubation, the cells were lysed, and equal amounts of total protein were loaded in Laemmli sample buffer on a 10% SDS-PAGE gel. The indicated samples were treated with *N*-glycosidase F. After Western blotting, specific protein bands were visualized using a monoclonal antibody against the HA tag present at the C-terminus of the LHR protein. The *asterisks* indicate a nonspecific band present in COS-1 cells. The *arrows* indicate the two lower molecular mass LHR proteins of 75 and 80 kDa. The abundance of the *N*-linked glycosylated 80-kDa protein is clearly reduced in both mutant receptors.

**TABLE 1.** Scatchard analysis of [ $^{125}$ I]hCG binding to intact COS-1 cells expressing wild-type and mutant LH receptors

	Wild-type hLHR	hLHRC343S	hLHRC543R
$K_d$ (nM)	3.5	—	—
$B_{max}$	25	— <sup>a</sup>	— <sup>a</sup>

$B_{max}$  is expressed as femtomoles per mg total protein.

<sup>a</sup> Number of binding sites not detectably different from empty vector-transfected COS-1 cells. Specific binding to intact empty vector transfected COS-1 cells was undetectable to low.

**TABLE 2.** [ $^{125}$ I]hCG binding to solubilized COS-1 cells expressing wild-type and mutant LH receptors

	Wild-type hLHR	hLHRC343S	hLHRC543R
Specific binding <sup>a</sup>	1177 ± 22	28 ± 12 <sup>b</sup>	99 ± 43 <sup>b</sup>

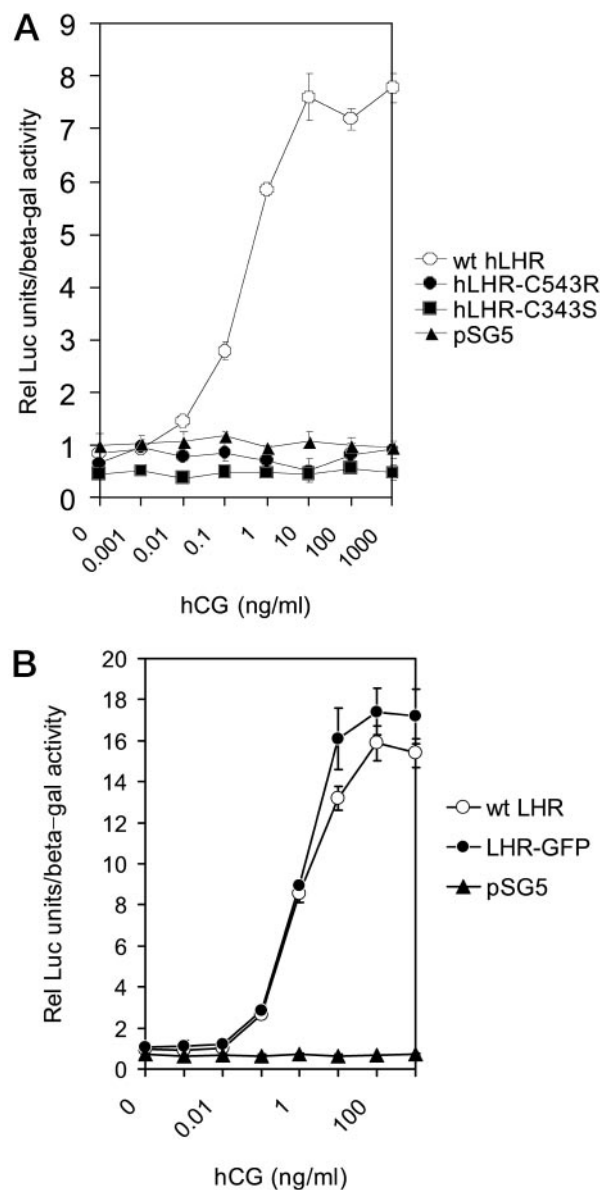
<sup>a</sup> Specific binding is expressed as counts per min/ $\mu$ g total protein.

<sup>b</sup> Binding not detectably different from empty vector-transfected COS-1 cells. Specific binding to membranes of empty vector-transfected COS-1 cells was 31 ± 16 cpm/ $\mu$ g total protein.

cate that the number of mutant receptor molecules present on the cell surface was too low to be detected. Subsequently, we determined the total hCG binding capacity of wild-type and mutant LHR-transfected COS-1 cells (Table 2). The binding capacity of solubilized extracts derived from COS-1 cells transfected with either mutant receptor was not different from that of cells transfected with the empty expression vector. However, solubilized extracts from cells transfected with the wild-type LHR showed considerable binding capacity.

We determined whether the mutant receptors have a residual ability to transduce the hormonal signal despite undetectable cell surface binding and absent cell surface

localization. Cells transfected with pSG5-hLHR showed a vigorous response to increasing concentrations of hCG, with an  $ED_{50}$  of approximately 5 ng/ml (Fig. 3A). In contrast, cells expressing hLHRC343S or hLHRC543R did not respond to hCG, indicating that the mutant receptors are completely deficient in signaling (Fig. 3A). To mimic the heterozygous phenotype of this patient *in vitro*, we also transfected both



**FIG. 3.** hCG induction of cAMP response element reporter activity by different LHR cDNAs expressed in COS-1 cells. COS-1 cells, cotransfected with the indicated LHR expression vector, a cAMP-responsive luciferase reporter plasmid, and a  $\beta$ -galactosidase reporter plasmid driven by a constitutively promoter, were incubated in the presence of the indicated concentrations of hCG. Luciferase activity measured in the cell lysates and normalized for  $\beta$ -galactosidase activity is presented. The results of one experiment of two performed are shown and are presented as the mean ± SD ( $n = 4$ ). A, hLHRC343S and hLHRC543R, but not the wild-type receptor, are completely devoid of hCG-dependent signal transduction. B, Wild-type hLHR and its EGFP-extended analog display identical hormone-dependent CRE activation.

mutant receptors simultaneously (not shown). Cells transfected with a single defective LHR as well as cells transfected with the two mutant LHR cDNAs together showed no detectable hCG-induced reporter activity. Thus, these two mutant receptors cannot complement each other *in vitro*, in line with the complete LCH phenotype observed in this patient. The absence of complementation contrasts with previous findings that severely N- and C-terminal truncated LHRs were able to complement each other (27). The difference may be that point mutations that block signaling are not sufficiently disruptive to induce the formation of receptor hybrids.

The complete absence of ligand binding and, therefore, signaling of both mutant LHRs contrasted with their near-normal protein expression levels, as demonstrated by Western blotting. To localize the mutant receptor protein in the cell we constructed LHR isoforms that were extended at the C-terminal end with an EGFP fusion protein that can be visualized using fluorescence microscopy. In COS-1 cells the wild-type LHR fused to EGFP displayed similar cell surface binding as the wild-type LHR without EGFP (not shown). Moreover, hormone (hCG)-dependent signal transduction, as measured by cAMP-responsive element activation, was also indistinguishable from that of wild-type LHR (Fig. 3B), indicating that extension of the LHR molecule with an EGFP fusion at the C-terminus has no detectable effect on receptor function. Subsequently, localization of mutant and wild-type LHR was studied at different time points after transfection in COS-1 cells (Fig. 4). Interestingly, 24 h after transfection both the two mutant and the wild-type receptors showed similar localization. At this time, localization resembled en-

doplasmic reticulum (ER) staining consisting mainly of intense vesicular perinuclear and nuclear membrane fluorescence (28–29a). At 48 h the pattern for the wild-type receptor, however, had significantly changed. At that time cell surface fluorescence appeared (Fig. 4, *arrow*) while the intense perinuclear staining remained visible. At 72 h the picture was similar, except that cell surface staining was more pronounced. The pattern for both mutant receptors was clearly different from that of the wild-type LHR. At 48 h the vesicular phenotype was still visible, but seemed less closely localized to the perinuclear area, with more widespread distribution throughout the cytoplasm. In addition, some small and large foci appeared, suggesting that the mutant receptor molecules may have trafficked in part beyond the ER compartment. At 72 h, these foci were more visible, particularly the large ones. These large vesicles were not observed in wild-type receptor-transfected cells. Cell surface fluorescence of EGFP was never observed with either mutant LHR.

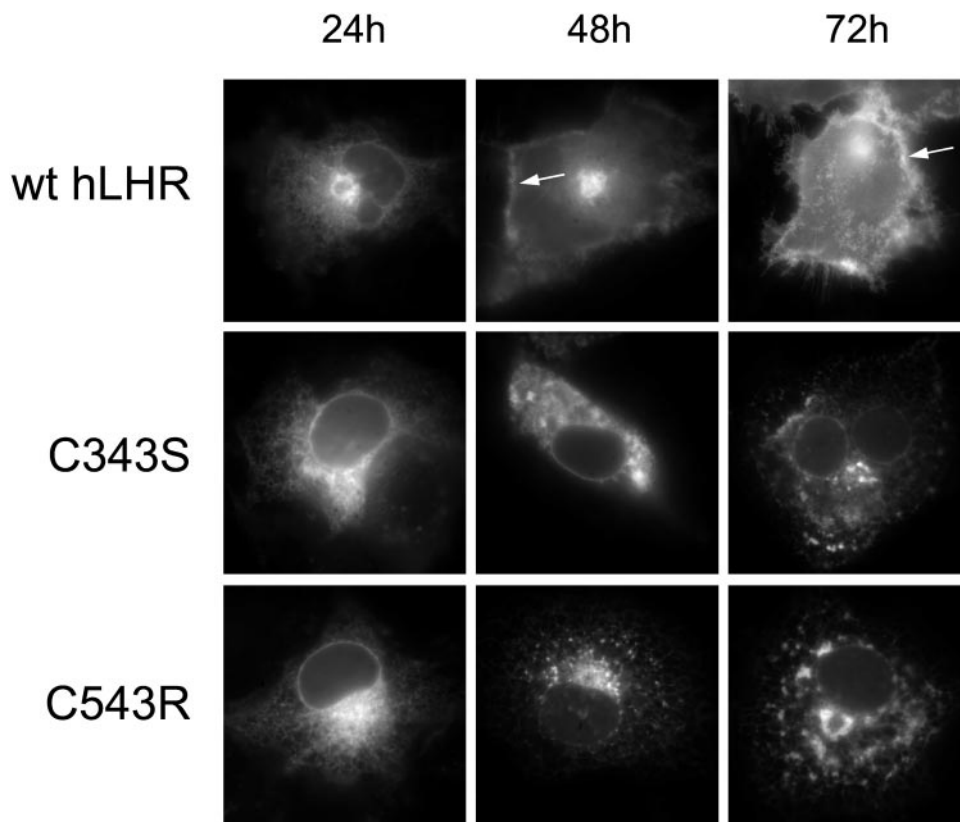
### Discussion

The present report describes two compound heterozygous missense mutations in the LHR gene that cause a complete form of Leydig cell hypoplasia and determines the molecular mechanism that underlies the signaling deficiency.

#### *Two new inactivating mutations in Leydig cell hypoplasia*

The C543R amino acid change is located in the fifth transmembrane segment of the LHR molecule. Interestingly, at a neighboring amino acid, I542, an activating mutation (I542L) has been described (30, 31). Thus, amino acid changes at

FIG. 4. Subcellular localization of EGFP-fused wild-type and mutant hLHRs in COS-1 cells. Cells were transfected with the indicated LHR expression vectors and observed using an inverted fluorescence microscope at 24, 48, and 72 h after transfection. At 24 h, wild-type and mutants receptors presented similar perinuclear distributions. Cell surface localization of wild-type receptor appeared at 48 h and increased at 72 h (indicated with an *arrow*). In contrast, no mutant processed to the cell surface. For both mutant receptors fluorescence remained cytoplasmic, with a widespread and heterogeneous distribution, including the appearance of small and large foci.



### *Improper processing beyond the endoplasmic reticulum*

The high molecular mass forms observed in the protein profile may represent dimeric and multimeric forms of the hLHR that play a direct role in signal transduction (39, 40), or they may represent a precursor of the mature protein, as has been shown for the rat LHR (41). Alternatively, the high molecular mass forms may also be SDS-PAGE artifacts, commonly observed for G protein-coupled receptors and other membrane-spanning proteins. Of the two lower molecular mass forms only the 80-kDa form appeared to be *N*-linked glycosylated. The 75-kDa protein clearly lacks *N*-linked glycosyl groups and does not show hormone binding or cell surface expression, as it is present in the mutant receptor protein profile. Often the addition of glycosyl groups to glycoproteins in the ER is required for most proteins to acquire the active conformation (26, 42), although the role of glycosylation in hormone binding to gonadotropin receptors

					*											+	+	←	hinge region					TM region →						
h LHR	339	K	T	P	R	C	A	P	E	P	D	A	F	N	P	C	E	D	I	M	G	Y	D	F	L	R	V	L	I	W
h FSHR	342	V	D	V	T	C	S	P	K	P	D	A	F	N	P	C	E	D	I	M	G	Y	N	I	L	R	V	L	I	W
h TSHR	394	E	D	M	V	C	T	P	K	S	D	E	F	N	P	C	E	D	I	M	G	Y	K	F	L	R	I	V	V	W
h LGR5	537	H	S	V	Q	C	S	P	S	P	C	P	F	K	P	C	E	H	L	I	D	G	W	L	I	R	I	G	V	W
consensus		d	v			<b>C</b>	s	<b>P</b>	k	P	D	A	<b>F</b>	<b>N</b>	<b>P</b>	<b>C</b>	<b>E</b>	D	I	M	G	Y		F	L	<b>R</b>	v/i	I	i/v	<b>W</b>

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remains controversial. Tunicamycin treatment showed that the rat LHR does not require any N-linked glycosyl groups for hormone binding (43), whereas the rat FSH receptor does (44). However, direct mutagenesis in the rat LHR showed that one particular N-linked glycosylation site that is conserved in the human receptor is required for hormone binding (45). In the present study we show that the absence of the glycosylated 80-kDa form of the LHR correlates with the lack of hormone binding.

In conclusion, we have identified two novel LHR mutations that collectively cause LCH in this patient. In addition, we have determined that both mutants, despite almost normal protein expression, completely lack the capacity to bind ligand and the ability to transduce the signal. In addition, the N-linked glycosylated form of the receptor was almost absent in protein profiles of both mutant receptors. Finally, using novel GFP fusion proteins, we showed a defective translocation of mutant LHR from the ER to the cell surface. Taken together our data demonstrate that improper maturation of LHR mutants is the primary cause of the complete signaling defect leading to the absence of Leydig cell differentiation in the patient.

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