Molecular mechanism of transcriptional control by nuclear vitamin receptors

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Nuclear receptors for vitamins A and D belong to the nuclear hormone receptor superfamily and act as ligand-inducible transcription factors. Therefore, most of the biological actions of vitamins A and D are now considered to be exerted through nuclear vitamin receptor-mediated gene expression. The vitamin A nuclear receptors compromise six members, three all-*trans* retinoic acid receptors (RARα, RARβ, RARγ) and three 9-*cis* retinoic acid receptors (RXRα, RXRβ, RXRγ) (Mangelsdorf *et al.* 1995). Unlike vitamin A receptors, only one member is identified for vitamin D. The present study investigating the vitamin D receptor function in gene expression in both cell culture and intact animals was undertaken to better understand the actions of the fat-soluble vitamin A and vitamin D at a molecular level.

Vitamin D: Vitamin A: Nuclear receptor: Transcription

25-hydroxyvitamin D_3 1α-hydroxylase[1α (OH)ase] as a key enzyme of vitamin D synthesis

The precursor of vitamin D, 7-dehydrocholesterol, is biosynthesized from cholesterol, then converted into vitamin D_3 by UV light in the skin. Vitamin D is also ingested from the diet, as Vitamin D_2 (ergocalciferol) mainly from plants, and vitamin D_3 (cholecalciferol) from animals (DeLuca, 1986). A hormonal form of vitamin D, $1\alpha,25(OH)_2D_3$, is metabolically formed through two steps of hydroxylation at the final stage (Fig. 1). First, vitamin D is hydroxylated in the liver to 25-dihydroxyvitamin D_3 ($25(OH)D_3$), which is subsequently hydroxylated in the kidney to $1\alpha,25(OH)_2D_3$ (see references in Kato *et al.* 1998). For metabolic inactivation of $25(OH)D_3$, or $1\alpha,25(OH)_2D_3$, the 24-hydroxylation to form $24,25(OH)_2D_3$ or $1\alpha,24,25(OH)_3D_3$, is the first step in degradation of vitamin D. The serum level of $1\alpha,25(OH)_2D_3$ is kept constant in the normal state, and is regulated in response to factors controlling calcium homeostasis. The regulation of $1\alpha,25(OH)_2D_3$ and $24,25(OH)_2D_3$ production by these factors is conducted by altering the activities of the enzymes that hydroxylate vitamin D derivatives. Vitamin D_3-25-hydroxylase (CYP27) catalyzes hepatic 25-hydroxylation, and renal 1α-hydroxylation is catalyzed by 25-hydroxyvitamin D_3 1α-hydroxylase [1α (OH)ase]. The first step of metabolic inactivation of vitamin D metabolites by 24-hydoxylation is catalyzed by $25(OH)D_3$-24-hydroxylase (CYP24) (Chen & DeLuca 1995).

Molecular mechanism of transcriptional control by vitamin D receptor

Vitamin D plays an essential role in a variety of biological events such as calcium homeostasis, bone formation and metabolism, and cellular differentiation (Bouillon *et al.* 1995; Walters, 1992; Chen & DeLuca, 1995). Hormonal form of vitamin D, $1\alpha,25$-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$), acts as a ligand for the vitamin D receptor (VDR), and the liganded VDR activates the target gene expression at the trancriptional level. VDR forms a homodimer or heterodimer with one of three retinoid X receptors (RXRα, RXRβ, RXRγ). The VDR homodimer or VDR-RXR heterodimer binds to specific enhancer elements referred to as vitamin D response elements (VDREs) for the $1\alpha,25(OH)_2D_3$-induced transactivation. For the ligand-induced transactivation by VDR, coactivators interacting with VDR in a ligand-dependent way have recently been shown to be essential for the formation of the initial transcription complex with RNA polymerase II, see Fig. 2 (Freedman, 1999). They include the SRC-1/TIF2 160 kDa protein family, CBP/p300 protein family and others (see references in Yanagisawa *et al.* 1999; Lanz *et al.* 1999). Most interestingly, these coactivators themselves are

Abbreviations: RAR, all-trans retinoic acid receptor; RXR, retinoid X receptors; VDR, vitamin D receptor.

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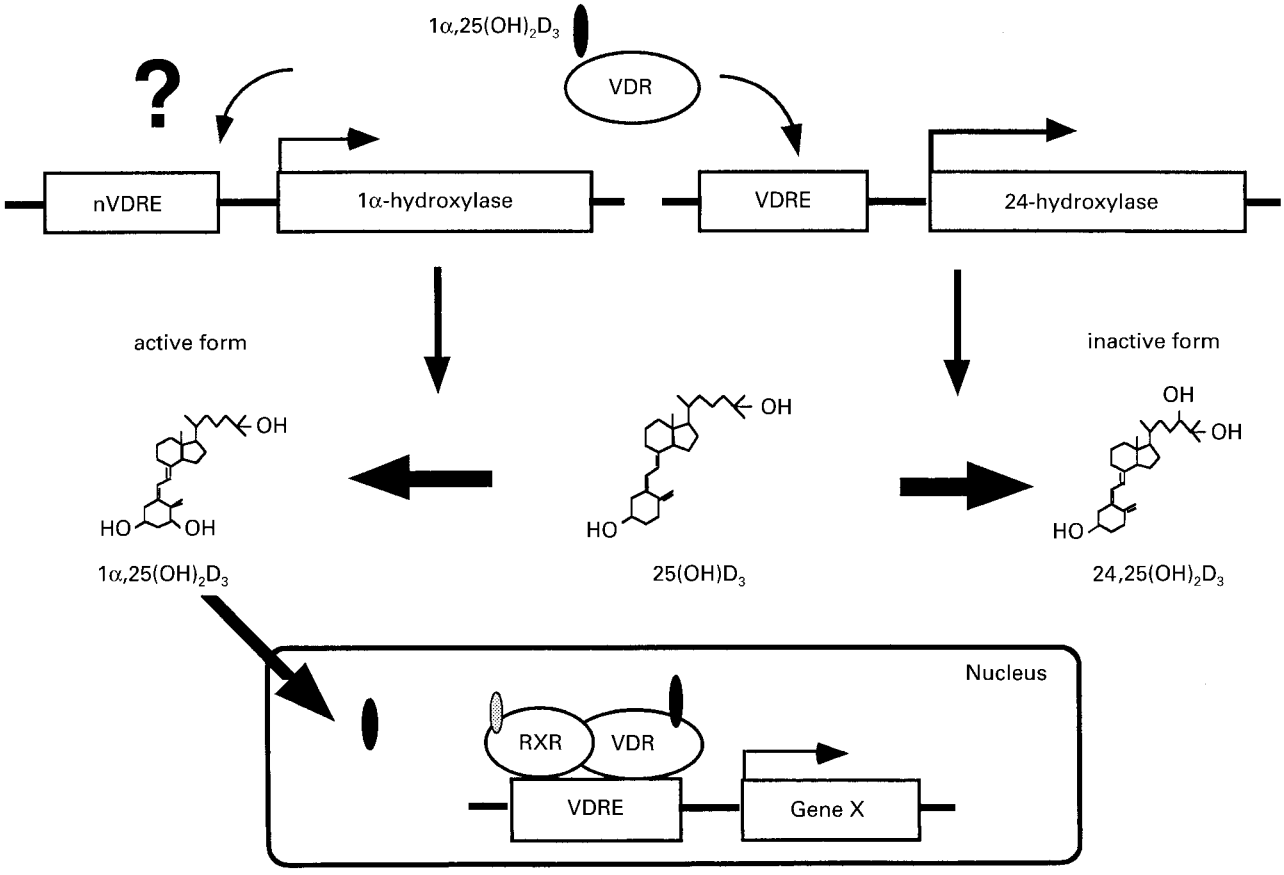

Fig. 1. A proposed molecular mechanism of regulations of $1\alpha,25(OH)_2D_3$ biosynthesis by 25-hydroxyvitamin D_3 1α-hydroxylase and $25(OH)D_3$-24-hydroxylase. The negative regulation of 1α (OH)ase gene expression by $1\alpha,25(OH)_2D_3$ did not occur in the mice lacking VDR (VDR knock-out mice), which raises a possibility that a negative VDRE is present in the promoter of the 1α (OH)ase gene. The positive VDRE has been identified in the promoter of the $25(OH)D_3$-24-hydroxylase gene. The levels of serum $1\alpha,25(OH)_2D_3$ is positively and negatively regulated through these VDREs binding linganded VDR.

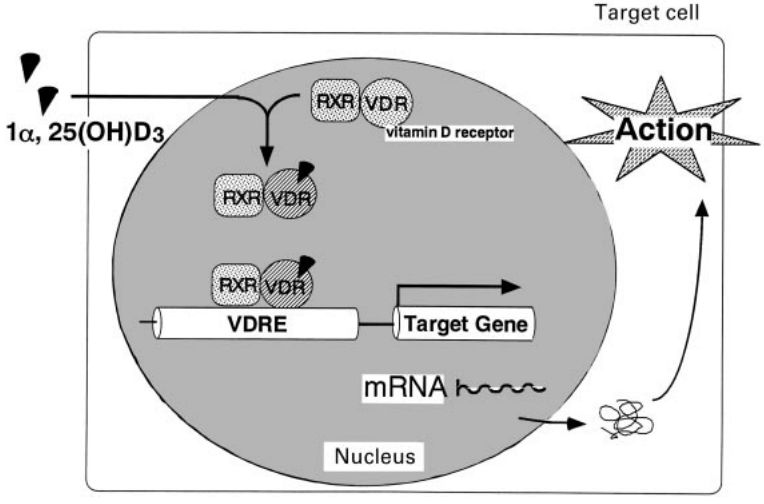

Fig. 2. Ligand-bound VDR forms a transcription initiation complex to activate gene expression. To ligand-induced transactivation, VDR forms a complex with basic transcriptional machinery and a coactivator complex in the promoter of the target gene for vitamin D. Two coactivator complexes with and without histone acetylase activity have been identified to date.

histone acetylases that modulate chromatin structure for activating gene expression. These coactivators are speculated to form a complex. More recently, another coactivator complex has been identified as DRIP/TRAP complex, which has no histone acetylase activity (Rachez *et al.* 1999; Freedman, 1999). In contrast, corepressors, SMRT, NCoR, were found to associate with ligand-unbound thyroid receptor and all-*trans* retinoic acid receptor (RAR) to repress their ligand-induced transactivation function (Horlein *et al.* 1995; Nagy *et al.* 1997). However, these corepressors did not show interaction with ligand-unbound VDR (Haussler *et al.* 1998). The products (proteins) of the target genes exert most of the biological actions of vitamin D.

Lessons from vitamin D receptor knock-out mice

As the molecular basis of the actions of $1\alpha,25(OH)_2D_3$ in bone formation and metabolism, and its role during development were uncertain, we generated VDR deficient mice by gene targeting in order to investigate the functions of VDR *in vivo* (Yoshizawa *et al.* 1997). Like vitamin D, vitamin A has been shown to play a critical role in growth and development, especially skeletal formation during embryogenesis (Kastner *et al.* 1995). In fact, the inactivation of one (RARγ) of six vitamin A nuclear receptors resulted in tracheal cartilage malformation and homeotic transformations along the rostral axial skeleton during embryogenesis. However, no bone malformation or overt phenotypic abnormalities were seen in the $VDR^{-/-}$ fetuses (data not shown).

Unexpectedly, the VDR null mutant mice did not differ from their heterozygous or wild-type littermates in growth rate or behavior, and seemed functionally normal after birth until weaning. However, after weaning (about 3 weeks), the VDR null mutant mice unexpectedly showed marked growth retardation, and the body weight of the null mutant mice at 6 weeks was about 50 % of those of the heterozygous and wild-type mice. After weaning the VDR null mutant mice developed rickets, and most of them died by 15 weeks due to an unknown reason. However, no overt abnormalities were found in the heterozygotes even at 6 months. By 7 weeks, all of the VDR null mutant mice developed alopecia and poor whiskers as typical features of rickets, and most of them displayed a flat face with a shorter nose. In the null mutant mice at 7 and 13 weeks, no apparent abnormalities were found by histological analysis in the VDR-expressing tissues other than bone and skin, including the intestine, kidney, brain and spleen. Uterine hypoplasia was found in the 10-week-old null mutant mice, though no abnormality was found in the male reproductive organs.

The observations in the VDR null mutant mice (Yoshizawa *et al.* 1997) are similar to a human hereditary and recessive disease, vitamin D dependency type II, in which mutations in the VDR gene have been identified in several families (Hughes *et al.* 1988), though unlike the VDR knock out mice, this disease is not lethal. Severe malformation induced by the inactivation of VDR only after weaning was detected in bone.

As these patients exhibit rickets with hypocalcemia, hypophosphatemia and elevated serum alkaline phosphatase, serum levels of these parameters were measured in the mice from 3 to 13 weeks old. During lactation (at 3 weeks), no effects of inactivation of VDR were seen in agreement with the phenotype and growth rate. However, at 4 weeks, only one week after weaning, the serum levels of calcium and phosphate were reduced with markedly elevated serum alkaline phosphatase activity in the VDR null mutant mice. At this stage, no defects such as alopecia were observed. In older VDR-deficient mice, these abnormalities became more prominent.

Functional cloning of 25-hydroxyvitamin D_3 1α-hydroxylase [1α (OH)ase] as a key enzyme of vitamin D synthesis

A marked increase in serum $1\alpha,25(OH)_2D$, and a clear reduction in serum $24,25(OH)_2D$ developed in the VDR null mutant mice at 4 weeks and persisted at 7 weeks, suggesting increased activity of 25(OH)D 1α-hydroxylase and reduced activity of 24-hydroxylase. Indeed, from the VDR knock out mice, we were able, for the first time, to clone the cDNA encoding mouse 25(OH)D 1α-hydroxylase by a newly developed expression cloning method (Takeyama *et al.* 1997). These observations establish that liganded VDR is essential for regulations of these enzymes by $1\alpha,25(OH)_2D_3$ after weaning, again supporting the idea that the $1\alpha,25(OH)_2D_3$-VDR system plays a critical role only after weaning (Kato *et al.* 1998).

Molecular cloning of cDNAs encoding mouse, rat and human (Fu *et al.*1997) 1α (OH)ase confirmed the reported biochemical characterization of 1α (OH)ase as a cytochrome p450. The predicted amino acid sequences revealed that 1α (OH)ase proteins harbor a mitochondrial target signal and two conserved regions (the sterol-binding domain and the heme-binding domain), and significant homology throughout the entire amino acid sequence is found among the p450 enzymes.

Mutations in the 1α (OH)ase gene cause hereditary type I rickets

Dietary vitamin D deficiency or hereditary defects cause rickets. Patients with rickets present clinical features like growth failure, impaired bone formation, hypocalcemia and weakness from early infancy. In any case, it is interpreted that blocking the vitamin D signaling pathway induces rickets (Fig. 3).

Using murine 1α (OH)ase cDNAs, we and others isolated the human 1α (OH)ase cDNA, and determined the genomic structure of the human gene (Kitanaka *et al.* 1998). FISH analysis with the human cDNA showed that this gene lies on chromosome 12q13·3 (Kitanaka *et al.* 1998). Interestingly, this locus matches well the chromosomal localization of a putative gene which had been mapped as responsible for type I rickets by linkage analysis in a group of Canadian patients (Labuda *et al.*1990).

We found that distinct homozygous missense mutations of the human 1α (OH)ase gene which abolish the 1α (OH)ase activity were found in four different type I rickets patients (Kitanaka *et al.* 1998), and more recently we

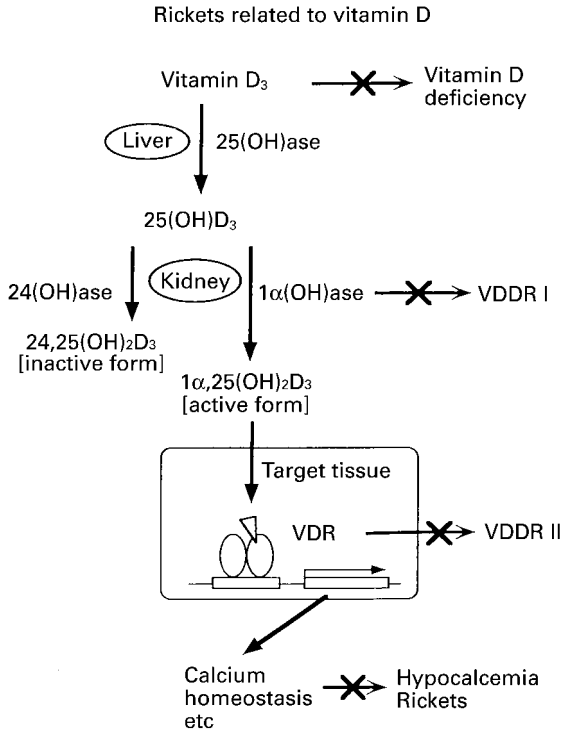

Fig. 3. Rickets related with vitamin D. The biosynthesis pathway of $1\alpha,25(OH)_2D_3$ and the mode of $1\alpha,25(OH)_2D_3$ action are illustrated. The defects in these processes cause rickets. Nutritional vitamin D deficiency, and defect of the renal 1α (OH)ase activity by genetic mutations (VDDRI patients) result in short supply of vitamin D. The mutated VDR in the VDDRII patients is unable to respond to $1\alpha,25(OH)_2D_3$, resulting in the rickets. The precursor of vitamin D, 7-dehydrocholesterol, is biosynthesized from cholesterol, then converted into vitamin D_3 by UV light in the skin. Vitamin D is also taken from diet, as Vitamin D_2 (ergocalciferol) mainly from plants and vitamin D_3 (cholecalciferol) from animals. A hormonal form of vitamin D acting as a ligand specific for VDR, $1\alpha,25(OH)_2D_3$, is metabolically formed through two steps of hydroxylations at the final stage. First, vitamin D is hydroxylated in the liver to 25-dihydroxyvitamin D_3 ($25(OH)D_3$) by the vitamin D_3-25-hydroxylase (CYP27). Subsequently, 25-hydroxyvitamin D_3 1α-hydroxylase [1α (OH)ase] in kidney mainly undergoes conversion into$1\alpha,25(OH)_2D_3$.

further identified a hetro-compound type mutation in an other patient (Kitanaka *et al.* 1999). Interestingly, the mutation sites, which cause complete loss in the enzymatic activity, were found widely in the entire region of 1α (OH)ase protein, though regions essential for the enzymatic activity have been identified by a biochemical approach (Sasaki *et al.* 1999). Therefore, the surrounding regions may support the enzymatic function, for instance, serving as an anchor in mitochondrial cell membrane. Thus, these observations establish that genetic mutations which inactivate the human 1α (OH)ase cause type I hereditary rickets.

Acknowledgments

I would like to thank Drs T. Yoshizawa and K. Sekine for the VDR knock-out mice study, and Drs K. Takeyama, S. Kitanaka, A. Murayama for the 1α (OH)ase gene project, and express sincere gratitude to all the members in my laboratory and the worldwide colleagues collaborating with these projects.

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