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Enzymes involved in the activation and inactivation of vitamin D

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Six cytochrome P450 (CYP) isoforms have been shown to hydroxylate vitamin D. Four of these, CYP27A1, CYP2R1, CYP3A4 and CYP2J3, are candidates for the enzyme vitamin D 25-hydroxylase that is involved in the first step of activation. The highly regulated, renal enzyme 25-hydroxyvitamin D-1α-hydroxylase contains the component CYP27B1, which completes the activation pathway to the hormonal form 1α,25-dihydroxyvitamin D_3. A five-step inactivation pathway from 1α,25-$(OH)_2D_3$ to calcitroic acid is attributed to a single multifunctional CYP, CYP24A1, which is transcriptionally induced in vitamin D target cells by the action of 1α,25-$(OH)_2D_3$. On the basis of alignments and crystal structures of other CYPs, homology models of vitamin-D-related CYPs have been generated. Two human forms of rickets caused by mutations of CYP2R1 and CYP27B1, as well as mouse knockout models of CYP27A1, CYP27B1 and CYP24A1, are helping us to establish the full *in vivo* physiological roles of the vitamin-D-related hydroxylases.

The history of the vitamin D hydroxylases began 37 years ago with the identification of the hydroxylated metabolites of vitamin D, including the principal circulating form, 25-OH-D_3 [1], and the hormonal form, 1α,25-$(OH)_2D_3$ (reviewed in Refs [2–4]). With the discovery of a bewildering array of different metabolites of vitamin D_3 and vitamin D_2 came the realization that specific 'mixed-function oxidases' must carry out these hydroxylation reactions. In fact, studies of the first tissue preparations containing the 25-OH-D_3-1α-hydroxylase, which showed that this enzyme is largely confined to the kidney [5], were done before the full structural characterization of the hormonal form was completed [6]. There followed several landmark papers describing the properties of the three main enzymes: vitamin D_3-25-hydroxylase, 25-OH-D_3-1α-hydroxylase and 25-OH-D_3-24-hydroxylase [7–9]. It quickly became apparent that partially purified vitamin D hydroxylases could be shown to contain CYP components that could be reconstituted in the test-tube with nicotinamide adenine dinucleotide phosphate (NADPH), ferredoxin and ferredoxin reductase to give vitamin D hydroxylase activity [10,11]. This review provides a historical perspective of the vitamin D hydroxylases, the biochemistry of their specific cytochrome P450 components and an overview of their physiological roles in the body.

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Cloning the CYP components of vitamin D hydroxylases

Studies on vitamin D metabolism during the 1980s simplified the myriad of metabolites into essentially three pathways (Figure 1): (i) the main two-step activation pathway in liver and kidney that produces 1α,25-$(OH)_2D_3$; (ii) a fairly ubiquitous, inducible carbon-24 oxidation pathway in vitamin D target cells for inactivating 25-OH-D_3 to 24,25-$(OH)_2D_3$ and 1α,25-$(OH)_2D_3$ to calcitroic acid [12–14]; (iii) and an enigmatic 26,23-lactone pathway for converting both 25-OH-D_3 and 1α,25-$(OH)_2D_3$ to lactone products [15–17].

The existence of these pathways implied that there were at least four or more CYPs responsible for vitamin D metabolism because the activation pathway (Figure 1) involved two different enzyme activities located in distinct tissues, and there seemed to be two catabolic pathways. In the early 1990s, Ohyama *et al.* [18] made a significant breakthrough with the first cloning of a vitamin-D-related CYP, rat CYP24A1, and the cloning of the human homolog of CYP24A1 quickly followed [19]. Subsequently, it was shown by using various transfection models and reconstitution systems with purified preparations that CYP24A1 was responsible for the multiple side chain hydroxylation and/or oxidation steps in pathways leading to calcitroic acid and 1α,25-$(OH)_2D_3$-26,23-lactone, respectively [20–22] (Figure 1).

In 1989, Russell's group [23] cloned a rabbit mitochondrial CYP, later named CYP27A1, one of the candidate isoforms vying for the title of vitamin D_3-25-hydroxylase, but it was not until 1992 with the cloning of the human isoform that the potential of this enzyme in vitamin D metabolism was realized [24]. Although CYP27A1 was found to be capable of the 25-hydroxylation of vitamin D_3 [25], it seemed more likely to be the low-affinity, high-capacity version of the 25-hydroxylase enzyme reported in previous tissue studies [7].

In 1997, St-Arnaud *et al.* [26] identified the second member of the CYP27 family when they cloned the rat CYP27B1 and showed that the gene encoding the human homolog mapped to the same locus as vitamin D dependency rickets (VDDR) type 1, which was postulated to be the result of mutations in the 25-OH-D_3-1α-hydroxylase. Work by several other groups led to the cloning of CYP27B1 from various species, including human, and unequivocally proved that CYP27B1 is the 1α-hydroxylase enzyme found in proximal renal tubules that carries out the 1α-hydroxylation of 25-OH-D_3 and is the enzyme that is defective in VDDR type 1 [27–31].

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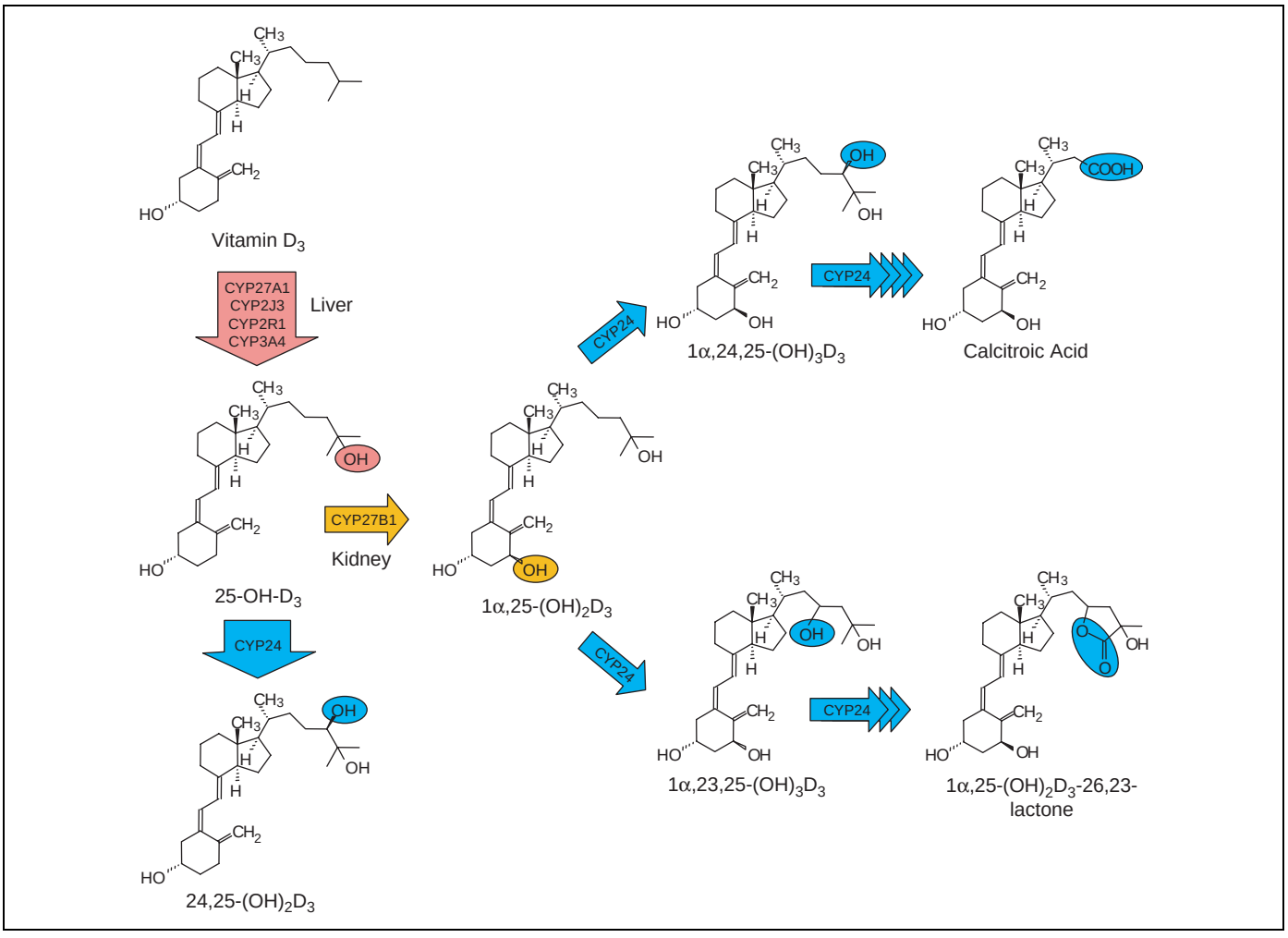

Figure 1. Pathways of vitamin D activation and inactivation. Vitamin D_3 and its chief metabolites are arranged in three pathways: an activation pathway from vitamin D_3 to $1\alpha,25$-dihydroxyvitamin D_3 [$1\alpha,25\text{-}(OH)_2D_3$] involving the 25-hydroxylase (red) and the 1α-hydroxylase (gold); and two catabolic pathways, the carbon-24 oxidation pathway from $1\alpha,25\text{-}(OH)_2D_3$ to calcitroic acid and the lactone pathway from $1\alpha,25\text{-}(OH)_2D_3$ to the $1\alpha,25\text{-}(OH)_2D_3$-26,23-lactone, which both involve the 24-hydroxylase (blue). Transport of vitamin D_3 to the liver, transport of 25-hydroxyvitamin D_3 from the liver to the kidney, and transport of $1\alpha,25\text{-}(OH)_2D_3$ from the kidney to the target cell are carried out by the plasma vitamin-D-binding protein (also known as Gc protein). Also shown in the figure (with the steps that they are known to carry out) are the cytochrome P450 (CYP) isoforms that are thought to be candidates for the 25-hydroxylase and the two established vitamin D hydroxylases, CYP27B1 and CYP24A1. Note that CYP24A1 is a multifunctional CYP that has been shown *in vitro* to catalyze the formation of the inactive blood metabolite 24,25-dihydroxyvitamin D_3 [$24,25\text{-}(OH)_2D_3$]. Note also that vitamin D_2 is an artificial form of the vitamin with an additional double bond between carbons 22 and 23, and an additional methyl group at carbon 24. Vitamin D_2 can be converted by the same enzymes, 25-hydroxylase, 1α-hydroxylase and 24-hydroxylase, into a set of products analogous to the ones shown here for vitamin D_3, except that there is no lactone formation and the carbon-24 oxidation pathway ends at 24,25-dihydroxyvitamin D_2 because of the 22–23 double bond.

Recently, the nature of the human microsomal vitamin D_3-25-hydroxylase has become clearer. At least five microsomal CYPs from various species have been shown to be capable of the 25-hydroxylation of vitamin D: namely, CYP2D11, CYP2D25, CYP2J2/3, CYP3A4 and CYP2R1; however, only the latter three of these seem to match the enzymatic properties and distribution pattern of the physiologically relevant human liver microsomal enzyme [32–34]. So far CYP2J3 has been reported only in rat, and there is no clearly defined human equivalent although CYP2J2 might be a possible candidate. CYP3A4 is a broad-specificity enzyme responsible for the catabolism of 50% of known drugs and shows enzyme kinetics, with vitamin D as a substrate, that are more similar to those of CYP27A1 than to those of the high-affinity 25-hydroxylase reported in the literature [7]. Alternatively, CYP2R1 shows high affinity for vitamin D, and Russell's group [35] has reported that a Leu99Pro mutation in CYP2R1 correlates with a form of hereditary rickets, thereby identifying the first human mutation of a liver CYP that is associated with this disease.

Structure and enzymatic properties of vitamin-D-related CYPs

The main activation and inactivation pathways of vitamin D are shown in Figure 1, along with the CYPs that are now thought to be responsible. As with all mitochondrial CYP-containing enzymes, the functional enzyme activity requires the assistance of two additional electron-transporting proteins to enable the reducing equivalents of NADPH to be transferred to the hydroxylation reaction via an electron chain consisting of a general-purpose ferredoxin reductase, a general-purpose ferredoxin, and a highly-specific CYP [36] (Figure 2a). Although there are about 60 CYP genes in the human genome, there is only one ferredoxin reductase gene and only one ferredoxin gene. The microsomal CYPs are simpler in that they

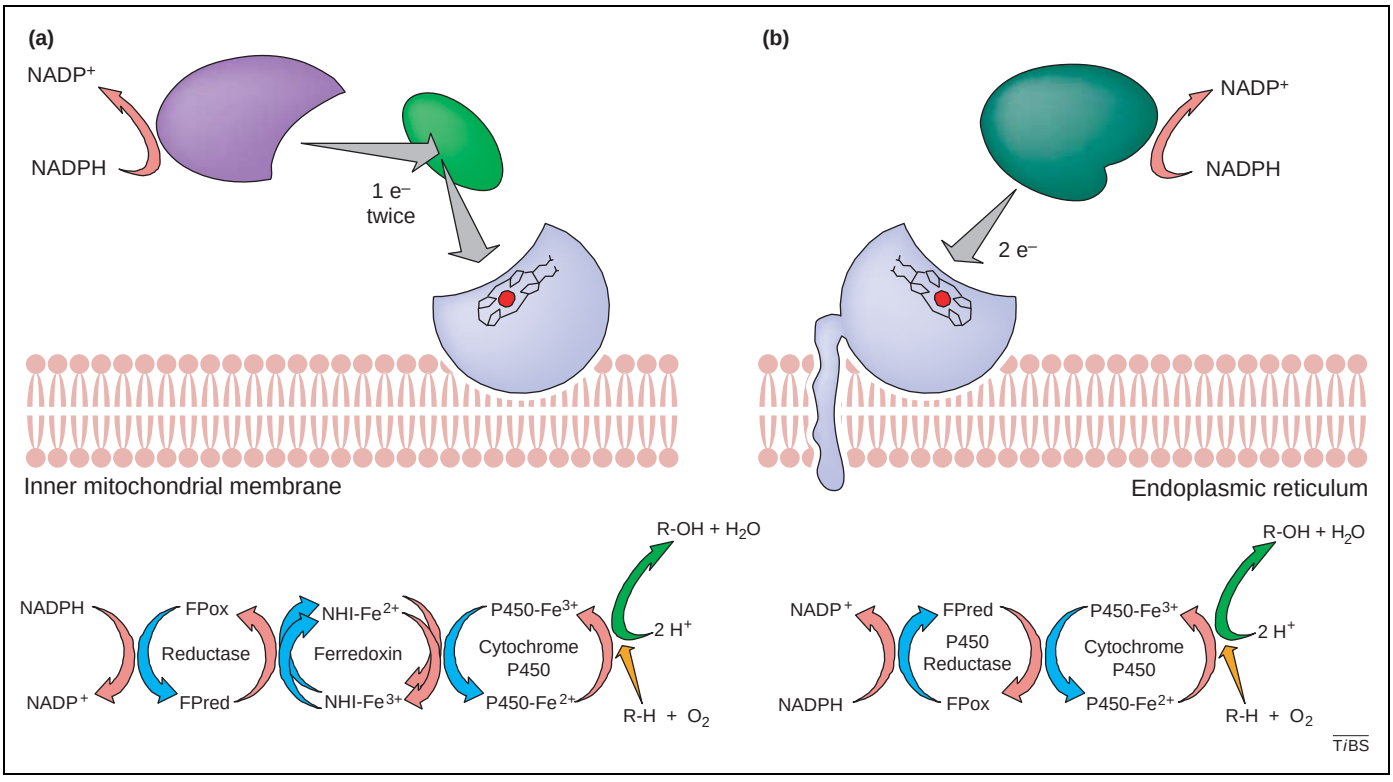

Figure 2. Electron transport chains and protein components of vitamin D hydroxylases. **(a)** In mitochondria, electron equivalents from nicotinamide adenine dinucleotide phosphate (NADPH) are captured by the flavoprotein (FP) ferredoxin reductase (purple) and transferred through a pool of ferredoxin (green), which contains non-heme iron (NHI) and carries a single electron, to the mitochondrial cytochrome P450 (CYP; pale purple). **(b)** By contrast, the reducing equivalents captured from NADPH by the flavoprotein NADPH–CYP reductase (dark green) are transferred sequentially to the microsomal CYP (pale purple). In both systems, the electron-transporting protein binds near the proximal heme face, and reducing equivalents are conveyed via the heme iron to reduce molecular oxygen and to hydroxylate (oxidize) the substrate (R-H).

require a single, associated, general-purpose protein, NADPH–CYP reductase (Figure 2b).

Because the CYPs provide the specificity of the vitamin D hydroxylation (or other oxidation) reaction, there has been a great deal of interest in defining their domain structure. Because CYPs in general are highly conserved across all phyla [37] and the vitamin D hydroxylases have remained largely unchanged from bony fishes to humans, multisequence alignments help to pinpoint functionally conserved motifs in the enzyme. Figure 3 presents an alignment of the principal human vitamin-D-related CYPs, CYP24A1, CYP27A1, CYP27B1 and CYP2R1, along with the other microsomal CYPs that are thought to possess 25-hydroxylase activity. Immediately apparent from the alignment are the functionally conserved domains that are common to both mitochondrial and microsomal enzymes.

Figure 3 also shows the predicted secondary structure of the vitamin-D-related CYPs derived from homology modeling with ten X-ray crystal structures of other CYPs that are currently available in the literature. Note that all CYPs are proteins of 50–55 kDa with a series of highly conserved helices (designated A–L) connected by loops and β-sheet structures. All CYPs contain a cysteine residue near the C terminus to which the heme group attaches; in addition, CYPs contain several other domains for interaction with the electron-transferring machinery such as ferredoxin or NADPH-CYP reductase. The N terminus is thought to insert into the endoplasmic reticulum membrane for microsomal CYPs or the inner mitochondrial membrane for mitochondrial CYPs. The substrate-binding pocket is formed by several secondary structures folded around the distal face of the heme-group so that the substrate can be brought to within 3.2-Å of the iron atom. Figure 4a,b shows the predicted folding around the substrate-binding pocket of CYP24A1. Current research is focused on defining the subtleties of the substrate-binding pocket for the enzymes CYP24A1, CYP27A1 and CYP2R1 that hydroxylate the side chains and on contrasting these structures with the binding pocket of CYP27B1, which hydroxylates the A-ring of the vitamin D molecule.

Regulation and physiological roles of 25-OH-D_3-1α-hydroxylase

The discovery of the 25-OH-D_3-1α-hydroxylase (CYP27B1) led immediately to the recognition of its central role in Ca^{2+} homeostasis [2]. The master switch of Ca^{2+} homeostasis is the Ca^{2+}-sensing receptor in the parathyroid cell, which regulates the secretion of parathyroid hormone (PTH). In turn, PTH is the principal activator of 25-OH-D_3-1α-hydroxylase gene expression, which represents the fine switch needed to titrate the plasma concentration of 1α,25-$(OH)_2D_3$ [38] [Box 1, Figure Ia and b(i)]. This regulation is independently augmented by low Ca^{2+} and PO_4^{3-} signals [39], although the signal transduction mechanism that mediates this upregulation of the CYP27B1 at the molecular level remains elusive.

By maintaining tight regulation of the concentration of 1α,25-$(OH)_2D_3$, and thereby giving rise to appropriate

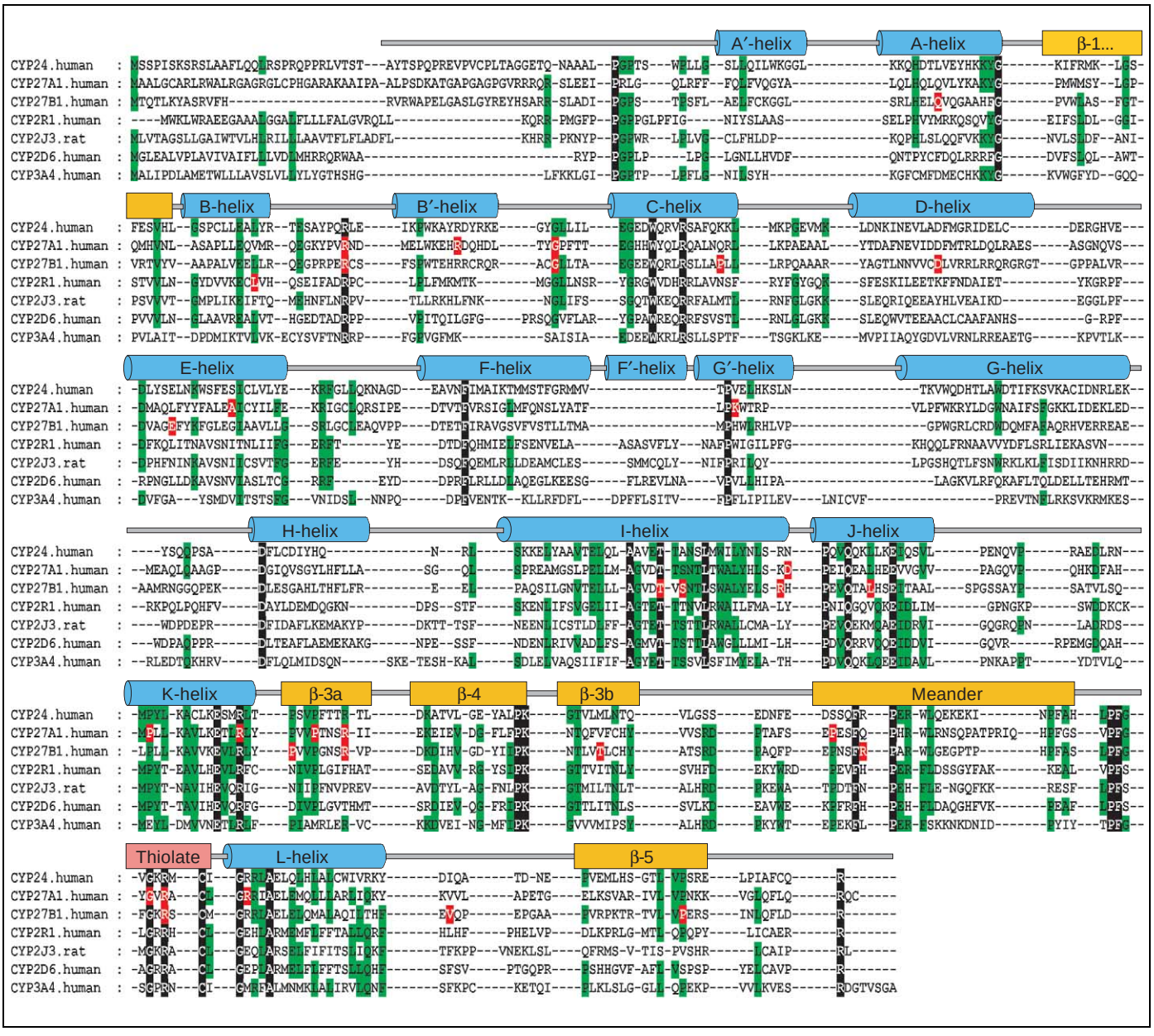

Figure 3. Sequence alignment with predicted secondary structure for vitamin D hydroxylases. Invariant (black-shaded) and partially conserved (green-shaded) residues in mitochondrial (CYP24A1, CYP27A1, CYP27B1) and microsomal (CYP2R1, rat CYP2J3, CYP2D6, CYP3A4) proteins are associated with the heme-binding site (thiolate) and structural motifs such as the ERR triad (not shown). Predicted secondary structures with helices (blue) and β-sheet structures (gold) are shown above the sequence alignments. Missense mutations (red-shaded) associated with cerebrotendinous xanthomatosis (CYP27A1 deficiency) and vitamin D dependency rickets type 1 (CYP27B1 deficiency) are described in Table 1. Sequences were retrieved from Entrez (www.ncbi.nlm.nih.gov/Entrez/index.html) and the Cytochrome P450 Homepage (drnelson.utmem.edu/CytochromeP450.html) and adjusted manually using GeneDoc v2.6 in a larger alignment (not shown) of species homologs and crystal structures, including CYP2B4, CYP2C5, CYP2C8, CYP2C9, CYP3A4, CYP51 (*Mycobacterium tuberculosis*), CYP119 (*Sulfolobus solfactaricus*) and P450Bm-3, cam, eryF and terp.

transcriptional activation of the genes involved in Ca^{2+} and PO_4^{3-} transport and cell differentiation [40], the 25-OH-D_3-1α-hydroxylase plays a vital role in vitamin D signaling. The hormone, 1α,25-$(OH)_2D_3$, accomplishes this modulation of gene transcription through a nuclear transcription factor, the vitamin D receptor (VDR), that operates in conjunction with several coactivators and corepressors known as vitamin D receptor interacting proteins (DRIPs) [41]. This vitamin-D-related transcriptional machinery targets appropriate genes for regulation through short repeating hexanucleotide sequences in the genome known as vitamin D response elements (VDREs), which are usually located upstream of the vitamin-D-dependent gene in its promoter.

A detailed description of the vitamin D transcriptional machinery and a comparison with that of other steroids and dietary lipids is beyond our scope here, but excellent reviews of vitamin D and general steroid hormones have been recently published elsewhere [39,41,42]. Suffice to say that encompassed within the numerous genes that are regulated by 1α,25-$(OH)_2D_3$ are the vitamin D hydroxylases themselves, and this set-up therefore constitutes a form of autoregulation [Box 1, Figure Ib(i,iii)]. The gene promoter of CYP27B1 contains negative regulatory VDRE elements that enable the 25-OH-D_3-1α-hydroxylase enzyme activity to be transcriptionally downregulated [43–45].

The physiological role of the renal 25-OH-D_3-1α-hydroxylase in vitamin D activation has not been

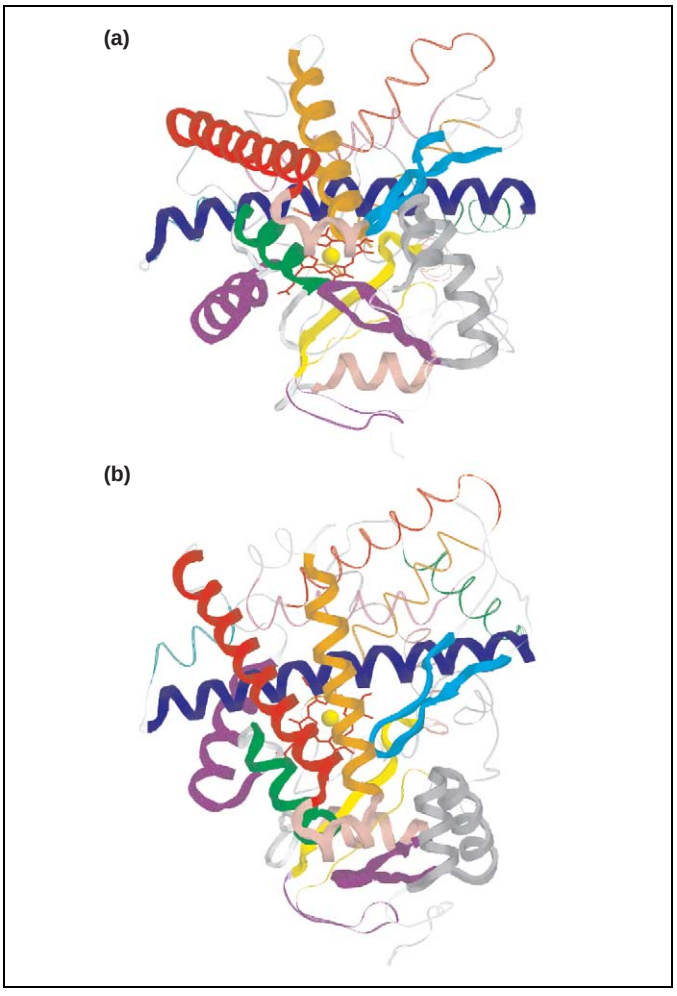

Figure 4. Different views of the homology model of CYP24A1. The homology model of CYP24A1 is viewed down the putative substrate access channel **(a)** and from above the distal heme face **(b)**. The tertiary structure of the heme distal cavity is bordered by the β1 sheet (center, purple), β3a strand (yellow), β5 hairpin (cyan), B′-helix (green), F-helix (orange) and the loop (center, pink) between the F-helix (orange) and the G-helix (red). Parts of the A- and A′-helices (gray) are positioned to contact a substrate access channel. The C-helix (purple) is also shown. Traces of other secondary structural elements are also visible in the figure. The model was constructed and energy-minimized using SYBYL6.8.

questioned since its discovery, and if anything the evidence for its function has been strengthened by its molecular characterization and the full elucidation of its clinical involvement. The recent genetic engineering of a *Cyp27b1* knockout mouse by two groups [46,47], coupled with experimentation involving dietary manipulation to exacerbate or to ameliorate the rickets phenotype, elegantly reinforces the impression that CYP27B1 has a crucial role in Ca^{2+} homeostasis and cell differentiation [48,49].

The story of CYP27B1 is not quite that simple, however, because it has been shown to be expressed in various extrarenal sites around the body including the keratinocyte [30], lung, colon [50] and macrophage [51], leading some to propose that extrarenal 25-OH-D_3-1α-hydroxylase has an autocrine or paracrine role in specific tissue differentiation [52]. Indeed, recent work has shown that cytokines, not PTH, upregulate expression of CYP27B1 in the macrophage [53] [Box 1, Figure Ib(ii)]. The concept of extrarenal 25-OH-D_3-1α-hydroxylase has not only physiological implications but also pathological ones. Sarcoidosis is a granulomatous condition that often involves hypercalciuria and eventually hypercalcemia caused by the overproduction of 1α,25-$(OH)_2D_3$ in sarcoid macrophages [51].

Regulation and physiological roles of 25-OH-D_3-24-hydroxylase

25-OH-D_3-24-hydroxylase (CYP24A1) is also regulated by 1α,25-$(OH)_2D_3$ but in the opposite direction to 25-OH-D_3-1α-hydroxylase (CYP27B1), owing to the presence of a strong 'double' positive VDRE in the CYP24A1 promoter [54] [Box 1, Figure Ib(iii)]. The outcome is a greater than 10- to 100-fold induction of CYP24A1 in all vitamin D target cells, a process that has been proposed to provide exquisite attenuation of the hormonal signal in the individual target cell when the gene transcriptional effects of 1α,25-$(OH)_2D_3$ need to be turned off [55] (Figure 5). Although a catabolic role for CYP24A1 is indicated by its profound transcriptional upregulation by 1α,25-$(OH)_2D_3$ and the reduced calcemic activity of the enzymatic products of 1α,25-$(OH)_2D_3$, there have been long-standing claims that CYP24A1 has an activating role in vitamin D metabolism. These claims revolve around the involvement of CYP24A1 in synthesis of the blood metabolite 24,25-$(OH)_2D_3$ (Figure 1), which has been proposed to have unique biological roles in bone fracture repair or cartilage maturation [56,57], although the details of the signal transduction pathway remain undefined and elusive.

The *Cyp24a1* knockout (*Cyp24a1*$^{-/-}$) mouse generated by St-Arnaud [58] is consistent with a catabolic role for CYP24A1, because it shows poor viability: 50% of homozygous mice die before weaning, showing hypercalcemia and signs of renal calcification. Surviving *Cyp24a1*$^{-/-}$ mice have a marked difficulty in excreting a bolus of [1β-^{3}H]1α,25-$(OH)_2D_3$, and isolated cultured keratinocytes from *Cyp24a1*$^{-/-}$ mice fail to synthesize calcitroic acid, whereas those of their heterozygous and wild-type littermates can make calcitroic acid [22].

An aspect of the *Cyp24a1*$^{-/-}$ mouse phenotype that is not completely consistent with a simple catabolic role for this enzyme is its unique bone pathology known as 'intramembranous bone' [58]. This condition has been reported in mice given excessive amounts of 1α,25-$(OH)_2D_3$ [59]; thus, the bone lesion in *Cyp24a1*$^{-/-}$ mice seems likely to be caused by an increase in 1α,25-$(OH)_2D_3$ and not by a lack of 24,25-$(OH)_2D_3$. Consistent with this theory is the fact that bone pathology is normal in *Cyp24a1*$^{-/-}$*VDR*$^{-/-}$ double knockout mice, in which an increase in 1α,25-$(OH)_2D_3$ levels cannot have deleterious effect owing the lack of any VDR-mediated signal transduction [60]. In conclusion, although an anabolic role for CYP24A1 has not been categorically ruled out, the data are overwhelmingly in favor of CYP24A1 having a crucial role in vitamin D inactivation *in vivo*.

Regulation and physiological roles of vitamin D_3-25-hydroxylase

The regulation of vitamin D_3-25-hydroxylase is poorly understood at the whole-animal level. Early work suggested that the enzyme activity might be partially downregulated by its product, 25-OH-D_3 [61], but the

Box 1. Regulation of the vitamin D hydroxylases

Regulation of the vitamin D hydroxylases revolves around their central role in fine-tuning concentrations of the hormone 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$] both in the circulation and inside the target cell (Figure Ia). By regulating the amount of ligand available for the vitamin D receptor (VDR), the hydroxylases are in effect regulating the extent of vitamin-D-dependent gene expression that occurs inside different target cells around the body, which in turn control Ca^{2+} homestasis and specific cell differentiation. Although the VDR is expressed in many cells around the body, differences in tissue-, differentiation-stage- and gene-specific transcription factors available at the various vitamin-D-dependent genes allow wide variability in the range of genes that are modulated in each tissue at any given time. Indeed, even the direction of the effect of 1α,25-$(OH)_2D_3$ on gene transcription, that is, whether it causes upregulation or downregulation, is gene-specific. For example, various Ca^{2+} homestatic genes (e.g. calbindins, Ca^{2+} channel proteins, osteocalcin, osteopontin, RANKL genes) are upregulated, whereas others [e.g. collagen and pre-pro-parathyroid hormone (PTH) genes] are downregulated by 1α,25-$(OH)_2D_3$.

The concentration of 1α,25-$(OH)_2D_3$ can be modulated by changes in both its rate of synthesis and its rate of catabolism. The generation of CYP27B1 and CYP24A1 knockout mice has illustrated the importance of both enzymes in regulating the concentration of 1α,25-$(OH)_2D_3$. Defects in the synthesis or catabolism of 1α,25-$(OH)_2D_3$ result in insufficient or excessive gene expression with the biological consequences of rickets or nephrocalcinosis, respectively. Notably, Repa *et al.* [42], recently reviewing a series of lipid-soluble, transcriptionally active hormones, pointed out the central role of CYPs in regulating the concentration and, hence, the biological effects of these hormones. Vitamin D, through its hormonal form 1α,25-$(OH)_2D_3$, fits this motif of lipid-soluble, transcriptionally active hormones very well and lacks only a designated efflux pump for its catabolic product, calcitroic acid.

Figure Ib(i,ii) shows how the renal and extrarenal 1α-hydroxylases are regulated. In Figure Ib(i), PTH is shown to be the chief upregulator of the renal enzyme, which mediates its effect on the renal proximal tubular cell through the PTH receptor by cyclic AMP and phosphatidylinositol 4,5-bisphosphate signal transduction. Transcriptional upregulation of the cytochrome P450 CYP27B1 and an increase in protein synthesis of the 1α-hydroxylase result from the actions of PTH. 1α,25-$(OH)_2D_3$ has been shown to downregulate the transcription of CYP27B1, leading to a reduction in 1α,25-$(OH)_2D_3$ synthesis. 1α,25-$(OH)_2D_3$ downregulates CYP27B1 gene expression through the direct interaction of its ligand-bound receptor with upstream elements in the promoter of the CYP27B1 gene.

Figure Ib(ii) shows the regulation of an extrarenal form of the 1α-hydroxylase, which is present in macrophages, by cytokines such as interferon-γ. The extrarenal 1α-hydroxylase might exist to provide a localized supply of 1α,25-$(OH)_2D_3$ for specific tissues. Note that inflammatory cytokines amplify the synthesis of 1α,25-$(OH)_2D_3$, but the negative factors that turn off this enzyme remain unknown. Granulomatous conditions such as sarcoidosis and tuberculosis involve extensive infiltration of tissues by macrophages, which cause an overproduction of 1α,25-$(OH)_2D_3$ that can leak into the bloodstream. These conditions suggest that negative regulation of 1α-hydroxylase might be absent in pathological states and that the regulation of normal extrarenal 1α-hydroxylase activity depends on controlling macrophage numbers. Some researchers think that the other forms of extrarenal 1α-hydroxylase are expressed in additional tissues (e.g. skin and prostate) and have important physiological functions in cell differentiation. CYP27B1 has been detected in several extrarenal sites by RT–PCR; however, the regulation of these extrarenal forms of the 1α-hydroxylase is poorly understood.

Figure Ib(iii) shows the regulation of the 24-hydroxylase (CYP24A1) by 1α,25-$(OH)_2D_3$. The 24-hydroxylase is found in kidney and in all vitamin D target tissues but is absent from the liver and osteoclasts. In the kidney, the 24-hydroxylase is thought to be expressed constitutively and to carry out the role of inactivating (wasting) the precursor 25-OH-D_3 when there is sufficient production of 1α,25-$(OH)_2D_3$. By contrast, expression of the 24-hydroxylase in the target cell is induced between 50- and 100-fold as part of the array of vitamin-D-dependent genes, and this induction leads to autoregulatory attenuation of the hormonal signal. 1α,25-$(OH)_2D_3$ is converted to calcitroic acid and sent to the liver for biliary excretion.

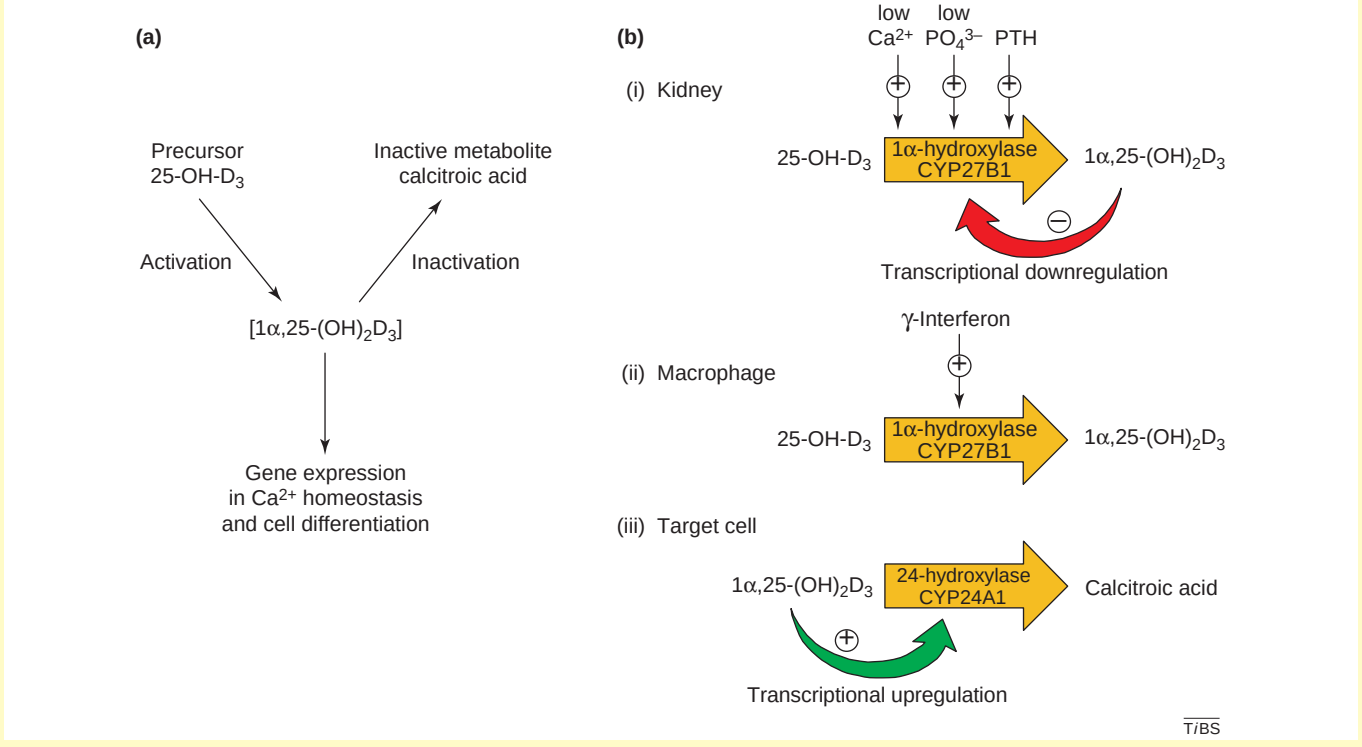

Figure I. Regulation of vitamin D hydroxylase expression and concentration. **(a)** Regulation of the 1α,25-$(OH)_2D_3$ concentration in vitamin-D-dependent gene expression. **(b)** Regulation of the expression of vitamin D hydroxylases: (i) renal 25-OH-D_3-1α-hydroxylase (CYP27B1); (ii) extrarenal 25-OH-D_3-1α-hydroxylase (CYP27B1); (iii) target cell 1α,25-$(OH)_2D_3$-24-hydroxylase (CYP24A1).

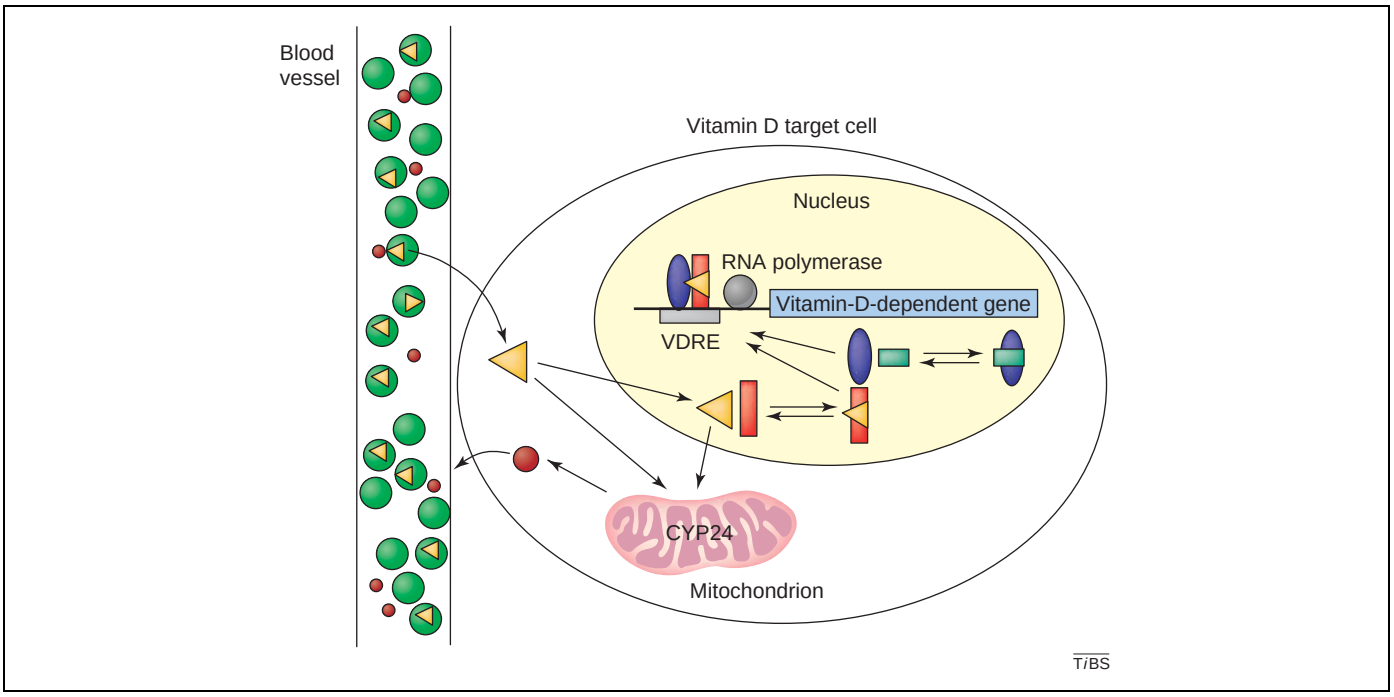

Figure 5. Role of CYP24A1 in vitamin D signaling to target cells. This model of the vitamin D target cell shows the importance of CYP24A1 in vitamin D signaling. The hormone 1α,25-$(OH)_2D_3$ (gold triangle) arrives at the target cell on the vitamin-D-binding globulin (green circle). Like most steroid hormones, 'free' 1α,25-$(OH)_2D_3$ is thought to gain entry, whereas its precursor 25-hydroxyvitamin D_3 (25-OH-D_3) remains sequestered on the cell exterior mainly because of its higher affinity for the vitamin-D-binding globulin. The 'resting' or naive cell lacks CYP24A1 and does not catabolize free 1α,25-$(OH)_2D_3$. The cell responds to free 1α,25-$(OH)_2D_3$ by altering the transcription of various vitamin-D-dependent genes (light blue), which are particular for a given cell type and depend on tissue-, temporal- and gene-specific factors. The transcription process usually involves a vitamin D receptor (VDR; red) and a retinoid X receptor (RXR; dark blue), which form a VDR–RXR heterodimer that operates at a vitamin D response element (VDRE) upstream of a vitamin-D-dependent gene. Among the spectrum of gene products documented by gene-array technology is CYP24A1 itself, which is greatly upregulated and eventually leads to an increase in 24-hydroxylase activity in the mitochondrion of the cell and the production of calcitroic acid (red circle). Presumably, the incoming 'free' or recycling 1α,25-$(OH)_2D_3$ ligand must now cross a 'minefield' of CYP24A1-laden mitochondria, which limits the hormonal response significantly. Use of a CYP24A1 inhibitor blocks the CYP24A1 enzyme activity, extending the half-life of the 1α,25-$(OH)_2D_3$. The efflux pump for calcitroic acid produced in the target cell remains unknown.

molecular details of this phenomenon have not been clarified, partly because of the elusiveness of the CYP moiety. A few CYPs have been examined as possible candidates for the vitamin D_3-25-hydroxylase, including CYP27A1, CYP3A4, CYP2R1 and CYP2J3. Although there are *in vitro* enzymatic data to suggest that these CYPs can all carry out the 25-hydroxylation of vitamin D_3, in some cases there is additional evidence that can be assembled arguing against a given CYP carrying out the same 25-hydroxylation step *in vivo*.

No meaningful regulation of CYP27A1 by vitamin D or Ca^{2+} homeostatic hormones has been reported in the literature, leading some to conclude that CYP27A1 is probably not the physiologically relevant 25-hydroxylase [4]. CYP27A1 does not 25-hydroxylate vitamin D_2, but efficient 25-hydroxylation of vitamin D_2 has been reported in most mammals *in vivo* [4]. The tissue distribution of CYP27A1 and its efficient hydroxylation of cholesterol side chains support the hypothesis that this enzyme is a key factor in biosynthetic pathways for acidic and neutral bile acids [62]. The phenotype of the *Cyp27a1* knockout mouse was not initially conclusive in defining the role of the enzyme *in vivo* [63], but a follow-up study has reinforced the essential role of this enzyme in cholesterol and bile acid metabolism and has shown that its deficiency does not affect serum levels of vitamin D metabolites [64]. Genetic mutations of CYP27A1 in humans cause cerebrotendinous xanthomatosis, a disorder of bile acid and lipid metabolism that sometimes presents with low 25-OH-D levels and a type of osteoporosis [65].

CYP3A4 contains a positive VDRE in its gene promoter [66] and this, coupled with the knowledge that it can 25-hydroxylate a synthetic analog, 1α-OH-D_2, *in vitro* [33], has led to the notion that CYP3A4 is important in vitamin D activation *in vivo*. The evidence is not very compelling because, first, CYP3A4 has a broad substrate specificity and a relatively low affinity for vitamin D as a substrate; second, there is a paucity of *in vivo* data to show that the hormonal form 1α,25-$(OH)_2D_3$ regulates 25-hydroxylase through a transcriptional mechanism; and third, the presence of the VDRE might not be very relevant given that current dogma holds that the VDR and thus VDR-mediated transcriptional effects are absent in liver cells [67].

The other candidates CYP2R1 and CYP2J3 are so new [32,34] that little is known about their regulation or gene promoters and knockout animals are not yet available. One clue that the CYP2R1 might be the physiologically relevant 25-hydroxylase has been provided in the recent report of a human mutation of CYP2R1 (Leu99Pro) present in a Nigerian individual affected with VDDR [35]. Thus, from a regulatory perspective, the physiological roles of CYP27A1, CYP3A4, CYP2R1 and CYP2J3 remain poorly defined. There is optimism, however, that the slate of new candidates will lead to some resolution of the exact nature of the vitamin D_3-25-hydroxylase.

Table 1. Natural mutations of human CYP27A1 and CYP27B1[a]

	Location	Putative function
CYP27A1 mutation		
Arg127Gln, Arg127Trp	B′–B loop	Heme binding
Arg137Trp	B′-helix	Structural
Ala216Pro	D-helix	OBS stability
Lys259Arg	F–G loop	Structural
Thr339Met	I-helix	OBS
Asp354Gly	I–J loop	Structural
Pro384Leu	K-helix	Stabilizes J-helix
Arg395Cys, Arg395Gln, Arg395His, Arg395Ser	K-helix	ERR triad
Tyr397His	K-helix	Stabilizes β5
Pro401Arg	β3	Stabilizes β3
Arg405Gln, Arg405Trp	β3	Heme binding
Pro441Cys	Meander	Stabilizes ERR triad
Gly472Ala	L-helix	Stabilizes Heme
Arg474Gln, Arg474Trp	L-helix	Heme binding
Arg479Cys	L-helix	Ferredoxin binding
CYP27B1 mutation		
Gln65His	A-helix	Stabilizes A′-helix
Arg107His	B′–B loop	Heme binding
Gly125Glu	B′–C loop	Stabilizes SRS
Pro143Leu	C–D loop	Structural
Asp164Asn	D-helix	Salt bridge Arg206
Glu189Leu	E-helix	Stabilizes start of helix
Thr321Arg	I-helix	OBS
Ser323Tyr	I-helix	Stabilizes OBS
Arg335Pro	I–J loop	Structural
Pro382Ser	β3	Stabilizes β3
Arg389His, Arg389Cys	β3	Heme binding
Thr409Ile	β3	Stabilizes ERR triad
Arg429Pro	ERR triad	Structural
Arg453Cys	L	Heme binding
Val478Gly	β5	Structural
Pro497Arg	β5	Stabilizes β3

[a]Abbreviations. ERR triad, structural motif formed between K-helix and meander region; OBS, oxygen-binding site centered on kink in I-helix; SRS, substrate recognition site in the heme distal cavity.

Clinical relevance of vitamin D hydroxylases

As alluded to in several sections above, there is now evidence that human mutations in two CYPs, CYP2R1 and CYP27B1, result in a deficiency of the hormone $1\alpha,25\text{-}(OH)_2D_3$, defective Ca^{2+} homeostasis and the classical bone lesions referred to as rickets [26,30,31,35]. In addition, the other liver mitochondrial CYP, CYP27A1, is mutated in cerebrotendinous xanthomatosis and this is sometimes accompanied by low 25-OH-D levels and a type of osteoporosis. Although this finding suggests that CYP27A1 might have a direct role in 25-OH-D synthesis, it is important to realize the fact that defects in bile acid metabolism can cause malabsorption of vitamin D, leading indirectly to lower levels of 25-OH-D [68]. Nevertheless, it is still informative to document the number of different mutations that have been reported for CYP27A1 and CYP27B1 and show in the homology model where these mutations are located in the CYP structure (Figure 3, Table 1). Many of the single-base substitutions affect heme-binding or ferredoxin docking, leading to a defective enzyme and thus disease. Notably, so far there are no known mutations of CYP24A1, and no inborn error of metabolism resulting in hypercalcemia has been connected to CYP24A1.

Problems of vitamin D hydroxylases can be also acquired, the main one being the loss of 25-OH-D_3-1α-hydroxylase in chronic kidney disease. A consortium of nephrologists assembled by the National Kidney Foundation (USA) has defined a five-stage process of disease, which ultimately leads to dialysis, in which there is a gradual reduction in glomerular filtration rate accompanied by a gradual loss of 25-OH-D_3-1α-hydroxylase enzyme activity with a concomitant fall in blood levels of $1\alpha,25\text{-}(OH)_2D_3$ [69]. As a result, individuals with renal failure slowly develop hypocalcemia and secondary hyperparathyroidism, which eventually leads to renal osteodystrophy [70,71].

There are currently 8 million individuals with stage-3 chronic kidney disease in the United States, and the number is expected to double in the next decade because renal failure is one of the chief complications of type II diabetes, itself a complication of the obesity epidemic. Individuals with renal failure need a form of replacement $1\alpha,25\text{-}(OH)_2D_3$ treatment to compensate for their loss of functional 25-OH-D_3-1α-hydroxylase enzyme activity, and this recommendation has been recently emphasized as part of the new K/DOQI guidelines for nephrologists treating individuals affected with chronic kidney disease [72].

Vitamin D analogs and vitamin D hydroxylases

These days, replacement $1\alpha,25\text{-}(OH)_2D_3$ therapy need not be confined to the hormone itself because the past two decades have seen the development of thousands of synthetic analogs (reviewed in Refs [3,4,73]), a select group of which have now reached the marketplace. These low-calcemic analogs are used not only in chronic kidney disease but also in the hyperproliferative skin disease psoriasis and other dermatological conditions. Further molecules are in clinical trial as treatments for various forms of cancer and autoimmune conditions. Several of these marketed analogs come with pre-existing 1α- and 25-hydroxyl groups and are fully active in their administered form, thereby circumventing the need for vitamin D_3-25-hydroxylase and 25-OH-D_3-1α-hydroxylase enzyme activities. Notably, these analogs, which include calcipotriol (Dovonex), 22-oxa-calcitriol (Maxicalcitol) and $19\text{-nor-}1\alpha,25\text{-}(OH)_2D_2$ (Zemplar or Paricalcitol), are still subject to catabolism, in part by CYP24A1.

Two other analogs, 1α-OH-D_3 (One-Alpha) and 1α-OH-D_2 (Hectorol), have been designed as prodrugs for use in individuals with chronic kidney disease. These molecules are synthesized with a 1α-hydroxyl function, which circumvents the need for the defective renal 25-OH-D_3-1α-hydroxylase, but they lack the side chain 25-hydroxyl, which makes them inactive in their administered form. An undefined liver CYP, probably one of the list of candidate 25-hydroxylases described above, adds the 25-hydroxyl function *in vivo* as the final step to activate the molecule, thereby reversing the normal physiology and also changing the pharmacokinetics of the drug. The pharmaceutical industry has therefore developed strategies both to circumvent and to exploit the enzyme activity of the vitamin D hydroxylases.

Another area of vitamin D hydroxylase research with particular interest to biochemists but also with clinical relevance is the development of CYP inhibitors. Inhibitors of CYPs with various degrees of specificity have been

shown to have clinical utility in other diseases. These include general antifungal agents (ketoconazole), aromatase inhibitors used in breast cancer (letrozole), and retinoic acid metabolism blockers used in prostate cancer (liarozole). Not surprisingly, then, the vitamin D hydroxylases with direct relevance to Ca^{2+} homeostasis and cell differentiation can be considered as prime targets for inhibition.

Blockers of CYP24A1 might extend the half-life of 1α,25-$(OH)_2D_3$ or vitamin D analogs within target cells, especially cells that overexpress CYP24A1, whereas blockers of CYP27B1 might have utility in conditions where 1α,25-$(OH)_2D_3$ is overproduced, such as sarcoidosis or nephrolithiasis. A class of inhibitors described by Schuster *et al.* [74,75] that are based on the same azole chemistry used so successfully in ketoconazole have provided us with molecules such as VID400, which selectively inhibits CYP27B1, as well as molecules that selectively block CYP24A1. Another approach to identifying potential inhibitors has been to screen libraries for molecules that selectively block CYP24A1 and to test the antiproliferative activity of these compounds [76].

Conclusions and future directions

In summary, by providing a well-regulated supply of 1α,25-$(OH)_2D_3$ and also equipping the target cell with a mechanism for inactivation, the vitamin D hydroxylases have an essential role in Ca^{2+} homeostasis and also regulate specific cell differentiation and proliferation. The vitamin D hydroxylase field is steeped in a rich history and there is much optimism that the new knowledge learned from these enzymes can be exploited clinically to help in diagnosis and treatment of disease. The picture emerging from the vitamin-D-related CYPs has many parallels to other small lipid signaling pathways, where activating and inactivating CYPs work in close conjunction with nuclear receptors to produce biological effects and then pass on their inactive products to cell efflux pumps for excretion [42].

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