Pediatr Nephrol (2000) 14:620–625

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Human 25-hydroxyvitamin D-1α-hydroxylase: cloning, mutations, and gene expression

Received: 12 October 1999 / Revised: 9 February 2000 / Accepted: 16 February 2000

Abstract The rate-limiting, hormonally regulated step in the bioactivation of vitamin D is the 1α-hydroxylation of 25-hydroxyvitamin D, which occurs in the kidney and other tissues and is catalyzed by the mitochondrial cytochrome P450 enzyme, P450c1α. After many years of effort, the cDNA and gene encoding this enzyme were cloned from mouse, rat, and human tissue in late 1997. The human gene encoding the 1α-hydroxylase is 5 kb in length, located on chromosome 12, and comprises nine exons and eight introns; its intron/exon organization is very similar to that of the other four mitochondrial P450 enzymes cloned to date. Mutations in P450c1α cause 1α-hydroxylase deficiency, also known as vitamin D-dependent rickets type 1, a rare autosomal recessive disease characterized by rickets and impaired growth due to failure of renal synthesis of $1,25(OH)_2D$. To date, 31 patients have been studied and 20 distinct mutations in the gene identified, including 13 mis-sense mutations, none of which encode a protein with significant enzyme activity. Recent studies in animals demonstrate that regulation of P450c1α gene expression by parathyroid hormone (PTH), low calcium diet, low phosphorus diet, and $1,25(OH)_2D$ occurs at the level of its mRNA. Transcriptional activity of the mouse and human P450c1α gene promoters can be stimulated by PTH, cAMP, and forskolin and suppressed by $1,25(OH)_2D$.

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Key words Vitamin D · 1α-hydroxylase · $1,25(OH)_2D$) · Vitamin D-dependent rickets type 1 · Rickets · P450c1α

Introduction

The hormone 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] is one of the principal hormonal regulators of calcium and phosphorus metabolism in the body and is critically important for normal growth and mineralization of bone. The classical actions of $1,25(OH)_2D$ are to stimulate calcium and phosphorus absorption from the intestine, thus maintaining plasma concentrations of these ions at levels sufficient for normal growth and mineralization of bone. $1,25(OH)_2D$ also has actions on bone, kidney, and parathyroid gland and on many other tissues unrelated to bone and mineral metabolism (for review see [1])

Biosynthesis of vitamin D

Vitamin D exists as either ergocalciferol (vitamin D_2) produced by plants, or cholecalciferol (vitamin D_3) produced by animal tissues and by the action of near ultraviolet radiation (290–320 nm) on 7-dehydrocholesterol in human skin. Both forms of vitamin D are biologically inactive pro-hormones that must undergo successive hydroxylations at carbons #25 and #1 before they can bind to and activate the vitamin D receptor (Fig. 1). The 25-hydroxylation of vitamin D occurs in the liver, catalyzed by the mitochondrial cytochrome P450 enzyme, P450c25. The activity of the hepatic 25-hydroxylase is not under tight physiologic regulation, and thus the circulating concentration of 25OHD is determined primarily by one's dietary intake of vitamin D and amount of sunlight exposure. Although 25OHD is the most abundant form of vitamin D in the blood, it has minimal capacity to bind to the vitamin D receptor and elicit a biologic response.

The active form of vitamin D, $1,25(OH)_2D$, is produced in the proximal renal tubule by the 1α-hydroxylat-

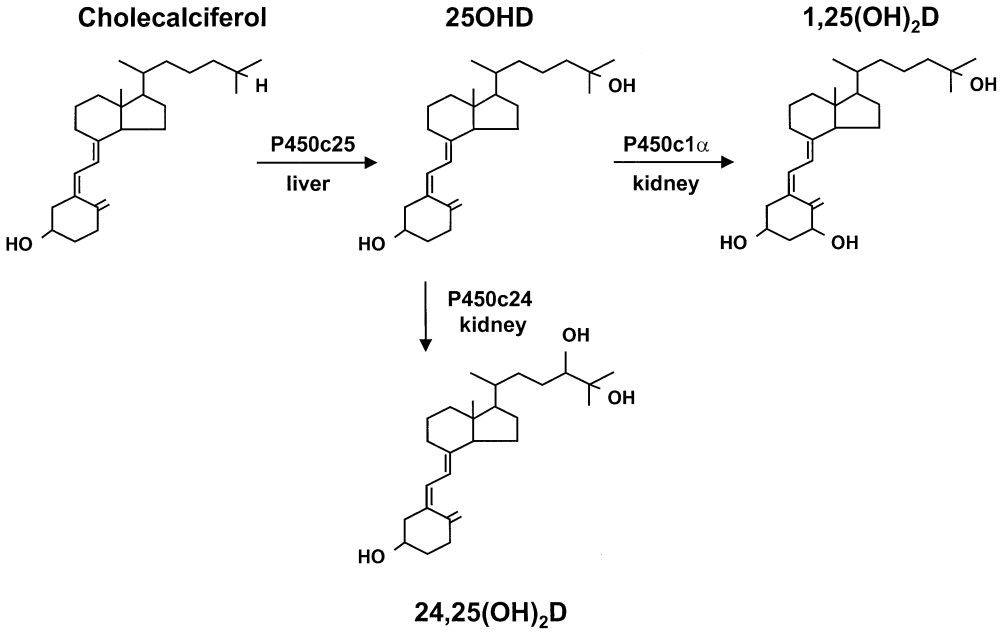

Fig. 1 Biosynthesis of vitamin D_3. Near ultraviolet light (290–320 nm) cleaves the B ring of 7-dehydrocholesterol in the skin to yield cholecalciferol (vitamin D_3). Vitamin D, which circulates in blood bound to 56-kDa vitamin D-binding protein, undergoes 25-hydroxylation in the liver. The resulting 25OHD, which is the most abundant form of vitamin D in the human circulation, may undergo 1α-hydroxylation in the kidney by P450c1α to yield the active hormonal compound $1,25(OH)_2D$. Both 25OHD and $1,25(OH)_2D$ also can undergo 24-hydroxylation in the kidney by P450c24 to yield either $24,25(OH)_2D$ or $1,24,25(OH)_3D$, respectively

ion of 25OHD (Fig. 1). The circulating concentration of $1,25(OH)_2D$ primarily reflects its synthesis in the kidney; however, 1α-hydroxylase activity also has been demonstrated in other tissues, including keratinocytes, macrophages, and osteoblasts. The 1α-hydroxylase is the rate-limiting step in the bioactivation of vitamin D, and enzyme activity in the kidney is tightly regulated by parathyroid hormone (PTH), calcium, phosphorus, and $1,25(OH)_2D$ itself [1]. Because of the importance of this enzyme in normal physiology and because synthesis of $1,25(OH)_2D$ is impaired in numerous disorders including chronic renal insufficiency, renal tubular diseases such as Fanconi syndrome and X-linked hypophosphatemic rickets, and autosomal recessive vitamin D-dependent rickets type 1, the 1α-hydroxylase has been the subject of intense study for nearly 30 years.

The other important vitamin D-metabolizing enzyme, the 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase), is found not only in kidney, but also in intestine, lymphocytes, fibroblasts, bone, skin, macrophages, and possibly other tissues [2]. The enzyme can catalyze the 24-hydroxylation of 25OHD to $24,25(OH)_2D$ and also of $1,25(OH)_2D$ to $1,24,25(OH)_3D$; both reactions are thought to initiate the metabolic inactivation of vitamin D via the C24-oxidation pathway. The kidney and intestine are major sites of hormonal inactivation of vitamin D by virtue of their abundant 24-hydroxylase activity.

The vitamin D biosynthetic enzymes

The vitamin D 25-hydroxylase, the 1α-hydroxylase, and the 24-hydroxylase are mitochondrial, or type 1, cytochrome P450 enzymes. Such enzymes receive electrons from NADPH via an electron-transfer chain consisting of two proteins, a flavoprotein termed ferredoxin reductase and an iron-sulfur protein termed ferredoxin. By contrast, type II P450 enzymes, such as the drug-metabolizing hepatic enzymes or the steroidogenic 21-hydroxylase enzyme, are found in the endoplasmic reticulum, and they receive electrons from a flavoprotein termed P450 oxidoreductase, sometimes with the allosteric assistance of cytochrome b_5 [3].

Vitamin D 25-hydroxylase and 24-hydroxylase

The hepatic 25-hydroxylase was the first of the three vitamin D-metabolizing enzymes to be cloned. This was accomplished by purifying vitamin D 25-hydroxylase from rat liver mitochondria and using polyclonal antisera raised against the purified protein to screen a rat liver cDNA expression library, yielding a P450c25 cDNA [4, 5]. This enzyme also can hydroxylate carbons 26 and 27 of sterols to initiate bile acid synthesis, and is often referred to as P450c27 [6]. The 24-hydroxylase was the second vitamin D-metabolizing enzyme to be cloned, again by purifying the protein from rat renal mitochondria, raising a polyclonal antiserum, and screening a rat kidney cDNA expression library [7]. Subsequently, the human cDNA [8] and gene [9] were cloned. Studies with the purified rat kidney enzyme [7] and with cells expressing the human P450c24 cDNA [8] confirmed that this enzyme can catalyze the 24-hydroxylation of both 25OHD and $1,25(OH)_2D$.

Vitamin D 1α-hydroxylase

Despite the cloning of the 25- and 24-hydroxylases in 1990 and 1991, it was not until the second half of 1997 that four groups of investigators working independently

using different approaches reported the cloning of the mouse, rat, and human vitamin D-1α-hydroxylase cDNAs, P450c1α, and the human gene [10–15]. Efforts to purify the 1α-hydroxylase enzyme were consistently unsuccessful, primarily because there is very little P450c1α protein in renal mitochondria. Hence the immunologic approaches used to clone the 24- and 25-hydroxylase enzymes could not be used.

We approached the problem of the low renal abundance of P450c1α by using a different tissue system, primary cultures of human keratinocytes [10]. These cells, when grown in serum-free medium in the presence of low concentrations of calcium, exhibit substantial 1α-hydroxylase activity [16]. Our efforts to screen a human keratinocyte cDNA library by probe hybridization and by expression cloning were unsuccessful. Using keratinocytes as a source of RNA enriched for P450c1α mRNA, we prepared degenerate-sequence oligonucleotides based on the relatively well conserved sequences of the ferredoxin-binding sites and heme-binding sites of P450c24 and P450c25, and used these for PCR amplification of the keratinocyte cDNA. The resulting 300-bp PCR product was then cloned and sequenced, yielding partial-length candidate clone for P450c1α. This was used to screen a keratinocyte cDNA library yielding a partial-length, 1.9-kb cDNA, whose complete sequence was obtained by rapid amplification of cDNA ends (5'-RACE) [10]. The human P450c1α cDNA is 2.4 kb in length and encodes a protein of 508 amino acids with a predicted molecular mass of 56 kDa [10].

Only five mitochondrial P450 enzymes have been identified to date, three of which are involved in the metabolism of vitamin D, the other two being P450scc, the cholesterol side-chain cleavage enzyme, and P450c11β-hydroxylase, and its isozyme P450c11AS, the aldosterone synthase. Within the heme-binding region of the P450c1α enzyme, the predicted amino acid sequence identity is 65%–73% to that of human P450c25 and P450c24; however, its overall identity to that of the other mitochondrial P450 enzymes is limited, 30%–39% [10].

After having cloned a P450c1α cDNA from human keratinocytes, we developed four lines of evidence that the keratinocyte and renal P450c1α enzymes are encoded by the same gene [10]. First, our P450c1α cDNA was transfected into mouse Leydig MA-10 cells, which contain abundant ferredoxin and ferredoxin reductase. The transfected cells catalyzed the conversion of $25OHD_3$ to $1,25(OH)_2D_3$, as measured by radioreceptor assay and as shown by comigration with authentic $1,25(OH)_2D_3$ using two different high-pressure liquid chromatographic solvent systems. Second, using this cell system, we synthesized substantial amounts of $1,25(OH)_2D_3$ and confirmed its chemical identity by gas chromatography/mass spectrometry. Third, we used reverse transcription/polymerase chain reaction (RT/PCR) to show that those sequences we had cloned from keratinocytes also were expressed in human kidney. Fourth, we obtained keratinocytes from a patient with vitamin D-dependent rickets type 1(VDDR-I), cloned the P450c1α cDNA from these cells,

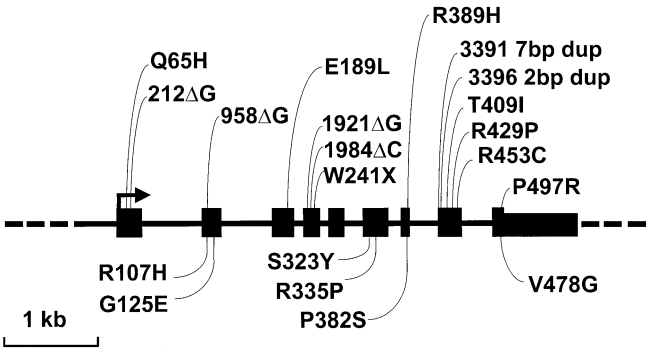

Fig. 2 Scale diagram of the intron/exon organization of the human gene encoding P450clα, as reported by Fu et al. [11]. All mutations causing 1α-hydroxylase deficiency reported through mid-1999 are shown. All mutations depicted above the gene are reported by Wang et al. [20]; ΔG958 and 3391 7 bp dup, also reported by Yoshida et al. [19]; R107H, G125E, R335P, and P382S, reported by Kitanaka et al. [18]; S323Y and V478G, reported by Smith et al. [21]

and demonstrated that the patient was a compound heterozygote for two deletion/frameshift mutations that resulted in premature truncation of the protein. We found that cultured skin keratinocytes from the patient were devoid of 1α-hydroxylase activity. Thus, we provided genetic proof of the identify of our P450c1α and the first proof that VDDR-I is caused by mutations in vitamin D-1α-hydroxylase gene [10].

After we cloned the human cDNA, we reported the cloning of the human gene, its localization to chromosome 12 by somatic cell hybrid analysis, the localization of the transcriptional start site, and a structural analysis of the genes for the four mitochondrial P450s now available (P450c1α, P450c24, P450scc, P450c11) [11]. The human gene for 1α-hydroxylase is of single copy and comprises nine exons and eight introns (Fig. 2) [11]. Although it is a substantially smaller gene, 5 kb, than those for other mitochondrial P450 enzymes, its intron/exon organization is very similar, especially to that of P450scc [11]. This strongly suggests that although the mitochondrial P450 enzymes retain only 30%–40% amino acid sequence identity with each other, they all belong to a single evolutionary lineage. Other groups of investigators also have reported cloning of the human P450c1α cDNA and gene [12, 15] and the mouse gene [17].

The molecular genetics of 1α-hydroxylase deficiency

Within recent months, the molecular genetics of 1α-hydroxylase deficiency has been studied by several groups [10, 18–21]. This autosomal recessive disorder, which has been termed vitamin D-dependent rickets type 1 (VDDR-I) or pseudovitamin D-deficiency rickets, is characterized by failure to thrive, muscle weakness, hypocalcemia, secondary hyperparathyroidism, and the bony changes of rickets [22]. The hallmarks of the dis-

ease are greatly reduced serum concentrations of $1,25(OH)_2D$ despite normal or increased concentrations of 25OHD, and the reversal of clinical and laboratory abnormalities by administration of physiologic amounts of $1,25(OH)_2D_3$ [22–24]. It has been assumed that the genetic defect results from defective renal 1α-hydroxylation of 25-OHD [22]. The first patient we studied, an American girl [10], and four unrelated Japanese patients studied by Kitanaka and colleagues [18] revealed that mutations in P450c1α could cause VDDR-I. However, it was not yet known whether all patients with the clinical syndrome of VDDR-I had mutations in P450c1α, nor was it known whether a genetic cluster of patients with VDDR-I in French Canada all had the same mutations.

We now have studied the P450c1α genes of 19 patients with VDDR-I from 17 families representing multiple ethnic groups, 5 French Canadian, 3 Polish, 4 Caucasian American, 1 Filipino, 1 Chinese, 1 Haitian, 1 African American, and 1 Hispanic families [20]. 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 or poor gross motor development. All patients had typical laboratory findings of 1α-hydroxylase deficiency: hypocalcemia, hypophosphatemia, increased serum concentrations of alkaline phosphatase and parathyroid hormone, normal serum concentrations of 25OHD, and low or undetectable concentrations of $1,25(OH)_2D$ (Table 1). All patients had radiographic evidence of rickets and all responded to physiologic replacement doses of $1,25(OH)_2D_3$. For each family, we identified the parental origin of all P450c1α mutations and correlated the mutations with the microsatellite haplotyping of chromosome 12q13 (where the P450c1α gene lies). We also measured the 1α-hydroxylase activity of the seven mis-sense mutations identified.

Although VDDR-1 is rare in most populations, it is more common among French Canadians, having an apparent carrier rate of 1/26 in the Charlevoix-Seguenay-Lac-Saint Jean region of Quebec [25]. In a prior study, Labuda et al. examined the microsatellite genetic markers on chromosome 12 and observed that the French Canadian patients with VDDR-I carried one of two haplotypes [26]. Patients from the Charlevoix region of Quebec (the "Charlevoix" population) carried haplotype 4-7-1, while patients from eastern Canada (the "Acadian" population) carried haplotype 6-7-2 [26]. Among the five French Canadian families we studied, nine of ten unique alleles carried the 4-7-1 haplotype and all nine of these carried the identical mutation in codon 88, deletion of G958, that changes the reading frame and leads to premature termination of translation; the resultant protein would have no enzyme activity. Thus, our finding that haplotype 4-7-1 is strongly associated with the ΔG958 mutation identifies it as the "Charlevoix" mutation. This mutation deletes the G in the sequence 5'ACGT3', a sequence normally recognized by the endonucleases *TaiI* and *MaeII*. This feature was used to design a rapid, accurate PCR-based diagnostic tactic that can detect this mutation in genomic DNA from any source [20].

Yoshida et al. characterized the P450c1α genes of four French Canadian patients [19], and found three to be homozygous for the ΔG958 mutation and one homozygous for the duplication of a 7-bp sequence in exon 8 [19]. Based on the geographic origins of each patient, Yoshida et al. suggested that mutation ΔG958 is the Charlevoix mutation and that the 7-bp duplication is the Acadian mutation, but did not perform microsatellite haplotyping to confirm this. We found this 7-bp duplication, upstream from codon 441, on seven separate alleles in six families. Four of these alleles carried the haplotype 9-7-2 but were found in different ethnic groups: Polish, Chinese, and Hispanic. The other three alleles bearing the 7-bp duplication carried the haplotypes 9-6-2, 9-3-3, and 6-6-1, and were found among Filipino, Caucasian American, and African American patients (Table 1). Only one of our patients (from Poland) carried the Acadian 6-7-2 haplotype, but that allele carried the mis-sense mutation P497R, rather than the 7-bp duplication. Smith et al. recently identified two unrelated patients from the United Kingdom who were homozygous for this same 7-bp duplication [21]. Thus the 7-bp duplication can arise de novo among many different ethnic groups, and the identity of the Acadian mutation remains to be established.

We identified a total of 14 different mutations in the 19 patients, including 7 amino acid replacement (mis-sense) mutations. To determine the effect of these mutations on enzyme activity, we expressed each mutant in transfected MA-10 cells, and found that none of the mis-sense mutations encoded a protein with 1α-hydroxylase activity significantly above the low endogenous activity of MA-10 cells [20]. Four additional mis-sense mutations were identified by Kitanaka et al. in four Japanese families, and the activities of these mutants were tested in a promoter/reporter transactivation assay based on activation of the vitamin D receptor by $1,25(OH)_2D$ [18]. None of the four mutants showed any activity consistent with the VDDR-I phenotype of the patients. Two other mis-sense mutations were identified by Smith et al. in a compound heterozygous patient from the United Kingdom [21]; 1α-hydroxylase activity in peripheral blood macrophages from the patient was undetectable although activity was present in cells from normal individuals and from the obligately heterozygous parents.

To date, a total of 31 patients with 1α-hydroxylase deficiency have been studied at a molecular genetic level and 20 distinct mutations, including 13 mis-sense mutations, have been identified. However, none of the mis-sense mutations encodes a protein with significant enzyme activity. The failure to identify mutations that can decrease rather than eliminate 1α-hydroxylase activity may reflect the small number of patients studied to date, particularly those with mild clinical findings; alternatively such mutations may not produce a clinically identifiable disorder.

Molecular regulation of the 1α-hydroxylase

With the cloning of P450c1α, attention has focused on understanding the molecular basis of regulation of the enzyme. In the kidney, 1α-hydroxylase activity is stimulated by PTH, insulin-like growth factor 1 (IGF-1), hypocalcemia, and hypophosphatemia; conversely, enzyme activity is suppressed by hyercalcemia, hyperphosphatemia, and $1,25(OH)_2D_3$ itself. The stimulatory effect of PTH on 1α-hydroxylase activity can be mimicked by cAMP and the adenylate cyclase activator, forskolin, suggesting that activation of the protein kinase A (PKA) signaling pathway is involved; such stimulation also requires an intact phospholipase C/protein kinase C signaling pathway. Studies to date in intact animals reveal that regulation of P450c1α gene expression by PTH, low calcium diet, and $1,25(OH)_2D$ occurs at the level of its mRNA [13, 14]. Brenza et al. cloned 1.7 kb of DNA flanking the 5' region of the mouse gene and, using a luciferase reporter gene construct, demonstrated that the gene's promoter activity was transcriptionally activated by PTH and forskolin [27]. Although $1,25(OH)_2D_3$ did not induce a decrease in basal promoter activity, it did inhibit by ~40% the increase otherwise induced by PTH [27]. These investigators could identify no consensus sequence for a vitamin D-responsive element within the 5'-flanking sequence of the gene. Murayama et al. [17] and recently Kong et al. [28] also found that transcriptional activity of the human gene promoter was induced by PTH, forskolin, and calcitonin. Suppression of basal promoter activity by $1,25(OH)_2D$ was observed by Murayama's group [17] but not by Kong's group [28], who rather observed that $1,25(OH)_2D_3$ suppressed PTH-stimulated promoter activity, findings similar to those of Brenza et al. [27].

The importance of phosphorus as a determinant of the renal production of $1,25(OH)_2D$ has been demonstrated in studies of experimental animals and healthy humans (for review see [1]). Hypophosphatemia induced by restricting dietary phosphorus induces an increase in the production rate and serum concentration of $1,25(OH)_2D$ [29–31] and in 1α-hydroxylase activity measured in vitro [32]. In healthy humans, changes in serum concentration of $1,25(OH)_2D$ induced by manipulating dietary phosphorus entirely reflect changes in its production rate; its metabolic clearance rate does not change [30]. In normal mice, hypophosphatemia induced by restricting dietary phosphorus induced a fivefold increase both in renal mitochondrial 1α-hydroxylase activity and in renal P450c1α mRNA abundance, whereas renal 24-hydroxylase mRNA abundance decreased by threefold [33]. These findings demonstrate that renal expression of the 1α-hydroxylase and 24-hydroxylase genes are regulated by phosphorus in a reciprocal fashion. The increase in $1,25(OH)_2D$ production induced by phosphorus restriction is abolished by hypophysectomy and is restored by administration of growth hormone or IGF-1 [34, 35], indicating that an intact growth hormone/IGF-1 axis is required for the effect. Although growth hormone and IGF-1 can enhance renal tubular reabsorption of phosphate, it is not yet known how changes in tubular transport of phosphate are linked to regulation of renal 1α-hydroxylase activity.

Acknowledgements Work in the authors' laboratories was supported by NIH grants DKAR54433 (A.A.P.), DK37922 (W.L.M.), DK42154 (W.L.M.), by grants from the March of Dimes Birth Defects Foundation (A.A.P. and W.L.M.), and by gifts from the David Carmel Trust (A.A.P.).

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