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Genetics of Vitamin D 1α-Hydroxylase Deficiency in 17 Families

Jonathan T. Wang,[1,2] Chin-Jia Lin,[1,2] Sandra M. Burridge,[1,2] Glenn K. Fu,[1,2] Malgorzata Labuda,[4] Anthony A. Portale,[1,3] and Walter L. Miller[1,2]

[1]Department of Pediatrics, [2]The Metabolic Research Unit, and [3]Department of Medicine, University of California, San Francisco; and [4]Centre de Recherche Louis-Charles Simard, Montreal

Summary

Vitamin D–dependent rickets type I (VDDR-I), also known as pseudo–vitamin D–deficiency rickets, appears to result from deficiency of renal vitamin D 1α-hydroxylase activity. Prior work has shown that the affected gene lies on 12q13.3. We recently cloned the cDNA and gene for this enzyme, mitochondrial P450c1α, and we and others have found mutations in its gene in a few patients. To determine whether all patients with VDDR-I have mutations in P450c1α, we have analyzed the P450c1α gene in 19 individuals from 17 families representing various ethnic groups. The whole gene was PCR amplified and subjected to direct sequencing; candidate mutations were confirmed by repeat PCR of the relevant exon from genomic DNA from the patients and their parents. Microsatellite haplotyping with the markers D12S90, D12S305, and D12S104 was also done in all families. All patients had P450c1α mutations on both alleles. In the French Canadian population, among whom VDDR-I is common, 9 of 10 alleles bore the haplotype 4-7-1 and carried the mutation 958ΔG. This haplotype and mutation were also seen in two other families and are easily identified because the mutation ablates a *Tai*I/*Mae*II site. Six families of widely divergent ethnic backgrounds carried a 7-bp duplication in association with four different microsatellite haplotypes, indicating a mutational hot spot. We found 14 different mutations, including 7 amino acid replacement mutations. When these missense mutations were analyzed by expressing the mutant enzyme in mouse Leydig MA-10 cells and assaying 1α-hydroxylase activity, none retained detectable 1α-hydroxylase activity. These studies show that most if not all patients with VDDR-I have severe mutations in P450c1α, and hence the disease should be referred to as "1α-hydroxylase deficiency."

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Address for correspondence and reprints: Dr. Walter L. Miller, Department of Pediatrics, Building MR-IV, Room 209, University of California, San Francisco, San Francisco, CA 94143-0978.

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Introduction

Vitamin D, either ingested in the diet or synthesized in the skin, is biologically inert and must be converted to its hormonally active form by a bioactivation pathway (for reviews see Strewler and Rosenblatt 1995; Feldman et al. 1996). Activation consists of an initial 25 hydroxylation by hepatic mitochondrial P450c25 (Su et al. 1990; Usui et al. 1990) to produce 25-hydroxyvitamin D (25OHD), which is largely inactive but is found abundantly in blood at ~10^{-7} M. The rate-limiting, hormonally regulated, biologically activating step is renal 1α-hydroxylation of 25OHD to 1,25 dihydroxyvitamin D [1,25$(OH)_2$D], which is found in blood at vastly lower levels, ~10^{-10} M. This 1α-hydroxylation is catalyzed by mitochondrial P450c1α (Fraser and Kodicek 1970; Fu et al. 1997*a*) in renal-proximal convoluted tubules (Brunette et al. 1978; Kawashima et al. 1981); another mitochondrial enzyme, P450c24 (Ohyama et al. 1991; Chen et al. 1993), catalyzes 24-hydroxylation, which may initiate an inactivation pathway.

Vitamin D–dependent rickets type I (VDDR-I), or pseudo–vitamin D–deficiency rickets (MIM 264700), is characterized by failure to thrive, muscle weakness, hypocalcemia, secondary hyperparathyroidism, and the bony changes of rickets (Fraser et al. 1973; Scriver et al. 1978; Delvin et al. 1981). Affected subjects have normal concentrations of 25OHD and very low concentrations of 1,25$(OH)_2$D. This disease, which can be treated with physiological replacement doses of 1,25$(OH)_2$D, can be caused by mutations in the gene encoding P450c1α, the renal and keratinocyte 1α-hydroxylase (Fu et al. 1997*a*). This gene has been fully sequenced (National Center for Biotechnology Information accession number AF 027152) and exists in a single copy, consisting of nine exons spanning only 5 kb (Fu et al. 1997*b*; Monkawa et al. 1997; Kitanaka et al. 1998). Linkage analysis of the disease phenotype (Labuda et al. 1990), gene localization via human/rodent somatic-cell hybrids (Fu et al. 1997*b*), and FISH (St.-Arnaud et al. 1997; Kitanaka et al. 1998) have shown that the gene maps to 12q13.3, a finding consistent with the autosomal recessive inheritance of 1α-hydroxylase deficiency.

Table 1

Oligonucleotide Primers Used for PCR Amplification and Sequencing of the P450c1α Gene

Exon	Sense Primer	Antisense Primer	Size (bp)
1	5′-CCTCACCCAAAGGTTAAATAG-3′ (61)	5′-CATTTGCTCTGCACTAGTCAG-3′ (64)	364
2	5′-TGGGCACAGGTCAAGCTGAAA-3′ (48)	5′-GATAGTTTCGGGACCCGCAGC-3’ (48)	333
3	5′-CGCGCTCCTTCACTGCAGCCA-3′ (55)	5′-AGGGGGCGCCGTCAGGGTTCC-3′ (54)	357
4	5′-CAGAGCTGGGCAGGCGTCGGG-3′ (52)	5′-GAAGGCACGTGAATACCTCGC-3′ (52)	348
5	5′-AGGCACAGGTCGAGGTGGAAA-3′ (60)	5′-TGGAGCCGGAGTCTGCGGAGG-3′ (43)	320
6	5′-TCTGCACCCTCTGCTGGGTTC-3′ (39)	5′-CCCTGCTTCCATCCACTAGTT-3′ (57)	312
7	5′-GAAGTTGGAAACAATGAGAAG-3′ (56)	5′-AGAGTGTTTGAGAACAGGGTt-3′ (56)	235
8	5′-TGCCTGCCCTATTCTGAGCCC-3′ (32)	5′-CACAACTTTAGAGGGTTAGGG-3′ (41)	315
9	5′-ACCAATTGGATTACCAATGAA-3′ (53)	5′-GTATACCTTGGTCTTGTGCCT-3′ (61)	272

NOTE.—Sense and antisense primers were in flanking or intronic DNA; the minimum distance between each primer and the corresponding exon is given in parentheses following the primer sequence. When an individual exon was amplified, the resulting PCR fragment had the size indicated in the far right column.

VDDR-I is rare in most populations, but it is particularly common among French Canadians, having an apparent carrier rate of 1/26 in the Charlevoix–Saguenay–Lac Saint Jean area of Québec (De Braekeleer 1991). Microsatellite analysis mapped the gene proximal to D12S312 and distal to (D12S305, D12S104) (Labuda et al. 1996). Analysis of 32 families, including 20 from Québec and 5 from eastern Canada, suggested (*a*) that a haplotype analysis of three markers—D12S90, D12S305, and D12S104—could distinguish the allelic contributions of various founder populations and (*b*) that the Charlevoix French Canadian subjects and the eastern Canadian (Acadian) subjects derived from independent founder events (Labuda et al. 1996).

After the publication of our initial finding that P450c1α mutations can cause this disease (Fu et al. 1997*a*), others confirmed and expanded this observation in four Japanese families (Kitanaka et al. 1998), but no extensive study of multiple ethnic groups has appeared. We have developed a rapid PCR–based approach to analyzing mutations in the P450c1α gene. Using this procedure, we describe 12 new mutations in 17 families from eight ethnic groups, identify the common French Canadian (Charlevoix) mutation and another common mutation, correlate these with the microsatellite haplotyping, and provide a rapid RFLP–based diagnostic for the Charlevoix mutation.

Material and Methods

DNA Preparation and PCR

Using reagents from Promega, we prepared genomic DNA from whole blood by salt deproteination of nucleated blood cells followed by alcohol precipitation, as described by Miller et al. (1988). The sense primer 5′-CACAGATCTCTCAGGAGGAGGGATTGGCTG-3′ was synthesized corresponding to bases −76 to −47 of the P450c1α gene (Fu et al. 1997*b*), except that the two underlined T's differed from the gene, so as to create a *Bgl*II cleavage site (AGATCT). The antisense primer 5′-GGAATTCAGATAGGCATTAGGGGAAGATGTA-3′ was synthesized antisense to bases 4034 to 4059, plus six 5′ bases, to create an *Eco*RI cleavage site (GAATTC). The PCR reaction included 200 ng of genomic DNA and 100 ng of each primer in 100 μl of 67 mM Tris-HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, and two units of a 20:1 mixture of *Taq* and *pfu* DNA polymerases that has better long-range processivity and higher fidelity than either polymerase alone (Cheng et al. 1994). PCR amplification was done with a program of 95°C for 3 min followed by 40 cycles at 95°C for 1 min and 65°C for 5 min on a TC1 thermal cycler (Perkin-Elmer).

Cloning and Gene Sequencing

The 4149-bp PCR product was either sequenced directly without cloning or cleaved with *Bgl*II and *Eco*RI, subcloned into *Bam*HI/*Eco*RI-cleaved vector pGEM3Zf+ (Promega), and prepared from single colonies. Each exon was sequenced on both strands with the primers shown in table 1 on an ABI Prism automated fluorescence sequencer. Putative mutations found on initial sequencing were checked twice by PCR amplification and sequencing of the affected exon directly from the genomic DNA of the patients and their parents, bypassing the 4-kb PCR.

Microsatellite Haplotype Analysis

PCR primers for the microsatellite markers D12S90, D12S305, and D12S104 (Labuda et al. 1996) were synthesized according to sequences obtained from the Whitehead Institute web page. The sense primer for each marker was end labeled with γ[^{32}P]-ATP (Amersham) and T4 polynucleotide kinase (New England Biolabs). The PCR reactions included 50 ng of genomic DNA, 5

pmol of each primer, and 0.5 units of *Taq* polymerase (Perkin Elmer) in 20 μl of 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, and 50 μM each dNTP, with 30 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s. The PCR products were displayed by electrophoresis on a 6% polyacrylamide sequencing gel, and the data were analyzed on a Storm 860 Phosphorimager (Molecular Dynamics). Unknown alleles were assigned by comparison with amplified patient DNA containing previously identified alleles (Labuda et al. 1996) and by comparison with an M13 sequencing ladder. Alleles were designated as described by Labuda et al. (1996). All analyses were done by one of us (S.M.B.), including confirmation of several previously assigned haplotypes (Labuda et al. 1996).

Site-Directed Mutagenesis and cDNA Expression

Identified mutations causing missense mutations were recreated by PCR-based oligonucleotide-mediated mutagenesis of our human P450c1α cDNA (National Center for Biotechnology Information accession number AF 020192) expression vector in pcDNA3 (Fu et al. 1997*a*), as described by Braman et al. (1996), using *pfu* DNA polymerase and selective digestion of wild-type parent plasmids with *Dpn*I. After confirming the mutations by sequencing, we transfected MA-10 cells at 50%–60% confluence with 2 μg plasmid DNA/ml using adenovirus-mediated transfection (Forsayeth and Garcia 1994), as described (Fu et al. 1997*a*). The cells were transferred to serum-free medium and incubated with 25-OHD_3; then the cells and medium were extracted with acetonitrite, and the 1,25$(OH)_2$D was determined by duplicate radio-receptor assay following C-18 and silica SepPack chromatography, all exactly as described (Reinhardt et al. 1984; Fu et al. 1997*a*).

Amino Acid Sequence Alignments

Sequences were aligned on the basis of secondary structures rather than linear amino acid sequence alignments. The structural alignments of P450cam, P450trp, and P450eryF were determined by combining the alignments of Hasemann et al. (1994, 1995) and of Cupp-Vickery and Poulos (1995). The presumed locations of P450c1α secondary structures were determined as described (Kneller et al. 1990), using an algorithm available from the web site of the University of California, San Francisco (UCSF), Department of Pharmaceutical Chemistry. Our previous alignment (Fu et al. 1997*b*) of the P450c1α sequence with P450c25 (CYP27) and alignment of rabbit CYP27 with P450cam and P450trp (Has-

Table 2

Clinical Data in Patients with 1α-Hydroxylase Deficiency

Patient	Age at Evaluation	Ca [8.5–10.5] (mg/dl)	P [4–6] (mg/dl)	Alk Phos [150–250] (IU/L)	PTH [10–60] (pg/ml)	25OHD [10–60] (ng/ml]	1,25$(OH)_2$D [20–60] (pg/ml)
1a[a]	9.8 years	9.6	3.8	275	265[b]	30	11
1b[a]	7.8 years	9.8	3.8	356	300[b]	59	16
2	13 mo	6.2	3.7	1042	171	103	55[c]
3	10 years	6.5	3.6	625	"High"	37	8
4	18 mo	6.1	2.5	1720	980[b]	30	<2
5a	23 mo	5.5	4.7	1900	…	…	…
5b	6 mo	"Low normal"	…	600	…	…	…
6[a]	18 mo	<8.0	2.6	1300	…	256	<20
7	11 mo	6.1	2.8	1958	2231	40	9
8[d]	12.5 years	7.4	3.3	920	524[b]	24	15
9[d]	8 years	8.4	2.0	3120	"High"	124	4
10	11.5 years	7.6	5.5	1730	…	304	44
11[a]	24 mo	5.8	3.0	1005	90	…	<17
12	12 mo	7.2	2.8	1515	101	58	<5
13	16 mo	5.7	4.4	1183	472	45	19
14	10.2 years	8.0	2.9	1113	945[b]	46	<5
15	9 mo	6.8	3.8	1660	…	45	<10
16	16 mo	8.1	2.2	3542	862	86	10
17[e]	13 mo	7.4	1.5	1880	377	110	<12

NOTE.—Values in brackets denote normal pediatric ranges.

[a] Had been receiving treatment since infancy; all medications were held for >1 week.

[b] C-terminal assays (normal range <330 ng/ml).

[c] Receiving calcitriol 0.25 μg/day.

[d] Described by Pronicka et al. (1992).

[e] Described by Fu et al. (1997*a*).

Table 3

Genetic Data in Patients with 1α-Hydroxylase Deficiency

Patient	Ethnicity	Microsatellite Haplotype (Maternal/ Paternal)	Mutations[a] (Maternal/Paternal)
1a	Filipino	4-1-1/9-6-2	T409I/7bpdup
1b	Filipino	4-1-1/9-6-2	T409I/7bpdup
2	White-USA	9-5-2/4-7-1	R389H/958ΔG
3	French Canadian	4-7-1/9-1-2	958ΔG/R389H
4	French Canadian	4-7-1/4-7-1	958ΔG/958ΔG
5a	French Canadian	4-7-1/4-7-1	958ΔG/958ΔG
5b	French Canadian	4-7-1/4-7-1	958ΔG/958ΔG
6	French Canadian	4-7-1/4-7-1	958ΔG/958ΔG
7	White-USA	4-7-1/4-7-1	958ΔG/958ΔG
8	Polish	6-7-2/4-1-2	P497R/E189L
9	Polish	9-5-2/9-5-2	W241X/W241X
10	Polish	9-7-2/9-7-2	7bpdup/7bpdup
11	Chinese	9-7-2/9-5-2	7bpdup/Q65H
12	French Canadian	4-7-1/4-7-1	958ΔG/958ΔG
13	White-USA	4-1-1/9-3-3	2bpdup/7bpdup
14	Black-USA	4-5-1/6-6-1	R429P/7bpdup
15	Haitian	4-7-2/4-7-2	R453C/R453C
16	Hispanic	4-1-1/9-7-2	212ΔG/7bpdup
17	White-USA	...	1921ΔG, 1984Δc[b]

[a] Amino acid replacement mutations are indicated according to codon number; nucleotide deletions are numbered from the transcriptional start site; 7bpdup refers to the insertion of an additional CCC ACC C sequence in codons 438–442.

[b] Parental alleles unavailable for assignment.

emann et al. 1995) facilitate manual alignment using the C-terminal region of the I-helix and the EXXR motif in the K-helix as initial landmarks.

Results

Clinical Findings in 1α-Hydroxylase Deficiency

Blood or DNA samples were obtained from 19 patients in whom the diagnosis of 1α-hydroxylase deficiency had been made. The patients are presently 1–35 years old and represent 17 presumably unrelated families from various ethnic backgrounds; 2 families had 2 affected siblings. Patient 17 has been reported by us previously (Fu et al. 1997*a*) and is included for completeness. There were 11 females and 8 males, consistent with the autosomal recessive nature of the disease. All of the patients were healthy at birth but came to medical attention within the first 24 months of life, most commonly because of growth retardation, slowing of growth, or poor gross motor development. Some infants had irritability or pain when held, developed pneumonia, or had seizures. Physical findings included enlargement of the costochondral junctions of the ribs, enlargement of the wrists and ankles, genu varus, and, in some children, hypotonia or frontal bossing. All patients had radiographic signs of active rickets. Laboratory data revealed hypocalcemia, hypophosphatemia, increased serum concentrations of alkaline phosphatase and parathyroid hormone (PTH), normal concentrations of 25OHD, and greatly reduced concentrations of $1,25(OH)_2D$. The available clinical data at the time of presentation for each patient are presented in table 2. All patients were treated with either $1,25(OH)_2D_3$ or $1\alpha OHD_3$, either initially at the time of diagnosis or after failure of treatment with vitamin D_2. Currently, all patients are receiving treatment with $1,25(OH)_2D_3$, have no clinical or radiographic signs of rickets, and have normal serum levels of calcium and phosphorus. PTH concentration is normal in those in whom it has been measured.

Microsatellite Haplotyping

In a previous study, the markers D12S90, D12S305, and D12S104 showed complete linkage disequilibrium with 1α-hydroxylase deficiency, but other markers on chromosome 12 showed some recombination (Labuda et al. (1996). These three markers are close to the gene for P450c1α, but the number of alleles is limited: seven for D12S90, five for D12S305, and only three for D12S104, so that use of these three markers can describe only 105 unique microsatellite haplotypes. Among the 16 families (31 alleles) that we haplotyped, only 10 microsatellite haplotypes were found (table 3). The 4-7-1 haplotype, previously associated with the founder effect in the Charlevoix–Saguenay–Lac Saint Jean area of Québec ("Charlevoix") (Labuda et al. 1996), was found in 9 of 10 French Canadian alleles (families 3–6 and 12), as well as in 2 of 3 alleles of families 2 and 7. Heterozygous patient 2, from the southeastern United States, had a French surname. Patient 7, from the Pacific Northwest, was the product of a consanguineous union; the patient had been adopted, and the ethnic origins of the biological parents were unknown. The 4-1-1 haplotype was found in three distinct ethnic groups: Filipino (family 1), American Caucasian (family 13), and Hispanic (family 16), and the 9-7-2 haplotype was similarly widely distributed among Polish (family 10), Chinese (family 11), and Hispanic (family 16) individuals. Thus, only the 4-7-1 Charlevoix haplotype appeared to be strongly associated with one ethnic group.

Mutation Analysis

The use of a mixture of thermostable DNA polymerases with proofreading subunits permitted us to amplify the entire 4.2-kb gene for P450c1α as a single fragment. Direct sequencing on both strands of all nine exons of this PCR product without prior subcloning generally required several 4.2-kb PCR reactions to yield enough ma-

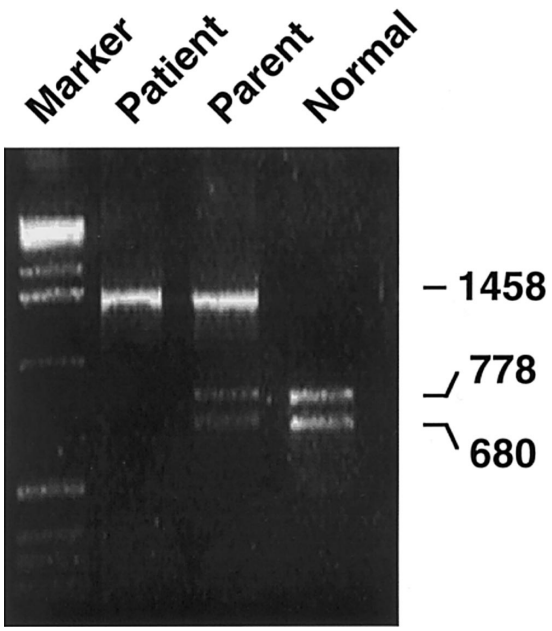

Figure 1 Genetic diagnosis of the 958ΔG (Charlevoix) mutation. Genomic DNA was amplified with oligonucleotides corresponding to bases 52–81 (sense) and bases 1612–1638 (antisense) and was digested with *Tai*I. The homozygously affected patient has only a 1458-bp band, the normal control has only the 778-bp and 680-bp bands, and the obligately heterozygous parent of the patient has all three bands. Markers: Gibco 1-kb ladder.

terial, but the resulting error rate was only ~1/12,000 bases, permitting the processing of several samples per week.

Mutations were found in all patients, but these did not always correlate well with the microsatellite haplotype analysis (table 3). The 4-7-1 Charlevoix haplotype was associated with the deletion of guanine 958, as numbered from the gene's transcriptional start site (Fu et al. 1997*b*). This 958ΔG mutation lies in codon 88, within the context 5′ ACGT 3′, which is the recognition sequence for the endonucleases *Tai*I and *Mae*II; the Charlevoix mutation destroys this site, permitting rapid diagnosis. There are three additional *Tai*I/*Mae*II sites in exon 3; however, PCR with the sense oligonucleotide 5′-ATGACCCAGACCCTCAAGTACGCCTCCAGA-3′ (bases 52–81, codons 1–10) and the antisense oligonucleotide 5′-GAGCGGGGCCAGGAGACTGCGGAGCCT-3′ (bases 1612–1638, codons 136–144) generates a 1,458-bp fragment that contains only the relevant *Tai*I/*Mae*II site. Digestion of the normal sequence yields fragments of 778 and 680 bp, digestion of the mutant sequence yields only the 1,458-bp fragment, and DNA from heterozygotes has all three fragments, thus providing rapid prenatal or other diagnosis (fig. 1). Thus, the 4-7-1 haplotype was associated with 958ΔG in all families tested.

The gene for P450c1α contains the duplicated sequence 5′-CCCACCC CCCACCC-3′ in exon 8, encoding residues 438–442 (Pro-Thr-Pro-His-Pro). In six families we found three rather than two copies of this 7-bp sequence, which alters the downstream reading frame and creates a premature TGA stop signal at codon 446. As this is upstream from the heme thiolate cystine at codon 455, the resulting truncated protein is devoid of activity. This 7-bp duplication was found in all alleles carrying the haplotype 9-7-2 (families 10, 11, and 16) but was also associated with the haplotypes 9-6-2 (family 1), 9-3-3 (family 13), and 6-6-1 (family 14). The 9-7-2 and 9-6-2 haplotypes are indeed distinct, as shown by the analysis of both D12S305 microsatellite haplotypes on a single gel (fig. 2); however, the difference could also be due to a microsatellite mutation. Furthermore, these families were of Filipino, Polish, Chinese, white, black, and Hispanic ancestry. The 7-bp duplication was associated with the 9-7-2 haplotype in families of Polish, Chinese, and Hispanic ancestry, while the possibly related 9-6-2 haplotype was in a Filipino family. It is not clear why the 9-7-2 haplotype and the 7-bp duplication are associated in such divergent populations, but it should be noted that the Généthon data indicate that this is a very common (10.6%) haplotype among Caucasians (Gyapay et al. 1994). Thus, the ethnic and haplotype diversity associated with the 7-bp duplication suggests that it has arisen by several independent de novo events and that the correlation between the microsatellite haplotypes and the mutations may be limited.

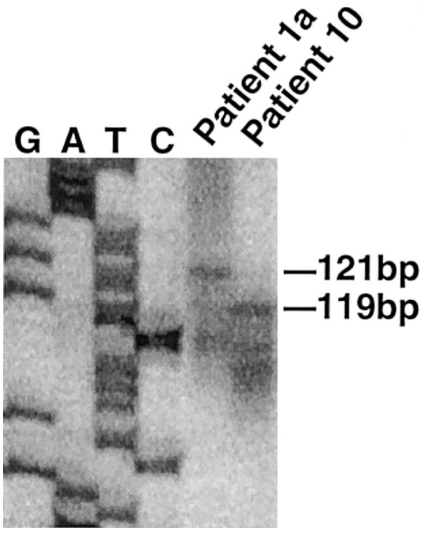

Figure 2 Microsatellite analysis. The microsatellite D12S305 was PCR amplified from DNA from patients 1a and 10 and was compared with a known DNA sequencing ladder to determine the exact sizes of the alleles. The PCR products are 121 bp and 119 bp, respectively, proving that the 9-6-2 haplotype of family 1 is different from the 9-7-2 haplotype of family 10.

Patient 13 was a compound heterozygote for the 7-bp duplication and for a 2-bp duplication (underlined) in the same region—5′-CCCACCCCCCACACCC-3′—again causing a frameshift and further suggesting that this area is a mutational hot spot. The mutation R389H was associated with two haplotypes, 9-1-2 in family 3 and 9-5-2 in family 2, and this 9-5-2 haplotype was associated with W241X in family 9. Thus, with the exception of the consistent correlation between 4-7-1 and 958ΔG, the haplotypes did not predict the mutations. Other mutations and haplotypes were confined to individual families. Patient 8 was a compound heterozygote for two haplotypes (6-7-2 and 4-1-2) and two mutations (P497R and E189L) seen in no other family. Patient 15 was homozygous for the mutation R453C and for the haplotype 4-7-2.

Activities of the Mutants

Each of the amino acid replacement mutations identified was recreated in a P450c1α expression vector by oligonucleotide-mediated site-directed mutagenesis, and its structure confirmed by complete sequencing of the mutant vector. These vectors were then transfected into mouse testicular Leydig MA-10 cells and assayed for 1α-hydroxylase activity. Like all mitochondrial P450 enzymes, P450c1α requires electron transfer from ferredoxin reductase and ferredoxin (Miller 1988). MA-10 cells contain the type I mitochondrial enzyme P450scc, which converts large amounts of cholesterol to pregnenolone and hence abundantly express the electron transport proteins ferredoxin and ferredoxin reductase. Expression of mitochondrial P450 enzymes in MA-10 cells yields 1,000-fold more activity than expression in monkey kidney COS-1 cells, which have small amounts of these proteins (Fardella et al. 1996), so that MA-10 cells provide a very sensitive assay system for the 1α-hydroxylase activity of P450c1α. As shown in table 4, none of these mutants yields significantly more activity than the low endogenous activity of the MA-10 cells (vector control), which we described previously (Fu et al. 1997*a*).

Table 4

Activity of the P450c1α Mutants

Construct	$1,25(OH)_2D$ (pg/plate)[a]
Vector	65
Q65H	68
E189L	85
R389H	45
T409I	52
R429P	58
R435C	65
P497R	60
Wild-type	1,530

[a] Detection limit, 8 pg/plate.

Discussion

The recent cloning of the cDNA and gene for human P450c1α showed that vitamin D 1α-hydroxylase activity was catalyzed solely by this enzyme and that mutation of the corresponding gene could cause VDDR-I (Fu et al. 1997*a*, 1997*b*; Kitanaka et al. 1998). We have now surveyed 19 patients from 17 kindreds with this disease and have found that all patients are homozygous or compound heterozygous for mutations in P450c1α that destroy all detectable 1α-hydroxylase activity. Thus, 1α-hydroxylase deficiency, caused by mutations in the gene encoding P450c1α, appears to be the only cause of this disease; hence, it is appropriate that the term "1α-hydroxylase deficiency" should now supersede the obsolete terms "vitamin D–dependent rickets, type I" and "pseudo–vitamin D–deficiency rickets."

P450c1α is a typical mitochondrial (class I) cytochrome P450. There are only four other known eukaryotic members of this class of enzyme: (1) P450scc, the cholesterol side-chain cleavage enzyme (Chung et al. 1986; Morohashi et al. 1987); (2) P450c11β, the steroid 11β-hydroxylase, and its isozyme P450c11AS, the aldosterone synthetase (Mornet et al. 1989); (3) P450c24, the vitamin D 24-hydroxylase (Chen et al. 1993); and (4) P450c25 (also termed "P450c27"), which catalyzes vitamin D 25-hydroxylation and the 26- or 27-hydroxylation of bile acids (Su et al. 1990; Cali and Russell 1991). To promote catalysis, all of these mitochondrial P450 enzymes require electrons from NADPH that are delivered to the P450 moiety through the intermediacy of ferredoxin (Chang et al. 1988; Picado-Leonard et al. 1988) and ferredoxin reductase (Solish et al. 1988; Lin et al. 1990). The tissue distribution of expression of these P450s is highly specific, but ferredoxin (Picado-Leonard et al. 1988) and ferredoxin reductase (Brentano et al. 1992) are found in all human tissues examined, albeit in highly varying quantities; thus, there may be other mitochondrial P450 enzymes that remain to be characterized. It may be noteworthy that all mitochondrial P450 enzymes characterized to date catalyze biosynthetic processes, in contrast to the many hepatic microsomal (class II) P450 enzymes, which are primarily involved in the catabolism of xenobiotics.

Like mitochondrial P450 enzymes, most bacterial P450s are also class I, receiving electrons from a ferredoxin and a ferredoxin reductase. The structures of several of these soluble bacterial P450 enzymes have been determined by X-ray crystallography, including the class I enzymes P450cam (Poulos et al. 1987), P450terp (Haseemann et al. 1994), and P450eryF (Cupp-Vickery and Poulos 1995) and the class II enzyme P450BM-P (Rav-

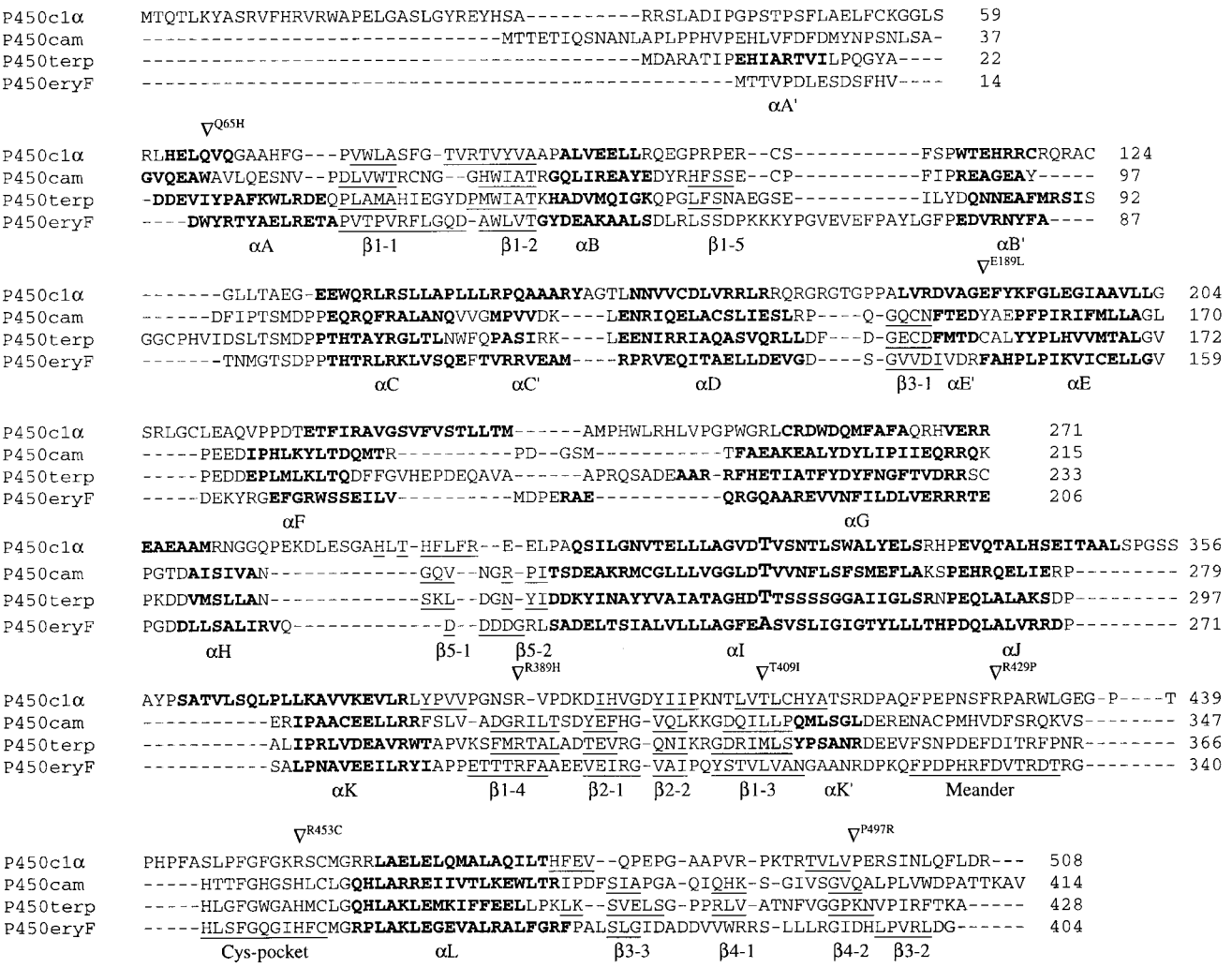

Figure 3 Alignment of the sequence of human P450c1α (*top*) with the sequences of the crystallographically solved bacterial class I P450 proteins P450cam, P450trp, and P450eryF. The names of the various α-helices and β-sheets are given below, with the α-helices highlighted in boldface and the β-sheets underlined. The locations of the "meander" and the Cys-pocket are also shown. The locations of the amino acid replacement mutations identified in P450c1α are shown above its sequence with downward-pointing arrows.

ichandran et al. 1993). Comparisons of the structures of these enzymes show remarkable conservation of their topology and their tertiary structures, despite low amino acid sequence identity (Hasemann et al. 1995). We aligned the sequence of P450c1α with the class I bacterial P450 enzymes on the basis of regions of predicted secondary structure rather than amino acid sequence identity (fig. 3). This permits a preliminary assignment of the locations of the amino acid replacement mutations we found in P450c1α. The mutation Q65H is in α-helix A′, T409I is in strand 3 of β-sheet 1, and R389H is in strand 4 of β-sheet 1. Although these mutations are distant from one another in terms of their amino acid numbers, they lie in the clustered β-sheet domain that interacts with the inner mitochondrial membrane and define the substrate entry channel. While it is not yet possible to define the exact lesion caused by each of these mutations, their locations suggest that they are likely to be conformational mutants that disrupt the ability of the enzyme to bind substrate rather than mutants that disrupt the catalytic site or the redox partner binding site. The mutant E189L lies in the E-helix and would disrupt the four-helix bundle consisting of the D, E, I, and L helices, and thus it would cause a significant disruption in the P450 structure. The mutant R429P inserts a proline at the junction of the K′ helix and the meander, changing the direction of the carbon backbone and grossly disrupting the meander. The mutant R453C, which is only two residues away from the thiolate cysteine 455, disrupts a salt bridge that interacts with the

heme proprionate, much like the corresponding R440C mutation of P450c17, which causes complete 17α-hydroxylase deficiency (Fardella et al. 1994), and like the R435C mutation in P450arom, which causes complete aromatase deficiency (Conte et al. 1994). Finally, the P497R mutant lies near strand 3 of β-sheet 3, which participates in defining the top of the substrate-binding pocket; the directional change in the α-carbon backbone resulting from insertion of a proline probably disrupts substrate binding. Thus, each of the identified P450c1α missense mutations is in a location predicted to disrupt enzymatic activity, consistent with the experimental demonstration that each lacks 1α-hydroxylase activity.

All of the frameshift and premature translation-arrest mutants eliminate the heme-binding site of P450c1α and hence cannot have 1α-hydroxylase activity. Furthermore, the seven missense mutations identified all lacked significant 1α-hydroxylase activity when expressed in transfected cells. However, despite this equivalent lack of enzymatic activity, the patients had varying degrees of severity of their clinical and laboratory findings. This finding is probably associated with other factors that can mitigate hypocalcemia, including early treatment with varying doses of calcium and with other sterols that may have a modest capacity to bind to the vitamin D receptor. Thus, the severity of the P450c1α mutation cannot be predicted from the clinical presentation of the disease.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

National Center for Biotechnology Information, Entrez, http://www.ncbi.nlm.nih.gov/Entrez (for P450c1α gene [accession number AF 027152] and cDNA [accession number AF 02092])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for VDDR-I [MIM 264700])

UCSF Department of Pharmaceutical Chemistry, http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html (for the amino acid alignment algorithm)

Whitehead Institute, http://www.genome.wi.mit.edu/cgi-bin/contig/phys_map (for sequences for microsatellite markers D12S90, D12S305, and D12S104)

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