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Differential Inhibition of 17α-Hydroxylase and 17,20-Lyase Activities by Three Novel Missense CYP17 Mutations Identified in Patients with P450c17 Deficiency

ERICA L. T. VAN DEN AKKER, JAN W. KOPER, ANNEMIE L. M. BOEHMER, AXEL P. N. THEMMEN, MIRIAM VERHOEF-POST, MARIANNA A. TIMMERMAN, BARTO J. OTTEN, STENVERT L. S. DROP, AND FRANK H. DE JONG

Department of Pediatrics, Division of Endocrinology (E.L.T.v.d.A., A.L.M.B., S.L.S.D.), and Department of Internal Medicine, Section of Endocrinology (J.W.K., A.P.N.T., M.V.-P., M.A.T., F.H.D.J.), Erasmus Medical Center, 3000 DR Rotterdam, The Netherlands; and Department of Pediatrics, Division of Endocrinology, Radboud University Hospital (B.J.O.), 6500 HB Nijmegen, The Netherlands

The microsomal enzyme cytochrome P450c17 is an important regulator of steroidogenesis. The enzyme has two functions: 17α-hydroxylase and 17,20-lyase activities. These functions determine the ability of adrenal glands and gonads to synthesize 17α-hydroxylated glucocorticoids (17α-hydroxylase activity) and/or sex steroids (17,20-lyase activity). Both enzyme functions depend on correct steroid binding, but it was recently shown that isolated lyase deficiency can also be caused by mutations located in the redox partner interaction domain. In this article we present the clinical history and molecular analysis of two patients with combined 17α-hydroxylase/17,20-lyase deficiency and four patients with isolated 17,20-lyase deficiency. In these six patients, four missense *CYP17* mutations were identified. Two mutations were located in the steroid-binding domain (F114V and D116V), and the other two mutations were found in the redox partner interaction domain (R347C and R347H). We investigated the activity of these mutated proteins by transfection experiments in COS-1 cells using pregnenolone, progesterone, or their hydroxylated products as a substrate and measuring 17α-hydroxylase- and 17,20-lyase-dependent metabolites in the medium. The mutations in the steroid-binding domain (F114V and D116V) of P450c17 caused combined, complete (F114V), or partial (D116V) 17α-hydroxylase and 17,20-lyase deficiencies, whereas mutations in the redox partner interaction domain (R347C and R347H) displayed less severe 17α-hydroxylase deficiency, but complete 17,20-lyase deficiency. These findings are consistent with the clinical data and support the observation that the redox partner interaction domain is essential for normal 17,20-lyase function of P450c17. (*J Clin Endocrinol Metab* 87: 5714–5721, 2002)

IN THE STEROIDOGENIC pathway, cholesterol is converted into pregnenolone, which subsequently can be processed to either mineralocorticoids (no 17α-hydroxylation) or glucocorticoids (17α-hydroxylation) in adrenal glands or to sex steroids in adrenals and gonads (17α-hydroxylation and 17,20-lyase activity). The microsomal enzyme cytochrome P450c17 is an important switchpoint in this steroidogenic pathway because it has both 17α-hydroxylase and 17,20-lyase activities (Fig. 1). The first step necessary for P450c17 enzyme activity is steroid binding; then electron transfer occurs, enhanced by oxidoreductase to catalyze the hydroxylase reaction. The lyase activity is dependent on facilitation of the interaction of oxidoreductase with the redox partner-binding site (1). This interaction is enhanced by cytochrome *b5* (2) or phosphorylation of phosphoserine residues (3). Optimal functioning of the redox partner-binding site is especially essential for the lyase reaction.

The P450c17 enzyme is encoded by the *CYP17* gene, which is located on chromosome 10q24.3 (4). *CYP17* gene mutations are known to cause either complete or partial, combined, or isolated 17α-hydroxylase/17,20-lyase enzyme deficiencies. The study of these mutant enzymes found in patients with 17α-hydroxylase/17,20-lyase enzyme deficiencies can help us to understand the factors involved in P450c17 enzyme function. Until now 15 single base pair *CYP17* gene mutations have been found, causing combined 17α-hydroxylase and 17,20-lyase deficiencies (Table 1) (5–19). Only 2 mutations identified in patients with isolated complete 17,20-lyase deficiency were examined *in vitro* (Table 1) (14). These 2 mutations (R347H and R358Q) are located in the redox partner interaction domain. The observation of differential residual enzyme activity in naturally occurring mutations in various regions of the *CYP17* gene supports the hypothesis that 17,20-lyase activity depends on normal function of the redox partner interaction site of the P450c17 enzyme (Fig. 1) (20).

In this article we present the clinical and molecular data of six patients with 17,20-lyase deficiency. In the *CYP17* gene of these patients four different missense mutations were identified. Two mutations were located in the steroid-binding domain and two in the redox partner interaction domain. The effects of these four mutations on the enzymatic activity of the protein were examined by *in vitro* expression studies.

Abbreviations: AIS, Androgen insensitivity syndrome; DHEA, dehydroepiandrosterone; hCG, human chorionic gonadotropin; V_{max}, maximum velocity.

Subjects and Methods

Patients

The clinical picture and hormone levels of patient 1 have been described previously (21). This 17-yr-old female patient was referred be-

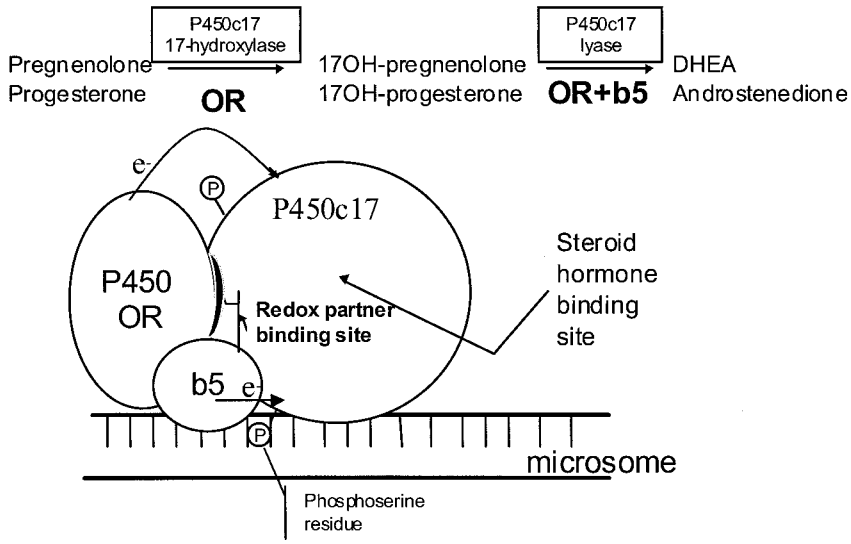

FIG. 1. Model of the mechanism of action of microsomal P450c17 and its differential regulation of 17-hydroxylase and 17,20-lyase activities. OR, Oxidoreductase; b5, cytochrome *b5*; e−, electron transfer [adapted from Auchus *et al.* (2)].

TABLE 1. All single base pair missense mutations in the *CYP17* gene described until now, their location, and their residual enzyme activity studied *in vitro*

Mutation	17α-Hydroxylase (%)	17,20-Lyase (%)	Site of mutation[a]	Reference
R35L	38	33	Membrane	15
Y64S	15		Membrane	8
G90N	<1	<1		11
F93C	11	10	Steroid	19
R96W	25	25		12
S106P	<1	<1	Steroid	5
F114V	2.2	<1	Steroid	This article
D116V	37.7	10.7	Steroid	This article
N177D	10	10	Steroid	15
P342T	20	20		6
R347H	65	<5%	Redox	14
	44.1	<1		This article
R347C	13.6	<1	Redox	This article
R358Q	65	<5	Redox	14
H373L	<1	<1	Heme	9
P409R	<1	<1	Steroid	16
R415C	8	10	Heme(?)	18
F417C	<1	<1	Heme	13 and 14
R440H	<1	<1	Heme	10
R496C	<10	<10	Heme	7
R496H	38	33	Heme	15

[a] Membrane, Membrane attachment domain; Steroid, steroid binding domain; Redox, redox partner interaction domain; Heme, heme binding domain; and Heme(?), uncertainty of affected domain (28).

cause of primary amenorrhea and lack of secondary sexual development (Tanner stage M1P1). She had one sister with normal secondary sexual development. The patient had female external genitalia, but no uterus and a 46,XY karyotype. Based on these results, the presumptive diagnosis of androgen insensitivity syndrome (AIS) was made. Secondary sexual development began after the start of estrogen substitution therapy. The patient underwent a hormonal evaluation before undergoing bilateral gonadectomy at the age of 20 yr. With the exception of fatigue, she had no complaints. Her blood pressure was elevated (150/110 mm Hg) despite low renin levels. Basal levels of androgens were low, and progesterone levels were high; basal cortisol was within the normal range, but rose insufficiently after ACTH (Table 2). These findings led to the diagnosis of combined 17α-hydroxylase/17,20-lyase deficiency. Results of *in vitro* studies of testis tissue of this patient, using pregnenolone, 17-hydroxypregnenolone, and dehydroepiandrosterone (DHEA) as substrate, followed by measurement of metabolites confirmed the absence of 17α-hydroxylase and 17,20-lyase activities (21).

Patient 2 is a 46,XY individual, who was born with an enlarged clitoris and no uterus. Her gonads were removed when she was 3 yr old. Based on the combination of ambiguous genitalia, lack of pubic hair at puberty, low-normal levels of androgens, and cortisol (Table 2), combined partial 17α-hydroxylase/17,20-lyase deficiency was diagnosed.

Patient 3 was born with complete female external genitalia and raised as a girl. She was the first child of consanguineous parents. The family history did not reveal any sexual differentiation disorders. At the age of 2 months, she had bilateral inguinal hernias, which contained testes, and subsequently, her karyotype was shown to be 46,XY. The presumptive diagnosis of AIS was made, and gonadectomy was performed. She was reevaluated at the age of 10 yr. Her blood pressure was 140/80 mm Hg despite normal renin levels. She underwent uneventful surgery twice. Basal levels of androgens were low, and progesterone was elevated. An ACTH test showed a low-normal basal level of cortisol that did not respond to ACTH (Table 3), suggesting partial 17α-hydroxylase deficiency with complete 17,20-lyase deficiency.

Patient 4, raised as a girl, was evaluated at age 14 yr because of delayed puberty. She had a 46,XY karyotype and complete female external genitalia with an absent uterus. The presumptive diagnosis of AIS was made, and she underwent gonadectomy. At age 28 yr she was

TABLE 2. Serum hormone concentrations of patients 1 and 2 with *CYP17* mutations in the steroid binding domain

Hormone	Patient 1 (F114V)[a]		Patient 2 (D116V)[a]
	Basal	ACTH	Basal
LH (U/liter)	107.4 (1.5–9.4)		
FSH (U/liter)	38.4 (2.6–7.4)		
Progesterone (nmol/liter)	14.5 (0.5–2)	14.9	3.2 (0.5–2)
17OH-progesterone (nmol/liter)	0.29 (<10)	0.31	0.1 (<10)
Cortisol (nmol/liter)	220 (200–800)	240 (>500)	248 (200–800)
DHEA (nmol/liter)	<0.1 (3.5–25)	<0.1	3.6 (3.5–25)
Androstenedione (nmol/liter)	<0.1 (2–10)	<0.1	1.67 (2–10)
Testosterone (nmol/liter)	<0.2 (0.5–3)	<0.2	0.3 (0.5–3)
Renin (pg/ml)	<0.1 (0–2.5)	<0.1	

Normal age-adjusted reference values are shown in *parentheses*. In the ACTH test, venous catheters were inserted, and serum levels of several hormones were determined in blood samples taken before and at 60 min after the iv administration of 0.25 mg ACTH.

[a] Age: patient 1, 17 yr; patient 2, 27 yr.

TABLE 3. Serum hormone concentrations of patients 3 and 4 with *CYP17* mutation R347C in the redox partner interaction domain

Hormone	Patient 3 (R347C)[a]		Patient 4 (R347C)[a]	
	Basal	ACTH	Basal	ACTH
LH (U/liter)	6.4 (<1)	5.4	10.6 (1.5–8)	
FSH (U/liter)	41.5 (<1)	46.5	37.3 (2–7)	
Progesterone (nmol/liter)	7.4 (0.5–2.0)	8.4	6.1 (0.5–2)	12.2
17OH-progesterone (nmol/liter)	2.4 (0.4–2.1)	2.6 (3.5–6.0)	1.9 (<10)	2.4
Cortisol (nmol/liter)	216 (200–800)	228 (>500)	232 (200–800)	285 (>500)
DHEA (nmol/liter)	0.3 (0.4–4.9)	0.5 (1.2–9.4)	0.0 (3.5–25)	0.0
Androstenedione (nmol/liter)	0.11 (0.24–0.8)	0.13 (0.4–1.6)	0.57 (2–10)	0.51
Testosterone (nmol/liter)	0.1 (<1)	0.1	0.1 (0.5–3.0)	
Renin (pg/ml)	12.5 (60–300)		10.2 (60–300)	

Normal age-adjusted reference values are shown in *parentheses*. The ACTH test is described in the legend to Table 2.

[a] Age: patient 3, 10 yr; patient 4, 28 yr.

reevaluated. Her blood pressure was 170/90 mm Hg despite normal renin levels. She underwent surgery several times without complications. She developed breasts on estrogen substitution. Her main complaint was the complete absence of pubic hair, which developed with testosterone propionate ointment therapy. Basal levels of androgens were extremely low with elevated progesterone. She had low-normal basal levels of cortisol, which did not rise after ACTH stimulation (Table 3). These data are consistent with partial 17α-hydroxylase deficiency and complete 17,20-lyase deficiency.

Patients 5 and 6 are siblings from consanguineous parents. Both 46,*XY* siblings were born with ambiguous external genitalia (Prader stage III). Patient 5 was assigned the male sex, and patient 6 was assigned the female sex on the basis of the sex designated by the parents at birth. There were no clinical signs of insufficient cortisol secretion. Both siblings underwent surgery several times without complications. Their basal serum levels of androgens were low and rose insufficiently after human chorionic gonadotropin (hCG) stimulation. Basal plasma renin activity was normal. Progesterone and 17-hydroxyprogesterone levels were high after hCG stimulation, and basal serum levels of cortisol were normal with some, but insufficient, rise during ACTH treatment (Table 4). On the basis of these observations the diagnosis of isolated 17,20-lyase deficiency was made.

Mutation analysis of the CYP17 gene

Genomic DNA was isolated from leukocytes according to standard procedures (22). Exons 1–8 and their flanking intron sequences of the *CYP17* gene were amplified individually by PCR using the primers and PCR conditions described by Monno *et al.* (5), followed by single strand conformation polymorphism analysis (23) and sequencing of the fragments that showed abnormal single strand conformation polymorphism patterns. To determine whether two mutations identified in the same patient were on separate alleles, allele-specific amplification was carried out (patient 3), or DNA of the parents was sequenced (patients 1, 2, and 4).

Construction of mutant expression plasmids

Mutant *CYP17* expression plasmids were constructed using the conditions described previously for the LH receptor (24). pcDNA3 was used as the expression vector. For the exchange of fragments containing the mutation in the wild-type *CYP17* expression vector, flanking primers were used: T7 forward, AATACGACTCACTATAG; and 638 reverse, CTGTATGACATTCAACTC for the F114V and D116V mutants; and 670 forward, GCAAAGACAGCCTGGTGGACC; and SP6 reverse, CTATAGTGTCACCTAAAT for the R347C and R347H mutations. The fragments were digested with the restriction enzymes *Bam*HI and *Bst*EII for the F114V and D116V mutants and *Bsp*EI and *Xho*I for the R347C and R347H mutations, respectively, and subsequently ligated into the expression vector that had been digested with the same enzymes. Primers that carry the mutation were as follows: F114V, GGGTATCGCCGTCGCTGACTCTG; D116V, CGCCTTCGCTGTCTCCGGAGCACACTGG; R347C, CAGTGACTGTAATCGATTGCTCCTGCTG; and R347C, CCAACTATCAGTGATCATAACCGTCTC and their reverse complements.

Culture and transfection of cells

COS-1 cells were grown in 24-well plates to 50% confluence and transfected with 0.4 μg/well (four wells per plasmid) of pcDNA expression plasmid containing wild-type or mutant *CYP17*. Transfection efficiency was monitored by cotransfection with a β-galactosidase expression plasmid. The transfected cells were washed and incubated in fresh medium. After 40 h when the COS-1 cells were 80–90% confluent, 1 μM pregnenolone, progesterone, 17-hydroxypregenenolone, or 17-hydroxyprogesterone was added to the medium. After 8 h, *i.e.* during the period of linear steroid production against time (data not shown), the medium was removed and assayed for products using RIAs for 17-hydroxypregnenolone (DRG Diagnostics, Marburg, Germany) and DHEA or 17-hydroxyprogesterone and androstenedione (Diagnostic Products, Los Angeles, CA). All transfections were performed at least twice.

TABLE 4. Serum hormone concentrations of patients 5 and 6 with *CYP17* mutation R347H in the redox partner interaction domain

Hormone	Patient 5 (R347H)				Patient 6 (R347H)			
	2 months old		7.5 yr old		2 wk old		2.5 yr old	
	Basal	hCG	Basal	ACTH	Basal	hCG	Basal	ACTH
LH (U/liter)	4.6 (2–12)				6.0 (2–26)			
FSH (U/liter)	2.9 (0–3.9)				4.7 (0–3.9)			
Progesterone (nmol/liter)	9.6 (0.5–2)		2.7 (0.5–2.0)	6.1			2.6 (0.5–2)	11.0
17OH-progesterone (nmol/liter)	4.5 (0.03–0.2)	14.9	1.3 (0.4–2.1)	1.5 (3.5–6)	13.4 (0.33–5.2)	19.6	1.3 (0.1–3.5)	2.1 (2.0–8.1)
Cortisol (nmol/liter)	194 (120–1158)	187	190 (157–414)	385 (>500)	270 (120–1158)	150	243 (157–690)	427 (>500)
DHEA (nmol/liter)	0.25 (0.9–8.2)	0.35	0.7 (0.4–4.9)	0.44 (1.2–9.4)	2.0 (0.9–8.2)	1.4	0.1 (0.3–1.5)	0.3 (0.7–3.3)
Androstenedione (nmol/liter)	0.14 (0.9–1.8)	0.15 (1.0–4.0)	0.10 (0.7–3.8)	0.09 (0.4–1.6)	0.3 (0.9–15)	0.4	0.07 (0.2–1.7)	0.05 (0.4–2.4)
Testosterone (nmol/liter)	0.7 (0.03–26)	4.1 (6.8–26)	0.02 (<0.5)	0.05	1.2 (0.03–26)	2.6 (6.8–26)	0.01 (<0.5)	0.09 (0.07–0.21)

Normal age-adjusted reference values are shown in *parentheses*. The ACTH test is described in the legend to Table 2. In the hCG test, serum hormone levels were determined in blood samples taken before and 72 h after the im administration of 1500 IU hCG.

In separate experiments, K_m and maximum velocity (V_{max}) were measured for the 17α-hydroxylase and 17,20-lyase activities of the enzyme by culturing the cells in the presence of 0.1, 0.2, 0.5, 1, or 2 μM pregnenolone or 17-hydroxypregnenolone, respectively, and measuring the concentration of the above-mentioned products using the same assays. Results were obtained with three or four wells of transfected cells per dose and were corrected for the concentrations measured in the medium of cells transfected with the empty vector.

Results

Mutations

The mutations identified in the *CYP17* gene of our six patients are shown in Table 5. Mutation analysis of patients 1, 2, and 4 revealed compound heterozygosity for three novel mutations: F114V (TTC→GTC), D116V (GAC→GTC), and R347C (CGT→TGT), combined with a frameshift mutation on the other allele: a 4-base duplication near codon 480. This duplication has previously been observed in several families of Dutch and German Mennonite descent and is known to completely abolish all P450c17 enzyme activity (25). The novel mutations identified in patients 1 and 2 (F114V and D116V) are in the steroid-binding domain. Patient 3 was a compound heterozygote for two new mutations: R347C (CGT→TGT) and a 25-bp deletion in exon 1 (nucleotides 204–228 of the coding sequence, counting from the A of the start codon). This deletion renders the message out of frame, resulting in a premature stop codon located at 28 residues after the deletion. We therefore expect that this deletion in allele 2 does not lead to the production of any functional protein. Patient 4 showed the same R347C mutation in one allele, whereas the 4-bp dup exon 8 mutation that was also present in patients 1 and 2 was present in the other. The mutation R347C is located in the redox partner interaction domain. Localization of two different mutations identified in the *CYP17* gene in patients 1–4 on separate alleles was confirmed by determining that each of their parents carried only one of these mutations (patients 1, 2, and 4) or by allele-specific amplification (patient 3). Finally, patients 5 and 6 (siblings) were homozygous for the R347H (CGT→CAT) mutation that has been described previously (14).

Expression of mutant proteins in COS-1 cells

The 17α-hydroxylase and 17,20-lyase activities of the mutated proteins were estimated using transient transfection in COS-1 cells and compared with those of the wild-type enzyme. The conversion of various concentrations of pregnenolone to 17-hydroxypregnenolone and DHEA was used as a measure for 17α-hydroxylase activity. Results are shown in Fig. 2A. Lineweaver-Burk plots calculated from these data are shown in Fig. 3. The r values for the regression lines were

TABLE 5. Mutations identified in the *CYP17* gene of six patients

Patient	Allele 1	Site of mutation[a]	Allele 2
1	F114V	Steroid	4-bp duplication exon 8
2	D116V	Steroid	4-bp duplication exon 8
3	R347C	Redox partner	25-bp deletion exon 1
4	R347C	Redox partner	4-bp duplication exon 8
5 and 6	R347H	Redox partner	R347H

[a] Steroid indicates steroid binding domain; redox partner indicates redox partner interaction domain (28).

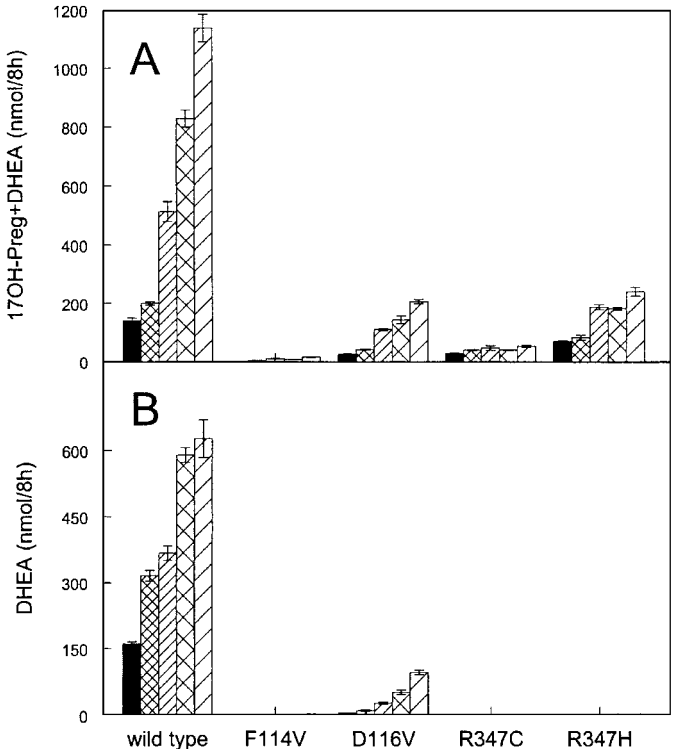

FIG. 2. Production of 17-hydroxypregnenolone (17OH-Preg) and DHEA after 8-h culture of transfected COS-1 cells in the presence of various concentrations of steroid precursors as a measure of 17α-hydroxylase activity (A; culture with pregnenolone) and of 17,20-lyase activity (B; culture with 17-hydroxypregnenolone). The cells were transiently transfected with wild-type *CYP17* and the *CYP17* mutants. Substrate concentrations were 0.1, 0.2, 0.5, 1, or 2 μM. Data are the mean ± SEM (n = 3 in A and n = 4 in B).

all above 0.95. Resulting apparent values for K_m and V_{max} have been summarized in Table 6. All V_{max} values for the mutated proteins were lower than that for the wild-type protein, whereas K_m values of the proteins with mutations in the steroid-binding domain were comparable to that of the wild-type protein, and the K_m values for the other two proteins were lower. As an example for the separate amounts of 17-hydroxypregnenolone and DHEA produced, the data obtained with 1 μM pregnenolone are plotted in Fig. 4. The use of a logarithmic axis allows a more clear indication of the amounts of DHEA. The wild-type enzyme and the F114V and D116V mutants converted between 10–20% of 17-hydroxypregnenolone to DHEA, whereas the R347C and R347H mutants did not produce measurable concentrations of DHEA despite the production of 17-hydroxypregnenolone.

Similar results were obtained using 17-hydroxypregnenolone as the substrate (Fig. 2B). Significant amounts of DHEA were only produced by the wild-type enzyme and the D116V mutant. The apparent V_{max} and K_m values for the wild-type enzyme were 1.55 min^{-1} and 0.35 μM, respectively. The production of DHEA by the mutated enzymes was too low to calculate V_{max} and K_m for the 17-hydroxypregnenolone to DHEA conversion by these enzymes. Conversion percentages at the highest concentration of 17-hydroxypregnenolone (2 μM) were 31.3%, 0.15%, 4.8%, 0.03%, and 0.12% for wild-type, F114V, D116V, R347C, and R347H, respectively.

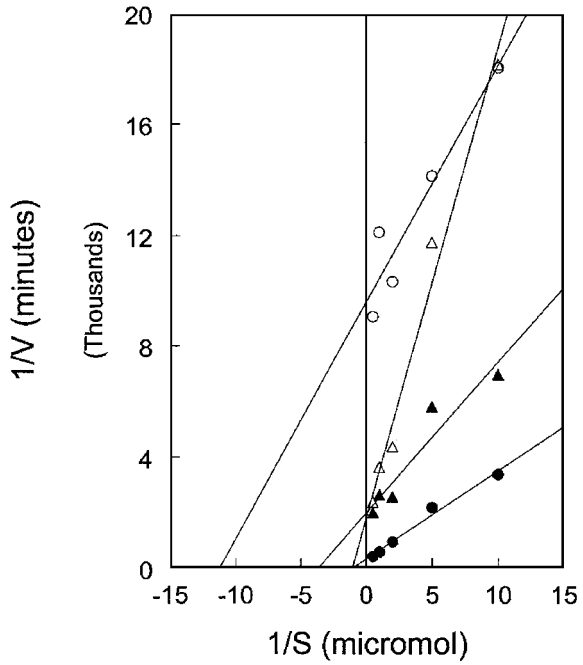

FIG. 3. Lineweaver-Burk plot for the conversion of pregnenolone to 17-hydroxypregnenolone plus DHEA, shown in Fig. 2A, by wild-type *CYP17* (●), and the D116V (△), R347C (○), and R347H (▲) mutants. Results for the F114V mutant are not shown, because all measured values are outside the axes used.

TABLE 6. Enzyme kinetic data on the 17α-hydroxylase activity of the *CYP17* mutants investigated

	Wild type	F114V	D116V	R347C	R347H
Km (μM)	0.97	0.76	0.95	0.08	0.27
Vmax (min^{-1})	3.0	0.04	0.56	0.10	0.50

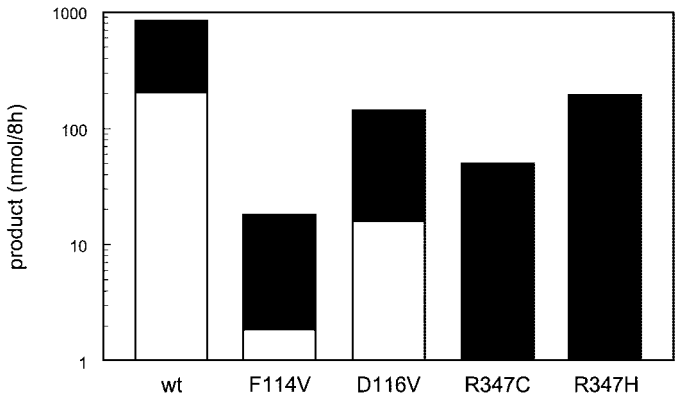

FIG. 4. Separate productions of 17-hydroxypregnenolone (■) and DHEA (□) from 1 μM pregnenolone by the COS-1 cells described in Fig. 2A. Note the logarithmic y-axis.

When progesterone was used as a substrate for testing the Δ^4-steroid biosynthetic pathway, the production of 17-hydroxyprogesterone, indicative of hydroxylase activity, showed a similar pattern as the production of 17-hydroxypregnenolone from pregnenolone, but at lower levels (Fig. 5). With neither progesterone nor 17-hydroxyprogesterone (results not shown) as substrate did we observe significant production of androstenedione by the mutant

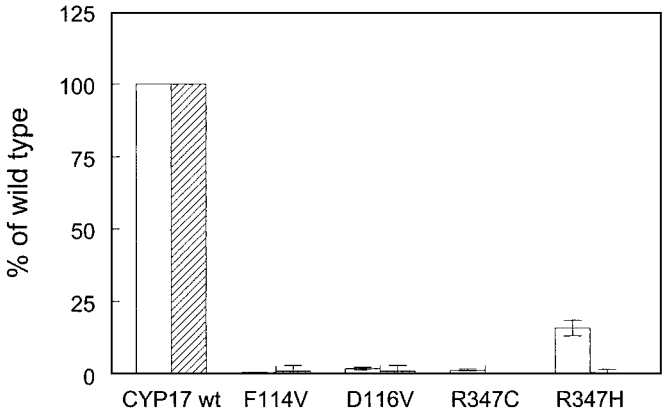

FIG. 5. Relative production of 17-hydroxyprogesterone as a measure of 17α-hydroxylase activity (□) and androstenedione as a measure of 17,20-lyase activity (▨) in COS-1 cells that were transiently transfected with wild-type (wt) *CYP17* or the *CYP17* mutants and cultured with 1 μM progesterone as substrate. For *CYP17* wild-type transfected cells, the 17-hydroxyprogesterone concentration was 760 nmol/liter, and the androstenedione concentration was 33 nmol/liter. Data are the mean ± SEM (n = 4).

proteins, indicating the absence of 17,20-lyase activity under these conditions.

Discussion

Combined 17α-hydroxylase/17,20-lyase deficiency (patients 1 and 2) is a well defined disorder (26). Isolated 17,20-lyase deficiency (patients 3–6) with subnormal 17α-hydroxylase function leading to a female phenotype or ambiguous genitalia, absence of sexual development, and normal basal cortisol levels without hypertension is extremely rare, with few clinical data. Isolated 17,20-lyase deficiency can be differentiated from combined 17α-hydroxylase/17,20-lyase deficiency by an ACTH test, showing elevated progesterone in 17α-hydroxylase-deficient patients and elevated 17-hydroxyprogesterone with subnormal rise of cortisol in patients with isolated 17,20-lyase deficiency. All of our patients had normal basal cortisol serum levels, but insufficient response to stimulation by ACTH. No guidelines are available for the hydrocortisone substitution treatment in these patients. In our view, daily hydrocortisone substitution treatment is not needed when there are no complaints, but in case of stress, a hydrocortisone stress dose is advised. Nevertheless, some of our patients underwent uneventful surgery without a hydrocortisone stress scheme.

In four of our six patients (patients 1–4), one allele of the *CYP17* gene was completely inactive due to insertions or deletions resulting in a frameshift. Patient 1 had a missense mutation in the steroid-binding domain in the other allele: F114V. This resulted in a combined complete 17α-hydroxylase/17,20-lyase deficiency, with a complete female phenotype. Patient 2 also had a mutation in the steroid-binding domain in the second allele (D116V), but had milder combined 17α-hydroxylase/17,20-lyase deficiency with low, but measurable, levels of androgens, explaining her ambiguous genitalia. The F114V mutation (patient 1) appears to affect steroid binding more seriously than the D116V mutation (patient 2) both *in vivo* as well as in the *in vitro* transfection experiments. Neither of these mutations has been described before. Like these two mutations, all other known mutations in the steroid-binding domain cause defects that affect 17α-hydroxylase and 17,20-lyase activities to a similar extent.

With regard to the patients with mutations in the redox partner interaction domain, special attention should be paid to the genotypic and phenotypic differences. Complete 17,20-lyase deficiency results in a complete female phenotype in 46,XY individuals (patients 3 and 4), but is theoretically not expected to result in ambiguous genitalia (patients 5 and 6). Patients 5 and 6 are, just like one of the previously described patients (10), homozygous for the R347H mutation and similarly had ambiguous genitalia at birth, indicating slight residual capacity for androgen synthesis. In contrast to this, patients 3 and 4 were heterozygous for another mutation in the same codon (R347C), the other allele being completely inactive due to a frameshift mutation. These two patients (both with an 46,XY genotype) showed female genitalia at birth and had barely detectable androgen levels. The differences between patients 5 and 6, on the one hand, and patients 3 and 4, on the other, indicate that the R347C mutation is more deleterious for the 17α-hydroxylase and/or 17,20-lyase reaction than the R347H mutation, as is also shown by the results of the transfection experiments. To explain the large difference in 17α-hydroxylase activity of mutations R347C and R347H, we hypothesize that the change of arginine to cysteine disrupts the function of the whole protein more seriously than a change to histidine, because of the possibility of the formation of abnormal cysteine dimers, causing not only disruption of 17,20-lyase activity, but also of 17α-hydroxylase. In addition, the functional allele in patients 3 and 4 may be haplo-insufficient and result in residual enzyme activity that is too low to stimulate male genital differentiation, whereas the homozygous presence of the R347H mutation may cause the residual presence of sufficient 17,20-lyase activity.

The partially virilized genitalia of patients 5 and 6 and the ability to synthesize some testosterone in the hCG test are not in accordance with our finding of absence of lyase activity *in vitro*. As described previously, R347 and R358, which are located in the redox partner interaction domain and contribute to the positive charges on the proximal surface of P450c17, are known to be key residues involved in the interaction with redox partner proteins. Geller *et al.* (20) reported that the absence of these charged amino acids selectively impairs 17,20-lyase activity without substantial reductions in 17α-hydroxylase activity or 17-hydroxypregnenolone binding. Coexpression of the R347 and R358 mutants with P450 oxidoreductase did not result in a significant increase in 17,20-lyase activity, but addition of excess cytochrome *b5* partially restored 17,20-lyase activity (20). Thus, the ambiguous genitalia in R347H homozygous patients might be explained by a partial rescue of the R347H mutation through *in vivo* accumulation of cytochrome *b5* in these patients.

To obtain a more detailed view of the P450c17 enzyme function in our patients, we compared the clinical data with *in vitro* expression studies. The K_m values obtained for the wild-type enzyme are in line with reported values (18, 27), and the R347H mutation yields a lower value as described

previously (20); these researchers also described an approximately 3-fold reduction of the V_{max} for this mutant enzyme. The enzyme activities of the mutated proteins *in vitro* were consistent with the clinical data and the hypothesis that mutations in the steroid-binding domain result in combined complete or partial 17α-hydroxylase and 17,20-lyase deficiency, whereas mutations in the redox partner interaction domain result in isolated 17,20-lyase deficiency. The latter point becomes especially clear from the results of the conversion of pregnenolone to DHEA; although the production of 17-hydroxypregnenolone by the cells transfected with the R347C and R347H mutated *CYP17* was larger than or comparable to that of the F114V and D116V cells, the amount of DHEA produced by the former two cell types was much lower than that secreted by the latter.

The *in vitro* results of the present study were obtained using the conversion of nonradioactive precursors to products, which were measured by RIA. Using this type of detection, Biason-Lauber *et al.* (13) showed that the F417C mutation in P450c17 can lead to isolated 17,20-lyase deficiency; these researchers used one dose of pregnenolone or progesterone as substrate. In contrast, using the conversion of tritiated precursors, Gupta *et al.* (17) indicated that this mutation affects both the 17α-hydroxylase and 17,20-lyase activities of the enzyme and argued that accurate enzymic studies of the mutant proteins should be performed to be able to conclude that isolated lyase deficiency is present. The latter researchers explained the discrepancies between the two studies on the basis of differences in the methods used to measure the products of the enzymic conversions; RIAs might lack the specificity needed to obtain reliable results. As we used RIAs of nonradioactive steroids for the detection of enzyme deficiencies of the mutated proteins, similar objections might be raised against our conclusions. However, it is highly unlikely that insufficient specificity of our methods plays a role, because we obtained straight lines in the Lineweaver-Burke plots with five doses of substrate. Cross-reacting substrates, if present, should show 100% cross-reaction in the RIAs for 17-hydroxypregnenolone or DHEA to explain these data. In addition, we found similar results for the experiments using pregnenolone and progesterone and their respective 17-hydroxylated derivatives as substrates for 17-hydroxylation and lyase activities. This could only be explained by similar problems in the assays by which we estimated the hydroxylated C_{21} steroids and the C_{19} products, respectively. Moreover, the argument of Gupta *et al.* (17) that substances cross-reacting in RIAs for 17-hydroxyprogesterone or DHEA were found in cord blood (29, 30) is not very strong, as the substance that showed the highest cross-reactivity in the assay for 17-hydroxyprogesterone was 17-hydroxylated itself (29), and it would be highly unlikely to underestimate the amount of DHEA present if other products would cross-react in the RIA for DHEA.

Finally, it is notable that all mutated amino acids described in this article were well conserved during vertebrate evolution. The phenylalanine of codon 114, aspartate of codon 116, and arginine of codon 347 are conserved in human, rat, guinea pig, pig, medaka, trout, and frog (GenBank accession no. NM_000102, NM_012753, S75277, Z81154, D87121, X65800, and AF042278, respectively). This suggests that the presence of these amino acids in these positions in P450c17 is essential for its function.

In conclusion, our clinical and *in vitro* expression results confirm the differential functions of various domains in the *CYP17* gene. The P450c17 gene mutations in the steroid-binding domain resulted in clinically combined 17α-hydroxylase/17,20-lyase deficiency; mutations in the redox partner interaction domain resulted in 17,20-lyase deficiency. Remarkably, the R347C and R347H mutations in the redox partner domain result in different phenotypes.

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Address all correspondence and requests for reprints to: Dr. Frank H. de Jong, Department of Internal Medicine, Section of Endocrinology, Erasmus Medical Center, Room Bd 289, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail: dejong@endov.fgg.eur.nl.

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