Inflamm. res. 47 (1998) 451–475
1023-3830/98/120451-25 $ 1.50+0.20/0

Inflammation Research

Review

Molecular mechanism of vitamin D receptor action

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Received 10 February 1998; returned for revision 21 April 1998; accepted by R. Day 24 August 1998

Abstract. The vitamin D system is unique in that distinct calcium homeostatic functions and cell growth regulatory activities are mediated through a single ligand, calcitriol, acting through a specific receptor exhibiting ubiquitous tissue expression, the vitamin D receptor (VDR). The VDR is a member of a superfamily of nuclear steroid hormone receptors which regulate gene transcription by interacting with response elements in gene promoters. Structure-function analysis of the VDR protein has defined distinct domains involved in DNA binding, ligand binding, receptor dimerisation and gene transactivation, including a C-terminal activation function domain (AF-2) that is important for cofactor interaction. A model for regulation of gene transcription by the VDR is evolving and proposes VDR interaction with various components of the basal transcriptional machinery, including newly defined coactivators and corepressors, which may act to regulate gene transcription by altering histone acetylation and chromatin structure. This review describes the vitamin D endocrine system and the role of the VDR in regulating this system, including the molecular basis for the diverse actions of synthetic calcitriol analogues in the treatment of autoimmune disease and cancer.

Key words: Vitamin D – Vitamin D receptor – Vitamin D analogues – Gene transcription

Introduction

Vitamin D_3 is essential for normal bone structure and the maintenance of serum calcium. Calcitriol (1,25-dihydroxy-vitamin D_3, Fig. 1) is the active metabolite of vitamin D_3. The genomic actions of calcitriol are mediated through its nuclear receptor, the VDR, which is a *trans*-acting transcriptional factor and member of the nuclear hormone receptor superfamily [1]. The VDR regulates gene transcription both positively and negatively by binding to hexameric core binding motifs in the promoter regions of target genes, designated vitamin D response elements, or VDREs.

In addition calcitriol has also been reported to modulate rapid non-genomic actions involving activation of bidirectional Ca^{2+} channels, activation of G-protein coupled receptors and downstream protein kinase C (PKC) and mitogen activated protein kinase (MAPK) pathways. These have been proposed to be mediated by a putative but as yet unidentified membrane receptor. The rapid non-genomic effects occur in concert with and in a cell-type specific manner with the genomic actions of calcitriol and are postulated to augment them (see Fig. 2) [2].

Structure-function studies of the VDR by deletion mutation analysis and amino acid sequence comparison with other nuclear hormone receptors has led to identification of key residues involved in ligand binding, DNA contact, receptor dimerisation and the characterisation of a transcriptional activation domain involved in interaction with transcriptional coactivators and corepressors (Fig. 2). Further insight into VDR gene transcription awaits the generation of three dimensional crystal structures, although recently three dimensional modelling of the VDR has identified amino acids within the ligand binding pocket which may interact with various pharmacophores of calcitriol. These models suggest that different analogues contact distinct sites within the VDR ligand binding pocket, and may thus modulate its configuration and subsequent interaction with the transcriptional machinery.

Calcitriol has also been shown in vitro to regulate the growth, differentiation and function of a number of specialised cells including bone, immune and haematopoietic cells, keratinocytes as well as cancer cells. However, in vivo these actions are achieved at doses that cause hypercalcaemia. Recent research has focused on development of analogues of calcitriol for clinical use in the treatment of auto-immune diseases such as multiple sclerosis and rheumatoid arthritis, psoriasis, breast, prostate and colon cancer, leukaemia, and osteoporosis. Although a number of vitamin D responsive

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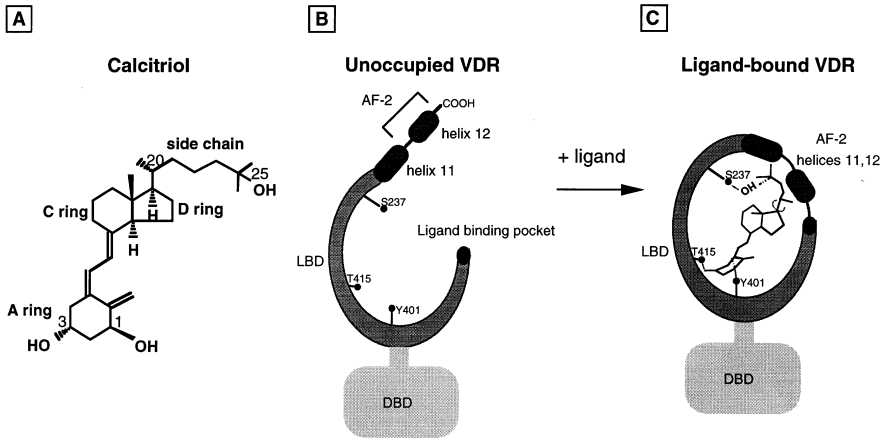

Fig. 1. Calcitriol and the VDR ligand binding domain. A) Generic structure of calcitriol. B) Schematic representation of the VDR ligand binding pocket in the unoccupied state and C) the effect of calcitriol docking on VDR conformation and repositioning of helix 12, the so-called 'mouse trap' model. Also indicated are the polar amino acids that potentially interact with various pharmacophores of calcitriol.

genes involved in cell cycle control, apoptosis and cell differentiation have been identified, the exact mechanisms underlying the growth regulatory actions of vitamin D analogues have not been completely defined. Through control of such genes vitamin D analogues could modulate a number of biological growth and signalling pathways.

The vitamin D endocrine system

The vitamin D hormone

The maintenance of a healthy normal skeleton and long-term regulation of serum calcium is dependent on conversion of vitamin D_3 to its active form calcitriol. The major source of vitamin D_3 is the dermis of the skin, where upon exposure to ultraviolet radiation 7-dehydrocholesterol is converted to previtamin D_3 before thermal conversion to vitamin D_3, the substrate for the subsequent hydroxylation steps. Hydroxylation occurs initially in the liver at the 25-carbon to produce 25-hydroxyvitamin D_3 (25-OHD) and then in the kidney at the 1-carbon in the alpha orientation. Renal 1α hydroxylase (CYP1), which has recently been cloned [3], is a mitochondrial cytochrome P450 mixed-function oxidase enzyme which is present in the proximal tubules of the kidney.

The major sites of action of calcitriol in calcium homeostasis are bone, kidney, gut and the parathyroid gland. Minute to minute homeostatic control of serum calcium levels is mediated through regulation of parathyroid hormone (PTH) secretion. Longer term maintenance of normocalcaemia involves the action of calcitriol. PTH released in response to hypocalcaemia stimulates the CYP1 to increase production of calcitriol which acts to increase calcium and phosphate absorption from the intestine, increase resorption at the distal tubule of the kidney and mobilise calcium stores from the skeleton [4].

Catabolism of 25-OHD and calcitriol to forms on the catabolic pathway are performed by the cytochrome P450 1α,25-$(OH)_2D_3$-24-hydroxylase (CYP24) in the kidney as well as the majority of target cells. Calcitriol stimulates CYP24 and inhibits CYP1 activity thus forming a negative feedback loop to maintain normal levels and prevent vitamin D_3 toxicity.

Biological and molecular actions of calcitriol on bone cell differentiation and bone development

The molecular role of VDR in control of bone development is unclear. With advancing genetic and molecular biology tools, including transgenic mice models, the complex control of bone development is beginning to be unravelled.

The osteoblastic lineage includes osteoblasts and osteocytes which undergo differentiation influenced by a wide variety of factors, including cytokines, growth factors and calcitriol. The osteoblast lineage originates from pluripotent stem cells which have the capacity to develop into adipocytes, myocytes, osteoblasts or chondroblasts depending on the tissue-specific and temporal pattern of expression of various factors [5].

The transcription factor Cbfa1 was recently cloned and shown to be an important osteoblast-specific differentiation factor [6]. Cbfa1, which binds a DNA element (OSE2) within the osteocalcin gene promoter, is expressed in the mesenchymal condensations of the developing skeleton and thereafter is restricted to cells of osteoblast lineage. Calcitriol treatment decreases its expression, while BMP7 treatment induces Cbfa1 expression. Two groups simultaneously reported the Cbfa1 gene knockout model in mice as a lethal phenotype in homozygous mice [7, 8]. These mice died soon after birth with a totally unmineralised skeleton lacking osteoblasts, while the heterozygous mice showed skeletal abnormalities similar to the human skeletal dysplasia syndrome, sterno-cleidocranial dysplasia [8]. In another study, examination of patients with sterno-cleidocranial dysplasia revealed gene deletions leading to heterozygous loss of the Cbfa1 gene, or insertions and missense mutations leading to premature translational stop codons in either the DNA binding or C-terminal transactivating domains of the protein [9]. Thus there is compelling evidence that Cbfa1 is a major early osteoblast specific developmental gene.

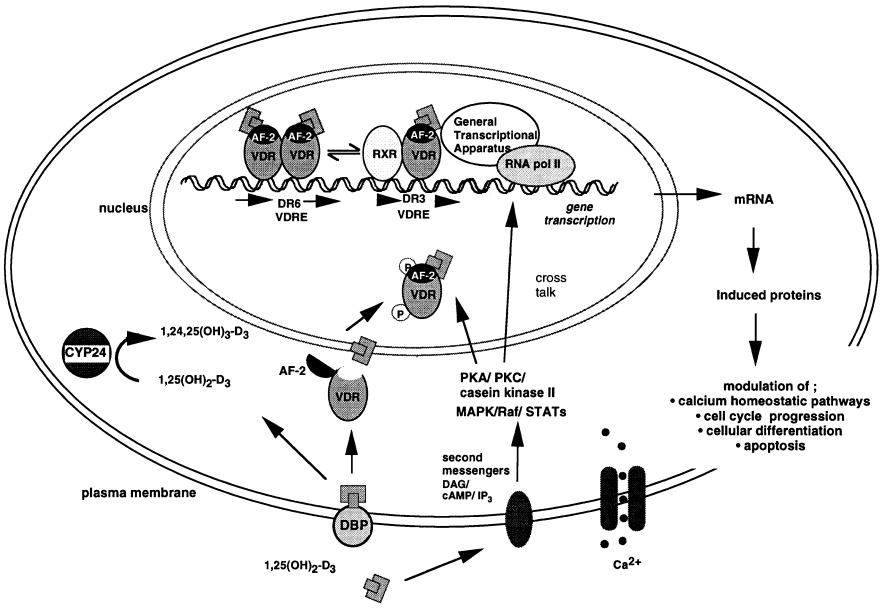

Fig. 2. Model for the integrated cellular pathways for calcitriol action through the VDR. Calcitriol enters the cell bound to the vitamin D binding protein (DBP). The rapid non-genomic actions of calcitriol include opening of Ca^{2+} channels and activation of second messanger pathways which engage in cross talk with the nucleus. Ligand activation results in VDR phosphorylation and nuclear transport. The VDR homodimerises or heterodimerises with RXR to interact with vitamin D response elements (VDREs) in gene promoters. Interaction with the general transcription machinery is essential to initiate gene transcription. The net result is regulation of expression of proteins involved in calcium homeostasis and cell growth regulatory pathways. Also shown is the calcitriol metabolic pathway through 24-hydroxylase (CYP24).

Other transcription factors whose expression in osteoblast cells is regulated by calcitriol include the helix-loop-helix family of transcription factors, such as HES-1 [10], the adipocyte-specific transcription factor ADD1 [11] and the inhibitor of differentiation Id1, a negative regulatory factor that is downregulated by calcitriol [12].

Osteocalcin, the most abundant non-collagenous protein in bone, is expressed only by cells of the osteoblast lineage. Regulation of osteocalcin expression by calcitriol is species-specific. In humans and rats calcitriol increases osteocalcin expression, while in mice it decreases osteocalcin expression [13]. This difference may relate to species-specific transcription factors and/or differences in the osteocalcin VDRE. Interestingly, osteocalcin knockout mice show increased bone formation, suggesting that osteocalcin has a negative regulatory role in bone development [14]. The VDR knockout mouse model not only exhibits all features of human rickets, notably severe bone formation, hypocalcaemia and alopecia, but also has marked growth retardation after weaning and uterine hypoplasia, implicating a role for VDR and calcium homeostasis in reproductive development and growth [15]. In order to study the molecular role of VDR in control of bone morphology our group has produced a transgenic mouse model in which the expression of the hVDR transgene is under the control of the osteoblast-specific osteocalcin promoter, thus directing VDR transgene expression to osteoblast cells [13].

VDR gene alleles and bone mineral density

Our group originally described the association between VDR gene alleles and bone mineral density (BMD) [16]. Correction of some genotyping errors suggests the effect is smaller than originally observed. However, although several studies have found no VDR effect, other studies in many different populations and age groups with varying calcium-intakes have supported this association. Overall, the effects of the VDR gene polymorphisms may contribue 5–10% of the genetic difference in bone mineral density in the normal population [17]. Recently, a study on pre-pubertal Mexican children found a correlation at the hip and spine between the VDR alleles and BMD [18], thus further emphasising two important points: 1) detection of any gene effect may be easier in a more homogeneous population like the Mexican childhood population, and 2) differences may be masked by differences in gonadal hormonal effects on growth by aging.

Structure of the VDR gene and its regulation

The human VDR gene

The human VDR cDNA was cloned from a human jejunal poly(A)+ RNA library using the avian VDR cDNA probe [19, 20]. A single 4.6 kb human transcript, found in most human tissues tested, contains a 1281 nucleotide open reading frame that codes for the full length VDR protein of 427 amino acids, a 115 bp non-coding leader sequence and a 3.2 kb 3′ untranslated region (UTR). The VDR protein was first isolated from chick intestinal epithelium and shown to bind calcitriol selectively and with high affinity ($k_d \leq 0.1$ nM) [21]. The mammalian forms of the VDR protein range in molecular weight between 52–60 kD by

biochemical analysis, although the calculated molecular weight deduced from the amino acid sequence is 48.3 kD.

The human VDR coding sequence is highly homologous to the avian, amphibian, mouse and rat sequences, particularly the highly conserved nine cysteine residues of the DNA binding domain (DBD). The avian VDR possesses a 22 amino acid extension 5′ to the corresponding hVDR N-terminal methionine. This 5′ extension arises from an alternative translational start site and gives rise to the 60/58 KDa doublet observed for the avian VDR protein [22]. Evolutionary diversity is evident in the highly variable sizes of 3′ UTRs, which in the avian transcript is 1 kb and in the amphibian is 300 bp [22, 23].

The hVDR gene has been cloned from a human liver genomic library and its 11 exons span more than 75 kb [24, 25]. The 5′ non-coding region contains 3 exons, 1A, 1B and 1C, while the remaining eight exons encode the translation product. Three VDR mRNA transcripts in the kidney have been identified that appear to rise from the differential splicing of 5′-noncoding exons. The promoter is GC rich, without a TATA box, but has multiple Sp-1 recognition sites and an array of putative binding sites for transcription factors. Preliminary results suggest the promoter contains a retinoic acid response element downstream of exon IC, though no vitamin D response element (VDRE) has yet been identified, suggesting an indirect mechanism for autoregulation possibly through interaction with other regulatory transcription factors [24]. More recently our group has identified three novel 5′ exons in the VDR gene which encode for N-terminal variant receptor proteins. In addition, the distal promoter generates unique transcripts in tissues involved in calcium regulation by 1,25-$(OH)_2D_3$ [25]. This suggests a possible role for these variant VDR transcripts in the tissue-specific actions of the VDR in bone and calcium homeostasis.

Chromosomal localisation of the VDR gene

The human VDR gene has been localised to human chromosome 12q13-14 [26, 27]. Interestingly the genes for the transcription factors Sp-1, the gamma isoform of the retinoic acid receptor (RARγ) and CYP1 [28] are located on the same region of chromosome 12. Mutations in the CYP1 gene which result in reduced calcitriol synthesis are associated with pseudo-vitamin D deficiency rickets [3, 29].

Disease associated mutations in the VDR gene

Naturally occurring mutations in the hVDR are found in patients with hereditary 1,25-dihydroxyvitamin D resistant rickets (HVDRR). Phenotypes arising from these mutations provide information on the structure-function of the VDR. HVDRR is characterised by defective bone mineralisation, low intestinal calcium absorption, hypocalcaemia and increased serum calcitriol levels to compensate tissue resistance. Patients with this disorder have reduced or no response to vitamin D_3, 25-OHD or calcitriol treatment. The VDR knockout mouse provides an animal model of HVDRR [15].

HVDRR associated VDR mutations fall into three classes (Table 1). The first and most abundant leads to substitutions of highly conserved amino acids in the zinc finger region of the DBD [30–33]. These mutations generally affect VDR DNA binding ability and transactivation. The second class of mutations gives rise to non-sense stop codons or mis-sense mutations in the ligand binding domain (LBD), resulting in a truncated or mutated receptor that lacks ligand binding activity [34, 35]. The third class comprises mutations in the extreme C-terminus and affect primarily receptor heterodimerisation and consequently calcitriol inducible gene transactivation [36].

VDR subcellular localisation

The VDR is predominantly a nucleophilic macromolecule in the ligand-bound state. There is controversy regarding the subcellular distribution of the unoccupied VDR. Cell fractionation studies in physiological ionic strength buffers and immunocytochemical studies suggest that a small fraction of unoccupied VDR may partition into the cytosol [37–39]. Studies by Pike et al. [40] and Barsony et al. [41] suggested that unoccupied VDR may exist in equilibrium between the nucleus, where it is loosely associated with chromosomal DNA, and the cytosol. Upon ligand diffusion across the cell membrane, the VDR redistributes to the

Table 1. Naturally occurring mutations in the VDR.

Class		Mutation	Domain	Function	Ref.
1	i)	Gln-33-Asp	tip of 1st zinc finger	no DNA binding or transactivation	[30]
	ii)	Lys-45-Glu	knuckle of 1st zinc finger	"	[280]
	iii)	Phe-47-Ile	"	"	[280]
	iv)	Arg-50-Gln	"	"	[31]
	v)	Arg-73-Gln	tip of 2nd zinc finger	"	[30]
	vi)	Arg-80-Gln	"	"	[281]
2	i)	Arg-73-stop	"	truncated-non-functional	[30]
	ii)	Gln-152-stop	hinge domain	"	[35]
	iii)	Arg-274-Leu	LBD	reduced ligand binding	[35]
	iv)	Tyr-295-stop	LBD	truncated & unstable	[282]
	v)	His-305-Cys	LBD	reduced ligand binding & transactivation	[33]
	vi)	Ile-314-Ser	LBD, heptad 2	"	[33]
3	i)	Arg-391-Cys	LBD	no RXR heterodimerisation	[36]

nucleus where it associates with dimerisation partners to bind target genes with high affinity. This is supported by findings that ligand-bound VDR elutes from DNA at a higher KCl concentration than the unoccupied form [40, 42].

More recently Barsony et al. [43] showed, using fluorescent labelled calcitriol, that a substantial amount of unoccupied VDR resides in the cytoplasm, mainly in the Golgi complex, endoplasmic reticulum and microtubules, but not in the plasma membrane. In this study equilibrium between cytoplasmic and nuclear VDR developed within 5 minutes of calcitriol addition. Mutation of the LBD prevented nuclear and cytoplasmic hormone binding, while mutations in the DBD decreased the nuclear retention of VDR and prevented localisation to nuclear loci, supporting the idea that nuclear import signals reside in the DBD.

Regulation of VDR expression

VDR protein expression has been identified in most human tissues, exemplifying the diversity and importance of VDR function. Homologous regulation of VDR expression, or autoregulation by calcitriol, and heterologous regulation by other hormones is species-, tissue- and cell type-specific and is altered during development, aging and in disease states (for review [44, 45]).

Cellular responses to calcitriol treatment may not only be related to the state of proliferation and differentiation, but the amplitude of response reflects VDR abundance and its regulation by other hormonal and physiological signals. For instance, changes in VDR abundance in response to forskolin (an adenylate cyclase stimulator), which increases VDR, or phorbol diesters (activators of PKC), which reduce VDR, are paralleled by the amplitude of calcitriol induction of gene transcription [46].

The mechanism of homologous VDR regulation is unclear but may involve transcriptional, post-transcriptional (alteration of mRNA stability), or post-translational mechanisms such as ligand induced alterations in receptor half-life. Homologous VDR regulation is linked to the auto-regulation of calcitriol synthesis and metabolism. Clearly this mechanism is cell type-specific. In human T-47D breast cancer cells calcitriol down-regulates the number of VDR binding sites, increases the rate of turnover of the occupied receptor and decreases its half-life relative to the unoccupied state [47]. By contrast, in rat ROS 17/2 osteosarcoma cells, no change in receptor half-life was observed following ligand binding, however there was an increase in VDR mRNA [48], while Arbour et al. [49] observed the opposite effect in a subclonal cell line ROS 17/2.8. In mouse fibroblasts and rat intestinal epithelial cells VDR protein levels are increased by calcitriol without changes in mRNA levels [50]. These examples indicate that homologous regulation is cell type-specific and has both a transcriptional and post-transcriptional component.

VDR expression in ameloblasts and odontoblasts of developing rodents implicates a role for the VDR in regulation of enamel and dentine formation [51]. In addition, regulation of VDR expression by calcitriol and PTH in rodent growth plate cartilage cells implicates a role for VDR in bone growth [52]. However, given that VDR knockout mice exhibit bone deformities only after weaning, the role of VDR in foetal bone development is unclear. Modulation of osteoblastic cell function by PTH and calcitriol can also involve feedback regulation of VDR expression by these hormones [53, 54]. Interestingly, the protective effect of oestradiol on age related bone loss may be mediated in part by increasing VDR expression in osteoblastic cells [55, 56].

Altered VDR expression is found in some disease states including secondary hyperparathyroidism and osteopetrosis. For instance, in animal models and patients with renal failure leading to secondary hyperparathyroidism elevated PTH is associated with reduced VDR expression in the parathyroid gland [57, 58]. Furthermore, changes in dietary calcium and phosphate levels result in changes in target tissue VDR expression. For example, phosphorus restriction in rodents results in upregulation of VDR in the intestine but not the kidney [59]. These examples of homologous and heterologous modes of VDR regulation suggest that studies of the VDR gene promoter are required for a better understanding of the molecular mechanisms underlying hormonal regulation of VDR gene expression.

Modular structure of the VDR and structure-function studies

The amino acid sequence of the VDR shows significant homology with other members of the nuclear hormone receptor superfamily which includes the receptors for glucocorticoids (GR), oestrogen (ER), androgen (AR), progesterone (PR), thyroid hormone (T_3R), retinoic acid (RAR), retinoid X (RXR) and over 150 orphan receptors [1, 60]. In general all members possess five functional domains, of which the DBD and several distinct regions of the LBD including the C-terminal AF-2 domain are highly conserved (Fig. 3). These domains appear to act as distinct modules that can operate independently in mutation experiments using truncated receptors, but in normal physiology probably act co-operatively. On the basis of similarities in amino acid sequence and mode of action the VDR, T_3Rs and RARs form a subfamily within the nuclear hormone receptor superfamily. This distinction is based mainly on observations that 'classic' hormone receptors, GR, ER and PR, function primarily as homodimers. The VDR subfamily members heterodimerise predominantly with RXR, and although these receptors also form homodimers, the homodimers may not be transcriptionally active.

The DNA binding domain

The DBD (domain C) of the VDR has been mapped to amino acid residues 22-114 [61] (Fig. 3). A defining feature of the DBD of nuclear hormone receptors is eight positionally conserved cysteine residues that tetrahedrally coordinate two zinc atoms to form zinc finger DNA binding motifs. Recognition of specific VDREs may involve amino acids that reside within the α-helical region at the base of the first zinc finger, the so-called 'P-box'. Solution structural analysis of the DBDs of the ER, GR, RARβ and RXRα indicate that the amino acids surrounding the zinc fingers are folded to form two α-helices. The first N-terminal α-helix is though to lie across the major groove of DNA and make specific

contact with DNA response elements [62]. The DBD is also rich in positively charged amino acids which favour electrostatic interactions with the negatively charged phosphate backbone of the DNA helix. The reverse β-turn formed by amino acids at the base of the second zinc finger, the so-called 'T-box', may form a dimerisation interface [63].

The hinge region is the stretch of amino acids between the DBD and LBD and confers flexibility to the protein and changes in structural conformation upon ligand activation. The VDR DBD and hinge region contain sequences homologous to nuclear localisation signals of other nuclear hormone receptors and of the SV40 T-cell antigen. These lie between amino acids 49-58 (nuclear import region 1, N1, Fig. 2) and 102-111 (N2), corresponding to a region between the two zinc fingers [64, 65]. Interestingly, N1 includes Ser-51 which is phosphorylated by PKC thus implying a connection between phosphorylation and nuclear import.

The ligand binding domain

The LBD or E domain, involved primarily in ligand binding, varies considerably between the nuclear hormone receptors. Homology is highest between the VDR and the T_3R (23%). Subdomains within the LBD appear to be involved in nuclear import signalling, dimerisation, transcriptional inhibition, transactivation and interaction with the transcriptional machinery. Delection mutation analysis has defined the N-terminal boundary of the LBD to lie between residues 114 and 166. A Δ1-114 mutant bound calcitriol with normal affinity while a Δ1-166 showed no ligand binding capacity [66]. Another study showed high affinity calcitriol binding requires amino acids 382 to 402, as deletions of amino acids 382-427 abolished ligand binding capacity while deletions of amino acids 403-427 retained 1/10 of the affinity of the wild type receptor, without changing ability to dimerise with RXR [67]. This C-terminal region is highly variable among family members and seems most likely to be involved in ligand-specific interactions with various nuclear cofactors.

The ligand binding pocket and docking of calcitriol

Based on published crystal structures of apo-hRXRα [68], ligand-bound hRARγ [69], hT_3Rα1 [70] and amino acid sequence comparisons, three dimensional models of hVDR LBD bound to several ligands have been generated [71]. The LBDs of nuclear hormone receptors form 12 highly conserved α-helices although in these models helices 1-3 of the VDR are omitted to achieve a better alignment with the other nuclear receptors. The VDR ligand binding pocket is lined mostly by hydrophobic residues with His-305 and His-397 buried within the ligand binding pocket. The importance of these two residues to high affinity ligand binding has been previously confirmed [33, 72]. Hydrophilic residues Ser-235, Ser-237, Ser-275, Try-401 and Thr-415 have their side chains oriented towards the cavity.

Calcitriol is a highly flexible molecule (Fig. 1A). The C6-C7 bond allows interconversion between 6-s-*trans* (extended) and 6-s-*cis* (fixed) conformations. The A-ring adopts A (below) and B (above) chair like conformations depending on the orientation of the C10-C19 double bond in relation to the conjugated triene system. In addition, the aliphatic side chain at C17 of the D-ring is highly flexible and can adopt a number of spatial orientations. Hence calcitriol can accommodate side chain modifications without major loss of VDR binding capacity and biological activity.

The A-ring is postulated to bury deep in the ligand binding pocket with the ligand side chain pointing toward the opening of the cavity (Fig. 1C). Hydrogen bonds potentially form between 1α-OH and Thr-415, 3β-OH and Tyr-401 and 25-OH and Ser-237. Three dimensional modelling with VDR bound to the analogues MC 903, EB 1089 and KH 1060 suggest that the ligand binding pocket can accommodate sterically altered side chains but these induce subtle deformations. Calcitriol side chain orientation favours contact with helices 11 and 12. The 20-*epi* conformation of KH 1060, however, favours contact with residues of helix 5, thus shifting the C/D rings deeper into the ligand binding pocket. These differences in side chain contact sites with the ligand binding pocket may account for differences in binding on-off rates, ligand-induced receptor conformational change and changes in receptor stability.

A recent study by Liu et al. [73] lends support to the above model. Deletion of helices 11 and 12 (amino acids 396-427) of the VDR abolished ligand binding to calcitriol but not to the 20-*epi* analogues MC 1288, MC 1301 and KH 1060. Thus amino acid residues N-terminal of Leu-390 may be important for 20-*epi* analogue binding. These analogues exhibited slower dissociation rates and induced distinct changes in VDR conformation as assessed by protease digestion assays.

The A/B domain

The amino terminal A/B domain varies among family members in both amino acid identity and size. The A/B domain of the VDR is short, consisting of 21 amino acids compared with 421, 185, 135, 102 and 88 amino acids in length for the hGR, hER, hRXRα, hT_3R and hRARα, respectively. The A/B domain of the hER, hRARs and hRXR contain a transcriptional activation function (AF-1), which unlike the C-terminal AF-2 domain, functions ligand-independently. The presence of a corresponding AF-1 function in VDR A/B domain is uncertain, as removal of the A/B domain of the VDR does not appear to affect ligand binding, DNA binding or transactivation function [61]. A possible additional function for this region is relaying inputs from other signal transduction pathways to the AF-2 domain analogous to the ER AF-1 domain which is phosphorylated on conserved serine residues in response to growth factor stimulation and input from membrane-bound receptor tyrosine kinases through the Ras/Raf/MAPK pathway [74]. Similarly, phosphorylation of hRARα AF-1 domain is involved in retinoic acid induced differentiation of F9 embryonic carcinoma cells [75]. However, the exact mechanism by which protein modification of the AF-1 region alters ligand-dependent function at the AF-2 region remains unclear.

VDR dimerisation interfaces

Regions within the VDR DBD and LBD are also important dimerisation interfaces, however, no single interface has been shown to be asbolutely necessary for VDR:RXR heterodimerisation. This includes the so-called 'E1' domain spanning amino acids 244 to 263. E1 is highly conserved among the nuclear hormone receptor superfamily, implicating this region in some shared biological function. Deletion mutation analysis of conserved amino acids within E1 of the VDR supports a role in dimerisation with RXR, however it does not account for all heterodimerisation function [76, 77].

The VDR LBD contains nine heptad repeats that form hydrophobic surfaces that are proposed to act as dimerisation interfaces [78]. The 'E2' region (325-332) includes heptad 4 and may also be a dimerisation interface. Simultaneous alterations of Leu-325 and Leu-332 inactivate all receptor functions: ligand binding, heterodimerisation and gene transactivation. Single mutations of either residues leave some functions intact [67]. The 'E3' region (381-399) encompassing the ninth heptad (383 to 390) is also important to heterodimerisation and acts in a cooperative manner with the E1 region [79]. VDR mutants bearing certain deletions in heptads 4 or 9 lacked the capacity to heterodimerise with RXR and failed to elicit calcitriol dependent transactivation of an osteocalcin gene reporter, despite retaining ligand binding function [67]. Another study found that deletion of two regions within the LBD important for heterodimerisation in yeast cells (amino acids 239-269 and 317-394) resulted in loss of transactivation, indicating that heterodimerisation is necessary for transactivation [80]. More recently, a natural mutation (Arg-391-Cys) in a HVDRR patient was shown to interfere with heterodimerisation with RXR [36].

Sites for VDR physophorylation

Hormone dependent phosporylation has been reported for most nuclear hormone receptors and may be involved in regulation of DNA binding, hormone binding, nuclear localisation and gene transactivation. The major sites for phosphorylation are serine residues. Calcitriol has been shown to stimulate phosphorylation of the VDR in mouse 3T6 fibroblasts, chick duodenal organ culture and COS cells transfected with VDR expression vectors, however there is some uncertainty regarding the functional consequence of this phosphorylation [81–83]. Darwish et al. [84] found that modulation of PKA by 8-Br-cAMP could enhance calcitriol mediated transactivation and that this could be abolished by addition of phosphatases. The VDR was also phosphorylated at Ser-51 by PKC in vitro and in vivo, however this resulted in a reduction in specific interaction of the VDR with VDREs [85].

Ser-208 is a preferred site for phosphorylation of the VDR by casein kinase II (CKII) [86, 87]. Jurutka et al. [88] found co-expression of the catalytic subunit of human CKII with hVDR in COS-7 cells led to a significant enhancement of calcitriol mediated gene transactivation, without affecting DNA binding, hormone binding and nuclear localisation. It would appear, then, that phosphorylation of Ser-51 or Ser-208 are not essential for VDR function but may reflect kinase specific inputs that can be positive or negative. The functional consequence of phosphorylation on VDR activity is dependent on both the cellular context and the signal transduction pathway or specific kinase involved and may represent a means by which signals from the cell membrane, in response to growth factor stimulation, modulate nuclear hormone receptor function.

The AF-2 transcriptional activation function domain

The AF-2 domain is suggested to provide an interactive surface for transcriptional corepressors and coactivators which links nuclear receptor activity with the preinitiation complex (PIC). The VDR AF-2 domain has been mapped to the extreme C-terminus of the VDR between amino acids Arg-402 to Gln-423 [89]. Deletion of amino acids 403-427 generated a transcriptionally inactive receptor that retained RXR heterodimerisation, but had somewhat reduced ligand binding capacity [67], suggesting that a transcriptional activation function resides within this region.

Sequence comparison with other nuclear hormone receptor AF-2 domains has identified highly conserved residues which may mediate transactivation function [89]. Double point mutations of residues Leu-417 and Glu-420 of the VDR to alanine residues produced a receptor that behaved as a dominant negative receptor, retaining ligand binding capacity, DNA binding capacity, the ability to heterodimerise with RXR and interact with TFIIB, but which could not transactivate a vitamin D responsive gene. This finding demonstrates that the transcriptional activation function of the VDR is independent and separate from its other activities and occurs following recruitment of ligand bound VDR:RXR heterodimers to the VDRE.

Mutational analysis of corresponding amino acids in the human $T_3R\beta1$, mouse RARα1 and RXRα receptors also produced transcriptionally inactive receptors, supporting the idea of a defined AF-2 domain within the LBD [90–93]. In comparison, mutation of residues in the mouse ER corresponding to Leu-417 or Glu-420 had a modest effect on transactivation, and this function was only lost by deletion of both AF-1 and AF-2 domains [94, 95]. The dominant negative phenotype of the Leu-417-Aln and Glu-420-Aln VDR mutant is strong evidence that all the transactivation function of the VDR resides in the AF-2 domain and that the VDR is unlikely to possess a functional AF-1 domain.

The twelfth α-helix of nuclear hormone receptor LBDs, which encompasses the highly conserved residues of the AF-2 domain, is structurally conserved [68–70]. It has been hypothesised in the so-called 'mouse trap' model [69] that in the unoccupied state the twelfth α-helix projects away from the core of the LBD. Upon ligand binding a conformational change in the receptor repositions helix 12 such that it covers the opening of the ligand binding pocket and exposes an interface for potential interaction with coactivators (Fig. 1B, 1C).

A recent study demonstrated autonomous ligand-independent transcriptional activity by a truncated VDR containing only the AF-2 domain (amino acids 408-427), thus lending support to the mouse trap model for VDR function [96]. Other VDR deletion mutants, Δ166-427, Δ281-427, Δ374-427 and Δ387-427, failed to transactivate a reporter in the absence or presence of ligand, as would be

expected with removal of the LBD. Nevertheless, L417S and E420Q mutants abolished the autonomous activity of the minimal AF-2 domain. Hence, in the unoccupied state full length VDR is folded in a way which possibly prevents interaction of the AF-2 domain with transcriptional coactivators such as SRC-1 and GRIP-1 [96, 97]. Whether the unoccupied VDR in a similar fashion to unoccupied T_3R or RAR interacts with a co-repressor, such as NCo-R/SMRT, remains unclear [98, 99].

Mechanism for the regulation of gene transcription by the VDR

VDR heterodimerisation with RXR

Heterodimerisation between a limited number of nuclear receptors generates a diversity of functionality different complexes and increases the complexity of transcriptional response to hormonal stimuli. VDR transcriptional activation requires nuclear auxiliary factors, one of which was subsequently cloned and identified as RXR [61, 100]. Various isoforms of the RXR serve as dimeric partners for VDR binding to DR3 elements but one study suggests VDR binds RXRγ more avidly than other RXR isoforms [80]. Similarly, T_3R and RAR require dimerisation with RXR to bind their cognitive responsive elements [101, 102]. The orientation and spacing of the response elements directs the polarity of heterodimer binding [103]. The RXR molecule binds the 5′ half site while the dimeric partner, VDR, T_3R or RAR, binds the 3′ half site [104, 105]. The 5th nucleotide position of the 3′ half site is thought to be necessary for heterodimer binding [106, 107]. Ligand induced VDR:RXR heterodimerisation is thought to enhance interaction with DNA [79].

Structure of vitamin D response elements

The VDR, as with other nuclear hormone receptors, regulates gene transcription by binding *cis*-acting elements, termed hormone response elements, in the promoter regions of target genes. Several factors direct nuclear hormone receptor binding to target sequences including the sequence and orientation of core hexameric half-site motifs, the intervening nucleotide spacing and the 5′-nucleotide sequences that precede the hexameric half-site. Nuclear hormone receptors recognise the ER consensus core motif AGGTCA (ERE) arranged as direct repeats or motifs arranged as palindromes and inverted palindromes [108]. The 1-5 spacing rule has been proposed to define the preference of VDR, T_3R and RAR for direct repeats spaced by 3, 4 and 5 intervening nucleotides, respectively (DR3, DR4 and DR5) and includes RXR homodimer and peroxisome proliferator-activated receptor (PPAR) preference for DR1 and the RAR alternative DR2 response element [103, 109].

The VDR can recognise and bind hexameric half sites with (A/G)GGTGA and AGTTCA sequences [110–112]. VDR homodimers show binding specificity for DR6 type elements, in both a ligand-dependent and independent manner [112]. VDR:RXR heterodimers bind to DR3 type elements, possibly due to conformational differences arising from rotation of the DBD and LBD about the hinge region (Fig. 2). Sequence changes in the third position of the ERE consensus motif may determine selectivity for homodimer to heterodimer complex formation. Half-sites bearing PuGTTNN sequences may act as recognition for hVDR homodimers and heterodimers, whereas those containing PuGGTNN can only bind VDR:RXR heterodimers [112]. The dimeric species capable of transactivation at any given point is also governed by the relative levels of receptors within the cell and the concentration of receptor specific ligands including 9-*cis* retinoic acid.

Novel vitamin D responsive genes

VDREs of numerous vitamin D responsive genes have been identified and characterised. More recent genes include novel elements involved in the cell regulatory functions of the VDR. Some VDREs are simple direct repeats while others are complex and often overlapping multimeric structures (Table 2). The most extensively studied are the human and rat osteocalcin [110], mouse osteopontin (also known as secreted phosphoprotein 1 or Spp1), [113] and the rat CYP24 [114–117]. In the human osteocalcin gene promoter a DR6 element (−511 to −493) overlaps a DR3 element (−499 to −485). Hence only one VDR dimer could bind at any given point since the 3′ VDR binding half site of the DR6 is shared by the 5′ RXR binding half site of the DR3. Yet both VDR homodimers and heterodimers exert positive transcriptional regulation. The 5′ half site of the DR6 element also overlaps an AP-1 consensus sequence (TGACTCA) and, therefore, access to the AP-1 element by the c-*jun*/c-*fos* complex may be obstructed in the presence of VDR:VDR homodimer binding and vice versa [118].

Table 2. Vitamin D responsive genes.

Gene	VDRE	Ref.
	−499 −485	
h. osteocalcin	GGGTGAacgGGGGCA	[110]
	−511 −494	
	TGGTGActcaccGGGTGA	[115]
	−154 −171	
m. fibronectin	GGGTGAcgtcacGGGGTA	[126]
	−779 −763	
h. p21^{WAF1}	AGGGAGattGGTTCA	[249]
	−108 −122	
h. PTH	GGTTCAaagCAGACA	[133]
	−786 −803	
h. PKCγ	AGGTCAgaccacTGGACA	[127]
	−28 −14	
r. BSP	AGGGTTtatAGGTCA	[138]
	−770 −756	
a. β_3 integrin	GAGGCAgaaGGGAGA	[128]
	−150 −136	
r. 24-(OH)ase	CGCCCTcacTCACCT	[283]
	−245 −258	
	GGTTCAgcgGGTGCG	[120]
	−233 −250	
	GGTCGAgcccaagGGTTCA	[114]
	−1121 −1075	
r. PTHrP	GGGTGGAnnnGGGTGA	[132]

Activation of AP-1 has been reported to synergistically enhance activation by calcitriol, however, overexpression of c-*jun* and c-*fos* in ROS 17/2.8 cells has been reported to reduce both basal and calcitriol inducible promoter activity [118, 119]. Thus binding of the activating complex, and whether these complexes act synergistically or in opposition, must depend on the state of cell differentiation and the relative abundance of individual transcription factors.

Calcitriol upregulated CYP24 gene expression in the intestine and kidney, as well as in other target cells. The rat CYP24 gene promoter has been reported to contain three VDREs, two DR3 elements located between nucleotides −150 to −136 (VDRE1) and −258 to −244 (VDRE2), and a DR6 element spanning nucleotides −231 to −250 [114, 120]. The human CYP24 gene promoter also contains two VDREs [121]. Calcitriol induced VDR binding to both VDRE1 and VDRE2 of the rat CYP24 promoter produces synergistic transactivation [117, 122]. VDRE1 is thought to exert stronger responsiveness to calcitriol due to a potential accessory enhancer element located upstream of VDRE1, yet VDR:RXR was shown to bind VDRE2 with higher affinity [123, 124]. Basal activity of VDR:RXR on the rat CYP24 VDREs in yeast cells and monkey kidney COS-1 cells exerts transcriptional repression and involves VDR:RXR interaction with the corepressor RIP13Δ1. Ligand activation causes dissociation of the corepressor and replacement with a transcriptional coactivator [125].

A calcitriol-inducible DR6 VDRE bound specifically by a VDR homodimer has been identified in the murine, rat and human fibronectin gene promoters [126]. A DR6 type element has also been identified recently in the calcitriol responsive phospholipase C-γ1 gene promoter. Although this promoter is bound by a VDR homodimer, VDR:RXR heterodimer binding to this element has not been ruled out [127]. The avian β3 integrin gene promoter contains three hexameric half sites spaced by nine and three nucleotides which are recognised by RAR:RXR and VDR:RXR heterodimers, respectively [128, 129]. Competition for binding to the common central half site may be an important regulatory mechanism.

Parathyroid hormone-related peptide (PTHrP) is produced in keratinocytes, cells of the osteoblast lineage, lactating mammary glands and by cancer cells involved in malignancy associated hypercalcaemia. PTHrP functions physiologically as a paracrine factor in bone and inhibits the proliferation of osteoblastic cells [130, 131]. PTHrP expression in osteoblastic cells is down-regulated by calcitriol through VDR:RXR heterodimer interaction with a repressor VDRE in the PTHrP gene promoter [132]. Interestingly, the 5′ half site motif is characterised by a 7 base pair sequence, GGGTGGA, which confers transcriptional down-regulation while the 3′ half site is identical to the rat osteocalcin VDRE. Transcriptional inhibition by calcitriol is more pronounced in the presence of mitogenic stimuli, indicating that VDR interaction with PTHrP promoter sequences may act to block interaction by other hormone activated transcriptional factors. The human PTH gene promoter is also down-regulated in response to calcitriol via a VDRE located between nucleotides −108 to −122 [133].

Is the VDR homodimer a functional complex?

Analogous to the ER, GR and PR, the VDR, T_3Rs and RARs may function as homodimers. Homodimerisation of the T_3R is both ligand-dependent and destabilised by ligand depending on the target response element [134]. Additionally RAR homodimer formation may be ligand independent [116]. VDR homodimers have been proposed to interact on direct repeats spaced by six nucleotides [111, 126], however a VDR homodimer has been reported to interact with the DR3 type response element of the mouse osteopontin gene promoter and was found to be reduced in the presence of calcitriol [112, 135].

The functional significance of a VDR homodimer is unclear. Kinetic studies in solution indicate that clacitriol decreases the amount of VDR homodimer bound to DNA, and causes dissociation of the homodimer by decreasing the rate of monomer to homodimer formation up to 5-fold. Thus calcitriol shifts the equilibrium in favour of VDR:RXR complex formation on a DR3 element [135]. However, it has not been tested whether the equilibrium would be shifted toward a VDR homodimer on a DR6 element. In a cell free transcription assay the VDR LBD alone did not transactivate the osteopontin gene DR6 element [136]. VDR LBD-mediated transactivation was ligand-dependent and required the presence of RXR and nuclear coactivators [137]. However, sequences in the DBD or hinge regions may be important for VDR homodimer formation, or may provide additional interfaces for interaction with coactivators.

In vitro experiments indicate that ligand-bound VDR and RAR homodimers often display lower affinity for DNA response elements than corresponding ligand-induced heterodimeric complexes. However, in the case of the bone sialoprotein (BSP) VDRE a VDR homodimer showed higher affinity than a VDR:RXR heterodimer in gel mobility shift assays [138]. Formation of homodimers in the absence of ligand and the apparent instability of some homodimers in the presence of their ligands suggests that nuclear hormone receptor homodimers may act to sequester transcription factors to DNA elements.

Alternatively, as with calcitriol suppression of BSP gene expression, homodimer binding to DNA may preclude access of other transcription factors to their putative enhancer elements. Since some vitamin D responsive gene promoters possess both DR3 and DR6 VDREs, homodimer-heterodimer cooperation may be necessary for a full transcriptional response. Further characterisation of homodimer specific vitamin D responsive genes in cells which lack RXR, such as yeast, are required to study the functional significance of VDR homodimers in gene transcription.

VDR interaction with the preinitiation complex (PIC)

Transcriptional regulation by steroid nuclear receptors involves direct interaction with the general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, indirect interaction via bridging proteins such as TATA-binding-protein (TBP) associated factors (TAFs) or via various newly characterised coactivator or corepressor proteins. This interaction is postulated to assemble general transcription factors to the PIC and subsequently enhance the transcriptional process, providing a physical link between nuclear receptors at enhancer elements and the PIC (Fig. 3c).

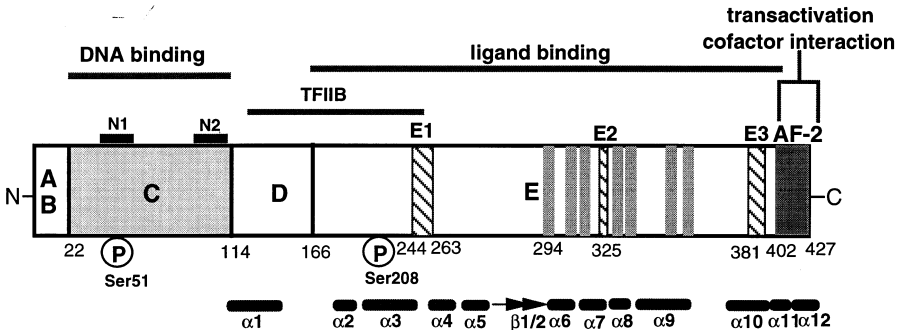

Fig. 3. Modular structure of the VDR. Common to all NHRs are five functional domains. The DNA binding domain (C) contains two potential nuclear transfer signals denoted N1 and N2. The ligand binding domain (E) contains nine heptad repeats (grey columns) and putative heterodimerisation interfaces denoted E1, E2 (overlapping heptad 4) and E3 (overlapping heptad 9). Also shown are phosphorylation sites Ser-51 and Ser-208. A TFIIB interactive interface is also indicated. The 12 α-helices and 2 β-sheets of the ligand binding domain are shown below as grey tubes and arrows, respectively.

The AF-2 domain of the ER has been shown to specifically interact with TFIIB and $TAF_{11}30$ in vitro [139, 140]. Similarly the VDR, $T_3R\beta$ and $RAR\alpha$ have been shown to bind TFIIB in solution [141, 142]. In mammalian cells $TAF_{11}135$ potentiates AF-2 dependent transcriptional activation of VDR, T_3R and RAR, but not ER or RXR [143]. Leong et al. [144] showed in vivo that $mRXR\beta$ interaction with TFIIB was ligand-dependent and AF-2-dependent. Moreover, an AF-2 domain truncated RXR acts as a dominant negative partner in VDR-mediated transcription [145]. Thus RXR participation in transcriptional activation by the VDR may therefore be through association with distinct coactivators or enhance VDR interaction with coactivators.

Evidence regarding the functional role of calcitriol in VDR-TFIIB interaction is conflicting. In the Blanco et al. [141] study, cotransfection of P19 mouse embryonal carcinoma cells with hVDR and hTFIIB resulted in synergistic 30-fold induction of transcription in the presence of calcitriol. This response is cell-type specific as in NIH3T3 Swiss mouse embryo cells TFIIB squelched calcitriol mediated transcription. In the study by Masuyama et al. [146] VDR-TFIIB interaction, both in yeast cells and in solution, was disrupted by calcitriol. These studies imply that a ligand induced VDR conformational change is not required to expose TFIIB contact sites on the VDR, in contrast to its requirement for stable complex formation with RXR and coactivators. The TFIIB contact sites on the VDR are mapped to amino residues 123-257, the hinge region-LBD boundary [141]. Hence calcitriol binding may be precluded when VDR is complexed to TFIIB.

VDR-TFIIB interaction in the absence of calcitriol may act to prime TFIIB in position to interact with the PIC, or to squelch TFIIB from the PIC during transcriptional repression. Ligand binding to the VDR may be important for releasing sequestered TFIIB, although VDR-TFIIB interaction subsequent to ligand activation of the VDR may also occur.

VDR interaction with transcriptional coactivators

It was first demonstrated with the ER that efficient transcription by nuclear hormone receptors requires the association of ligand-dependent and AF-2-dependent cofactors, the p140 and p160 (ERAP140 and ERAP160, also known as RIP140 and RIP160, respectively) and the adenovirus E1A-associated p300 protein [147–150]. These proteins interact with the LBD of the ER in a ligand- and AF-2-dependent manner, while p140 and p160 also interact with the RAR and RXR. A host of other cofactors, both coactivators and corepressors, that interact with numerous nuclear hormone receptors including the VDR have now been cloned (Table 3). Some of these cofactors seem to be receptor-specific while others have less specificity and interact with a wide range of nuclear receptors and other factors.

The RIP160 coactivator complex probably includes the steroid receptor coactivator-1 (SRC-1) or nuclear-receptor coactivator 1 (N-CoA1) protein. SRC-1/N-CoA-1 was originally isolated using the yeast dual hybrid system with the PR as a bait [151] and has been shown to interact in a ligand-dependent manner with the PR, ER, RAR, RXR, T_3R, GR and, recently, the VDR [96]. Highly related to SRC-1/N-CoA1 are the proteins GRIP-1/TIF-2 which have been shown to act as coactivators with VDR, GR, TR and RAR [97, 152, 153]. TIF-2 and SRC-1 share a common N-terminal region containing a PAS-a-basic helix-loop-helix (bHLH) homology domain for protein-protein interactions [154]. Interestingly, SRC-1 has recently been demonstrated to interact with the p50 subunit of NF-κB, though not the p65 subunit, implying a role for SRC-1 in regulation of genes involved in inflammation [155]. Furthermore, RIP140 specifically down-regulates coactivation mediated by SRC-1 in mammalian cells, suggesting a model in which RIP140 competes with SRC-1 for nuclear receptors [156]. There is also evidence that SRC-1 may interact with the N-terminus of nuclear receptors in addition to the AF-2 domain. Onate et al. recently demonstrated that SRC-1 interacts with the A/B domains or AF-1 containing domain of the PR, GR and ER, as well as their respective D/E or AF-2 containing domains [157].

A third coactivator, RAC-3, which shares high protein homology with SRC-1 and TIF2, has SRC-1-like bHLH domains in the N-terminus and has been shown to interact with the VDR, as well as with GR, RAR, RXR, TR and ER [158]. RAC-3 also shares 78% amino acid homology with another coactivator protein, p/CIP. p/CIP is complexed in the

Table 3. Steroid hormone receptor cofactors.

Cofactor	Interaction with NHRs	Comments	Ref.
ERAP140 & 160	ER	AF-2 dependent	[147–150]
TFIIB	VDR, ER, PR, TR, RXR		[139, 144, 158]
TAF(II) 135	VDR, RAR, TR	AF-2 dependent	[143]
N-CoR/SMRT	TR, RAR	corepressor	[98, 99]
CBP/p300	RAR, RXR, ER, TR, PR	HAT activity	[150, 154, 163]
p/CAF	RXR/RAR	HAT activity, interacts with SRC-1 and CBP/E1A	[164, 165, 284]
SRC-1/N-CoA1	PR, GR, ER, TR, RXR, VDR	HAT activity	[96, 168]
GRIP-1/TIF2	VDR, GR, TR, RAR	AF-2 dependent	[97, 152, 153]
RAC-3	VDR, RAR, RXR, PPAR, TR	AF-2 dependent	[158]
ACTR	GR, RAR, RXR, TR, ER	HAT activity	[167]
DRIP complex	VDR	HAT activity	[285]
NCoA-62/hSkip	VDR, RAR, RXR, GR, ER	interacts with v-ski oncoprotein	[286]
TRAP220 complex	TR, VDR, RAR, RXR, PPAR, ER	AF-2 dependent	[287]
L7	VDR, RXR	80S ribosome component	[288]
p/CIP	RAR, ER, PR		[159]
TAFII250	?	HAT activity	[166]

cell nucleus with the CREB binding protein (CBP), a large protein which mediates cyclic-AMP regulated gene expression, and with a closely related protein called p300, which binds selectively to the protein kinase A-phosphorylated form of CREB [159].

The CBP/p300 complex is required for transcriptional activity of a large number of factors with a wide range of biological roles, including nuclear hormone receptors, transcription factors important for inflammation such as the STATs (STAT1 and STAT2) and -p65 of NF-κB, the oncoproteins c-*jun*/*fos* and EIA, the negative gene regulator YY1, the general transcription factors TFIIB and TBP, and muscle differentiation factors MyoD and myb [154]. CBP/p300 belong to a family of bromodomain-containing proteins and contain an N-terminal domain for interaction with the RAR, ER, TR and RXR, though an interaction with VDR has yet to be described [160, 161]. Cellular CBP levels, which are tightly regulated, have been shown to have developmental importance, as individuals with Rubinstein-Taybi syndrome, a genetic disorder associated with mental retardation is thought to be caused by a haplo-insufficiency of CBP [162]. CBP/p300 [163] and severnal other factors, including p/CAF [164, 165], TAFII 250 [166], ACTR [167], and SRC-1 [168] have all been shown to possess intrinsic chromatin histone acetyltransferase (HAT) activity suggesting these cofactors play a role in chromatin remodelling [169].

Chromatin remodelling and nuclear hormone function

The eukaryotic genome is compacted within the cell into nucleosomes, the primary structural units of chromatin. Each nucleosome is composed of an octameric protein core containing two copies of histones H2A, H2B, H3 and H4 around which approximately 200 bp of DNA winds. Each core histone has two domains: a histone fold domain which is involved in wrapping DNA into nucleosomes and an amino acid tail domain that lies on the outside of the nucleosome and can interact with other regulatory proteins and with DNA. The N-terminal tail lysine-rich domains are targets for acetylation, which leads to a modest relaxation in the wrapping of DNA around the histone octamer resulting in loosley packed nucleosomes. Recent evidence suggests this is one mode by which coactivator/HAT complexes allow transcription factor access to promoter DNA sequences and enhance transcription [170].

Human histone deacetylase (HD1) continually deacetylates histones in chromatin leading to a repressed chromatin state. It is thought, therefore, that persistent activity of coactivator/HAT is required to maintain gene activity presumably by coactivator enhancement of assembly of basal transcription factors into a stable PIC. Histone acetylation thus provides a molecular mechanism by which DNA can be rendered generally accessible to transactivating factors whilst still maintaining a nucleosome architecture [170].

As different regions of CBP seem to be required for interaction with most of these transcription factors it is possible that, on an appropriately configured promoter, CBP/p300 could interact simultaneously with more than one class of transcription factor and hence may act as a transcriptional integrator of multiple signal transduction pathways. The VDR has not yet been shown to interact with CBP/p300. However, other coactivators such as SRC-1 and ACTR interact with CBP/p300, and may therefore act as a bridging factors for VDR, TR, ER, RXR and RAR [167, 171]. Hence the VDR, cofactors and basal transcription factors are potentially linked with CBP/p300 and p/CAF in a multimeric complex (Fig. 3c). Different nuclear hormone receptors possibly use various members of the histone acetyltransferase class of proteins/coactivators in a tissue specific manner to increase their regulatory complexity and their control of gene regulation [172].

Mechanism for transcriptional repression by the VDR

Negative control of gene function provides another important regulatory mechanism. Transcriptional repression by nuclear hormone receptors occurs through a number of mechanisms, including: 1) low affinity DNA binding in the unoccupied

state; 2) cross-coupling to non-receptor transcription factors such as TBP and AP-1 to inactivate them and 3) binding to and preventing access to transcription factor binding sites. For example, expression of the rat BSP gene is suppressed by calcitriol in osteoblastic cells, by a mechanism involving VDR interaction with a putative DR3 VDRE that overlaps an inverted TATA box, thus precluding access by TBP [138] (Fig. 3b). The VDR exerts transcriptional repression on the interleukin-2 (IL-2) gene by a similar mechanism. VDR and VDR:RXR block the association of the T-cell specific transcription factor NFATp and AP-1 binding to a positive regulatory element in the IL-2 gene promoter in solution [173]. NF-κB-mediated repression of cytokine gene expression (IL-8 and IL-12) has also been shown to be vitamin D-dependent [174, 175]. Inhibition of transcription factor function may be one mechanism by which calcitriol inhibits T-cell proliferation and exerts some of its immunosuppressive effects.

Regulation of rat growth hormone gene transcription by thyroid hormone and RA is disrupted by the VDR. This 'transrepression' phenomenon involves squelching of RXR from dimeric interactions with the T_3R and RAR on response elements [176]. Similar VDR-mediated transrepression of T_3R transcriptional activity has been reported [177]. VDR has also been suggested to inhibit GR-mediated gene transcription by squelching the coactivators GRIP-1 and SRC-1 [178]. Interestingly, an AF-2 domain deficient VDR did not affect GR-mediated transcription and lacked the capacity to interact with coactivators. The VDR disrupts RAR regulation of TNFα gene expression by competing for dimerisation with RXR, or possibly squelching coactivators [179].

The transcriptional function of the VDR can be inhibited by similar transrepression phenomena. This has been demonstrated with AP-1 using transient gene expression systems [173] and mouse gene knockout models [180]. c-*fos* knockout mice are growth-retarded and develop osteopetrosis, with deficiency in bone remodelling and tooth eruption [180], while overexpression of c-*fos* or with c-*jun* in bone tissue results in the development of osteosarcomas [181]. c-*jun* is widely expressed at low levels in many cell types and its expression is elevated in response to many stimuli, including growth factors and cytokines. The phosphorylation of c-*jun* enhances its ability to activate transcription [182]. This enhancement is most likely due to a higher affinity of phosphorylated c-*jun* for the transcriptional coactivator CBP. Thus competition between AP-1 and nuclear receptors for CBP/p300 could account for transrepression seen with for example the GR, RAR and VDR [161].

Another protein that negatively regulates VDR function is the ubiquitous regulator Ying-Yang 1(YY1) [183]. YY1 binds specifically to YY1 recognition sequences (5′-CAT-3′) within the vitamin D responsive element in the bone-specific osteocalcin promoter, and may compete with VDR:RXR heterodimers for binding to this HRE [184], as well as competing for VDR binding with the general transcription factor, TFIIB [183]. Interestingly, it has recently been shown that YY1 binds to CBP/p300, as well as to TAF55 and TBP, thus further integrating the complex array of positive and negative factors controlling nuclear hormone receptor gene function [185].

Model for initiation of gene transcription by VDR

Based on our current knowledge and by drawing on models for ER and PR interactions with the transcriptional machinery, a model for VDR gene transcription is proposed (Fig. 3). It is unclear how nuclear receptors can access their putative response elements on DNA packaged into nucleosomes, since most receptor-DNA interactive studies are performed in vitro. Possibly the 3′ VDRE half-site is exposed on the outer ridge of the nucleosome such that in the unoccupied state the VDR monomer or homodimer is primed in position to respond to hormonal signals (Fig. 3a). Calcitriol 'activation' of the VDR, resulting in conformational change, could shift the position of the AF-2 domain and initiate dimerisation with RXR at interfaces in the VDR LBD. This would initiate recruitment of TFIIB and coactivators, such as SRC-1, RAC-3 and GRIP-1, to the activated transcription complex, thus bridging contact between the receptors, HATs and RNA polymerase II (Fig. 3c). Histone acetyltransferases would then loosen the nucleosome to expose 5′ response element half sites, allowing RXR to interact with the VDR DBD and DNA, thus 'locking' the dimer in place and initiating transcription.

Calcitriol actions on non-classic target tissues and therapeutic applications of vitamin D analogues

Anti-inflammatory and immunosuppressive properties of calcitriol

In addition to its role in calcium homeostasis and bone metabolism calcitriol exhibits anti-inflammatory and immunomodulatory properties. Thus calcitriol and its analogues are potential therapeutics in psoriasis, multiple sclerosis, rheumatoid arthritis, diabetes and transplantation (Table 4). The discovery of VDR expression in peripheral blood monocytes and activated T-lymphocytes, and the observation that T-cell mediated delayed hypersensitivity response is impaired in vitamin D deficiency but suppressed by calcitriol suggests a role for calcitriol in modulating cellular immune responses [186–188]. Calcitriol inhibition of B and T-lymphocyte proliferation, IL-2 and IFN-γ secretion by T-lymphocytes and immunoglobulin secretion by B lymphocytes has been well established [189–191]. This section reviews studies on the use of calcitriol in psoriasis, multiple sclerosis and arthritis, paying particular attention to animal models of human disease. It should be stressed that the therapeutic application of calcitriol is limited by its hypercalcemic effects and therefore studies of synthetic non-hypercalcemic analogues is an active field.

Psoriasis

Psoriasis is a chronic or chronically relapsing skin disease characterised by erythematous patches covered by thick scales. In psoriasis, an increase in the activity of Langerhans cells, the antigen-presenting cells (APC), results in constituently activated T-cells within the skin. T-cells release lymphokines such as interleukin-2 (IL-2) and interferon-γ (IFN-γ) causing excessive epidermal stem cell growth

Table 4. Clinical applications of vitamin D analogues.

Analogue	Company	Target disease
Calcitriol	Roche	hypocalcaemia, osteoporosis, renal osteodystrophy, osteomalacia, psoriasis, autoimmune disease
$1,25(OH)_2$-16-ene,23-yne-D_3 (Ro23-7553)	Roche	leukaemia
$1,25(OH)_2$-16-ene,23-yne. 26.27-F_6D_3 (Ro24-5531)	Roche	breast cancer, leukaemia
$1,25(OH)_2$-16-ene-D_3 (Ro24-2637)	Roche	lupus erythematus, cardiac allografts
ED-71	Chugai	osteoporosis
22-oxacalcitriol	Chugai	psoriasis, hyperparathyroidism
Calcipotriol (MC 903)	Leo/Bristol Myers-Squibb	psoriasis, cancer
EB 1089	Leo	breast and prostate cancer
KH 1060	Leo	breast cancer, autoimmune graft rejection

(hyperplasia), abnormal keratinisation and dermal inflammation. Use of calcitriol in psoriasis was sparked by a case study in which an osteoporotic patient receiving $1\alpha OHD_3$ showed improvement in her psoriatic lesions [192]. This led to a myriad of studies demonstrating that the skin is a target for calcitriol action and demonstrating the presence of nuclear VDR in virtually all cell types of the skin, including keratinocytes which convert previtamin D to vitamin D_3 [193–195].

Although some studies in psoriasis patients have suggested an improvement with oral use of calcitriol this is dependent on the dose and duration of treatment and the risk of hypercalcemic toxicity requires patients be constantly monitored. Topical application of low doses of calcitriol ointment is safer and well tolerated, however it has not been approved for marketing [196, 197]. The synthetic analogue calcipotriol (calcipotriene, MC 903) binds the VDR with equal affinity as calcitriol, is a potent cell regulatory agent in vitro, yet is rapidly metabolised in vivo making it a weak calcemic agent [198]. Calcipotriol ointment has proved very effective and well tolerated in both short and long term studies and has been marketed as an antipsoriatic agent [191, 199].

The mechanism of calcitriol action on psoriasis has been the subject of many studies. Calcitriol modulates the three key processes in the pathogenesis of the disease, notably: epidermal hyperproliferation; incomplete epidermal terminal differentiation; and activated immunocytes. Calcitriol exhibits a dual effect on keratinocytes in culture where it stimulates proliferation of keratinocytes committed to

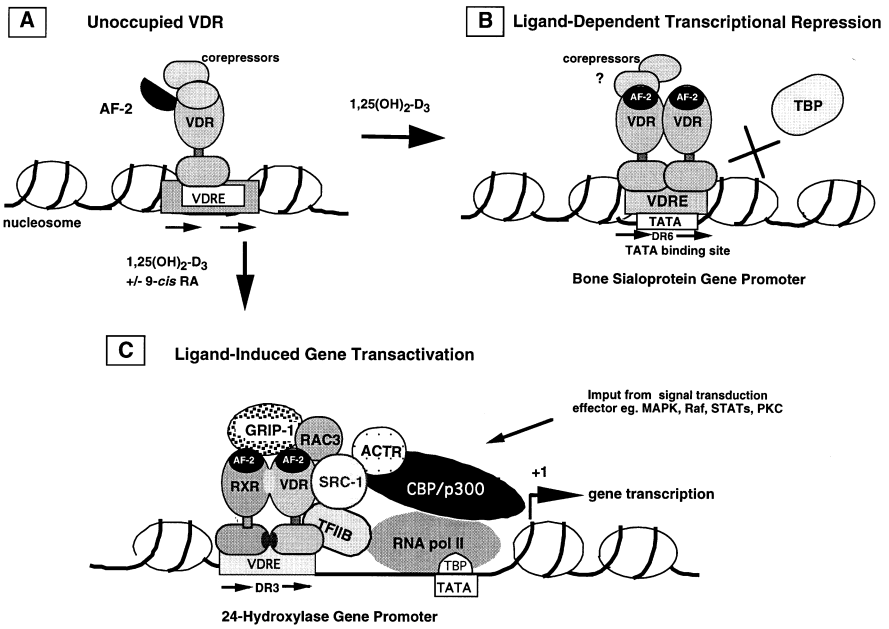

Fig. 4. Model for VDR regulation of gene transcription. A) Unoccupied VDR may be associated with transcriptional corepressors. B) Ligand-induced transcriptional repression may involve blocking access of transcription factors, such as TATA binding protein (TBP), to their binding sites. C) Ligand-induced transactivation involves dissociation of corepressors, AF-2 domain reconfiguration and interaction with transcriptional coactivators, acting as histone acetyltransferases, such as SRC-1, GRIP-1 and RAC-3, to form a stable receptor-coactivator preinitiation complex.

differentiate, while inhibiting the proliferation of undifferentiated cells [200, 201]. Topical application of calcitriol and 20-epi vitamin D analogues MC 1288, MC 1301 and KH 1060 induce epidermal cell proliferation of normal mouse skin [202, 203]. Clacitriol decreases monocyte HLA-DR expression, antigen presentation and promotion of T-cell proliferation. In addition calcitriol suppresses the number of Langerhans cells and infiltrating neutrophils in psoriatic epidermis [204–207].

Experimental autoimmune encephalomyelitis (EAE)

EAE is a mouse model of human multiple sclerosis (MS), a demyelinating disease of the central nervous system (CNS). The aetiology of MS is possibly persistent neurotropic or peripheral infection triggering an inflammatory demyelinating attack on the CNS and a breakdown in immunological self-tolerance. Currently it is hypothesised that, in addition to a genetic component of MS, environmental factors may also contribute to the onset of disease. Notably poor sunlight exposure, resulting in reduced production of vitamin D_3 in the skin, is thought to be a risk factor in MS [208]. This hypothesis is born out of epidemiological studies into the geographic distribution of MS which indicate higher disease prevalence at high latitudes and low altitudes. In addition, vitamin D deficiency is more prevalent in MS patients, while vitamin D_3 supplements can repress the symptoms of MS [209]. Current knowledge of the immunomodulatory properties of calcitriol, and evidence that it can inhibit EAE, support this hypothesis. Hence, the use of calcitriol in MS holds promise for the prevention of disease onset in genetically susceptible individuals.

In the EAE model rodents are immunised with CNS proteins, such as myelin basic protein, and develop a paralytic disease mimicking MS. Lemire et al. [210] and Branisteanu et al. [211] demonstrated the immunosuppressive effect of high doses of calcitriol in an EAE-induced strain of mice in which the disease is lethal. In these studies calcitriol prolonged survival compared with untreated controls and low dose calcitriol treated mice. In a more detailed study, Cantorna et al. [212] demonstrated the preventative effects of calcitriol on EAE induction and progression and that vitamin D deficiency in mice accelerated the onset of paralytic symptoms. Hence, when administered before and during induction of EAE calcitriol exhibits a protective effect. The immunosuppressive effects of calcitriol on EAE mice is most likely mediated by the genomic functions of the VDR and is distinct from and not a consequence of its hypercalcemic effects [213]. However, since high doses of calcitriol are required to elicit an inhibitory effect, the use of efficacious non-hypercalcemic analogues would be more apropriate.

The CNS as a target tissue for calcitriol is supported by discovery of calcitriol binding sites in the rat forebrain, hippocampus, cerebellum, brainstem, spinal cord and perivascular tissue [213–215]. In a model of chronic relapsing EAE in the Lewis rat, Nataf et al. [216] investigated the effects of post-immunisation administration of calcitriol. Immunohistochemistry of rat brain sections demonstrated a profound down regulation of complement receptor type 3 (CR3, OX42-positive) expression and CD4 antigen expression (W2/25-positive) by infiltrating T-cells, monocytes/macrophages and parenchymal activated microglia, and to a lesser extent, down regulation of expression of MHC class II molecules. These changes may suggest an inhibition of the antigen-presenting function of these cells. Most striking was the observed region-specific changes in cellular function following calcitriol treatment. CR3 positive cells decreased in number in the anterior brain but not spinal cord and cerebellum. There was reduction in CD4 antigen expression in all regions, this was most pronounced in the cerebellum and brainstem. These regional effects of calcitriol may reflect functional heterogeneity of neurons and their ability to mount a specific immune response and/or differences in the distribution of VDR expression [216].

The question of whether calcitriol can modulate the production of pro-inflammatory agents by CNS cells was partly addressed in a study by Garcion et al. [217]. This study demonstrated inhibition of inducible nitric oxide synthase (iNOS) immunostaining and mRNA expression in monocyte/macrophages, activated microglia and astrocytes in the cerebellum, brainstem and spinal cord of calcitriol treated EAE rats compared with EAE controls. iNOS gene expression is upregulated during EAE and production of nitric oxide is believed to contribute to progression of inflammatory lesions in EAE and MS, and the destruction of oligodendrocytes [218]. It is unclear whether inhibition of iNOS expression by calcitriol is mediated through the genomic actions of VDR or an indirect consequence of calcitriol inhibition of pro-inflammatory cytokines involved in iNOS gene activation. Although it is interesting to note that both macrophages and astrocytes express VDR [190, 219].

Rheumatoid arthritis

Investigation into the use of calcitriol in the treatment of rheumatoid arthritis (RA) is in its early stages however, existing studies are promising and warrant further investigation. Rheumatoid arthritis patients show a loss of bone mineral density with increasing disease severity. A recent study by Oelzner et al. [220] showed a negative relationship between serum calcitriol and PTH and disease activity. Given that calcitriol is an effective immunomodulator, RA patients would benefit from treatment with calcitriol or its non-hypercalcemic analogues. In addition, calcitriol may dampen the loss of bone following corticosteroid therapy in RA patients, presumably by reversing the steroid-induced decrease in intestinal calcium absorption and promoting osteoblastic cell function [221].

In the rat model of adjuvant arthritis (AA) low dose calcitriol cotreatment with cylosporine produced an additive inhibition of disease onset and severity compared with either agent alone [212]. In murine models of Lyme arthritis (infection with *Borrelia burgdorferi*) and collagen-induced arthritis (cartilage-derived collagen II immunisation) calcitriol supplementation at the time of disease induction prevents the development of arthritic symptoms, and halts further progression when given after the lessions have developed [223]. These studies were performed using calcitriol doses that did not alter serum calcium, thus ruling out the calcemic properties of calcitriol as a mechanism of action. In another study in the rat the

non-hypercalcemic analogue, MC 1288, exhibited a protective effect on disease onset, as well as reducing disease severity when given post-immunisation [224]. This was reflected in a reduction in titters of IgG2a antibodies to collagen II. The exact mechanism of calcitriol action is not clear, however it appears that it can inhibit both the immune and inflammatory components of RA.

Breast cancer

Cancer cell replication is stimulated by low physiological concentrations of calcitriol but inhibited by higher concentrations. The antiproliferative effects of calcitriol on breast cancer cells cause an accumulation of cells in the G_0/G_1 phase of the cell cycle [225, 226]. However, the dose required to achieve suppression of solid tumours in vivo is accompanied by hypercalcaemia, unless accompanied by marked restriction of calcium intake [227]. Newly synthesised non-hypercalcaemic analogues have potent antiproliferative effects in vitro and chemopreventive and chemotherapeutic effects in vivo, making them useful for cancer therapy (Table 4).

One promising agent is the analogue Ro 23-7553, 1,25-$(OH)_2$-16-ene,23-yne-D_3, which reduces the rate and occurrence of nitrosomethylurea (NMU) induced mammary tumours in mice and has now been approved for clinical trial in breast cancer treatment [228]. Another analogue, Ro 24-5531, 1,25-$(OH)_2$-16-ene,23-yne-26,27-F_6-D_3, is 10-100 times more potent than calcitriol at inhibiting proliferation of human breast cancer cells and primary cells from acute myelogenous leukaemia patients and reduces tumour growth and incidence in rats without affecting serum calcium. Ro 24-5531 also significantly enhances the antitumour effects of tamoxifen and in addition, when given as a dietary supplement, is a chemopreventive agent in the azoxymethane model of experimental colonic carcinogenesis [229, 230].

Interestingly from a therapeutic point vitamin D analogues such as KH 1060 (20-*epi*-22-oxa-24a,26a,27a-tri-homo-calcitriol) inhibit the growth of both ER-positive and negative breast cancer cells in culture [231]. This holds promise for the treatment of the more aggressive oestrogen-independent (ER-negative) breast cancers.

In addition calcitriol, EB 1089, KH 1060, 22-oxa-calcitriol (OCT) and Ro 23-7553 exhibit higher antiproliferative efficacy in breast cancer cells and tumours when used in combination with retinoic acid (RA) or dexamethasone [232], tamoxifen [233], 9-*cis* retinoic acid (9-*cis*-RA) [234] or the antioestrogen ICI 164-384 [235]. Combination chemotherapy permits the use of lower concentrations of cytotoxic agents and thereby reduces the development of drug resistance and side effects. Combination therapy with ICI 164-384 or tamoxifen has the added benefit of reducing calcitriol and analogue induced bone resorption [235].

More recently, a novel vitamin D_5 series analogue, 1α-hydroxy-24-ethyl-cholecalciferol, exhibited potent chemopreventive properties against the development of preneoplastic lesions in carcinogen-treated mammary glands in organ culture [236], thus making it a candidate chemopreventive agent against development of primary cancers in high risk groups as well as secondary lesions.

The more widely studied analogue EB 1089 which is effective in rat mammary tumours models [234] has been approved for phase 1 trial in breast cancer treatment. Preliminary studies in normal subjects show EB 1089 is not completely devoid of calcaemic effect, although less than calcitriol [237]. EB 1089 induces apoptosis in MCF-7 cells but not in cells lacking a functional p53 tumour suppressor protein which are growth inhibited or induced to differentiate (see review [231, 238]). Induction of apoptosis was associated with upregulation of p53 and bax protein, a suicide switch protein, and the concomitant reduction in the expression of bcl-2, a suppressor of apoptosis [234, 239]. Similar changes in protein expression were observed in MCF-7 cells treated with KH 1060 [231]. However, the mechanism behind changes in expression of these proteins by vitamin D analogues is unclear.

Changes in proteins involved in apoptosis in MCF-7 cells following vitamin D analogue treatment occur long after the changes in cell cycle phase distribution. This indicates that cells are first growth arrested and then proceed down either differentiation or apoptotic pathways depending on the abundance and activity of certain regulatory proteins. Growth inhibition of cells lacking the p53-mediated apoptotic pathway indicates that multiple VDR signalling pathways are operational. Analogue effects on cyclin and cyclin dependent kinase (Cdk) activity in cell cycle control has not been addressed in breast cancer cells. Possibly, like the antioestrogens, vitamin D analogues may exert antiproliferative effects by inducing the expression of Cdk inhibitor proteins $p21^{WAF1CIP1}$, $p27^{Kip1}$ and p16, which consequently bind to and inactivate cyclin-Cdk complexes, thus blocking progression through the cell cycle. However, the mechanism for stimulated cell growth at low concentrations of calcitriol is unclear.

Leukaemia

Interest in vitamin D analogue use in leukaemia has expanded over recent years. The response of haematopoietic cells to calcitriol treatment is dependent on cell type, the differentiation state and the dose of calcitriol used. In combination with RA or 9-*cis*-RA the response is dependent on the order of administration. Mouse M1 myeloid leukaemic, HL-60 promyeloid and U937 myelomonocytic cells treated with RA or 9-*cis*-RA differentiate to neutrophils or granulocytes. Treatment with nanomolar concentrations of calcitriol drives cells down the monocyte/macrophages differentiation pathway [240–242].

In vitro and in vivo studies support the use of 9-*cis*-RA and calcitriol combination chemotherapy to counteract the development of resistance in patients with promyelocytic leukaemia. HL-60 cells that have developed resistance to RA show greater responsiveness to clonal inhibition by 1,25-$(OH)_2$-Δ16-ene-D_3 than the parent cell line [243, 244]. Other candidate antileukaemic drugs include Ro 24-5531 and Ro 23-7553, both are more potent than calcitriol in vitro and in animal models of leukaemia but have reduced calcaemic activity [245–247]. It is unclear whether the higher potencies of Ro 24-5531 and Ro 23-7553 in vivo are

due to accumulation of active metabolites or related to altered VDR-mediated activation effects.

In leukaemic cells calcitriol regulates numerous genes at both transcriptional and translational levels. These include c-*fos*, c-*myc*, IL-1, IL-6, TNFα and carbonic anhydrase [248]. However, calcitriol-regulatory elements have been characterised only in a few gene promoters. By mRNA subtractive hybridisation Liu et al. [249] identified calcitriol inducible genes involved in U937 cell differentiation, including the Cdk inhibitor p21, the transcription factor Mad1 and the homeobox gene Hoxa10. Others have also demonstrated upregulation of p21 and p27 mRNA in U937 and HL-60 cells by calcitriol [250, 251]. These changes probably mediate the observed increase in number of cells blocked in G_0/G_1 and concomitant increase in cells expressing cell surface markers of differentiation. Characterisation of the p21 gene promoter has identified four putative VDREs that are bound by VDR:RXR heterodimers [249]. Mad1 gene promoter analysis has also identified a VDRE bound by a VDR:RXR heterodimer [136]. Transcriptional regulation of Mad1 gene, a pro-differentiating gene, was found to be cell type-specific. These findings suggest that calcitriol action on leukaemic cells is mediated through a number of pathways via regulation of genes involved in both cell cycle control and differentiation.

Prostate cancer

Use of vitamin D analogues in prostate cancer treatment is a recent area of investigation. Primary prostatic malignancies and some cell lines are growth inhibited by calcitriol [252, 253]. Schwartz et al. [254] demonstrated a modest 15% reduction in tumour volume by the analogue Ro 23-7553 in athymic nude mice inoculated with PC-3 human prostate cancer cells. The analogues KH 1060, EB 1089, Ro 23-7553, 1,25-$(OH)_2$-16-ene-D_3 (Ro 24-2637) and the 20-epimer of calcitriol, MC 1288, inhibited the clonogenic growth of PC-3 and LNCaP prostate cancer cells to varying degrees, while DU-145 prostate cancer cells are generally resistant to vitamin D analogue treatment [255]. The mechanism of this growth inhibition is unknown, but may involve induction of differentiation, as suggested by an increase in the production of prostate-specific antigen, a possible marker of differentiation, and E-cadherin, a cell adhesion protein [253, 256].

A study of fluorinated analogue effects on the clonal growth of LNCaP, PC-3 and DU-145 cells suggested an association between expression of the AR, tumour suppressor p53, Cdk inhibitor p16 and functional retinoblastoma (Rb) protein with responsiveness to vitamin D analogue treatment [256]. Generally, loss of AR expression leads to reduced responsiveness, with the AR negative cell line DU-145 being least responsive and LNCaP most responsive. However, there was no direct correlation between expression of cell cycle regulatory proteins and vitamin D analogue effects, as 1,25-$(OH)_2$-16-ene,23-yne-26,27-F_6-19-nor-D_3 was equipotent in all three cell lines. DU-145 is the most highly differentiated cell line and lacks expression of all the above mentioned proteins, indicating that they are not necessary for the inhibitory action of 1,25-$(OH)_2$-16-ene,23-yne-26,27-F_6-19-nor-D_3. However, growth inhibition of DU-145 by this analogue involved upregulation of p21 [256]. Clearly, the actions of vitamin D analogues are mediated through the regulation of genes involved in numerous biological pathways, in a cell type-specific manner. It is not clear whether different analogues exhibit specific gene promoter regulation.

Other clinical applications of vitamin D analogues

Vitamin D analogues have clinical application in a number of other cell proliferative disorders (Table 4). Calcitriol in the non-obese diabetic mouse model of type 1 diabetes is protective against insulitis (infiltration of islets of Langerhans with immune cells), and in combination with cyclosporin A halts further islet destruction [257, 258]. OCT, 22-oxa-calcitriol may be useful in suppressing secondary hyperparathyroidism resulting from renal failure and in breast cancer [2, 259]. Another possible application is the use of calcitriol in combination with isotretinoin in squamous and basal cell carcinomas and Bowen's disease [260]. Ro 24-2637 and KH 1060 have been proposed for use in autoimmune diseases such as lupus erythematosus and graft rejection.

ED-71 (2-β(3-hydroxypropoxy)-1α,25-$(OH)_2D_3$) in animal models has been proposed to protect against age-related, corticosteroid and post-ovarectomy bone loss. ED-71 depressed bone loss in ovariectomised rats and increased bone formation in both normal and oestrogen-deficient rats [261]. These positive effects on bone are mediated through its higher serum Ca^{2+} raising effects relative to calcitriol [262]. In addition, ED-71 counteracts prednisone-induced bone loss by increasing intestinal Ca^{2+} absorption, reducing bone resorption and enhancing mineralisation [263].

Pharmacological and molecular basis for the differential actions of vitamin D analogues

Factors that influence the biological effects of vitamin D analogues fall into three categories: 1) pharmacokinetic influences, including bioavailability, cellular access and metabolic clearance; 2) pharmacodynamic influences resulting from the effect of a ligand on receptor function, these may be mediated through differential VDR binding affinity, ligand-induced receptor stabilisation, receptor conformational change and/or phosphorylation and 3) pharmacogenetic factors which include the action of a ligand on gene transcription in the context of specific genetic and cellular environments. Thus the net effect of any analogue is due to numerous factors.

Pharmacokinetic influences

Target cell bioavailability, tissue distribution and metabolic clearance rates of vitamin D_3, its metabolites and analogues may be influenced by affinity for the serum vitamin D binding protein (DBP). The ligand binding specificity of DBP is different to that of the VDR. High affinity binding to DBP requires the presence of a C25 hydroxyl whereas introduction of a 1α-hydroxyl reduces binding. Thus

25-OHD, the most abundant circulating form of vitamin D_3, is the best ligand for DBP. Introduction of a double bond at C16 of 25-OHD markedly reduces its affinity for DBP, and modifications to C11 and C14 of the C and D rings of the molecule, respectively, decrease binding [264]. Most newly synthesised analogues with modifications of the side chain, including those with unsaturated bonds at C22 or C23, C26 and C27 fluorination and side chain elongated compounds, have reduced or absent binding to DBP.

DBP binding capacity determines the amount of free ligand available to the cell. Lack of binding to DBP enhances the potency of some vitamin D analogues in in vitro cell culture experiments, however, it may reduce in vivo potency due to rapid hepatic clearance [265]. In addition, the concentration of serum in cell culture experiments influences biological responses to analogues. Analogues may bind specifically to other unidentified plasma carrier proteins which may influence tissue distribution. For instance, the analogue OCT is ineffective if given orally due to rapid clearance, however, recent studies indicate that OCT binds β-lipoprotein in vivo and accumulates in the parathyroid gland at greater concentrations than in other tissues. This may be important for its suggested therapeutic role in secondary hyperparathyroidism [266].

Differential modes and cell type-specific metabolism

Some analogues exhibit differential modes or rates of metabolism in comparison to calcitriol, undergo catabolism to more active or stable byproducts, or exhibit cell type-specific metabolism. Calcitriol undergoes a series of metabolic conversions, in the kidney and target cells, to produce inactive metabolites. Following 24-hydroxylation, further oxidation produces 1,25-$(OH)_2$-24-oxo-D_3 which is further hydroxylated at C23 before being transformed into calcitroic acid. A recent study showed OCT is metabolised to inactive metabolites in target cells such as keratinocytes and bone, and less efficiently in liver cells. The production of C23-27 truncated metabolites in the liver is suggestive of alternative metabolic pathways for OCT [267].

MC 1288 has increased VDR binding affinity and correspondingly higher transactivational potency than calcitriol due to reduced DBP binding and a 10-fold slower rate of catabolism [268, 269]. Despite greater in vitro potency MC 1288 is predicted to be less useful in vivo due to rapid hepatic clearance, however, recent studies show it is metabolised to a stable 24-oxo intermediate which is almost as active as the parent compound in gene transactivation [270].

By comparison, the analogue EB 1089 has been shown to be metabolised at a slower rate in vitro in cultured liver and keratinocyte cells and displays a longer half life in vivo [271]. This is reflected in its high biological potency both in vitro and in vivo. More recently the metabolic byproducts of EB 1089 in vivo in rats and in vitro in cultured cells have been identified. The enhanced potency of EB 1089 may be due to a different mode of metabolism that results in the accumulation of a series of 26-hydroxy-EB 1089 metabolites [272]. These metabolites displayed 10 to 100-fold lower potency in cell growth assays but were more stable.

Ro 24-5531 has one-third the affinity for DBP as calcitriol, which increases its target tissue bioavailability but may lead to more rapid hepatic clearance in vivo [244–246]. However increased in vivo potency suggests Ro 24-5531 may be converted to an active metabolite, as has been suggested for MC 1228. In vitro target cell metabolism of Ro 24-5531 by leukemic WEHI-3 cells results in a comparable half life to that of calcitriol. Yet in addition to the generation of labile 24-OH metabolites, Ro 24-5531 apparently yields a number of more stable metabolites [273].

Ro 24-2637 is also metabolised to a stable, active intermediary 1,25-$(OH)_2$-24-oxo-16-ene-D_3 via the C-24 oxidation pathway, both in a perfused rat kidney model and in the human myeloid leukemic cell line RWLeu-4 [270]. Ro 24-2637 and its 24-oxo metabolite display equipotent antiproliferative activity on RWLeu-4 cells and trans-activational efficacy in ROS 17/2.8 cells and are equally effective in suppressing experimentally induced autoimmune encephaloyelitis in mice [213, 270]. In contrast to the 24-oxo metabolite of calcitriol, the 24-oxo metabolite of Ro 24-2637 is resistant to subsequent hydroxylation by 23-hydroxylase. The analogue KH 1060 has been reported to be metabolised to twenty-two different compounds in vitro, of which the 24α-OH-KH 1060 and 26-OH-KH 1060 metabolites are active, although less so than the parent compound [274].

Cell type-specific metabolism may arise from differential expression of cytochrome P450 enzymes or the existence of alternative metabolic pathways. The effects of calcitriol, KH 1060, 1,25-$(OH)_2$-16-ene,23-yne,26-F_3-D_3 (Ro 23-6010), 11α-methyl-calcitriol (CD99) and 1α-(OH)-20-epi,23-yne, 25,26-epoxy-D_3 (ZXY 835) on MCF-7 breast cancer cell proliferation are all enhanced by the cytochrome P450 inhibitor ketoconazole [275]. Ketoconazole also enhances the activity of calcitriol but not the other analogues in MG-63 cells, while analogue effects on HL-60 cell growth are not altered by ketoconazole. This suggests existence of analogue-specific and cell type-specific modes of metabolism and can partly explain the different potencies of vitamin D analogues in these cells. Hence side chain modified analogues that are highly potent in vitro may be useful in vivo.

Pharmacodynamic influences

Pharmacokinetic factors explain only part of the biological differences between analogues. In fact studies performed in vitro in the absence of serum indicate distinct differences in analogue activation of the VDR. Lack of correlation between binding affinity and biological potency implies analogues may modulate VDR dimerisation, DNA binding affinity and interactions with cofactors, presumably through distinct changes in VDR conformation. A study by Nayeri et al. [276] suggests that ligand induced VDR resistance to proteolysis is a better predictor of biological potency than competitive binding, suggesting that ligand induced stability and/or conformational changes affect function. MC 1288 and KH 1060, which enhanced VDR:RXR heterodimerisation and the binding of VDR:RXR to an osteopontin VDRE, induced distinct VDR conformational changes [277].

Enhanced VDR-DNA binding affinity is a potential mechanism for higher transactivational potency of vitamin D

analogues. In gel shift assays 1,25-$(OH)_2$-26,27-F_6-D_3 induced the DNA binding of VDR:RXR heterodimer to a DR3 VDRE at a 10-times lower concentration than required for calcitriol without affecting the dissociation kinetics [278]. Cheskis et al. [279] have examined the kinetics of analogue induced VDR-DNA binding and dimerisation in solution using surface plasmon resonance and found distinct differences between analogues.

Pharmacogenetic influences

The pharmacogenetic influences on vitamin D analogue action have not been studied, largely due to technical limitations. The dissociated actions of analogues on calcium homeostasis versus cell differentiation and proliferation may potentially be mediated through specificity for regulation of different genes, or promoter selectivity. Different ligand-induced conformations of the VDR may direct promoter selectivity. Such conformational changes may also influence VDR interaction with different coactivators and the PIC depending on the cellular environment. These potential but relatively poorly studied mechanisms may act in concert with the pharmacokinetic and pharmacodynamic factors to determine the cell-specific actions of analogues.

Summary

The VDR mediates the diverse biological effects of calcitriol and its analogues by regulating the expression of various genes involved in numerous signalling pathways. VDR exerts both positive and negative regulation on gene transcription through complex ligand-dependent and independent interaction with VDREs in gene promoters. Regulation of gene transcription by the VDR involves interaction with different components of the basal transcription machinery. How these factors come together to form multimeric complexes is unclear. Determining which of these factors are important to the regulation of specific gene promoters, in mammalian cells and in vivo, will increase our understanding of the mechanism for tissue specific gene regulation.

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