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CYP11B1 mutations causing non-classic adrenal hyperplasia due to 11β– hydroxylase deficiency

Karin Joehrer, Stephan Geley, Elisabeth M.C. Strasser-Wozak, Ricardo Azziz[1], Hartmut A. Wollmann[2], Klaus Schmitt[3], Reinhard Kofler and Perrin C. White[4,*]

Institute for General and Experimental Pathology, Division of Molecular Pathophysiology, University of Innsbruck, Medical School, Innsbruck, Austria, [1]Departments of Obstetrics and Gynecology and of Medicine, The University of Alabama at Birmingham, Birmingham, Alabama, USA, [2]Department of Pediatrics, University of Tübingen, Tübingen, Germany, [3]Childrens' Hospital, Linz, Austria and [4]Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, TX 75235-9063, USA

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Steroid 11β-hydroxylase deficiency is the second most common cause of congenital adrenal hyperplasia, the inherited inability to synthesize cortisol. Severely affected patients carry mutations in the CYB11B1 gene that destroy enzymatic activity. Such patients have signs of androgen excess and usually have hypertension. Mild or non-classic 11β-hydroxylase deficiency has been reported previously but not studied genetically. In this study we report analysis of the CYP11B1 genes of three patients thought to suffer from non-classic 11β-hydroxylase deficiency. Mutations were detected in the CYP11B1 genes of two patients. One was a compound heterozygote for missense mutations N133H and T319M, whereas the other carried a nonsense mutation (Y423X) on one allele and a missense mutation (P42S) on the other. All three missense mutations affected enzymatic activity when expressed *in vitro.* No mutations were detected in the coding regions or intron–exon boundaries of the CYP11B1 genes of the other putative non-classic patient. In addition, we were unable to detect CYP11B1 mutations in two hirsute women with mildly elevated levels of 11β-hydroxylase precursors who had previously been identified in a screening study of patients in a reproductive endocrinology clinic. We conclude that non-classic 11β-hydroxylase deficiency is a rare disorder. It is not a significant cause of hyperandrogenism in women and relatively stringent criteria should be used to prevent its misdiagnosis.

INTRODUCTION

Congenital adrenal hyperplasia (CAH), the inherited inability to synthesize cortisol, is one of the most common inherited endocrine disorders (1). Cortisol is normally synthesized in the zona fasciculata of the adrenal cortex in five enzymatic steps: cleavage of the cholesterol side chain to convert cholesterol to pregnenolone, dehydrogenation at the 3β position to yield progesterone and a series of hydroxylations at the 17α, 21 and 11β positions to form 17α-hydroxyprogesterone, 11-deoxycortisol and cortisol respectively.

Whereas >90% of cases of CAH are caused by 21-hydroxylase deficiency, steroid 11β-hydroxylase (P450c11, EC 1.14.15.4) deficiency accounts for 5–8% of cases and has a frequency of 1/200 000 in the general Caucasian population (2). In classic 11β-hydroxylase deficiency decreased or absent cortisol secretion stimulates ACTH secretion which, in turn, leads to accumulation of steroid precursors that are shunted into the androgen synthesis pathway. Typical signs of androgen excess include masculinization of female external genitalia and precocious pseudopuberty in both sexes. Patients undergo rapid somatic growth with premature epiphyseal closure resulting in short adult stature. Moreover, elevated metabolites with mineralocorticoid activity, such as deoxycorticosterone and its derivatives, cause hypertension in about two thirds of patients. Patients are treated with glucocorticoid replacement and with antihypertensive therapy if necessary.

A non-classic form of 11β-hydroxylase deficiency has been reported that causes milder androgen excess than the classic form. This may produce menstrual cycle abnormalities, hirsutism and acne in previously asymptomatic women. These problems resemble those found in women suffering from polycystic ovary syndrome (3,4). Whereas the frequency of non-classic 11β-hydroxylase deficiency is not known, it has been hypothesized that some women thought to have polycystic ovary syndrome might in fact have non-classic 11β-hydroxylase deficiency (5).

CYP11B1, the gene encoding 11β-hydroxylase, is located on chromosome 8q21–22, tandemly arranged with the CYP11B2 gene encoding aldosterone synthase (P450aldo) (6). These enzymes are 93% identical in their amino acid sequences but differ in their expression patterns and catalytic activities (7). Mutations in CYP11B1 causing classic 11β-hydroxylase deficiency have been identified (8–14), but no molecular investigations of the non-classic disorder have been performed thus far.

*To whom correspondence should be addressed. Tel: +1 214 648 3501; Fax: +1 214 648 9772; Email: pwhit2@mednet.swmed.edu

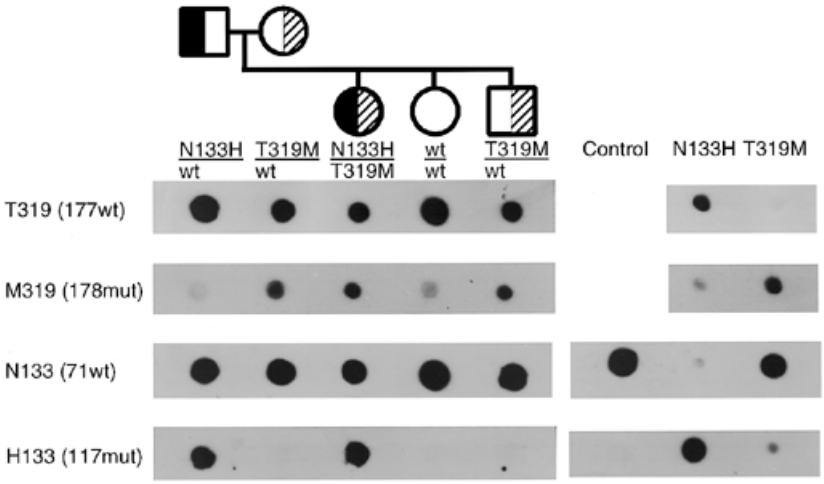

Figure 1. Dot blot analysis of the family of patient 1. Dot blot hybridizations are aligned below a pedigree diagram. In the diagram alleles carrying N133H are in black and those carrying T319M are hatched. The genotype of each individual is shown (wt, wild-type). Control indicates a PCR fragment of a normal control DNA. Columns labeled N133H and T319M represent hybridization to genomic clones harboring the respective mutations. Each row is labeled with the oligonucleotide used for that hybridization; the first two rows are for the wild-type and mutant alleles of codon 133 and the last two rows for alleles of codon 319 (the numbers in parentheses permit reference to Table 4).

Therefore, we sequenced the CYP11B1 genes of three putative non-classic 11β-hydroxylase deficiency patients and expressed the identified mutations *in vitro*. We also attempted to identify CYP11B1 mutations in selected hirsute women seen in a reproductive endocrinology clinic.

RESULTS

Genomic cloning of the CYP11B1 genes of patient 1 followed by sequence analysis revealed compound heterozygosity for missense mutations N133H (exon 3, AAT→CAT) and T319M (exon 6, ACG→ATG). Her father was heterozygous for N133H, her mother and a brother carried T319M on one allele and a sister was homozygous normal (Fig. 1). Complete sequence analysis of the genomically cloned CYP11B1 genes of patient 1 led to identification of the hitherto unknown nucleotide sequences of intron 2 and intron 8, with a total length of 1798 and 469 bp respectively (EMBL Databank accession nos X85218 and X85219).

Sequence analyses of PCR-amplified DNA fragments from patient 2 also revealed two mutations, one missense (exon 1, CCC→TCC, P42S) and the other nonsense (exon 8, TAT→TAG, Y423X). Dot blot analysis showed that the patient and her mother were heterozygous for P42S, whereas the patient inherited Y423X from her father, as demonstrated by sequence analysis (data not shown). Y423X leads to a premature stop in exon 8 yielding a truncated enzyme lacking the essential cysteine residue (C450) that interacts with the heme prosthetic group (9).

To demonstrate that the missense mutations in patients 1 and 2 affected enzymatic activity, normal and mutant cDNAs were expressed in cultured COS-1 cells. P42S, N133H and T319M reduced 11β-hydroxylase activity in standard *in vitro* enzyme assays to 15, 17 and 37% of wild-type activity respectively (Fig. 2).

Sequence analysis of the PCR-amplified CYP11B1 genes of patient 3 did not reveal any deviations from the normal sequence.

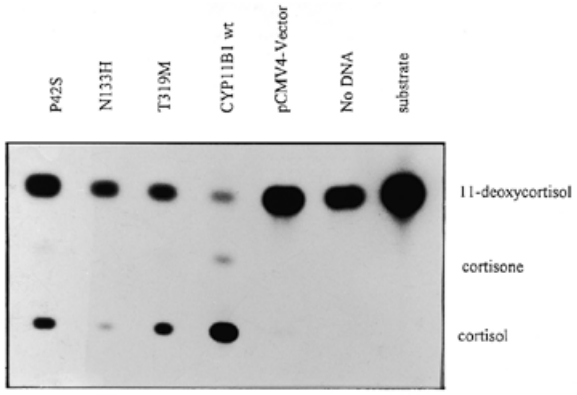

Figure 2. TLC analysis of the activity of CYP11B1 wild-type and mutated enzymes causing non-classic 11β-hydroxylase-deficient CAH (autoradiogram). This is a representative experiment out of seven (P42S and N133H) or four (T319M). The appearance of cortisone is due to an intrinsic 11-hydroxysteroid dehydrogenase activity of COS cells.

The possibility of CYP11B1 deletions was ruled out by Southern blotting (not shown).

Patients 4 and 5 were adult women with signs of androgen excess who had levels of 11-deoxycortisol greater than three times the 95th percentile of normal after ACTH stimulation (5). No mutations were detected in samples from either patient. Based on the negative molecular findings, the hormonal status of each patient was re-evaluated by repeated ACTH testing. Whereas the stimulated 11-deoxycortisol levels remained above normal for both patients (11 μg/l for both patients), the levels were markedly less than initially measured (Table 1).

Table 1. Biochemical characterization of patients with suspected non-classic 11β-hydroxylase deficiency

	Patient					Controls
	1	2	3	4	5	
THS	–	1690	4421	–	–	<50
17α-OHP	2.2/6.7	–	–	–/4.2	–/1.4	<2/<3
11-deoxycortisol	12/41	–	84/171	6/24	5/22	<5/<8
Cortisol	151/101	–	121/141	180/234	55/246	<190/<300

THS, urinary tetrahydro-11-deoxycortisol excretion (μg/24 h). Other steroid levels are measured in plasma in μg/l. Two values separated by a slash (/) represent basal and ACTH-stimulated levels. 17α-OHP, 17α-hydroxyprogesterone. Control values represent upper 95th percentile limits.

Table 2. Homology alignments between members of the CYP11 family (taken from the EMBL Databank)

	Sequences framing P42	Sequences framing N133	Sequences framing T319
Human P450c11	PFEAM**P**RRPGN	GVFLL**N**GPEWR	GSVDT**T**VFPLL
Human P450aldo	PFEAM**P**QHPGN	GVFLL**N**GPEWR	GSVDT**T**AFPLL
Bovine P450c11	PFEAM**P**RCPGN	GVFLL**N**GPQWR	GSVDT**T**AFPLL
Rat P450c11	PFEAI**P**QYSRN	GVFLL**N**GADWR	GSVDT**T**AISLV
Mouse P450c11	PFEAI**P**QYSRN	GVFLL**N**GPEWR	GSVDT**T**AIPLV
Rat P450aldo	PFEAI**P**QYSRN	GVFLL**N**GAEWR	GSVDT**T**AIPLV
Human P450scc	PFNEI**P**SPGDN	GVFLK**K**SAAWK	GGVDT**T**SMTLQ
Bovine P450scc	PYSEI**P**SPGDN	GVFLK**K**SGTWK	GGVNT**T**SMTLQ
Pig P450scc	PFSEI**P**SPGDN	GVLLK**K**SGAWK	GGVDT**T**SMTLQ
Rat P450scc	SFNEI**P**SPGDN	GVLFK**S**SDAWR	GGVDT**T**SMTLQ
Trout P450scc	NFSEI**P**GLWRN	GVLLK**N**GEDWR	GGVDT**T**SITLL

Aligned portions of amino acid sequences are listed in single letter code and amino acid residues homologous to mutated residues found in non-classic CAH patients are bold and underlined.

DISCUSSION

Functional effects of mutations seen in non-classic 11β-hydroxylase deficiency

We aligned the predicted amino acid sequence of human CYP11B1 with related P450 enzymes, particularly bovine CYP11A (P450scc), for which a 3-dimensional model exists (19). One of the mutated amino acid residues, N133, is conserved in all CYP11B isozymes but not in CYP11A (Table 2). This residue is thought to lie within a region that may form part of the access route for the substrate; point mutations in this region significantly alter substrate specificity in different P450s (20,21).

T319 is conserved in all CYP11B and CYP11A isozymes and in many but not all other P450s. It is situated within a region that may be critical for binding of molecular oxygen and/or proton transfer (21,22). The adjacent residue, T318, is completely conserved in P450s and the analogous T318M mutation of CYP11B1 destroys enzymatic activity and causes classic 11β-hydroxylase deficiency (9). However, there is an intron in CYP11B1 between T318 and T319 (6) and it is also possible that the T319M mutation interferes with proper splicing of pre-mRNA. Expression of minigene constructs would be required to assess this possibility.

P42 is conserved in all CYP11B isozymes and in rather distantly related enzymes such as P450cam. Several proline residues are found in most P450 enzymes in this region (23). They may be essential for proper orientation of the enzyme with respect to the membrane, as is the case for microsomal P450s (24). A mutation of proline to leucine in the corresponding region of CYP21 (P30L) causes mild 21-hydroxylase deficiency, consistent with this hypothesis (25).

Comparisons between non-classic deficiencies of adrenal enzymes

The most common cause of classic CAH is steroid 21-hydroxylase deficiency. Non-classic 21-hydroxylase deficiency occurs even more frequently than the classic form of the disease, being found in >1% of certain populations such as Jews of central or eastern European origin (26). This disorder can be asymptomatic or can cause signs of androgen excess during childhood or at puberty.

Non-classic 11β-hydroxylase deficiency has been described on the basis of clinical investigations (27), although no molecular studies have been reported. It appears to cause similar clinical symptoms to those seen in non-classic 21-hydroxylase deficiency but differs in the accumulated precursors that are detected and in the possible appearance of mildly elevated blood pressure in some 11β-hydroxylase-deficient patients. Because this disorder causes relatively mild signs and symptoms of androgen excess, it is possible that its apparent rarity reflects problems of ascertainment. Consistent with this, the two patients in whom we found

mutations were compound heterozygotes. Because the degree of compound heterozygosity in recessive genetic disorders that do not have stereotypic mutations is roughly proportional to the frequency of mutant alleles in the general population, this suggests that there may be a relatively high frequency of non-classic 11β-hydroxylase deficiency alleles in the population. We found that the degree of enzymatic compromise associated with the non-classic form of 11β-hydroxylase deficiency is similar to that seen in non-classic 21-hydroxylase deficiency. The less affected alleles in two of our patients encode enzymes that have ~15 and 40% of the 11β-hydroxylase activity of the wild-type enzyme respectively, whereas mutations in CYP21 that cause non-classic 21-hydroxylase deficiency decrease 21-hydroxylase activity to 20–60% of normal (25,28–30). Thus, the non-classic form of 11β-hydroxylase deficiency is a true allelic variant of this disorder in at least some individuals.

We were unable to detect mutations in three of the individuals that we analyzed. Patient 3 was a male who presented at 8 years of age with signs of androgen excess and clear hormonal evidence of 11β-hydroxylase deficiency. Possibly this patient carries mutations in introns or in transcriptional regulatory regions that affect expression of the intact enzyme. The other two patients were adult women who were the only subjects identified as possibly having 11β-hydroxylase deficiency upon consecutively screening 260 women with hyperandrogenism (5). Since subsequent genotyping did not identify mutations in CYP11B1 and since repeated ACTH stimulation produced less marked elevation of 11-deoxycortisol levels than those originally obtained, our results suggest that non-classic 11β-hydroxylase deficiency is a very uncommon cause of hyperandrogenism in women. We also conclude that the diagnostic criterion for 11β-hydroxylase deficiency used in screening the hyperandrogenic women, an 11-deoxycortisol level three times the 95th percentile of normal, is not sufficiently stringent. Considering that patient 1, who actually had 11β-hydroxylase deficiency, had an ACTH-stimulated 11-deoxycortisol level of 41 μg/l (approximately five times the 95th percentile of normal), a threshold value of five times the upper limit of normal would increase the specificity of diagnosing this disorder.

The failure to detect any actual cases of non-classic 11β-hydroxylase deficiency among women with hyperandrogenism is consistent with other studies that suggest that a related but more frequent defect in adrenal steroidogenesis, non-classic 21-hydroxylase deficiency, accounts for at most a small minority of cases of hyperandrogenism in women (31). Moreover, studies of putative non-classic 3β-hydroxysteroid dehydrogenase deficiencies failed to detect any mutations in the gene encoding this enzyme, suggesting that this disease, too, is not a significant cause of hyperandrogenism (32).

We conclude that non-classic 11β-hydroxylase deficiency is a rare but actual allelic variant of 11β-hydroxylase deficiency causing signs of androgen excess.

MATERIALS AND METHODS

Patients

We analyzed the CYP11B1 genes of three unrelated patients, two female (patients 1 and 2) and one male (patient 3), with a clinical diagnosis of non-classic 11β-hydroxylase deficiency. They were all diagnosed in childhood because of advanced bone age, accelerated growth, acne and precocious adrenarche (see Table 3). In addition, we studied two participants (patients 4 and 5) from a study screening for 11β-hydroxylase deficiency in women with signs of androgen excess (patients 229 and 506 in ref. 5). Both demonstrated 11-deoxycortisol levels 60 min after administration of 1.0 mg ACTH (1–24) (Cortrosyn; Organon, Orange, NJ) that were greater than three times the upper limit of normal.

Table 1 summarizes the steroid hormone analyses supporting the diagnosis of 11β-hydroxylase deficiency in these patients. Patients 1 and 3 had increased basal and ACTH-stimulated levels of 11-deoxycortisol in serum. The diagnoses of patients 2 and 3 were supported by measuring elevated 11-deoxycortisol metabolites in urine.

Genomic cloning, PCR amplification and sequencing

DNA was prepared from peripheral blood samples following standard or phenol-free protocols (15,16). The CYP11B1 genes of patient 1 were isolated from a genomic DNA library constructed in bacteriophage λGEM12 (Promega, Madison, WI) by standard methods and screened in duplicate with a radiolabeled 900 bp *Pst*I fragment of CYP11B2 (Helmberg *et al.*, 1992) and oligonucleotide probes 74 and 76 specific for CYP11B1 (see Table 4). Plasmid subclones were then sequenced.

In order to analyze the CYP11B1 genes of the other patients, 100 ng genomic DNA were amplified in three PCR reactions using each of three sets of primers amplifying exons 1 and 2 (primers 209/210), exons 3–5 (primers 211/212) and exons 5–9 (primers 213/214) respectively. Primers 210, 212 and 213 were 5′-biotinylated, allowing coupling of each fragment to streptavidin-coated paramagnetic beads (Dynabeads, Dynal M-280). PCR was carried out in a volume of 100 μl, containing 50 mM KCl, 10 mM Tris–HCl, 0.1% Triton X-100, 200 μM deoxy-NTPs, 0.2 μM each primer, 100 ng genomic DNA and 3 U Taq DNA polymerase at pH 9. $MgCl_2$ concentrations used were 1.0 mM for fragments A and B and 0.8 mM for fragment C. After initial denaturation at 94°C for 2 min, 35 cycles (94°C 1 min, 64°C 2 min and 72°C 3 min) were performed in an OmniGene Temperature Cycler (Hybaid Ltd, Teddington, UK). After amplification the reaction mix was purified using the Wizard™ PCR Purification System (Promega) following the producer's instructions. The samples were coupled to an equal volume of streptavidin-coated paramagnetic beads (6–7 × 10^8 beads/ml) (17). After washing in water the DNA strands were separated by denaturation using 0.2 N NaOH. Both strands served as templates in subsequent chain termination reactions.

Table 3. Clinical features of patients 1–3, who were diagnosed as children

	Patient 1	Patient 2	Patient 3
Sex	F	F	M
Chronologic age at diagnosis (years)	8 5/12	5 10/12	8 5/12
Bone age at diagnosis (years)	11	12[a]	13
Height percentile	>95	75	95
Acne	No	Yes	Yes
Pubic hair stage[b]	2	2	2

[a]Bone age at a chronologic age of 10 3/12 years.
[b]Stages of pubic hair development (33).

Table 4. Oligonucleotides and primers

Oligonucleotide	Sequence	Localization[a]	Purpose	Comment
209 (s)	CCCATGACGTGATCCCTCTCGAAGG	555–579	PCR	
210 (as)	AAACACAGGCCCTGACCCGTATCCC	3138–3163	PCR	Biotinylated
211 (s)	TCAGCACCTGTGGGCAGAAGCTACCA	2965–2990	PCR	
212 (as)	AGCGTCATCAGCAAGGGAAACACCG	4548–4572	PCR	Biotinylated
213 (s)	CGCCCTCAACAGTACACCAGCATCG	4069–4093	PCR	Biotinylated
214 (as)	AAACCACAGCACCCTTGCATGGCCA	6344–6368	PCR	
322 (s)	TTTGGATCCACCATGGCACTCAGGGCAAAGGCA	CYP11B1-5′-UT	PCR	5′ PCR
323 (as)	GAATCTAGAGACGTGATTAGTTGATG	CYP11B1-3′-UT	PCR	3′ PCR
74 (s)	GAAGGCAAGGCACCAGG	575–591	Screen	CYP11B1
76 (s)	TCAACTAATCACGTCTC	5730–5746	Screen	CYP11B1
262mut (s)	GAAGCCATGTCCCGGCGTCC	Codon 42	mut	
281wt (s)	GAAGCCATGCCCCGG	Codon 42	hyb	T_H 58°C
282mut (s)	GAAGCCATGTCCCGG	Codon 42	hyb	T_H 57°C
345mut (s)	TCTTGCTGCATGGGCCTGAATGGCG	Codon 133	PCR-mut	
344mut (as)	AGGCCCATGCAGCAAGAACACGCCA	Codon 133	PCR-mut	
71wt	TCTGTAGGAATGGGCCTGAA	Codon 133	hyb	T_H 61°C
117mut	TCTGTAGGCATGGGCCT	Codon 133	hyb	T_H 50°C
316mut (as)	GAAAGACCATCGTGTCCACGCTCCC	Codon 319	PCR-mut	
317mut (s)	GGACACGATGGTGTTTCCCTTGCTG	Codon 319	PCR-mut	
177wt (s)	CCTGCAGACGGTGTTTC	Codon 319	hyb	T_H 50°C
178mut (s)	CCTGCAGATGGTGTTTC	Codon 319	hyb	T_H 51°C
252	CTGGTGAGTATTCAACCAAGTC	β-Lactamase	mut	Selection

[a]Position numbers are based on CYP11B1 sequences published in the EMBL Databank (GenBank™ accession no. J05140: M32863, M32878 and M32179). The missing sequences of intron 2 and 8 were determined in the course of our studies and submitted to the EMBL Sequence Database (accession nos X85218 and X85219). The fragments were merged at positions 1482 (M32863 and X85218) and 2834 (X85218 and M32878), in order to include the missing intron 2 sequence, and at positions 5467 (M32878 and X85219) and 5751 (X85219 and M32179), closing the gap in intron 8. The orientation of each oligonucleotide is indicated as sense (s) or antisense (as). Oligonucleotides were used for PCR (PCR), site-directed mutagenesis according to the method of Deng *et al*. (mut) or PCR (PCR-mut) and dot blot analysis (hyb). Mutated bases are underlined in the respective primer. T_H, high stringency washing temperature.

Genetic segregation analysis

Allele-specific oligonucleotide hybridization of dot blots was performed using wild-type and mutant-specific oligonucleotides (see Table 4). PCR-amplified DNA fragments of patients and parents were purified as above and dotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Dot blots were denatured in 0.5 N NaOH, 1.5 M NaCl, neutralized in 0.5 M Tris–HCl, pH 8.0, 3 M NaCl and 6× SSC (2 min each step; 1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) and baked for 2 h in a vacuum oven at 80°C. Filters were hybridized with ^{32}P-labeled oligonucleotides at 37°C in 5× Denhardt's solution, 6× SSC and 0.05% SDS to analyze the inheritance patterns of the respective mutations. Blots were washed in 2× SSC, 0.1% SDS at high stringency washing temperatures (see Table 4) and autoradiographed on Agfa RP-1 film (Agfa, Vienna, Austria) with intensifying screens at –90°C for 12 h. Genetic segregation analysis for the Y423X allele was performed by sequencing the parental CYP11B1 genes.

Site-directed mutagenesis and *in vitro* expression studies

Mutation P42S was introduced into the CYP11B1 expression vector pCMV4-11B1 wild-type by means of mutation-specific primer 262 and by eliminating the unique *Sca*I site in the β-lactamase gene of pCMV4-11B1 using primer 252 as described (18). Site-directed mutagenesis of mutations N133H and T319M were performed using a PCR-based protocol (9). Oligonucleotides used to introduce the respective mutations are listed in Table 4.

For *in vitro* expression analysis COS-1 cells were transiently co-transfected with pCMV4-11B1 wild-type or mutated construct and human adrenodoxin and adrenodoxin reductase expression vectors pCMV4-Adx and pCMV4-AR (gifts from Dr W.L.Miller), using Lipofectamin (Gibco BRL, Gaithersburg, MD) as described (7,9). Approximately 10^5 cells at 80% confluence in 35 mm wells were incubated with 20 µg Lipofectamin, 1–2 µg pCMV4-AR and pCMV4-Adx alone or together with 5 µg pCMV4-11B1 wild-type or pCMV4-11B1 mutated vector. Cells were allowed to recover for 18 h in

Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum.

Enzyme activities were analyzed by incubating the transfected cells with 0.1–1.0 μM [^{3}H]11-deoxycortisol (New England Nuclear, Boston, MA) for 15–24 h and resolving the extracted steroids by thin layer chromatography on silica-coated plates (Merck, Darmstadt, Germany) in methylene chloride, methanol, H_2O (300:20:1). Plates were treated with En^{3}Hance™ spray (NEN, Boston, MA) and autoradiographed for at least 12 h at –90°C. The portion of the plate corresponding to each spot was scraped off and counted using a liquid scintillation counter.

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