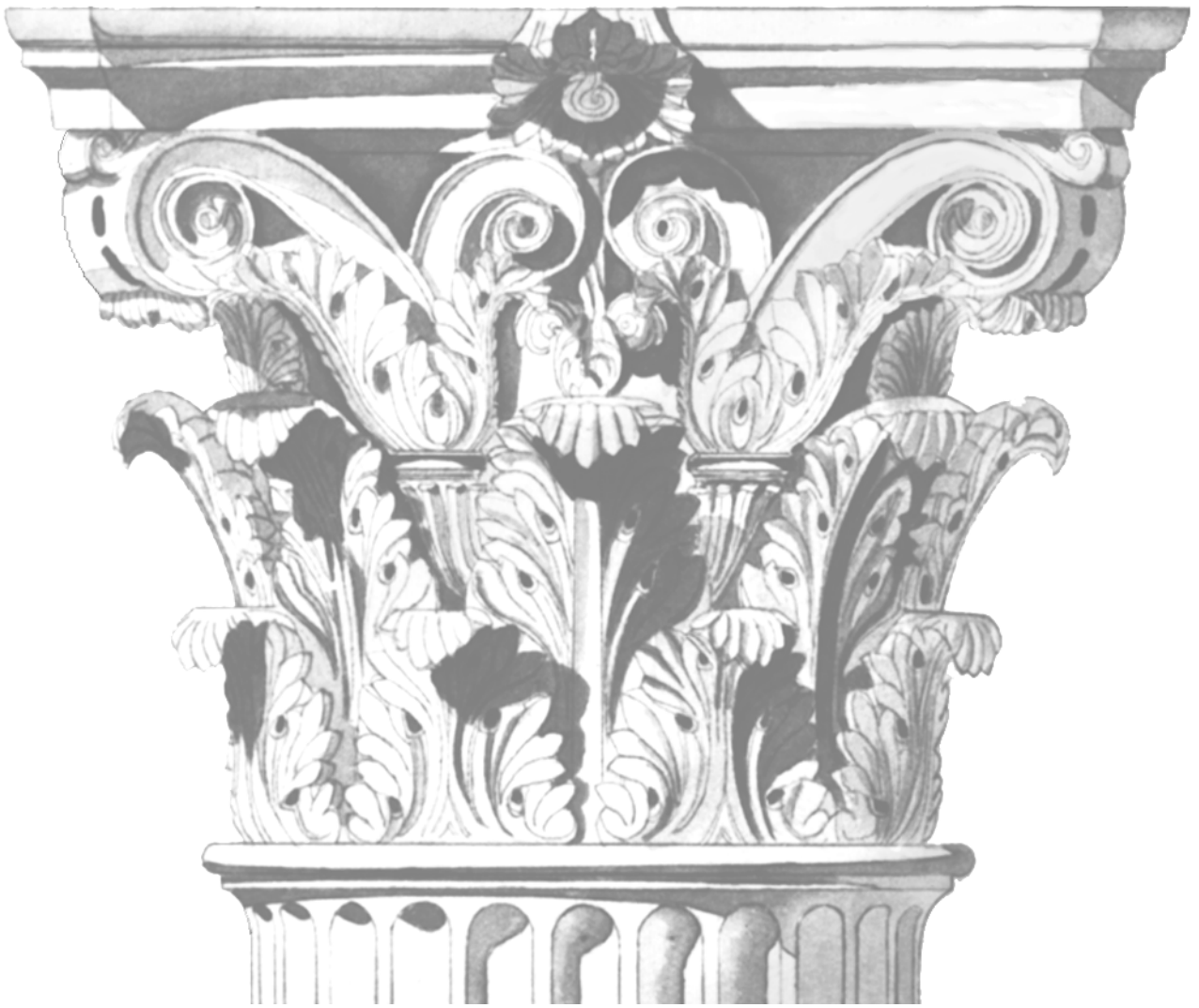
FUNCTIONAL AND STRUCTURAL CHARACTERIZATION OF NUCLEAR VITAMIN D RECEPTOR AND ITS LIGAND BINDING DOMAIN

KARI JUNTUNEN

Research Center for
Molecular Endocrinology,
Faculty of Medicine and
Biocenter Oulu,
University of Oulu

OULU 2002



KARI JUNTUNEN

FUNCTIONAL AND STRUCTURAL CHARACTERIZATION OF NUCLEAR VITAMIN D RECEPTOR AND ITS LIGAND BINDING DOMAIN

Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in the Auditorium 9 of the University Hospital of Oulu, on November 29th, 2002, at 12 noon.

OULUN YLIOPISTO, OULU 2002

Supervised by
Professor Pirkko Vihko

Reviewed by
Docent Christel Lamberg-Allardt
Professor Pentti Tuohimaa

ISBN 951-42-6878-4 (URL: http://herkules.oulu.fi/isbn9514268784/)

ALSO AVAILABLE IN PRINTED FORMAT
Acta Univ. Oul. D 703, 2002
ISBN 951-42-6877-6
ISSN 0355-3221 (URL: http://herkules.oulu.fi/issn03553221/)

OULU UNIVERSITY PRESS
OULU 2002

Juntunen, Kari, Functional and structural characterization of nuclear vitamin D receptor and its ligand binding domain
Research Center for Molecular Endocrinology, Faculty of Medicine and Biocenter Oulu, University of Oulu, P.O.Box 5000, FIN-90014 University of Oulu, Finland
Oulu, Finland
2002

Abstract

The hormonally active form of vitamin D, 1,25$(OH)_2D_3$, is involved in many biological functions throughout the body, such as regulation of calcium and phosphate homeostasis, bone remodeling and controlling cell proliferation and differentiation. Vitamin D receptor (VDR), a member of the nuclear hormone receptor (NHR) super family, mediates those genomic actions of 1,25 $(OH)_2D_3$ by actively repressing or activating its target genes. In the present study recombinant human nuclear VDR and its ligand binding domain (LBD) were expressed in *Spodoptera frugiperda* (*Sf*9) insect cells and in *E.coli*. Recombinant proteins were purified and their biochemical and biophysical properties were characterized.

Recombinant VDR was shown to bind to the vitamin D response element (VDRE) of osteopontin and osteocalcin genes as a homodimer or as a heterodimer with the retinoid X receptor (RXR)-αΔAB.

Full-length VDR and its LBD were demonstrated to bind natural ligand 1,25 $(OH)_2D_3$ with high affinity. The binding affinities of several vitamin D analogs were also determined. Ligand binding induced conformational change within the receptor was studied using several methods such as partial proteolytic digestion, small angle neutron scattering (SANS), native gel electrophoresis and circular dichroism (CD) spectroscopy. Results indicate that ligand binding induces conformational change within VDR and different 1,25$(OH)_2D_3$ analogs might induce a somewhat different conformation within the receptor. This is seen as an unequal capacity of analogs to stabilize receptor against proteases or heat and as differences in the promotion of receptor homodimerization.

Compared to other nuclear hormone receptors, VDR presents a large insertion region at the N-terminal part of the LBD between helices H1 and H3, encoded by an additional exon. In the present study this additional exon was deleted and the properties of mutated LBD were compared to the wild type LBD.

Biochemical analyses indicated that the mutant protein exhibits the same ligand binding, dimerization with RXR and transactivation properties as the wild-type VDR, suggesting that the insertion region does not affect these main functions. Furthermore, solution studies by small angle X-ray scattering indicated that the insertion region in the VDR locates on the surface of molecule and it is not structurally well ordered.

Keywords: mutated LBD, RXR, steroid receptor, vitamin D analogs

Acknowledgements

The present study was carried out at Biocenter Oulu and at the Recearch Center for Molecular Endocrinology, which also acts as WHO Collaborating Centre for Research on Reproductive Health, Faculty of Medicine, University of Oulu.

I wish to express my deepest gratitude to my supervisor Professor Pirkko Vihko for providing excellent working facilities, and for her guidance and never-ending optimism throughout this study.

I wish to acknowledge Docent Christel Lamberg-Allardt and Professor Pentti Tuohimaa for their critical reading of the manuscript. I also express my gratitude to Anna Vuolteenaho for the language revision of this thesis manuscript.

I am very grateful to my co-authors Sami Väisänen, Arsi Itkonen, Professor Pekka Mäenpää, Natacha Rochel, Dino Moras, Guiseppe Tocchini-Valentini, Pascal Egea, Jean-Marie Garnier, Fabio Falsone, Riitta Kurkela, Andreas Steinmeyer and Andreas Kungl.

I am grateful for the skilled technical assistance of Sirpa Rannikko, Pirkko Ruokojärvi, Marja-Liisa Norrena, Marja-Riitta Hurnasti and Mirja Mäkeläinen.

I warmly thank all my colleagues in protein lab Annakaisa Herrala, Helena Kaija, Ritva Isomäki and Anita Pulkka for their friendship and Anja Raatikainen and Sirpa Annanperä for their administrative help.

I thank all my relatives and friends for their support and understanding. I thank my son Touko and my daughter Iiris who always remind me that there are more important things in human life than science.

Above all, I owe my deepest gratitude to my wife Annamari Vuorela for her love and encouragement. I would also like to thank her for critical reading of the manuscript.

This work was supported financially by Biocenter Oulu, the Research Council of Finnish Academy, the Sigrid Juselius Foundation and the Vihuri Foundation.

Oulu, November 2002 Kari Juntunen

Abbreviations

1α-hydroxylase	25-hydroxyvitamin D_3-1-α-hydroxylase
$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
$25OHD_3$	25-hydroxyvitamin D_3
CBP	CREB-binding protein
CD	circular dichroism
COS cell	SV40-transformed African Green monkey kidney cell
CRE	cAMP-responsive element
CREB	cAMP-responsive element binding protein
DBD	DNA binding domain
DBP	vitamin D binding protein
D_{max}	maximum dimension
DR	direct repeat
DRIP	D-vitamin receptor interacting protein
GTF	general transcription factor
HAT	histone acetyl transferase
HDAC	histone deacetylase complex
HRE	hormone response element
HVDRR-II	hereditary vitamin D resistant rickets type II
LBD	ligand binding domain
N-CoR	nuclear co-repressor
NHR	nuclear hormone receptor
NGF	nerve growth factor
NGFI-B	NGF-induced B receptor
PCAF	p300/CBP-associated factor
PIC	preinitiation complex
PTH	parathyroid hormone
RAR	retinoic acid receptor
RevErb	orphan receptor encoded on the noncoding strand of the thyroid receptor gene
R_G	radius of gyration
RXR	retinoid X receptor
SANS	small angle neutron scattering
SAXS	small angle X-ray scattering

*Sf*9	*Spodoptera frugiperda* 9 cell
SIN3	negative regulator of the yeast HO gene
SMTR	silencing mediator for thyroid and retinoid receptors
SRC	steroid receptor coactivator
SWI/SNF	switching of yeast maiting type/sucrose nonfermenting
TFIID	transcription factor IID
Tm	thermal unfolding transition
TR	thyroid hormone receptor
TRAP	thyroid hormone receptor associated protein
VDR	nuclear vitamin D receptor
VDRE	vitamin D responsive element
VDR_{mem}	putative cell membrane receptor for 1,25-dihydroxyvitamin D_3

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Väisänen S, Juntunen K, Itkonen A, Vihko P, Mäenpää PH (1997) Conformational studies of human vitamin-D receptor by antipeptide antibodies, partial proteolytic digestion and ligand binding. Eur J Biochem 248: 156-162.

II Juntunen K, Rochel N, Moras D, Vihko P (1999) Large-scale expression and purification of the human vitamin D receptor and its ligand-binding domain for structural studies. Biochem J 344: 297-303.

III Rochel N, Tocchini-Valentini G, Egea PF, Juntunen K, Garnier JM, Vihko P & Moras D (2001) Functional and structural characterization of the insertion region in the ligand binding domain of the vitamin D nuclear receptor. Eur J Biochem 268: 971-979.

IV Falsone SF, Juntunen K, Kurkela R, Steinmeyer A, Vihko P & Kungl AJ (2002) Binding of synthetic vitamin D analogs to the human vitamin D receptor: Effects on homodimerization and unfolding. Manuscript.

Contents

1 Introduction

The hormonally active form of vitamin D, $1,25(OH)_2D_3$, is involved in many biological functions throughout the body, such as regulation of calcium and phosphate homeostasis, bone remodeling and controlling cell proliferation and differentiation. Nuclear VDR mediates those genomic actions of $1,25(OH)_2D_3$. Vitamin D receptor is a member of the nuclear hormone receptor (NHR) superfamily, which is composed of over 350 different receptors. Crystallographic structures and functional studies of NHRs indicate that the same general structural and functional features are present through out the NHR superfamily.

Vitamin D receptor acts as a ligand-dependent transcription factor which is able to actively repress or activate its target genes. Ligand binding to VDR is an initial step in transactivation. It induces a conformational change within the receptor that enhances heterodimerization with retinoid X receptor (RXR) and binding with high affinity and specificity to the vitamin D-responsive element (VDRE), locating at the 5' flanking region of the target gene. In addition to that, the conformational change induces releasing of corepressor molecules from the surface of VDR and allows receptor interaction with coactivator molecules. Coactivators modulate the local structure of chromatin and mediate the receptor interaction with basal transcription machinery in order to activate transcription of the target gene.

Because the active metabolite of vitamin D is also involved in the regulation of cellular growth and immune responses, there is increasing interest towards the use of $1,25(OH)_2D_3$ in treatment of several diseases such as psoriasis, autoimmune diseases, osteoporosis, hyperparathyroidism and cancer. However, the therapeutic applications of $1,25(OH)_2D_3$ are limited due to the hypercalcemic and phosphataemic activity of this compound. In order to avoid these unwanted side effects, a lot of studies have been carried out aimed at synthesizing analogs that exhibit weaker effects on calcium metabolism while retaining growth and immune regulating properties.

In the present study we describe the expression and purification of recombinant VDR and its ligand binding domain (LBD). The study focuses on the characterization of biochemical and biophysical properties of those proteins such as ligand and DNA binding, dimerization and conformational changes induced by natural ligand and synthetic analogs. A further aim was to create a VDR mutant lacking the large insertion region at the N-terminal part of the LBD between helices H1 and H3 and to characterize the biological and structural role of this domain.

2 Review of the literature

2.1 The vitamin D endocrine system

2.1.1 Vitamin D metabolism

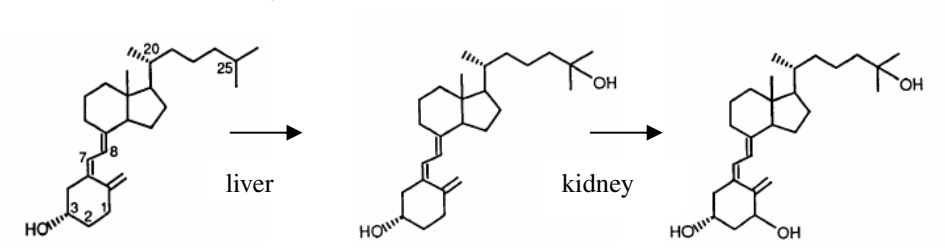
Vitamin D_3 is a fat-soluble prehormone which plays an important role in many biological functions throughout the body. Two thirds of the vitamin D_3 content of the human body is synthesized from the precursor molecule 7-dehydrocholesterol in the skin by the action of sunlight and one third is obtained from the diet. The importance of sunlight in the synthesis of vitamin D_3 can be seen in decreased serum levels of vitamin D_3 compounds among northern human population during winter (Jones *et al*. 1998, Brown 1999, Malloy & Feldman 1999).

Once vitamin D_3 enters the blood circulation it binds to the vitamin D binding protein (DBP) (Haddad *et al*. 1993), which carries vitamin D_3 to the liver and kidney (Wikvall 2001) for bioactivation. In the first activation step vitamin D_3 is hydroxylated by the enzyme 25-hydroxylase to 25-hydroxyvitamin D_3 ($25OHD_3$) mainly in the liver (Fig. 1). This reaction is poorly regulated, which is why the level of $25OHD_3$ in the serum increases in proportion to vitamin D_3 intake (Okuda 1994). In the second step the biologically active hormone 1,25-dihydroxyvitamin D_3 $1,25(OH)_2D_3$ is generated by hydroxylation of $25OHD_3$. This reaction is catalyzed by the enzyme 25-hydroxyvitamin D_3-1-α-hydroxylase (1α-hydroxylase) and it occurs mainly in the kidney (Okuda *et al*. 1995, Henry 1992). The active hormone stays in blood circulation for about 7 hours. As a fat soluble molecule $1,25(OH)_2D_3$ penetrates easily the plasma membrane of its target cells, where it is catabolized (Dusso *et al*. 1991).

Vitamin D compounds are catabolized primarily by oxidation of the side chain. The 24-hydroxylation of $1,25\text{-}(OH)_2D_3$ is the first catabolic step in the elimination of active hormone leading to the formation of 1,24,25-trihydroxyvitamin D_3, which is 10 times less potent than $1,25\text{-}(OH)_2D_3$ (Okuda *et al*. 1995) (Fig. 1). Further oxidative reactions of 1,24,25-trihydroxyvitamin D_3 lead to progressive loss of biological activity and finally to

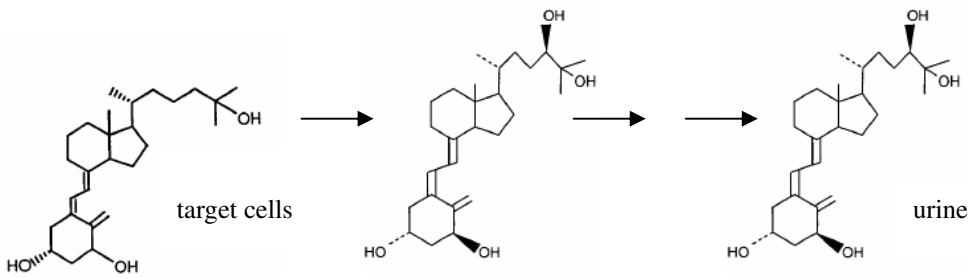
the production of water-soluble calcitroic acid, which is excreted in urine (Makin *et al*. 1989).

Synthesis of $1,25(OH)_2D_3$



vitamin D_3 $25(OH)D_3$ $1,25(OH)_2D_3$

Elimination of $1,25(OH)_2D_3$



$1,25(OH)_2D_3$ $1,24,25(OH)_3D_3$ calcitroic acid

Fig. 1. The schematic representation of key metabolic and catabolic steps of vitamin D_3 is illustrated in the figure.

2.1.2 Regulation of $1,25(OH)_2D_3$ concentration in serum

$1,25(OH)_2D_3$ has high potency to elevate serum calcium and phosphate levels. Therefore it is important that the circulating concentration of $1,25(OH)_2D_3$ (~75 pM) is under tight regulation. This is achieved by controlling the reciprocal rates of synthesis and degradation of $1,25(OH)_2D_3$ (Jones *et al*. 1998, Brown *et al*. 1999).

Major regulators of $1,25(OH)_2D_3$ concentration in serum are parathyroid hormone (PTH), calcium, phosphate and $1,25(OH)_2D_3$ itself (Table 1). The parathyroid gland

monitors the concentration of calcium in serum and secretes PTH in response to hypocalcemia (Jones *et al*. 1998). Parathyroid hormone raises the level of $1,25(OH)_2D_3$ in serum by increasing the transcription of 1α-hydroxylase gene (Brenza *et al*. 1998, Murayama *et al*. 1999) and slowing down the degradation of $1,25(OH)_2D_3$ by decreasing 24-hydroxylase activity (Shinki *et al*. 1997). There is evidence that calcium and phosphate are able to regulate the level of $1,25(OH)_2D_3$ also in a PTH-independent manner (Weisinger *et al*. 1989, Bland *et al*. 1999, Brown *et al*. 1999). This regulation seems also to be mediated through changes in the mRNA level of 24-hydroxylase and 1α-hydroxylase genes (Weisinger *et al*. 1989, Shinki *et al.* 1992, Wu *et al*. 1996).

$1,25\text{-}(OH)_2D_3$ inhibits its own production by suppressing the synthesis and secretion of PTH (Demay *et al*. 1992, Mackey *et al*. 1996). High level of $1,25\text{-}(OH)_2D_3$ also downregulates its own synthesis by inhibiting the expression of renal 1α-hydroxylase (Takeyama *et al*. 1997, Kato *et al*. 1999, Murayama *et al*. 1999) and speeds up its own degradation by increasing D-24-hydroxylase gene expression. The promoter of the D-24-hydroxylase gene contains two sets of vitamin D response elements, which are involved in this upregulation (Ohyama *et al*. 1994).

Table 1. A summary of key factors and their target enzymes that are involved in controlling the concentration of 1,25-$(OH)_2D_3$ in serum.

Inductor	Mediator	Target enzyme	$[1,25\text{-}(OH)_2D_3]$
High Ca^{2+} PO_4^{-3}		1α-hydroxylase ↓	$1,25\text{-}(OH)_2D_3$ ↓
High Ca^{2+}, PO_4^{-3}	PTH ↓	1α-hydroxylase ↓	$1,25\text{-}(OH)_2D_3$ ↓
High Ca^{2+}, PO_4^{-3}	PTH ↓	24-hydroxylase ↑	$1,25\text{-}(OH)_2D_3$ ↓
Low Ca^{2+}, PO_4^{-3}	PTH ↑	1α-hydroxylase ↑	$1,25\text{-}(OH)_2D_3$ ↑
Low Ca^{2+}, PO_4^{-3}	PTH ↑	24-hydroxylase ↓	$1,25\text{-}(OH)_2D_3$ ↑
High $1,25\text{-}(OH)_2D_3$	PTH ↓	24-hydroxylase ↑	$1,25\text{-}(OH)_2D_3$ ↓
High $1,25\text{-}(OH)_2D_3$		1α-hydroxylase ↓	$1,25\text{-}(OH)_2D_3$ ↓
High $1,25\text{-}(OH)_2D_3$		24-hydroxylase ↑	$1,25\text{-}(OH)_2D_3$ ↓

2.1.3 Physiological actions of $1,25(OH)_2D_3$

$1,25(OH)_2D_3$ regulates several functions in the body by modulating genomic events via its nuclear receptor. Classically the main role of $1,25(OH)_2D_3$ is the regulation of calcium and phosphorous concentrations in serum via actions in bone, parathyroid gland, kidney and intestine that are conceived as classical target organs for $1,25(OH)_2D_3$. In addition, $1,25(OH)_2D_3$ is able to generate several other biological responses (nonclassical actions of vitamin D) that are not related to the control of mineral homeostasis. Today there are over 30 nonclassical target tissues for $1,25(OH)_2D_3$ (Bouillon *et al*. 1995).

In addition to the genomic actions, $1,25(OH)_2D_3$ is also able to generate rapid biological responses, which do not require any protein synthesis as genomic actions do (Baran 1994, Norman *et al.* 1999). It has been thought that putative cell membrane receptor for $1,25(OH)_2D_3$ (VDR_{mem}) mediates those responses by activating a variety signal transduction systems, such as several protein kinases and phospholipase C pathways (Nemer *et al*. 1998, Sitrin *et al*. 1999). There is also evidence that rapid

responses are able to modulate the genomic pathway of $1,25(OH)_2D_3$ actions via phosphorylation of nuclear VDR (Norman *et al*. 2001). Receptor phosphorylation could increase the affinity of VDR to coactivator complexes and thus enhance gene activation (Barletta *et al*. 2002).

2.1.3.1 Control of mineral homeostasis

The principal function of $1,25(OH)_2D_3$ and PTH is to control the calcium and phosphate status to ensure the availability of the minerals for biological functions of the host as well as skeletal mineralization. This is achieved by coordinated actions of the parathyroid, kidney, intestine and bone. Parathyroid hormone is the principal hormone involved in the minute-to-minute regulation of ionized calcium levels in the extracellular fluid, whereas $1,25(OH)_2D_3$ plays a key role in the day-to-day maintenance of calcium balance (Brown *et al*. 1999). These hormones exert important regulatory effects on each other.

The most critical role of $1,25(OH)_2D_3$ in mineral homeostasis is to enhance the efficiency of the small intestine to absorb dietary calcium and phosphate (Li *et al*. 2001). $1,25(OH)_2D_3$ takes part in several steps in that process; it increases the entry of calcium through the plasma membrane into the enterocytes, it enhances the movement of calcium through the cytoplasm and across the basolateral membrane into the circulation (Wasserman & Fullmer 1995). $1,25(OH)_2D_3$ is capable of reducing the excretion of calcium to urine by increasing the readsorbtion of calcium in kidneys (Li *et al*. 2001). Moreover $1,25(OH)_2D_3$ is able to increase the mobilization of calcium from bone into the circulation if the calcium supply from food is not sufficient. This is achieved through the enhancement of osteoclastogenesis and osteoclastic activity (Takeda *et al*. 1999).

2.1.3.2 Bone and $1,25(OH)_2D_3$

Bone is one of the classical target organs for $1,25(OH)_2D_3$ action. $1,25(OH)_2D_3$ not only plays a role in bone calcium mobilization but it has an essential role in bone development, mineralization and maintaining the dynamic nature of bone. $1,25(OH)_2D_3$ facilitates those functions by controlling the availability of calcium and phosphate and by regulating the level of hormones such as PTH, parathyroid hormone related peptide and insulin-like growth factor. It is also involved in the synthesis of bone matrix proteins such as type I collagen, alkaline phosphatases, osteocalcin, osteopontin and matrix-Gal protein (Gallagher & Riggs 1990, Glenville *et al*. 1996, Goltzman *et al*. 2001).

2.1.3.3 New functions of $1,25(OH)_2D_3$

In recent years it has been shown that vitamin D has many additional target cells where it is involved in a wide array of new functions that are unrelated to its actions on mineral metabolism. Three independent methods have been widely used to find out those new target cells and functions for $1,25(OH)_2D_3$; autoradiographic localization of radioactive ligand, immunohistochemical detection of VDR in putative target cells and responsiveness of specific cell types in culture to $1,25(OH)_2D_3$ or its analogs. In many

cases, these data are coupled to biological responses in animals or to loss of function in natural VDR mutations and in VDR null mice (Li *et al*. 1997, Haussler *et al*. 1998, Malloy *et al*. 1999, Verstuyf *et al*. 1999). The precise molecular mechanisms as well as the target genes mediating the diverse functions are currently not very well known (Haussler *et al*. 1998, Brown *et al*. 1999). It has been shown that vitamin D target cells have their own enzyme machinery for the local regulation of $1,25(OH)_2D_3$ concentration that enables a separate regulation of mineral homeostasis and other actions of $1,25(OH)_2D_3$ (Hewison *et al*. 2000).

Table 2. A representation of the target tissues and functions of vitamin D hormone.

Tissue/cell	Action
Hematopoietic tissues	differentiation
myeloid cell precursors	
colony forming units	
Immune system	enhancement of immune function to control viral and
monocyte/macrophages, lymphocyte	bacterial infections and tumor growth
	immunosuppression
Skin	antiproliferative, differentiation
keratinocytes, fibroblasts, hair follicle, Langerhans	
cells and melanocytes	
Muscle	antiproliferative, differentiation
smooth muscle cell, myoblast, heart cardiac muscle	inhibition of antinatriuretic factor synthesis
cell and atrial myocytes	
Pancreas β cells	enhancement of insulin synthesis and secretion
Mammary gland	growth regulation
Cancer cells	antiproliferative, differentiation
Adrenal gland medullary cells	control of catecolamine metabolism
Prostate	antiproliferative, differentiation
Brain hippocampus/selected neurons	neuronal regeneration, enhancement of nerve growth
	factor and neurotrophin synthesis, control of
	sphingomyelin cycle
Cartilage chondrocyte	antiproliferative, differentiation
Female reproductive	antiproliferative, control of foliculogenesis organs
ovarian, myometrial and endometrial cells	
Liver parenchymal cell	enhancement of liver regeneration, control of
	glycogen and transferrin synthesis
Lung	enhancement of maturation, phospholipid synthesis
fetal pneumocytes	and surfactant release
adult pneumocytes	cell growth
Male reproductive organs	enhancement of sertoli cell function and
sertoli/semminiferus tubule	spermatogenesis
Pituitary production	control of T_3-induced growth hormone, prolactin and
	tyrotrophyn
Thyroid	inhibition of calcitonin synthesis
follicular cells	

2.1.4 Vitamin D receptor gene knockout mice studies

Vitamin D receptor gene knockout mice have been generated to investigate the functional role of VDR in living animal by Li *et al*. 1997 and Yoshizawa *et al*. 1997. They found that VDR-null mutant mice displayed no defects in development and growth before weaning. After weaning, however, mutants displayed a phenotype that resembles human hereditary vitamin D resistant rickets type II (HVDRR-II). It is a rare recessive genetic disorder caused by mutation in the VDR that results in end organ resistance to $1,25(OH)_2D_3$ action. The major symptoms of HVDRR-II include $1,25(OH)_2D_3$ resistance, hypocalcemia, secondary hyperpharathyroidism and osteomalacia (Malloy *et al*. 1999). It has been suggested that the main reason for hypocalcemia is low expression level of renal calbindin D9k, which causes impaired calcium reabsorption in the kidney (Li *et al*. 2001). Infertility and uterine hypoplasia with impaired folliculogenesis was also found in female mice. However, the defects in reproduction could be avoided by eating a high calcium diet, indicating that infertility of VDR null mice is indirectly caused by hypocalcemia (Johnson & DeLuca 2001).

In addition to defects in the calcium homeostasis VDR null mice have abnormalities in the function of several nonclassical target tissues and cells. The mice have disturbances in the initiation of hair cycle leading to the development of alopecia (Sakai & Demay 2000, Sakai *et al*. 2001, Kong *et al*. 2002).

Pre-malignant changes, such as diminished growth control and DNA damage, could be found in the mammary gland and sigmoid colon. Those findings confirm the role of $1,25(OH)_2D_3$ in the prevention of cancer (Kallay *et al*. 2001, Narvaez *et al*. 2001, Zinser *et al*. 2002).

Recent studies of VDR null mice have also shown that $1,25(OH)_2D_3$ has a role in the regulation of the immune system by modulating cytocine production and maturation of macrophages and dendrite cells (Griffin *et al.* 2001, O'Kelly *et al*. 2002).

2.2 Nuclear vitamin D receptor, receptor for $1,25(OH)_2D_3$

2.2.1 Nuclear VDR a member of nuclear hormone superfamily

The genomic actions of $1,25(OH)_2D_3$ are mediated by its nuclear receptor, whose cDNA was first cloned from chicken in 1987 (McDonnell *et al*. 1987) and shortly thereafter from human (Baker *et al*. 1988). The examination of primary sequences of vitamin D receptor cDNAs revealed that the VDR protein belongs to the superfamily of NHRs. A common feature for all NHRs is that they act by regulating gene expression (Mangelsdorf *et al*. 1995). Nuclear hormone receptors form the largest group of eukaryotic transcription factors including more than 350 members derived from both vertebrate and invertebrate

sources (Duarte *et al*. 2002). Receptors can be divided into six major classes according to molecular phylogeny methods (Laudet 1997). One class includes receptors for classical steroid hormones like estrogen, progesterone and androgen. Another large class contains receptors like all-*trans* retinoic acid receptor (RAR), VDR and thyroid hormone receptor (TR). The nuclear hormone receptor superfamily also contains a number of receptors (orphan receptors) for which ligands have not yet been identified. Many of the orphan receptors may not even require any ligand for being functional (Bruce & Evans 1998, Weatherman *et al*. 1999).

2.2.2 Human VDR gene and its promoter

The human VDR is a product of the single chromosomal gene which locates on chromosome 12 at 12q13-14 (Labuda *et al.* 1992). The gene is comprised of 11 exons that, together with intervening introns, span approximately 75 kb. The noncoding 5'-end of the gene includes 3 exons 1A, 1B, and 1C. Eight additional exons (exons 2-9) encode the structural portion of the VDR gene product. Three VDR mRNA transcripts are synthesized depending on how exons 1A, 1B, and 1C are spliced during transcription. The promoter sequence lying upstream of exon 1A is GC rich and does not contain an apparent TATA box. A unique feature for the VDR gene is an additional exon (V) that is not found in other NHR genes. It codes an insertion peptide about 40 amino acid long that locates in the LBD of receptor (Miyamoto *et al*. 1997).

2.3 Modular structure of theVDR protein

In man, the VDR protein consists of 427 amino acids, with a molecular mass of ~48 kDa. Like the other NHRs, VDR can be divided by function into several domains (Fig. 2). At the amino terminus there is an A/B domain 20 amino acids long. DNA-binding domain (DBD), termed also C domain, locates between amino acids 21 and 92. D or flexible linker region locates approximately between amino acids 93 and 123, followed by the E- or ligand binding domain between amino acids 124 and 427. Unlike some other NHRs, VDR does not have a C-terminal F domain at all (Jones *et al*. 1998).

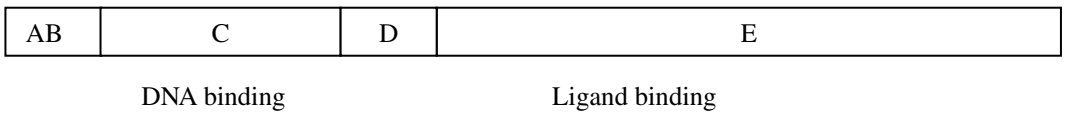


Fig. 2. Schematic representation of VDR domain structure is given in the figure.

2.3.1 A/B domain

The amino terminal A/B domain varies a lot among the NHR family members in both amino acid identity and size. The A/B domain of VDR is short compared with other members of family, e.g. the A/B domain of the human glucocorticoid receptor is about 400 amino acids longer than in VDR. The A/B region of many receptors contains an autonomous transcriptional activation function that contributes to constitutive ligand independent receptor activation (Aranda & Pascual 2001). It has been shown that p160 coactivators and components of D-vitamin receptor interacting protein (DRIP) complexes are able to interact with the A/B domain of certain steroid receptors and enhance A/B domain mediated transactivation (Allen *et al*. 1999, Hittelman *et al*. 1999). The removal of the A/B domain from VDR does not appear to affect the ligand binding, DNA binding or transactivation properties of VDR (Issa *et al*. 1998).

2.3.2 DNA binding domain (DBD)

Nuclear hormone receptors regulate transcription by binding to specific genomic sequences known as hormone response elements (HREs). The DNA binding domain mediates this vital interaction. In addition to that DBD plays a role in receptor dimerization, although isolated DBDs do not dimerize in solution without appropriate HRE (Hard *et al*. 1990).

The core sequence of DBD domain is highly conserved among NHRs. It has over 40% amino acid identity over a 67-residue region (Rastinejad *et al*. 2000). The three dimensional structure of several NHR DBDs is known, including TR (Rastinejad *et al*. 1995), RXR, RAR (Rastinejad *et al*. 2000, Fraydoon *et al*. 2000), glucocorticoid receptor (Lusi *et al*. 1991, Baumann *et al*. 1993), estrogen receptor (Schwabe *et al*. 1993) and VDR (Shaffer & Gewirth 2002). The overall architecture of all known DBD core structures is very similar, suggesting that the same general structural features are present through the NHR superfamily (Khorasanizadeh & Rastinejad 2001). The core of DBD is composed of two zinc-finger motifs, each containing four highly conserved cysteine molecules which coordinate the binding of the zinc atom. Zinc atoms and cysteine residues are necessary for maintaining a three dimensional structure whose core is composed of two helices (helix I and II) oriented at approximately right angles to each other. Helix I is critical for the specific binding to the major groove of HRE on a DNA and helix II is involved in receptor dimerization (Khorasanizadeh & Rastinejad 2001).

Shaffer & Gewirth 2002 recently solved the crystal structure of homodimeric VDR DBD with three different VDREs. They found that the key interactions between DBD subunits and DNA half-sites are similar to those seen in previous structures of NHR DBD-DNA complexes. Conserved Glu42, Lys45, Arg49 and Arg50 residues in the recognition helix make sequence-specific base contacts in the major groove of VDRE half-site. Shaffer & Gewirth were also able to demonstrate how naturally occurring VDREs are able to form unique contacts with VDR DBD and how this is reflected in affinity and stability of certain VDR/VDRE complexes.

In the crystal structure of VDR DBD-DNA complex protein subunits are arranged in a head-to-tail orientation where protein subunits are separated by center-center distance of 9 bp and rotated 45^{o} relative to each other. Pro61, Phe62 and His75 of the upstream protomer and residues Asn37, Glu92 and Phe93 of the downstream subunit form a dimerization interface, which is forced together mainly by van der Waals forces. These six residues are conserved among VDRs from various species. They form a unique combination among NHRs that specifies the uniqueness of the homodimer interface (Shaffer & Gewirth 2002). Biochemical and mutagenesis studies also support the role of those residues in receptor dimerization (Towers *et al.* 1993, Quack *et al*. 1998).

The C-terminal extension of DBD also takes part in receptor dimerization and contributes to response element binding affinity and specificity by sterically blocking a receptor dimer assembly on incorrectly spaced HREs. In contrast to the core DBD, whose structure is conserved in all NHRs, the structure of each C-terminal extension has a more unique character (Shaffer & Gewirth 2002). It is also suggested that the C-terminal extension of VDR DBD (amino acids 76-102) has an additional role in the nuclear accumulation of VDR (Luo *et al*. 1994).

2.3.3 Ligand binding domain (LBD)

The C-terminal LBD is a globular multifunctional domain. It is responsible for hormone binding, strong receptor dimerization and interaction with co-repressors and co-activators, which all together are critical for the regulation of transcriptional activities (Hsieh *et al*. 1998, Weatherman *et al*. 1999).

The crystal structures of several LBDs of NHRs have been solved, including RXR (Bourguet *et al*. 1995), RAR (Renaud *et al*. 1995), TR (Wagner *et al*. 1995), peroxisome proliferator-activated receptor (Nolte *et al*. 1998), estrogen receptor (Pike *et al*. 1999), progesterone receptor (Williams & Sigler 1998), androgen receptor (Sack *et al*. 2001) and VDR (Rochel *et al*. 2000). Despite the rather large differences in amino acid sequence composition all the known structures fold in a similar manner. All of them are composed of 11-13 α-helices and one β-turn, which are arranged as a three-layered antiparallel α-helical sandwich (Moras & Gronemeyer 1998, Weatherman *et al*. 1999, Egea *et al*. 2000b).

The three dimensional structures and biochemical analyses indicate that LBDs are able to form three distinct structures depending on the ligand binding state of the receptor; apo receptor receptor without ligand, holo agonist and holo antagonist structure receptor with agonist or antagonist ligands (Nayeri & Carlberg 1997, Moras & Gronemeyer 1998). The existence of the large conformational differences between apo and holoreceptors suggests that hormone binding triggers structural changes within the LBD. The most striking ligand induced conformational change is seen in C-terminal helix 12, which is completely repositioned upon ligand binding. The apo form of the receptor has a ligand binding cavity open and helix 12 is exposed to the solvent, whereas in the holo agonist receptor helix 12 is rotated back against ligand binding cavity. In addition to relocation of helix 12 minor structural changes are seen in helices 3, 6 and 11 (Bourguet *et al*. 1995, Moras & Gronemeyer 1998, Blondel *et al*. 1999, Egea *et al*. 2000a). Those structural

rearrangements generate a transcriptionally active receptor by disrupting the binding of corepressors and generating surfaces that allow interaction with coactivator molecules (Darimont *et al*. 1998, Nolte *et al*. 1998, Shiau *et al*. 1998).

Ligands having antagonist properties bind into the same ligand binding pocket as agonistig ligands, but in a way that prevents the correct assembly of helix 12. The wrong location of the helix 12 abolishes the binding of coativators leading to inactivation of transcription (Brzozowski *et al*. 1997, Shiau *et al*. 1998, Pike *et al*. 1999, Bourguet *et al*. 2000).

Vitamin D receptor is unique among the NHR superfamily. It contains a large insertion in the peptide connecting helices 1 and 3 of the LBD. This insertion domain is encoded by an additional exon in the VDR chromosomal gene and it is not found in other NHRs (Miyamoto *et al*. 1997). The length of the region connecting helix 1 and 3 varies between 72 and 81 residues within VDRs, compared to 15–25 residues within other nuclear receptors (Rochel *et al*. 2000). The crystallographic structure of VDR LBD without this extra domain (amino acids 165-215) has recently been solved as a complex with its natural ligand and synthetic analogs MC1288 and KH1060. The overall fold of this protein is similar to that of the agonist fold of other NHRs (Rochel *et al*. 2000, Tocchini-Valentini *et al*. 2001).

The crystal structure of VDR LBD illustrates the molecular basis of ligand binding and its relationship to the transactivation. In structure helix H12, whose position is critical for coactivator binding and transactivation, makes two direct Van der Waals contacts with the methyl group of 1,25-$(OH)_2D_3$ (Val-418 and Phe-422). The position of helix 12 is further stabilized by several hydrophobic contacts with amino acid residues of helices 3, 5 and 11. Two polar interactions are also involved in stabilization, salt bridge between Lys-264 (H4) and Glu-420 and a hydrogen bond between Ser-235 of H3 and Thr-415. Some of the residues that stabilize the position of the helix H12 are also interacting directly with 1,25-$(OH)_2D_3$ (Val-234, Ile-268, His-397, and Tyr-401), suggesting that 1,25-$(OH)_2D_3$ directly controls the position of helix 12 (Rochel *et al*. 2000).

In comparison to other steroid hormone ligands 1,25$(OH)_2D_3$ is a unique molecule having long and flexible structural features, mainly caused by an eight carbon side chain, seco B-ring which permits rotation about the 6-7 single carbon bond, and A-ring which undergoes chair-chair conformational interconversion (Norman *et al*. 2001). It is supposed that 1,25$(OH)_2D_3$ can generate at least three functionally different shapes which effectively adapt in the ligand binding pocket of DBP, VDR_{mem} and nuclear VDR (Norman *et al*. 1999). In the crystal structure 1,25$(OH)_2D_3$ is adopted as a curved shape in a hydrophobic pocket of VDR LBD. The conjugated triene connecting the A and C ring of 1,25-$(OH)_2D_3$ is tightly fitted in a hydrophobic channel sandwiched between Ser-275 and Trp-286 on one side and Leu-233 on the other. The C6–C7 single bond of 1,25-$(OH)_2D_3$ exhibits trans conformation that deviates by 30° from the planar geometry. In the complex the A ring adopts a chair B conformation with the 1-OH and 3-OH groups in equatorial and axial orientations, respectively. The hydroxyl groups of 1,25-$(OH)_2D_3$ have a key role in ligand binding. The hydroxyl moiety at position 1 forms two hydrogen bonds with Ser-237 (H3) and Arg-274 (H5), while the 3-OH group forms two hydrogen bonds with Ser-278 (H5) and Tyr-143. The 25-hydroxyl group, which is located at the other end of 1,25-$(OH)_2D_3$ is hydrogen bonded to His-305 (loop H6–H7) and His-397 (H11) (Rochel *et al*. 2000).

2.4 Hormone response element (HRE)

Nuclear hormone receptors regulate transcription by binding to HREs is normally located in the 5'-flanking region of the target gene. Hormone response elements are composed principally of two hexameric half-site core sequences (Aranda & Pascual 2001). Diversity among HREs is achieved by modifying the location of the half-sites relative to one another. For dimeric HREs, the half-sites can be configured as palindromes, inverted palindromes or direct repeats (DR). This influences the receptor dimerization pattern on an HRE. Steroid receptors, for example, form symmetrical head to head arranged homodimers on a palindromic HRE (Lusi *et al*. 1991) and type II receptor like VDR binds always to the DR elements arranged asymmetrically in head to tail orientation as a homodimer or as a heterodimer with RXR. Further selectivity is created between HREs by a varying number of neutral base pairs separating the half-site repeats. According to inter-half-site spacing (1-5 bp), these elements are also known as DR1-DR5 (Umesono *et al*. 1991, Mangelsdorf & Evans 1995, Zhao *et al*. 2000). This is the key identity factor contributing to the binding specificity of different RXR heterodimer pairs. It provides the geometry that is needed for two subunits to interact specifically. The insertion of even one extra base pair in the inter-half-site spacing displaces the interacting subunits by nearly 3.4 Å and re-orients them by ~35°. This leads to the disruption of supportive protein-protein and protein-DNA interactions (Rastinejad *et al*. 2000). Sequence composition of the spacer nucleotides has been shown to play a less critical role in the recognition of HRE (Zhao *et al*. 2000, Khorasanizadeh & Rastinejad 2001, Rastinejad 2001).

A number of natural VDREs have been identified and most of them represent the DR3 type structure, which is the preferential form of the binding site for VDR-RXR heterodimer. However, other VDRE structures such as DR4, DR6 and inverted palindrome with nine intervening nucleotides have been shown to be functional (Calberg *et al.* 1993, Rhodes *et al*. 1993, Schräder *et al*. 1995).

2.5 Molecular mechanism of nuclear hormone receptors in the control of transcription

Hormone binding to NHR initiates the series of events that leads to active repression or activation of target gene expression. Tens of proteins and several protein-protein and protein-DNA interactions participate in those complicated chain of events.

Nuclear hormone receptors are structurally and functionally very similar. In order to be able to give a more complete view of their mechanism of action this section of the review is not entirely restricted to VDR but contains additional information from studies dealing with other NHR.

2.5.1 Releasing of nucleosomal repression and the initiation of gene transcription

Eukaryotic DNA is highly packed into the chromatin. The fundamental subunit of chromatin, the nucleosome, is composed of two molecules of each of the four core histones (H2A, H2B, H3, H4), about 180 bp of DNA and a single molecule of a linker histone H1. Nucleosomes have a capacity to self-associate into higher order structures such as chromatin fiber and chromosomes (Kornberg & Lorch 1999, Wolffe & Hayes 1999). Dense packing of DNA represses transcription by limiting the access of general transcription factors (GTFs) and RNA polymerase II to the DNA (Collingwood *et al.* 1999).

The initial step in transactivation is the binding of NHR dimers to their HRE within the promoter region of the hormone responsive gene. Nuclear hormone receptors have a unique ability to bind to DNA although it is wrapped around the histone core (Li & Wrange 1995, Wong *et al*. 1997). Ligand binding to the NHR starts the series of events that arrives at the releasing of nucleosomal repression and the initiation of gene transcription. Conformational change induced by ligand has an important role in this process. It promotes the tight association of receptor to its HRE, it enhances receptor dimerization and generates new surfaces on the receptor that allow the binding of coativator molecules, which are essential factors in a gene activation cascade (Nolte *et al.* 1998, Shiau *et al*. 1998).

2.5.2 Coactivators

Coactivators are distinct from GTFs in that most of them do not directly bind to the DNA but are associated with the promoter region via a gene specific activator molecule like NHR. Over 30 potential coactivators have been identified by their ability to bind and alter the transcriptional activity of ligand activated NHRs (Wallberg *et al*. 2000). Two distinct steps in target gene activation turn up to be regulated by coactivators. Firstly, coactivators remodel the chromatin structure of the promoter region in order to facilitate binding of other activators and the component of the RNA polymerase II transcriptional machinery. Secondly, coactivators recruit protein complexes (mediator complex) that interact with one or more subunits of the RNA polymerase II and enhance the initiation of transcription by stabilizing the preinitiation complex (PIC) (Näär *et al*. 2001, Urnov & Wolffe 2001).

There are two general classes of enzyme complexes that appear to a play critical role in nucleosome remodeling mediated transcriptional activation. These are:

1. Histone acetyl transferases (HATs) which regulate nucleosome structure by altering the histone acetylation pattern of core histone tails (see Sterner & Berger 2000, Wu & Grunstein 2000).
2. ATP-dependent chromatin remodeling factors (see Sudarsanam & Winston 2000, Varga-Weisz 2001).

These two enzyme complexes appear to act synergistically to establish a local chromatin structure that is the proper environment for the next events leading to PIC formation (Bevan & Parker 1999, Fry & Peterson 2001, Urnov & Wolffe 2001).

2.5.2.1 Histone acetyltransferases

Histone acetyl transferases are the best-characterized group of enzymes that covalently modify the structure of chromatin. They acetylate basic lysine residues locating at the N-terminal tail of histones (Sterner & Berger 2000). The acetylation of histones is thought to reduce electrostatic interactions between histones and DNA (Hong *et al*. 1993) and between separate nucleosome particles leading to the destabilization of the higher-order folding of chromatin (Tse *et al*. 1998). Acetylation might also disrupt the secondary structure of histone N-termini, which might further destabilize interaction with DNA and the nucleosome itself (Hansen *et al*. 1998).

Additionally, it has been shown that acetylation of specific lysine residues in the core histones provides novel recognition surfaces for proteins having bromodomain structures. This lead to the conclusion that, histone acetylation may enhance the initiation of transcription by two distinct mechanisms: by remodeling the structure of nucleosomes which leads to increased access of transcription factors to the promoter, and by creating the specific binding sites for bromodomain containing transcriptional coregulators such as TFIID and SWI/SNF (DiRenzo *et al*. 2000, Hassan *et al*. 2000, Jacobson *et al*. 2000, Syntichaki *et al*. 2000).

The acetylation state of chromatin is a dynamic phenomenon. Acetylated lysines of hyper acetylated histones turn over rapidly with a half-live of minutes within the trancriptionally active chromatin, whereas hypo acetylated histones of transciptionally silent regions do so much less rapidly. This is thought to provide mechanism for reversible activation and repression of transcription (Wolffe & Hayes 1999).

A number of coactivators that are recruited by activated NHRs contain intrinsic HAT activity including the p160 family of coactivators also known as steroid receptor coactivators (SRC) (Leo & Chen 2000) and general coactivators CBP/p300 and PCAF (Yao *et al.* 1996).

The p160 coactivators communicate with virtually all NHRs in a hormone dependent manner, suggesting a common pathway of hormone-induced gene activation among the NHR family (McKenna *et al*. 1999, Leo & Chen 2000). The p160 proteins bind to the LBD of nuclear receptors via receptor interacting domain, which contains three short LXXLL binding motifs. These motifs are conserved in both sequence and spacing in each p160 family member (Heery *et al*. 1997). Biochemical and X-ray crystallography studies indicate that the LXXLL motif is a part of the short α-helix, which binds to the hydrophobic groove locating on the surface of NHR LBD. Two out of three LXXLL binding motifs are needed for cooperative interaction with NHR dimer (Darimont *et al.* 1998, Shiau *et al*. 1998, Wisely *et al*. 1998). The mechanisms of interaction seem to be receptor specific and several combinations of LXXLL binding motifs are differently required for interaction with the different composition of NHR dimers (Darimont *et al.* 1998, Mak *et al*. 1999, Heery *et al*. 2001).

In addition to having enzymatic HAT activity p160 coactivators have an important role as platform molecule, which recruit other proteins such as CBP/p300 and PCAF complexes. CBP/p300 is one of the most potent acetyltransferases. Unlike p160 family members CBP/p300 is able to acetylate all four histones within nucleosomes and it is able to communicate with numerous promoter-binding transcription factors such as CREB, NHRs, STATs, Ets, c-Fos, c-Jun and c-Myb. Therefore CBP/p300 could be seen as a global coactivator in higher eukaryotes (Ogryzko *et al*. 1996, Yang *et al.* 1996, Shikama *et al*. 1998). In addition to histone acetylation p300/CBP can acetylate non-histone proteins such as the p160 family of coactivators, transcription factors such as p53 and components of general transcription machinery such as TFIIE and TFIIF (Sterner & Berger 2000). This acetylation mechanism is thought to mediate the auto regulation of coactivation process. For example, it is documented that the acetylation of lysine residues of p160 proteins in the vicinity of the LXXLL motif abolishes p160 interaction with NHR, which in turn causes the dissociation of coactivator complex including p300/CBP from the receptor and target gene promoter, leading to the attenuation of transcription (Chen *et al.* 1999). Thus CBP/p300 could have a dual role, firstly to catalyze histone acetylation required for gene activation and secondly to attenuate the process by acetylating p160 proteins (Bevan & Parker 1999).

2.5.2.2 ATP-dependent chromatin remodeling factors

SWI/SNF, ISWI and Mi-2 complexes form another important class of coactivators involved in NHR mediated chromatin remodeling (Dilworth *et al*. 2000, DiRenzo *et al*. 2000, Varga-Weisz 2001). Unlike HATs, these complexes do not carry out covalent modification of histones. Instead they catalyze the uncoupling of ionic interactions between histones and DNA using the energy supplied by ATP hydrolysis. They are able to alter nucleosome conformation by sliding histone octamers to another site on the DNA or by changing the helical torsion of the DNA twist (Havas *et al*. 2000, Sudarsanam & Winston 2000, Fry & Peterson 2001).

It has been proposed that ATP-dependent chromatin remodeling factors are involved in the early events on activation of transcription by creating localized alteration in the chromatin fiber that helps tight binding of NHR dimer to its HRE and makes the histone tails accessible to HAT complexes (Cosma *et al*. 1999, Krebs *et al*. 2000). Moreover they may mediate the final event in the transcriptional initiation process by permitting binding of basal transcriptional machinery to the DNA template (Dilworth & Chambon 2001). Recent studies have also indicated that in addition to gene activation ATP-dependent chromatin remodeling factors are able to mediate transcriptional repression by restructuring the chromatin fiber from an open state to a closed conformation (Sudarsanam & Winston 2000, Urnov and Wolffe 2001).

2.5.2.3 Mediator complexes

Mediator type coactivator complexes such as TRAP/DRIP do not modify the structure of chromatin (Fondell *et al*. 1996, Rachez *et al*. 1998). Instead they act as a direct link between the ligand activated receptor and RNA polymerase II holoenzyme complex and possibly recruit limiting components into the preinitiation complex. Multiple protein-protein interactions formed this way may enhance the stability of the PIC and thereby facilitate the initiation of transcription (Chiba *et al*. 2000, Rachez & Freedman 2000).

In summary, in NHR mediated gene activation various coactivator complexes with distinct activities enter and exit their target promoter in an ordered manner and the action of one complex sets the stage for the arrival of the next one.

Histone acetyltransferases and SWI/SNF complexes are recruited first by NHR in response to hormone binding leading to the remodeling of the local structure of chromatin. Dissociation of the HAT complex occurs trough direct acetylation of p160 coactivator by CBP/p300. This would allow the mediator type coativator complex binding to NHR and the recruitment of RNA polymerase II holoenzyme (Bevan & Parker 1999, McKenna *et al*. 1999, Chen *et al.* 1999, Rachez & Freedman 2000). At the moment there are conflicting results concerning the precise sequential action of various cofactors in gene activation. The differences in the nucleoprotein architecture of studied promoters might be the reason for that. However, there is a consensus emerging that the co-operation of several types of chromatin remodeling enzymes can lead to a more profound alteration of the infrastructure of chromatin than could be obtained by these enzymes acting independently (Urnov & Wolffe 2001).

2.5.3 Mechanism for transcriptional repression

Several members of the NHR family appear to exert their critical biological function also by actively repressing the transcription of target genes (Glass & Rosenfeld 2000, Hu & Lazar 2000). In the literature there are diverse mechanisms for NHR mediated repression and some of them are shortly described below.

NHRs (RAR, TR and VDR) are able to repress the basal transcription of the target gene by binding to HREs in the absence of the ligand (Horlein *et al*. 1995, Chen *et al*. 1995). Aporeceptors have been found to interact with nuclear receptor corepressor proteins such as N-CoR (nuclear co-repressor) (Tagami *et al*. 1998), Alien (Dressel *et al*. 1999, Polly *et al*. 2000) and SMTR (silencing mediator for retinoid and thyroid receptors) (Chen *et al* 1995, Horlein *et al*. 1995). Corepressors have been proposed to mediate repression by recruiting SIN3/HDACs (histone deacetylase complexes), which possess histone deacetylase activity. Histone deactylases counteract the actions of HATs, leading to the formation of more compactly folded DNA (Kao *et al*. 2000, Wen *et al*. 2000). N-CoR/SMTR corepressors and LXXLL motif containing coativators are thought to utilize overlapping binding surfaces on NHR LBD. Ligand binding to NHR releases the corepressor and allows the binding of coactivator (Lin *et al*. 1998, Nagy *et al*. 1999).

Nuclear hormone receptors might also form dimer pairs that are transcriptionally inactive (Garcia-Villalba *et al*. 1996, Jimenez-Lara & Aranda 1999, Yoh & Privalsky

2001). For example, it has been reported that TR homodimers assembled on DNA exhibit particularly strong interactions with the SMRT corepressor, whereas TR/RXR heterodimers are inefficient in binding to SMRT, suggesting that TR homodimers, and not heterodimers, may be important mediators of transcriptional repression. Thus the nature of the DNA binding site can influence the ability of the receptor to recruit corepressor by selecting for the receptor homodimers or heterodimers (Yoh & Privalsky 2001). There are also reports that altered heterodimer polarity can result in constitutive repression by recruiting corepressors even in the presence of the ligand (Kurokawa *et al*. 1995).

Several NHRs can inhibit the activity of other transcription factors in a ligand-dependent manner by up-regulating the expression of the inhibitory factor or by competing for coativator proteins or heterodimerization partners (Raval-Pandya *et al*. 1998, Jimenez-Lara & Aranda 1999, Glass & Rosenfeld 2000, Rosenfeld & Glass 2001). In addition, NHRs can compete for DNA binding sites with other transcriptional activators, e.g. monomeric VDR outcompetes NFAT1 for binding to response site in the granulocyte-macrophage colony-simulating factor promoter (Towers & Freedman 1998, Towers *et al*. 1999).

Moreover, it has been reported that NHRs can down-regulate transcription even without binding to DNA by interacting with other transcription regulators in a ligand dependent manner and prevent their function. For example, liganded RARs are able to interfere with c-Jun/c-Jun homodimerization and c-Jun/c-Fos heterodimerization and, in this way, prevent the formation of AP-1 transactivation complex (Zhou *et al*. 1999). Ito *et al*. 2000 proposed another novel mechanism where ligand activated GR is able to inhibit transcription by recruiting HDAC to the interleukin-1β activated promoter complex without binding to DNA.

In conclusion, ligand, cell type and the context of the promoter area have a great influence on gene activation and repression by NHRs. The diversity and complexity of NHR mediated regulation of cellular processes allows coupling of different signals and signaling pathways and gives flexibility to control gene expression.

2.6 Vitamin D synthetic analogs and their therapeutic applications

The active metabolite of vitamin D, $1,25(OH)_2D_3$, has a wide range of nonclassical actions in the body, such as regulation of cell growth, differentiation and the immune system. This has led to increased interest in using $1,25(OH)_2D_3$ in the treatment of several diseases such as psoriasis, autoimmune diseases, osteoporosis, hyperparathyroidism and cancer. However, the therapeutic applications of $1,25(OH)_2D_3$ are limited due to the hypercalcemic and phosphataemic activity of this compound (Norman 1995). The elevated level of calcium and phosphate in serum causes soft tissue calcification especially in the kidney, heart, aorta and intestine that can lead to organ failure and death (Vieth 1990). In order to avoid these unwanted side effects, a lot of work has been done to synthesize analogs that exhibit weaker effects on calcium metabolism while retaining growth and immune regulating properties (Norman 1995). It is estimated that over 1,500 analogs have already been synthesized and biologically evaluated. A number of them exhibit potent anti-proliferative activity while still having reduced hypercalcemic toxicity

at therapeutic dosages; however, only few analogs are currently in clinical trials or in active use in medicine (Gulliford *et al*. 1998, Jones *et al*. 1998, Diaz *et al*. 2000, Hansen *et al*. 2001).

Clinically the most promising results were obtained by using analogs, which contain modification in the side chain of $1,25(OH)_2D_3$. It appears that VDR is relative tolerant of changes in this part of the molecule (Jones *et al*. 1998). The recently published crystal structure of VDR LBD shows that ligand occupies only 56% of the volume of ligand-binding cavity and there is an additional space around the aliphatic side chain, which would allow analogs with different chain lengths to fit in (Rochel *et al*. 2000).

2.6.1 Chronic plaque psoriasis

$1,25(OH)_2D_3$ has a vital role in skin-tissue function through its effects on proliferation and differentiation of keratinocytes. Development of the new $1,25(OH)_2D_3$ analogs initially focused on treatment of psoriasis which is a skin disease where patients have defects in the normal cycle of epidermal development leading to epidermal hyper proliferation, altered maturation of skin cells, vascular changes and inflammation. Calcipotriol (MC903) is the first synthetic, low calcemic analog of $1,25(OH)_2D_3$ that is successfully used in the topical treatment of chronic plaque psoriasis (Ashcroft *et al*. 2000). Animal studies have demonstrated that calcipotriol is 100-200 times less potent than $1,25(OH)_2D_3$ on calcium metabolism. It has been suggested that this is due to its rapid metabolism in the liver and epidermal keratinocytes. When calcipotriene is given topically as ointment it survives long enough to help in psoriasis but does not affect the calcium balance of the body (Kissmeyer & Binderup 1991). The mechanism of action of calcipotriene is similar to $1,25(OH)_2D_3$. It binds to VDR on epidermal cells leading to the inhibition of keratinocyte proliferation and induction of cell differentiation in the psoriatic skin. In addition, it suppresses the activation and proliferation of inflammatory T-cells and inhibits the production of some inflammatory mediators, which all contribute to the pathogenesis of psoriasis (Binderup & Bramm 1988, de Jong & van de Kerkhof 1991, Reichrath *et al*. 1997). More recently another vitamin D analogue tacalcitol (1α,24-dihydroxyvitamin D_3), has become available for the treatment of psoriasis (Gerritsen *et al*. 1994, van De Kerkhof 2001).

2.6.2 Hyperparathyroidism

Deficiencies in vitamin D metabolism cause secondary hyperparathyroidism, increased secretion of PTH, which leads to high bone turnover, bone loss, mineralization defects, hip and other fractures. Vitamin D analogs 19-nor-1α,25-dihydroxyvitamin D_2 (paricalcitol) (Martin *et al*. 1998), 22-oxy-$1\alpha,25(OH)_2D_3$ (OCT, Maxacalcitol) and 1α-hydroxyvitamin D_2 (doxercalciferol) are available for the control of secondary hyperparathyroidism (Brown 2001). 19-nor-1,25-dihydroxyvitamin D_2 is widely used and it has been extensively evaluated in animals, revealing that this analog has a selective

effect on suppressing PTH secretion and lesser effects on calcium and phosphorus metabolism (Martin *et al*. 1998, Steddon *et al*. 2001).

2.6.3 Cancer

There is numerous evidence that vitamin D plays a role in the prevention of cancer progression. Epidemiological studies have shown that people who live in the northern latitudes have a higher risk to get prostate, colon and breast cancer than individuals living closer to the equator. It has been presented a hypothesis that differences in the amount of vitamin D synthesised in the skin play a role in this phenomenon (Hanchette & Schwartz 1992, Mawer *et al*. 1997, Garland *et al*. 1999, Ahonen *et al*. 2000). In addition to that there are lot a of *in vivo* and *in vitro* studies indicating that $1,25(OH)_2D_3$ plays a role in the control of proliferation, differentiation and apoptosis of many cell types including cancer cells. Anti-cancer effects of $1,25(OH)_2D_3$ are mediated via several distinct pathways, which are briefly described below. Many of those diverse mechanisms and responses appear to be dependent on cell type and their growing environment (Hansen *et al*. 2001).

Vitamin D has been shown to inhibit cancer cell growth by arresting cell cycle progression. This blocking of cell division is associated with up-regulation of the cyclin-dependent kinases p21 and p27 and down regulation of cyclins A and D1 (Liu *et al*. 1996, Verlinden *et al*. 1998). Other important molecules which are involved in cell growth inhibition by $1,25(OH)_2D_3$ are c-fos and c-myc proto-oncogenes (Jensen *et al*. 2001).

Growth factors regulate growth and the differentiation of normal cells. In cancer this regulation is often disturbed, leading to malignant processes. Vitamin D compounds have been shown to block the mitogenic activity of insulin like growth factor I and II in cancer cells (Rozen & Pollak 1999, Nickerson & Huynh 1999). Other growth factors such as transforming growth factor-beta (Heberden *et al*. 1998, Wu *et al*. 1999,) epidermal growth factor (Lee *et al*. 2001), keratinocyte growth factor (Lyakhovich *et al*. 2000), vascular endothelial growth factor (Mantell *et al*. 2000) and interleukin 10 are also shown to be targets for the growth inhibitory effects of $1,25(OH)_2D_3$ (Peleg *et al*. 1997).

Cell death by apoptosis is a natural regulatory process in the body but in cancer, cells are often filed to undergo apoptosis leading to malignant outgrowth (Hansen *et al*. 2001). Vitamin D compounds can induce apoptosis in a number of different cancer cell types by several distinct pathways where MAP kinases, tumor necrosis factor-alpha and apoptosis regulatory protein bcl-2 have been demonstrated to play a critical role (Park *et al*. 2000, Blutt *et al*. 2000, Pirianov & Colston 2001).

Vitamin D can inhibit cancer progression also by interfering with specific steps such as angiogenesis and metastasis (Hansen *et al*. 1994, Yudoh *et al*. 1997, Mantell *et al*. 2000). $1,25(OH)_2D_3$ inhibits cancer cell invasion by decreasing the activity of certain proteases which degrade extracellular matrix and basement membrane (Koli & Keski-Oja 2000).

Many vitamin D analogs have been shown to have promising anti-cancer effects *in vivo* cancer models and a few analogs (EB1089, OCT and Ro23-7553) are currently under clinical trial (Diaz *et al*. 2000, Brown 2001, Hansen *et al*. 2001). However, we are

still looking forward to a real breakthrough in cancer therapy by vitamin D analogs. The main reason for these disappointing results is that the use of systemically applied vitamin D analogs causes severe side effects at the supraphysiological doses that are needed in order to reach clinical improvement (Feldman *et al*. 2000, Reichrath 2001).

2.6.4 Osteoporosis

Vitamin D, $1,25(OH)_2D_3$ and its synthetic analogs have also been used to treat osteoporosis. Analogs stimulate bone formation through direct action on bone forming cells and also suppress bone resorption mainly by indirect effect involving PTH suppression. However, the lack of bone specific analogs hampers their wide use in osteoporotic patients (Erben 2001).

In the future other possible clinical uses for vitamin D analogs include prevention of graft rejection and autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, scleroderma and insulin-dependent diabetes mellitus. This is based mainly on the immunosuppressive properties of $1,25(OH)_2D_3$, such as its ability to inhibit cytokine secretion, decrease the proliferation rate and activity of the helper T cells, and its property to induce the availability of suppressor T cells (Hayes 2000, Lemire 2000, Verstuyf *et al*. 2000, Deluca & Cantorna 2001, Riachy *et al*. 2001).

2.7 Pharmacological and molecular basis for differential actions of vitamin D analogs

Factors that influence the biological profile of the vitamin analogs can be divided into pharmacokinetic and pharmacodynamic factors. There are two main pharmacokinetic factors which affect the ligand availability for VDR, stability in blood and catabolism of the target cell. Binding of the analogs to DBP or other blood molecules such as albumin and lipoproteins affects half-life values of the analogs in the blood and the rate of analog uptake by target cell. Those analogs, which have a strong affinity to DBP possess the longest extra cellular half-lifes on the order of days. Analogs with reduced affinity to DBP are metabolized and excreted most rapidly (Bouillon *et al*. 1991, Brown 2000).

Catabolic enzymes of target cells are another important factor controlling the concentration of $1,25(OH)_2D_3$ and its analogs inside the target cell. Target cells might inactivate analogs in different ways or create new compounds which retain significant biological activity (Bouillon *et al*. 1996, Brown 1999, Peleg & Reddy 2001, Siu-Caldera *et al*. 2001). It has also been reported that various cell types have different ability for catabolic vitamin D compounds. This could cause cell specific differences in the action of the analogue and may explain why some analogs have a unique biological profile with favorable dissociation of differentiation versus calcemic potency (Brown 2000).

The pharmacodynamic influences of the vitamin D analogs are based on their ability to modulate VDR functions differently from the natural hormone. Vitamin D analogs could use different contact amino acid residues for binding into the ligand binding cavity

of VDR than 1,25$(OH)_2D_3$ does (Gardezi *et al*. 2001). This may result in the formation of additional bonds between the analogue and the ligand binding cavity that could lead to the prolonged half-lives of activated receptor and longer-lasting effects on gene activation (Peleg *et al*. 1998, Tocchini-Valentini *et al*. 2001).

Analogs might induce slightly different structural conformation within the hormone-receptor complex (Liu YY 1997), which may modulate the receptor dimerization (Zhao *et al*. 1997, Liu YY 2001), affect the DNA binding properties and even the promoter selectivity of VDR (Quack & Carlberg 1999). Moreover, analog induced different conformation may have influence receptor interactions with tissue specific cofactors and the stability of RXR/VDR/DNA/coactivator complex (Yang & Freedman 1999, Herdick *et al*. 2000, Issa *et al*. 2002). It might also increase the intracellular half-life of the receptor-ligand complex by interfering with the SUG1/proteosome mediated receptor degradation (Masuyama & MacDonald 1997, Jääskeläinen *et al*. 2000).

It has been shown that certain analogs are able to specifically activate nuclear VDR or membrane bound VDR. This could modulate the cross talk between receptors and thus alter genomic activity of the nuclear VDR (Brown 1999).

3 Aims of the present study

Nuclear vitamin D receptor mediates the genomic actions of the active form of vitamin D, $1,25(OH)_2D_3$. This study was aimed at characterization of the structure and the function of VDR.

The specific aims of the present study were:

1. to express and purify human VDR and its LBD,
2. to characterize functional properties of recombinant VDR and its LBD,
3. to study the natural ligand and its synthetic analogs induced conformational changes within the receptor, and
4. to characterize the structure and function of the insertion region of LBD.

4 Materials and methods

Detailed description of the materials and methods is presented in the original papers I-IV.

4.1 Plasmid construction (I-III)

In order to express human VDR or its truncated form in insect *Spodoptera frugiperda* (*Sf*9), *E. coli* or COS cells (SV40-transformed African Green monkey kidney cell) the cDNA of human VDR were amplified by PCR and the amplified fragments were cloned into the selected expression vectors (table 3.). All constructs were verified by automated DNA sequencing.

Table 3. Expression plasmids used in this work.

Expression plasmid	cDNA	Host
pVL 1392 VDR	VDR amino acids 3-427 with hexahistidine-tag	*Sf*9 cell
pVL 1392 VDR LBD	VDR amino acids 108-427 with hexahistidine-tag	*Sf*9 cell
PK503-9 VDR	FLAG peptide -VDR amino acids 3-427 with hexahistidine-tag	*Sf*9 cell
PK503-9 VDR LBD	FLAG peptide -VDR amino acids 108-427 with hexahistidine-tag	*Sf*9 cell
pET15b VDR LBD	VDR amino acids 118-427 with hexahistidine-tag	*E. coli* BL21
pET15b VDR LBD mt	VDR amino acids 118-427 Δ165-215 with hexahistidine-tag	*E. coli* BL21
pET3a RXRα LBD	RXRα LBD	*E. coli* BL21
PSG5 VDR	VDR amino acids 1-427	COS cell
PSG5 VDR mt	VDR amino acids 1-427 Δ165-215	COS cell
PXJ440 VDR	DBD of the yeast activator Gal4 (1-147) with VDR amino acids 1-427	COS cell
PXJ440 VDR mt	DBD of the yeast activator Gal4 (1-147) with VDR amino acids 1-427, Δ165-215	COS cell
pSP65 VDR	VDR amino acids 3-427	*In vitro* translation

pK503-9 (Schioth *et al.* 1996)

RXRα LBD (Egea *et al*. 2000)

4.2 Overexpression of VDR and its LBD in insect cells and in *E. Coli* (I-III)

In order to express VDR in insect cells recombinant baculoviruses were generated by using the Bac-To-Bac baculovirus expression system (Gibco BRL) or linearized wild-type BaculoGold-DNA (Pharmigen, San Diego, CA, U.S.A.).

Recombinant proteins were produced in exponentially growing *Sf*9 cells ~2 x 10^6 cells per ml at 27 °C infected by recombinant virus at a multiplicity of infection of 1 in 1000 ml spinner flasks (Bellco, Vineland, NJ, U.S.A.) containing 10% v/v fetal calf serum and antibiotics in complete TMN-FH insect medium (Sigma, St. Louis, MO, U.S.A.). For mass production of the recombinant proteins, *Sf9* cells were first scaled up to a 2-liter bioreactor (Biostat MD) and subsequently to a 30-liter bioreactor (Biostat UD 30; Braun Biotech International, Melsungen, Germany) as described previously (Vihko *et al*. 1993). Infection in the Biostat UD 30 bioreactor was produced by adding recombinant viruses at a multiplicity of infection of 1 with 2 liters of fresh medium once a cell concentration of ~2 x 10^6 per ml had been reached. Infected cells were allowed to grow for 3 days until harvesting. The expression of recombinant proteins was monitored in Western blots by using anti-VDR monoclonal antibodies together with a ProtoBlot AP system (Promega, Madison, WI, U.S.A.).

Human VDRwt (118-427) and VDRmt (118-427, Δ165- 215) were overproduced as hexahistidines-tagged proteins in *E. coli* BL21 (DE3) (Novagen). A 200-mL Luria-Bertani preculture containing ampicillin 200 μg/mL was grown overnight at 37 °C and 25-mL aliquots of the preculture were used to inoculate a 1-L culture containing ampicillin 200 μg/mL. Cells were grown at 37 °C to OD_{600} = 0.6 and after that they were allowed to grow for an additional 6 hours at 20 °C under induction of 1 mM isopropyl thio-β-D-galactoside. Cultured cells were harvested by centrifugation and cell pellets were frozen and kept at –80 °C.

4.3 Purification of VDR and its LBD (II-III)

Proteins were extracted by using Potter-Elvehjem homogenizer and ultrasonication in the buffer containing 30 mM Tris/HCl pH 8.0, 200 mM NaCl, 5 mM 2-mercaptoethanol and protein inhibitor cocktail. The cell extracts were ultracentrifuged at 100 000 ***g*** for 40 min at 4 °C. Clarified insect cell extracts were precipitated by ammonium sulphate at a final concentration of 1.5 M. Redissolved pellet was loaded into an immobilized metal affinity column (Talon, Clontech). Bacterial lysates were applied to the column without ammonium sulphate precipitation step. After washing, the column bound proteins were eluted with 0.2 M imidazole and the fractions of interest were pooled and concentrated using a Macrosep 10 K centrifugal concentrator (Filtron).

In order to remove the histidine tag from the bacterially produced VDR LBD, the sample was digested with bovine thrombin 1 unit per mg of protein overnight at 4 °C in the presence of 5 mM $CaCl_2$. The proteins were further purified by gel filtration on a Superdex 75 column (Pharmacia) equilibrated in buffer suitable for final use of proteins.

The fractions with the highest VDR or LBD contents were pooled. The purity of recombinant proteins was evaluated by SDS/PAGE and visualized by staining with Coomassie Blue. The protein concentration was measured by Bio-Rad Protein Assay (Bio-Rad, Richmond, CA, U.S.A.) using BSA as a standard or by UV spectrophotometry considering the absorption coefficients ($M^{-1} \cdot cm^{-1}$) ε = 16,410 and 16,290 for VDRwt LBD and VDRmt LBD, respectively, at λ 280 nm.

4.4 Formation and purification of receptor-DNA complexes (II)

In order to obtain RXR/VDR/VDRE complex for protein crystallization studies, synthetic oligonucleotides based on the VDREs of the genes for osteopontin (5´-**AGGTGA**ATG**AGGACA-**3´) or osteocalcin (5´-**AGGTCA**AGG**AGGTCA**-3´) were purified by reverse-phase and ion exchange chromatography. The reverse-phase R1H column (Poros) was washed with six-column volume of ammonium acetate. The oligonucleotide was directly detritylated on the column with 2% (v/v) trifluoroacetic acid. The detritylated oligonucleotide was eluted with an acetonitrile gradient and loaded on an anion exchange column (HqH, Poros). Fractions of interest were pooled, dialysed against water, freeze-dried and resuspended in 2 ml of annealing buffer 5 mM cacodylate pH 6.5, 50 mM NaCl. The purified single-stranded oligonucleotides were combined in equimolar amounts and annealed. To form DNA/receptor complexes, approximately 2-5 mg of purified VDR or VDR and RXR$\alpha\Delta$AB were mixed with an equimolar amount of double-stranded VDRE in an excess of 1α,25$(OH)_2D_3$ or 1α,25$(OH)_2D_3$ and 9-*cis*-retinoic acid (9-*cis*-RA). Monomeric RXRα lacking the N-terminal A/B domain (RXR$\alpha\Delta$AB) was obtained by over expression in *E. coli* and purified as described (Chen *et al*. 1994). The receptor/DNA mixture was dialysed overnight in buffer 10 mM Tris/HCl pH 8.0, 100 mM KCl, 10 mM DTT, 10 mM MgCl at 4 °C and formed receptor/DNA complexes were purified by Superdex 200 16/60 gel-filtration chromatography (Pharmacia). The column was pre-equilibrated with the same buffer as in the dialysed sample and eluted at a flow rate of 1 ml/min. The complexes were analyzed by SDS/PAGE and native PAGE, which was performed on a PhastSystem (Pharmacia) with PhastGel gradient medium (8-25%) and Coomassie-stained.

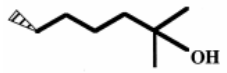
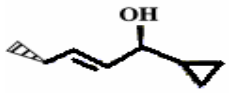
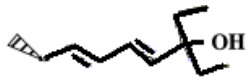
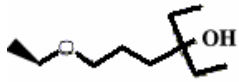
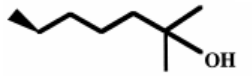
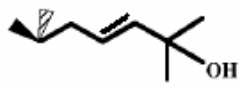
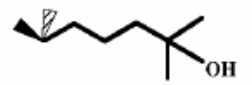
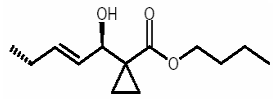
4.5 Partial proteolytic digestion of VDR (I)

In order to study ligand induced conformational change within VDR by partial proteolytic digestion, a [^{35}S] methionine-labeled biosynthetic human VDR was prepared *in vitro* by a coupled wheat germ extract system. *In vitro* translated or recombinat VDR was incubated with 1 µM 1α,25$(OH)_2D_3$ or its analogs (table 4) diluted with sterile water for 10 min at 22 °C before exposing it to increasing amounts (0-200 µg/ml) of trypsin or chymotrypsin. Samples were digested for 10 min at 22 °C and the reaction was stopped by adding 4 µl of six-fold SDS-loading buffer and boiling for 5 min. The digestion fragments were separated by 12% SDS/PAGE and autoradiographed. After autoradiography the main digestion products were further analysed by scanning the figures with a color scanner

using the VistaScan 2.3.1 program. The intensity of protein bands was measured by computer using the NIH Image 1.55 program.

Part of the samples were transferred onto an activated poly (vinyl difluoride) membrane by electro blotting and analysed with antipeptide antibodies; targeted against the N-terminal amino acids 5-18 (16p103), Zn-finger region amino acids 20-50 (8pZn), insertion region amino acids 172-186 (11p104) and C-terminal amino acids 412-426 (23p107) of VDR. Immune complexes were revealed using alkaline-phosphatase-conjugated secondary antibody and a standard colorimetric reaction. Edman N-terminal sequencing was used to identify fragments of 34 kDa and 28 kDa.

Table 4. The vitamin D compounds used in this work.

Name	Chemical structure of side chain
1α,25-dihydroxy-vitamin D_3	
MC903 1α,(24S)-$(OH)_2$-22-ene-26,27-cyclopropyl-vitamin D_3	
EB1089 1α,25-dihydroxy-22,24-diene-24,26,27-tri-homo-vitamin D_3	
KH1060 20-epi-22-oxa-24α,26,27α-tri-homo-1α,25-dihydroxy-vitamin D_3	
MC1288 20-epi-1α,25-dihydroxy-vitamin D_3	
ZK 157202 20-methyl-23-ene-1α,25 dihydroxy-vitamin D_3	
ZK 161422 20-methyl-1α,25 dihydroxy-vitamin D_3	
ZK 159222 (butyl-(5Z,7E,22E)-(1S,3R,24R)-1,3,24-trihydroxy-26,27-cyclo-9,10(19),22-tetraene-25-carboxylate	

4.6 Analytical gel filtration (II-III)

Analytical gel filtrations were performed on a Superdex 200-HR 10/30 column (Pharmacia) with a flow rate of 0.5 mL/min and an injection volume of 0.5 mL. The buffer was 10 mM Tris pH 8.0, 250 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 1 mM Chaps and 0.05 mM n-dodecyl α-d-maltoside, and the protein concentration of the

samples was 1-2 mg/mL. Fractions of 300 µl were collected and analysed by SDS/PAGE and Western blotting with anti-VDR antibodies. The column had previously been calibrated with proteins of known molecular mass.

4.7 Mass spectra analyses (III)

For electrospray ionization mass spectra, proteins were concentrated on Centricon 30 K to a concentration of 4 mg/mL and dialyzed extensively against 50 mM ammonium acetate pH 7.0.

4.8 Ligand binding assays (II-III)

For ligand binding analyses, the crude extracts of *E. coli* or *Sf9* cells expressing VDR LBD were diluted 1000 times and incubated with increasing amounts of [26,27-3H]1,25$(OH)_2D_3$ (Amersham) in 20 mM Tris, 250 mM NaCl, 5 mM dithiothreitol, 10% glycerol for 16 h at 4 °C. After incubation the amount of unbound and protein bound ligands was determined by liquid scintillation. Data were analyzed by nonlinear least square method for a model of one specific binding site and nonspecific binding sites as described (Claire *et al.* 1978). The competition assays with the analogs MC903, EB1089 and KH1060 (table 4.) were performed as described (Zhao *et al.* 1997). Ligand binding properties of VDR were also studied by using the steady state fluorescence isothermal titration method. Experiments were performed using a Perkin Elmer LS50B fluorometer (Baconsfield, UK) at 25 °C. The emission of highly purified VDR at a concentration of 60 nM was recorded over the range of 300-400 nm at the excitation wavelength of 285 nm in solution 50 mM Tris, 500 mM NaCl and 5 mM 2-mercaptoethanol. Ligand-induced fluorescence quenching was followed by increasing the concentration of the respective ligand in 2 nM steps, allowing equilibration for a period of 2 min after each addition. The concentration of the ligand stock solutions (20 µM in methanol) was kept high in order to minimize dilution of the sample under investigation. The slit widths were set at 10 nm and 15 nm for excitation and emission respectively, and the spectra were recorded with 200 nm/min. A 290 nm cut-off filter was inserted into the emission path to avoid stray light. The spectra were background-corrected and the areas were integrated between 300 and 400 nm. The mean values of normalized fluorescence intensity signals resulting from three independent experiments were plotted against the ligand concentration. The resulting binding isotherms were fitted by non-linear regression to the following equation describing a bimolecular association reaction (Nomanbhoy *et al.* 1996, Kristl *et al.* 2001)

$$F = F_i + F_{\max}\frac{K_d + |hVDR| + |L| - \sqrt{(K_d + |hVDR| + |L|^2) - 4|hVDR||L|}}{2|hVDR|}$$

where *Fi* is the initial fluorescence intensity and F_{max} is the maximal fluorescence intensity. K_d represents the dissociation constant, *[hVDR]* the concentration of protein and *[L]* the concentration of the respective ligand.

4.9 Dimerization with retinoid X receptor (RXR) (III)

In order to study the dimerization properties of VDR LBDmt the his-tagged VDR LBDmt and nontagged hRXRα LBD were coexpressed and purified in three steps. The cell pellets were homogenized by sonication. The heterodimer was purified on a metal affinity column (Talon, Clontech) using imidazole elution, followed by gel-filtration on a Superdex S75-HR 26/60 (Pharmacia). The excess of monomeric VDR LBDmt was removed in this step. Fractions containing the heterodimer were pooled together and the histidine tags were removed as described earlier. The purification of the dimer was finalized by gel filtration on a Superdex S200-HR 10/30 (Pharmacia). Ligands were added in excess throughout the first two steps.

4.10 Analytical ultracentrifugation studies (III)

The sedimentation equilibrium of the VDR LBDmt/RXRα LBD heterodimer complexed with $1,25(OH)_2D_3$ and 9-*cis* retinoic acid was performed on a Beckman XLA analytical ultracentrifuge. The complex was prepared in a buffer containing 20 mM Tris pH 8.0, 1 mM EDTA and 250 mM KCl. Experiments were carried out at 20 °C in 1.2 cm pathlength double-sector cells and run at 12,000 rpm. After 12 h centrifugation, scans were compared at 2 h intervals to ensure that equilibrium was reached. The data were analyzed by single or multifit analysis programs supplied by Beckman as described (Birck *et al*. 1996).

4.11 Characterization of transactivation properties of VDR Δ165-215 deletion mutant (III)

To study the ability of VDRΔ165-215 to activate transcription, two transactivation assay systems were performed in COS cells. In the first system cells were transfected (Xiao *et al*. 1991) with 250 ng of receptor expression vectors PXJ440 Gal4 DBD-VDR LBD or Gal4 DBD-VDRmt LBD, 2 mg of reporter gene 5 x 17m-TATA-CAT (chloramphenicol acetyltransferase), 2 mg of an internal control expressing β-galatosidase pCH110lacZ (Pharmacia) made up to 20 mg with carrier DNA. For the second system, 500 ng of pSG5 VDR (1-427) or VDR mutant (1-427, Δ165-215) were cotransfected with 2 mg of reporter gene rat osteopontin VDRE-tk (thymidine kinase)-Luc (luciferase), 2 mg of pCH110lacZ made up to 20 mg with carrier DNA. Cells were treated with 100 nM $1,25(OH)_2D_3$ or ethanol vehicle. During experiments COS cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% dextran-coated charcoal

stripped fetal bovine serum. Forty-eight hours after transfection cells were harvested by three freeze-thaw cycles.

β-Galactosidase activity was determined by colorimetric assay with o-nitrophenyl-β-D-galactopyranoside (Sigma) and normalized for transfection efficiency against β-galactosidase enzyme (Sigma) standards. Chloramphenicol acetyltransferase activity was quantified by ELISA (Boehringer Mannheim) according to the manufacturer's instructions. Luciferase activity was measured with luciferase assay reagent (Promega) in a luminometer. Luciferase and chloramphenicol acetyltransferase activities were both standardized using galactosidase activities. Results are given as a percentage of maximal VDR wild-type in the presence of 100 nM $1,25(OH)_2D_3$. Experiments were performed twice in triplicate.

4.12 Small angle X-ray scattering (SAXS) measurements (III)

In order to obtain structural information on the insertion domain of VDR LBD and a ligand induced conformational change within the receptor, small angle X-ray and neutron scattering studies were performed with native and mutated LBD.

To avoid aggregation problems, samples were subjected to gel filtration before analysis, and only the top of the elution peak was used for scattering measurements without any further concentrating steps.

The buffer for SAXS measurements was 10 mM Tris/HCl pH 8.0, 250 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1 mM di-isopropyl fluorophosphate, 1 mM Chaps and 0.05 mM n-dodecyl α-D-maltoside. The concentrations of the VDR wild-type were 3.8 and 4.9 mg/mL and the VDR mutant 2.0 mg/mL. Small angle X-ray scattering measurements were recorded at 5 °C on the D24 SAXS instrument using synchrotron radiation at LURE-DCI, Orsay, France (Depautex *et al*. 1987). The X-ray wavelength was 1.488 Å and the detector distance was 1,573 mm. An asymmetric experimental set up was used in order to simultaneously record data in small and high Q regions. The effective Q range extended from 0.01 to 0.48 $Å^{-1}$ but was reduced from 0.01 to 0.3 $Å^{-1}$. Nine frames of 200 s each were recorded for each sample. Each frame was individually visualized to check the absence of denaturation or aggregation during data collection. The scattering intensity of a reference sample (carbon black) was recorded before and after each sample and that was used to normalize all data. The scattering of the buffer was subtracted before analysis. The data acquisition was performed with the program OTOKO.

4.13 Small-angle neutron scattering measurements (II)

Neutron scattering measurements were performed in the buffer of 10 mM Tris/HCl, pH 8.0, 500 mM NaCl, 1 mM DTT, 14 mM 2-mercaptoethanol and 0.1 mM di-isopropyl fluorophosphate in ^2H_2O. The sample concentrations were 2 mg/ml for the apo and holo VDR LBDs. Neutron scattering data were obtained in one session on Instrument D11 at the Institute Laue-Langevin, Grenoble, France (Ghosh 1989). The size of the detector was 64 cm x 64 cm. Sample-detector distance was 2.5 m and the wavelength of the beam was

10 Å. The corresponding Q range was 0.01-0.03 $Å^{-1}$. Samples were measured at 4 °C in Hellma rectangular quartz cells 2 mm thick. The data acquisition time was 30 min. Data were processed with Grenoble software RNILS, XPOLLY, RGUIM and RPLOT (Ghosh 1989).

4.14 Scattering curve calculations (II-III)

X-ray scattering curves were computed from crystallographic coordinates of the VDR mutant (Rochel *et al*. 2000) using the program CRYSOL (Svergun *et al*. 1995, Svergun *et al*. 1998) which takes into account the hydration layer surrounding the protein surface and can be fitted against experimental curves.

The scattering intensity $I(Q)$ from a dilute monodisperse solution is an isotropic function proportional to the scattering from a single particle averaged over all orientations, and the experimental curves are obtained by subtracting the solvent contribution. Recording of the scattering intensities allows determination of the radius of gyration (R_G) that is a measure of structural elongation if the internal inhomogeneity of scattering densities within the protein can be neglected. Analysis of the $I(Q)$ curves at small Q in Guinier plots gives the R_G and the forward scattering at zero scattering angle I(0) (Guinier & Fournet 1955):

$\ln I(Q) = \text{In } I(0) - R_G^2 Q^2/3$ with $Q = 4\,\pi \sin\theta/\lambda$

where Q is the scattering vector for a scattering angle of 2θ and a wavelength λ. The expression is valid in a QR_G range up to 1.5. The relative value of $I(0)/c$ with c the sample concentration, gives the apparent molecular mass m of the particle in solution. m is calculated by using a lysozyme solution at 6 mg/mL in 100 mM NaCl, 50 mM sodium acetate pH 4.6, as a molecular mass standard reference. If the structure is sufficiently elongated, the averaged R_G of the cross-sectional structure R_{XS} and the averaged cross-sectional intensity at zero angle $[I(Q)\,Q]_{Q\to 0}$ are obtained from (Glatter & Kratky 1982):

$\ln [I(Q)Q] = \text{In } [I(Q)\,Q]_{Q\to 0} - R^2_{XS}Q^2/2$

The R_G and R_{XS} analyses lead to estimates of the length of the longest axis L of the particle in solution with $L = [12(R_G^2 R^2_{XS})]\}^{1/2}$ (Glatter & Kratky 1982).

The QR_{XS} ranges for VDRwt and VDRmt were $0.8 \bullet QR_{XS} \bullet 2.0$ and $0.7 \bullet QR_{XS} \bullet 1.8$, respectively.

Indirect transformation of the full scattering data $I(Q)$ in the reciprocal space into that in real space provides $P(r)$, the interatomic distances radial distribution function. The distance distribution function $P(r)$ corresponds to the distribution of distances r between any two volume elements within one particle and is obtained by indirect Fourier transformation algorithms that suppress truncation artefacts due to the limited reciprocal space of the scattering. In this study $P(r)$ functions were calculated using the program GNOM (Svergun *et al*. 1988). This function offers an alternative calculation of $I(0)$, its zeroth moment, and of R_G, its second moment, and gives the maximum dimension of the molecule D_{max}.

$$P(r) = \frac{1}{2\pi^2}\int_0^\infty I(Q)Qr \sin(Qr)\mathrm{d}Q$$

The criteria to evaluate correctly the $P(r)$ distribution function (Heidorn & Trewhella 1988) were satisfied in this study. D_{max} between 55 and 90 Å correspond to $Q_{\min}$ = 0.057 /Å and 0.034 /Å. D_{max} was determined from the value of r when P(r) became zero at large r. The X-ray $I(Q)$ curves contained 255 points ($0.01 < Q < 0.3$ /Å). D_{max} was varied in 1 Å steps between 50 and 100 Å to test the stability of the $P(r)$ transformation.

4.15 Fluorescence anisotropy measurements (IV)

The fluorescence anisotropy technique was used to analyze changes in the quaternary structure of VDR using different receptor concentrations with presence and absence of $1,25(OH)_2D_3$.

The fluorescence anisotropy data of VDR were collected in 10 mM Tris/HCl pH 8.0, 0.5 M NaCl, 5 mM DTT at an excitation wavelength of 282 nm and an emission wavelength of 346 nm. The fluorescence anisotropy was defined as

$r = (IVV - G \cdot IVH) / (IVV + 2G \cdot IVH)$

where IVV is the fluorescence intensity recorded with excitation and emission polarization in vertical position, and IVH is the fluorescence intensity recorded with the emission polarization aligned in horizontal position. The G factor is the ratio of sensitivities of detection system for vertically and horizontally polarized light $G = IHV/IHH$. For each point of dimerisation experiments, the anisotropy was recorded over 30 seconds and the mean r values for each VDR concentrations were utilized to determine a dimerisation constant for VDR.

4.16 Circular dichroism spectroscopy

Thermal stability of VDR was studied in presence and absence of the different ligands by using CD-spectroscopy. Far-UV CD spectra were recorded on a Jasco J-710 spectropolarimeter (Japan Spectroscopy, Tokyo, Japan). The temperature of a 7 µM hVDR solution was increased stepwise (10 °C/5 min) in the range of 15 °C- 90 °C and CD-spectra were recorded at a wavelength ranging from 195 to 250 nm with a response time of 0.25 sec and with a data point resolution of 0.1 nm using cuvettes with 0.1 cm pathlength. Three scans were averaged in order to obtain smooth spectra. The same measurements were repeated in the presence of the ligand. The change in ellipticity at 222 nm was plotted against the temperature and the curve thus obtained was fitted to the Boltzmann equation.

5 Results

5.1 Expression and purification of VDR and its LBD (I-III)

In order to study the structure and function of VDR we expressed full-length human VDR and its truncated forms in *E. coli* and in *Sf*9 insect cells. Hexahistidine tag was fused to the expression constructions to help the purification of the recombinant receptors. Receptors were purified by using $(NH_4)_2SO_4$, metal-chelating affinity chromatography and gel filtration. Samples taken at each purification step were subjected to SDS PAGE and native PAGE. Coomassie staining revealed that proteins were purified to apparent homogeneity with the yields; 1.5-3.5 and 0.2-0.5 mg/l of VDR and LBD produced in *Sf*9 cells, respectively, and 2 mg of LBD mutant protein (LBDmt), lacking the residues 165-215 was obtained from 1 L of *E. coli* culture. The removal of the insertion domain increased the solubility and stability of LBD mutant protein and thus improved the yield of the protein obtained from *E. coli*.

5.2 Characterization of VDR and its LBD

5.2.1 Ligand binding properties (II-IV)

Ligand binding ability of VDR, VDR LBD and VDR LBDmt were analyzed from crude cell lysate. The dissociation constant for $1,25(OH)_2D_3$ ranged 0.37 to 1.2 nM. To compare the relative binding affinities of VDRmt LBD for $1,25(OH)_2D_3$ and several $1,25(OH)_2D_3$ agonist analogs (table 4), ligand binding competition experiments were performed. $1,25(OH)_2D_3$ exhibited the highest affinity for VDRmt LBD. The relative affinities of the compounds MC903, EB1089 and KH1060 for VDRmt LBD, at the concentration of 2 nM were 86, 83 and 90%, respectively, compared to 100% of $1,25(OH)_2D_3$.

Ligand binding of the highly purified VDR was also monitored using steady state fluorescence isothermal titration, the dissociation constant 30 nM was calculated from

binding isotherms for the natural ligand $1,25(OH)_2D_3$ and for the analogs ZK 1572027 nM, ZK 161422 34 nM and ZK 159222 172 nM.

5.2.2 Ligand induced conformational change within VDR (I-II)

The ligand binding induced conformational change of VDR was studied using partial proteolytic digestion experiments. In the absence of $1,25(OH)_2D_3$, VDR was rapidly digested with trypsin into three main fragments of about 34, 21 and 20 kDa analyzed by SDS/PAGE. In addition, a weaker fragment of about 28 kDa was produced. In this respect, no qualitative differences were found between human VDR without a ligand, and after treatment with $1,25(OH)_2D_3$ or its analogs. However, the fragments generated after the addition of ligands differed in band intensity on gels, suggesting that the analogs protected the VDR with different efficiency. The 20-epi analogs KH1060 and MC1288 protected the 34 kDa fragment even better than natural ligand, whereas the analogs MC903 and EB 1089 did not differ appreciably from it in this respect. When digested with chymotrypsin, the digestion produced three main fragments of 30, 25, and 14 kDa. As with trypsin, the differences between the natural ligand and its analogs were in the intensity of the fragments, and the 20-epi analogs provided again better protection than the natural hormone.

The trypsin digestion fragments were identified by using Western blotting with antibodies generated against different parts of the receptor. The fragment of 34 kDa was identified to be derived from the C-terminus and the fragment of 21 kDa from the N-terminus of human VDR. The fragment of 34 kDa was also sequenced in order to locate the exact trypsin cleavage site. The obtained sequence His-Thr-Pro-Ser-Phe-Ser-Gly indicated that the fragment was cleaved between the amino acids Arg 173 and His 174. The minor fragment of 28 kDa gave an identical N-terminal sequence. The C-terminal antibody did not identify smaller fragments, indicating that the smaller fragment was generated when the fragment of 34 kDa was further cleaved from the C-terminal end.

The ligand binding induced conformational change of the LBD was also demonstrated by using the SANS method. The calculated R_G values for apo and holo LBD were in ^2H_2O 21.1±0.3 Å and 20.2±0.6 Å, respectively, indicating that the ligand induced conformational change within LBD generates more compact folding.

5.2.3 Modulation of thermal stability of VDR by ligand binding (IV)

Ligand binding induced modulation in the thermal stability of VDR was studied using CD spectroscopy. Results indicate that ZK157202 strongly destabilizes the structure of VDR, causing a shift in thermal unfolding transition (Tm) from 53 °C for the unliganded VDR to 44 °C for the liganded receptor. Only a minimal effect on the stability of VDR was observed in the presence of ZK 161422 and ZK 159222 resulting in Tms of 50.8±1.3 °C and 55.3±0.6 °C, respectively.

5.2.4 Dimerization properties of VDR (II-IV)

Dimerization properties of VDR and its LBD were studied by using several methods. On analytical gel filtration, all proteins studied (full length VDR, LBD, LBDmt), eluted as one peak corresponding to the monomeric form, whether or not preincubated with $1,25(OH)_2D_3$. Conversely, concentration-dependent fluorescence anisotrophy experiments indicated that the full-length VDR molecules are also able to form homodimers in the presence of natural ligand, but not without ligand at the receptor concentration 3.5 μM. In addition to that, synthetic analogs seem to have a different capacity to promote the homodimerization of the full length receptor. ZK 157202 was able to promote homodimerization at receptor concentration less than 500 nM, but ZK 164122 and ZK 159222 were not able to induce homodimerization even at receptor concentration 3.5 μM. Small angle scattering studies of LBD and LBDmt indicated that LBD and LBDmt exist as monomers at protein concentration 130 μM in the presence of the natural ligand.

Vitamin D receptor modulates gene activity by forming heterodimer complexes with RXR isoforms mainly via dimerization interface locating on the surface of LBD. It was therefore important to investigate wheather the recombinant VDR LBD and its mutant were able to form a heterodimer with RXR LBD. In order to do that VDR LBDmt and nontagged hRXRα LBD were coexpressed and purified in three steps. Analytical gel filtration and native electrospray ionization mass spectroscopy studies indicated that the purified VDRmt/RXRα LBDs complexed with $1,25(OH)_2D_3$ and 9-*cis* retinoic acid is a stable species and the two partners are able to bind their ligands. The stability of this complex was also demonstrated using analytical ultracentrifugation. Only one species of 53,000 ± 600(±2 σ) Da was detected by equilibrium sedimentation corresponding to the theoretical heterodimer.

5.2.5 Sequence-specific DNA-binding activity of VDR (II)

VDR modulates gene expression by binding to VDRE as homo or heterodimer with RXR isoforms. The sequence-specific DNA-binding activity of the recombinant VDR was studied *in vitro* by incubating VDR with double-stranded DNA oligonucleotides containing the VDRE of osteocalcin or osteopontin genes, in the presence of $1,25(OH)_2D_3$. Vitamin D receptor molecules formed stable homodimeric complexes with both binding sequences analyzed by gel filtration chromatography and by a native PAGE. The binding of VDR to DNA increased the solubility and stability of the receptor. This could be seen when receptors were concentrated alone or complexed with VDRE. Receptors alone tended to precipitate earlier than the receptor/VDRE complexes. Crystallization studies of the receptor and the receptor/DNA complex also showed that the complex is more soluble than receptor alone (Juntunen, unpublished observation).

Because VDR exerts its biological activity primarily as a heterodimer with RXR, we decided to investigate the association of these proteins. This was done by incubating equimolar amounts of purified VDR, human RXRαΔAB monomers and VDRE with either $1,25(OH)_2D_3$ alone or with $1,25(OH)_2D_3$ and 9-*cis*-RA. The protein/DNA complexes thus formed were purified by gel filtration chromatography. The protein

elution profile indicated that over 90% of the molecules took part in complex formation. The purified complex presented only RXRαΔAB/VDR/VDRE heterodimers. 9-*cis*-RA did not affect the formation of the complex, but the mobility of the complex was slightly higher on the native PAGE gel in the presence of 9-*cis*-RA. Similar results were obtained with the VDRE of the osteocalcin or osteopontin gene. Formation of the heterodimeric complex was also studied in the absence of DNA. The complexes formed under these conditions were eluted in gel filtration as a broad peak and gave rise to several bands in the native PAGE gel.

5.2.6 Transactivation properties of VDR Δ165-215 deletion mutant (III)

To study the role of the insertion domain in VDR driven transctivation, mutated LBD was fused to Gal4-DBD and this gene construct was transfected with Gal4 responsive reporter construct to COS cells. Transfected cells were treated with either vehicle alone or $1,25(OH)_2D_3$. The chloramphenicol acetyltransferase activity of VDRmt transfected cells was compared to the activity of wild type transfected cells. The expression of VDRmt in transfected cells was similar to that of the wild type, and the relative transactivation ability of the VDRmt was 80% of the activation of the VDRwt in the presence of ligand. The same results were obtained with the full length VDR cotransfected with a luciferase reporter plasmid containing the VDRE of osteopontin gene VDRE under the control of the thymidine kinase promoter.

5.2.7 Solution studies of VDR LBD and its mutant

To obtain structural information on the insertion domain of VDR LBD SAXS studies were performed. Linear Guinier plots were obtained for VDR LBD and VDRmt LBD in satisfactory QR_G ranges. No aggregation was detected at the concentrations used. VDR LBD and its mutant appear to be homogenous and monodisperse. Molecular mass calculations indicated that both proteins were monomeric. The radii of gyration for VDR LBD and VDRmt complexed to $1,25(OH)_2D_3$ were 26.2 and 23.4 Å, respectively. The difference of 2.8 Å observed between the R_G of the two proteins corresponds to an increase in volume of 35% for a spherical object. A similar difference (2.2 Å) in R_G values was observed in SANS measurements.

Experimental scattering curves for VDR LBD and VDRmt were fitted against the theoretical curve calculated from the crystal structure of VDRmt bound to $1,25(OH)_2D_3$, using the CRYSOL program. The experimental scattering curve of the VDRmt fits well with the curve calculated from the crystallographic structure, indicating that the conformation in solution for the VDRmt is similar to that observed in the crystal.

The interatomic distances radial distribution $P(r)$ was calculated from SAXS data for VDR LBD and VDRmt. The experimental curve of VDRmt also fits well with the curve calculated from the crystal structure, confirming the agreement of the solution and crystal structures of VDRmt. Due to the low concentration of the two proteins used in this study, only estimates of D_{max} were obtained. The D_{max} difference between molecules was 15 Å.

The R_G values calculated from $P(r)$ functions are in agreement with those calculated from Guinier analysis.

6 Discussion

6.1 Production and purification of recombinant VDR proteins (II, III)

Insect cell system has been a popular method for production of eukaryotic proteins which contain posttranslational modifications or are poorly soluble in *E. coli* protein production system. In the present study we developed a large-scale expression system for the recombinant VDR and its LBD. By using a bioreactor we were able to produce soluble full-length VDR over 3 mg/L in Sf9 insect cell culture, which is more than previously reported in insect cells (MacDonald *et al*. 1991, Nakajima *et al*. 1993) and yeast (Sone *et al*. 1990). The yield of purified VDR LBD was lower due to difference in expression level rather than differential losses during purification.

Vitamin D receptor and its LBD are poorly soluble proteins and they have a tendency to accumulate in inclusion bodies when expressed in *E. coli*. In previous reports on the expression of VDR LBD in *E. coli*, the protein was solubilized by renaturation of inclusion bodies (Nakajima *et al*. 1993, Schaefer-Klein *et al*. 1993), or the solubility of the protein was increased by expressing recombinant protein as a fusion with the glutathione S-transferase (Craig & Kumar 1996) or with the maltose binding protein (Motteshead *et al*. 1996).

Vitamin D receptor has a typical feature among NHRs. It contains an additional region at the N-terminal part of the LBD connecting the helices H1 and H3 (Baker *et al*. 1988, Labuda *et al*. 1992). The length of this connecting region varies between 72 and 81 residues in the VDR family compared to 15-25 residues for the other nuclear receptors (Rochel *et al*. 2000). We created a VDR LBD deletion mutant lacking residues 165-215 of this region and expressed it in *E. coli*. The deletion of the insertion domain increased the stability and solubility of the protein and thus 2 mg of soluble purified VDR LBD mutant protein could be obtained from 1 L of culture. According to secondary structure prediction, insertion domain is not well ordered and forms only a few short β strands. The removal of the insertion domain probably decreases the number of conformations in VDRwt and thus stabilizes the mutant protein.

6.2 Characterization of biochemical properties of recombinant proteins

6.2.1 Dimerization (II-IV)

Nuclear hormone receptors contain two dimerization interfaces, one in the DBD and another in the LBD. In gel filtration studies, the recombinant full length VDR exists as monomeric without VDRE in the presence or absence of natural ligand and no stable homodimeric species were detected under the conditions that were used. This is in according with the results obtained by Craig *et al*. 1999. Contrary to gel filtration results we were able to detect ligand induced dimerization of the full length receptor by using a fluorescence anisotrophy method. The differences between these findings are most probably due to methodological differences. Nishikawa *et al*. 1995 found weak ligand dependent dimerization activity of VDR by using a maltose-binding-protein-based protein binding assay with radiolabeled proteins. Cheskis and Freedman 1996 detected also VDR homodimers and determined their dimerization constant to be 600 nM by using the surface plasmon resonance method. This value is almost the same that we obtained with natural ligand and ZK157202 analogue. Our studies indicate also that synthetic analogs have a different capacity to promote the homodimerization of the full length receptor. This may be due to the different conformation of receptor ligand complexes. It has been demonstrated previously that the RXR/VDR/VDRE complex occupied with ZK159222 analogue had a different conformation than the complex with the natural ligand (Toell *et al*. 2001).

It is known that the protein-protein interaction leading to the formation of RXR-VDR heterodimer is about 1,000 order stronger than that for VDR homodimerization. The dimerization constant of RXR-VDR heterodimer is determined to be 0.16 nM (Dong & Noy 1998). In our studies, VDR LBD and LBDmt formed stable heterodimers with RXRα LBD, indicating that the insertion domain of VDR LBD does not take part in the formation of heterodimerization interface.

Vitamin D receptor binds to target VDRE on DNA as a heterodimeric complex with RXR or as a homodimer. The biological significance of VDR homodimerization is still under debate, although there is an increasing amount of evidence that homodimeric complexes are also capable of activating transcription in a ligand dependent manner (Takeshita *et al*. 2000, Shaffer & Gewirth 2002). We established conditions for the formation and purification of both types of complexes in the presence of oligonucleotides representing known VDREs. Complex formation increases the stability and solubility of the VDR in solution. The RXR/VDR/VDRE complex is considerably more stable than the homodimeric one. Homodimeric VDR complexes were not detected if both receptors were present in equimolar concentration. Jurutka *et al*. 2002 obtained similar results. They reported that in competition studies, the relative dissociation of homodimeric VDR complex from the VDRE was extremely rapid t½ under 30 s compared to the dissociation of heteromeric complex t½ over 5 min. On the other hand, Shaffer & Gewirth 2002 found that VDR DBD formed preferentially a homodimeric complex on the VDRE. This

indicates that LBD of VDR has a dominant role in the determination of dimerization partner for the full length receptor.

We found also that the addition of 9-*cis*-RA to the preformed 1,25$(OH)_2D_3$ occupied RXR/VDR/VDRE complex slightly increased its mobility on the native PAGE gel. This observation could mean that the preformed RXR/VDR/VDRE complex is able to bind 9-*cis*-RA, and that this binding causes a conformational change in the complex. This would be in contrast with the results of Thompson *et al.* 1998. They postulated that VDR, if previously bound to 1,25$(OH)_2D_3$, influences the conformation of RXR in the heterodimer in a manner that makes it resistant to binding by 9-*cis*-RA.

Several biophysical studies using surface plasmon resonance (Cheskis & Freedman 1996), fluorescence anisotropy (Dong & Noy 1998) and electrospray mass spectra (Craig *et al*. 1999), have shown that 1,25$(OH)_2D_3$ favors VDR/RXR heterodimer binding to DNA while 9-*cis* retinoic acid reduces it, but maximal binding is observed in the presence of both ligands (Dong & Noy 1998).

6.2.2 Ligand binding (II, III)

We analyzed the ligand binding ability of the recombinant proteins VDR, LBD and LBDmt. We did not find any significant differences between the values for dissociation constants for 1,25$(OH)_2D_3$. The values obtained in this study agreed well with those previously obtained for the recombinant VDR from *E. coli* (Mottershead *et al*. 1996), yeast (Sone *at al*. 1990) and insect cells (Nakajima *et al*. 1993, Schaefer-Klein *et al*. 1993, Craig & Kumar 1996). Neither did any ligand-binding competition experiments with several 1,25$(OH)_2D_3$ agonist analogies detect any differences between VDR and VDRmt on the ligand binding properties, and the relative affinities of MC903, EB1089 and the 20-epi analog KH1060 are comparable with previously published results (Bouillon *et al*. 1995). These results indicate that the insertion domain does not play any role in the ligand binding. The crystal structure of VDRmt bound to 1,25$(OH)_2D_3$ shows that the truncated insertion domain locates distant from the ligand. Thus it is unlikely that it could affect the binding of the ligand (Rochel *et al*. 2000).

6.2.3 Transactivation properties of VDR deletion mutant (III)

Transactivation assays were performed in COS cells in order to study the role of insertion domain in gene activation. VDRmt LBD (Δ165-215) fused to Gal4-DBD and the full length VDRmt were able to activate transcription in a hormone dependent manner, indicating that the insertion region of VDR is not involved in the gene activation function of this nuclear receptor. Because reporter gene constructions did not integrate to chromosome we can not exclude the possibility that in certain promoter contents the insertion domain could have an effect on the transactivation. The insertion domain could have a role in receptor interaction with comodulators that are involved in the modification of chromatin structure needed for gene activation or repression.

One mutation associated with the genetic disease vitamin-D resistant rickets has been found in the area of the insertion region (Cys190Trp), but the mechanism behind that has not been reported (Malloy *et al.* 1999). In addition to that phosphorylation sites have been identified at serines 182 and 208 which are also located in the insertion region (Jurutka *et al*. 1996, Hsieh *et al*. 2001, Jurutka *et al*. 2002). It has been postulated that $1,25(OH)_2D_3$ induced rapid responses could modulate the genomic pathway via protein kinases which phosphorylate nuclear VDR at serine 208, and this could lead to enhanced gene transcription (Fleet 1999). Phosphorylation at serine 182 appears to downregulate gene activation by inhibiting heterodimer formation (Hsieh *et al.* 2001).

6.3 Structural studies

6.3.1 Limited protease digestion experiments (I)

Several different techniques were used to analyze ligand binding induced conformational change within LBD. Limited protease digestion experiments are widely used for characterization of functional conformations of NHRs (Leng *et al.* 1993, Keidel *et al.* 1994). Treatment with $1,25(OH)_2D_3$ or its agonist analogs protected the VDR LBD against partial proteolysis, indicating that the aporeceptor and holoreceptor have different conformation. The protease cleavage site is more exposed in the aporeceptor than in the holoreceptor. In the presence of saturating concentration of ligand two typical VDR LBD fragments of the molecular mass of 34 and 28 kDa were obtained. The use of too low percentage SDS/PAGE gel led to a sligth overestimation of molecular weight of those fragments in the present study. The actual sizes of the fragments are about 28 kDa and 23 kDa, obtained by calculating the molecular weight of fragments 174-427 and 174-391 and reported by others (Peleg *et al*. 1995, Nayeri & Carlberg 1997, Carlberg *et al*. 2001).

All studied ligands generated a similar pattern on the SDS/PAGE gel but quantitative differences in protection ability were seen between natural hormone and its synthetic analogs. The 20-epi analogs KH1060 and MC1288, which exhibit an inverted stereochemistry at position 20 in flexible aliphatic chain, appear to protect VDR better than natural hormone, MC903 and EB1089 analogs. Koszewski *et al.* 1999 reported similar observations. The relative affinity of KH1060 and MC1288 for VDR is less than that of the natural hormone as seen in competition experiments. Thus, the differential sensitivity to partial proteolysis between VDR treated either with $1,25(OH)_2D_3$ or analogs may not be due to higher or lower affinities of the ligands towards receptor.

The crystal structure of VDR LBDmt complexed with KH1060 and MC1288 was published recently by Tocchin-Valentini *et al.* 2001. They found that the protein conformation with analogs is identical to those of the natural hormone, indicating that better protection against proteases by epi-analogs are not due to different conformation of receptor-ligand complexes. Instead of that KH1060 generates additional interactions with ligand binding pocket compared with natural hormone and this could provide an explanation for the higher stability of this complex, whereas the higher stability of the VDR MC1288 complex might be due to the low energy conformation of the ligand in the

conserved pocket compared to the more tense conformation of $1\alpha,25(OH)_2D_3$. Both the more favorable conformation of ligand and the additional interactions of 20-epi analogs would result in higher stability and longer half-life of active complexes as seen in limited protease digestion experiments and *in vivo* studies (Liu *et al*. 1997) which also demonstrate that the dissociation rate of 20-epi analogs is slower than that of natural hormone. Those observations might partly explain the high potency of 20-epi analogs to induce VDR dependent transcription. 20-epi analogs induce VDR-dependent transcription at concentrations at least 100-fold lower than the natural ligand and present antiproliferative activity several orders of magnitude higher than the natural ligand (Binderup *et al*. 1991, Peleg *et al*. 1995).

6.3.2 Scattering studies (II, III)

Scattering studies of neutrons and X-rays provide a low resolution information compared to crystallization and NMR techniques, but on the other hand they do have several advantages. Because scattering experiments can be carried out under solution conditions that mimic specific physiological conditions, they can yield unique insight into the structures of proteins and DNA in solution, domain interactions within proteins, and protein-protein and protein DNA within their functional complexes. They can also provide information about conformational transitions of molecules. Scattering experiments can overcome the problems caused by flexibility of protein that can inhibit protein crystallization. Moreover, the molecular weight of the studied molecule is not a limiting factor in scattering studies as it is in NMR studies. On the other hand, the relatively high concentration of the protein studied may be a limiting factor, as it was in the case of full-length VDR. The full length VDR starts to aggregate at the concentration needed for neutron scattering studies.

The neutron and X-ray scattering experiments of the VDR LBD and VDRmt reveal that hormone binding to LBD results in a closure of the hormone binding cleft. This closure leads more compact folding, which is seen as a reduced R_G value of LBD. That observation is in agreement with previous results obtained from solution studies of RARα LBD (Egea *et al*. 2001) and crystal structures of several NHRs that indicate a conformational change in the orientation of the AF-2 motif (Egea *et al*. 2000a). Circular dichroism spectroscopy experiments of NHRs also support this finding (Falsone *et al*. 2001).

Compared to other NHRs VDR contains a large insertion region at the N-terminal part of LBD between H1 and H3, encoded by an additional exon. Scattering experiments of the VDR mutant and VDR wild type reveal that the volume of VDR wild type is 35% bigger than that of deletion mutant. This difference is rather large for an additional domain of 50 residues, suggesting that this insertion domain is not well ordered and may adopt several conformations in solution.

Many groups have put in a lot of effort into crystallizing native VDR LBD without any success, but the removal of the insertion domain has led to success in crystallization (Rochel *et al*. 2000). This indicates also that the insertion domain is inherently flexible.

The experimental scattering curves of native VDR LBD and LBD mutant were fitted against the theoretical curve calculated from the crystal structure of VDR LBD mutant bound to natural hormone. Experimental scattering curve of LBD mutant fits well with the curve calculated from the crystallographic structure, indicating that the solution structure of VDR LBD mutant is similar to that observed in the crystal. Our structural model for native VDR LBD proposes that the insertion domain is a highly mobile structure and the rest of the LBD is structured similarly to mutated VDR LBD.

7 Conclusions

In this study recombinant VDR and its LBD were produced in insect cells and purified to homogeneity. The biochemical properties of proteins were extensively characterized. The result show that the recombinant VDR is biologically active and it display the same ligand binding, dimerization with RXR and transactivation properties as the wild-type VDR.

Structural studies of purified receptors reveal that the binding of hormone results in conformational change within the LBD that is most probably due to the closure of the hormone binding cleft.

The present study also shows that the insertion domain of VDR is not involved in the main biological functions of VDR, leaving the clear biological role of this domain still open.

8 References

Ahonen MH, Tenkanen L, Teppo L, Hakama M & Tuohimaa P (2000) Prostate cancer risk and prediagnostic serum 25-hydroxyvitamin D levels (Finland). Cancer Causes Control 11: 847-852.

Allen P, Claessens F, Verhoeven G, Rombauts W & Peeters B (1999) The androgen receptor aminoterminal-domain plays a key role in p160 coactivator-stimulated gene transcription. Mol Cell Biol 19: 6085-6097.

Aranda A & Pascual A (2001) Nuclear hormone receptors and gene expression. Phys Rev 81: 1269-1304.

Ashcroft DM, Po AL, Williams HC & Griffiths CE (2000) Systematic review of comparative efficacy and tolerability of calcipotriol in treating chronic plaque psoriasis. BMJ 320: 963-967.

Baker AR, McDonnell DP, Hughes M, Crisp TM, Mangelsdorf DJ, Haussler MR, Pike JW, Shine J & O'Malley BW (1988) Cloning and expression of full-length cDNA encoding human vitamin D receptor. Proc Natl Acad Sci USA 85: 3294-3298.

Baran DT (1994) Nongenomic actions of the steroid hormone 1α,25-dihydroxyvitamin D_3. Cell Biochem 56: 303-306.

Barletta F, Freedman LP & Christakos S (2002) Enhancement of VDR-mediated transcription by phosphrylation: correlation with increased interaction between the VDR and DRIP205, a subunit of the VDR-interacting protein coactivator complex. Mol Endocrinol 16: 301-314.

Baumann H, Paulsen K, Kovacs H, Berglund H, Wright AP, Gustafsson JA & Hard T (1993) Refined solution structure of the glucocorticoid receptor DNA-binding domain. Biochemistry 32: 13463-13471.

Bevan C & Parker M (1999) The role of coactivators in steroid hormone action. Exp Cell Res 253: 349-356.

Binderup L & Bramm E (1988) Effects of a novel vitamin D analogue MC903 on cell proliferation and differentiation *in vitro* and on calcium metabolism *in vivo*. Biochem Pharmacol 37: 889-895.

Binderup L, Latini S, Binderup E, Bretting C, Calverley M & Hansen K (1991) 20-epi-vitamin D_3 analogs: a novel class of potent regulators of cell growth and immune responses. Biochem Pharmacol 42: 1569-1575.

Birck C, Vachette P, Welch M, Swaren P & Samama JP (1996) Is the function of the cdc2 kinase subunit proteins tuned by their propensities to oligomerize? Conformational states in solution of the cdc2 kinase partners p13suc1 and p9cksphy. Biochemistry 35: 5577-5585.

Bland R, Walker EA, Hughes SV, Stewart PM & Hewison M (1999) Constitutive expression of 25-hydroxyvitamin D_3-1α-hydroxylase in a transformed human proximal tubule cell line: evidence for direct regulation of vitamin D metabolism by calcium. Endocrinology 140: 2027-2034.

Blondel A, Renaud J-P, Fischer S, Moras D & Karplus M (1999) Retinoic acid receptor: A simulation analysis of retinoic acid binding and the resulting conformational changes. J Mol Biol 291: 101-115.

Blutt SE, McDonnell TJ, Polek TC & Weigel NL (2000) Calcitriol-induced apoptosis in LNCaP cells is blocked by overexpression of Bcl-2. Endocrinology 141: 10-17.

Bouillon R, Allewaert K, Xiang DZ, Tan BK & van Baelen H (1991) Vitamin D analogs with low affinity for the vitamin D binding protein: enhanced in vitro and decreased in vivo activity. J Bone Miner Res 6: 1051-1057.

Bouillon R, Okamura WH & Norman AW (1995) Structure-function relationships in the vitamin D endocrine system. Endocr Rev 2: 200-257.

Bouillon R, Verstuyf A, Zhao J, Tan BK & Van Baelen H (1996) Nonhypercalcemic vitamin D analogs: interactions with the vitamin D-binding protein. Horm Res 45: 117-121.

Bourguet, W, Ruff M, Chambon P, Gronemeyer H & Moras D (1995) Crystal-structure of the ligand-binding domain of the human nuclear receptor RXR-α. Nature 375: 377-382.

Bourguet W, Vivat V, Wurtz JM, Chambon P, Gronemeyer H & Moras D (2000) Crystal structure of a heterodimeric complex of RAR and RXR ligand-binding domains. Mol Cell 5: 289-298.

Brenza, HL, Kimmeljehan C, Jehan F, Shinki T, Wakino S, Anzawa H, Suda T & DeLuca HF (1998) Parathyroid hormone activation of the 25-hydroxyvitamin D_3 1α-hydroxylase gene promoter. Proc Natl Acad Sci USA 95: 1387-1391.

Brown AJ, Dusso A & Slatopolsky (1999) Vitamin D. Am J Physiol 277: 157-175.

Brown AJ (1999) Regulation of vitamin D action. Nephrol Dial Transplant 14: 11-16.

Brown AJ (2000) Mechanisms for the selective actions of vitamin D analogs. Curr Pharm Des 6: 701-716.

Brown AJ (2001) Therapeutic uses of vitamin D analogs. Am J Kid Dise 38, No 5, Suppl 5: 3-9.

Bruce B & Evans RM (1998) Orphan nuclear receptors new ligands and new possibilities. Genes Dev 12: 3149-3155.

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA & Carlquist M (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389: 753-758.

Carlberg C, Bendik I, Wyss A, Meier E, Sturzenbecker LJ, Grippo JF & Hunziker W (1993) Two nuclear signalling pathways for vitamin D. Nature 361: 657-660.

Carlberg C, Quack M, Herdick M, Bury Y, Polly P & Toell A (2001) Central role of VDR conformations for understanding selective actions of vitamin D_3 analogs. Steroids 66: 213-221.

Chen H, Lin RJ, Xie W, Wilpitz D & Evans RM (1999) Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. Cell 98: 675-686.

Chen, JD & Evans RM (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377: 454-457.

Chen ZP, Shemshedini L, Durand B, Noy N, Chambon P & Gronemeyer H (1994) Pure and functionally homogeneous recombinant retinoid X receptor. J Biol Chem 269: 25770-25776.

Cheskis B & Freedman LP (1996) Modulation of nuclear receptor interactions by ligands: kinetic analysis using surface plasmon resonance. Biochemistry 35: 3309-3318.

Chiba N, Suldan Z, Freedman LP & Parvin JD (2000) Binding of liganded vitamin D receptor to the vitamin D receptor interacting protein coactivator complex induces interaction with RNA polymerase II holoenzyme. J Biol Chem 275: 10719-10722.

Claire M, Raffestin-Oblin ME, Michaud A, Corvol P, Venot A, Roth-Meyer C, Boisvieux JF & Mallet A (1978) Statistical test models and computerized parameter estimation for aldosterone binding in rat kidney. FEBS Lett 88: 295-299.

Collingwood TN, Urnov FD & Wolffe AP (1999) Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription. J Mol Endocrinol 23: 255-275.

Cosma MP, Tanaka T & Nasmyth K (1999) Ordered recruitment of transcription and chromatine remodelling factors to a cell cycle and developmentally regulated promoter. Cell 97: 299-311.

Craig TA & Kumar R (1996) Synthesis and purification of soluble ligand binding domain of the human vitamin D_3 receptor. Biochem Biophys Res Comm 218: 902-907.

Craig TA, Benson LM, Tomlinson AJ, Veenstra TD, Naylor S & Kumar R (1999) Analysis of transcription complexes and effects of ligands by microelectrospray ionization mass spectrometry. Nat Biotechnol 17: 1214-1218.

Darimont BD, Wagner RL, Apriletti JW, Stallcup MR, Kushner PJ, Baxter JD, Fletterick RJ & Yamamoto KR (1998) Structure and specificity of nuclear receptor–coactivator interactions. Genes Dev 12: 3343-3356.

de Jong EM & van de Kerkhof PC (1991) Simultaneous assessment of inflammation and epidermal proliferation in psoriatic plaques during long-term treatment with the vitamin D_3 analogue MC903: modulations and interrelations. Br J Dermatol 124: 221-229.

Deluca HF & Cantorna MT (2001) Vitamin D: its role and uses in immunology. FASEB 15: 2579-2586.

Demay MB. Kiernan MS, DeLuca H F &. Kronenberg HM (1992) Sequences in the human parathyroid hormone gene that bind to the 1,25-dihydroxyvitamin D_3 receptor and mediate transcriptional repression in response to 1,25-dihydroxyvitamin D_3. Proc Natl Acad Sci USA 89: 8097-8101.

Depautex C, Desvignes P, Leboucher P, Lemonnier M, Dagenaux D, Benoit JP & Vachette P (1987) The small angle X-ray scattering instrument D24. CNRS annual report LURE, Orsay France.

Diaz GD, Paraskeva C, Thomas MG, Binderup L & Hague A (2000) Apoptosis is induced by the active metabolite of vitamin D_3 and its analogue EB1089 in colorectal adenoma and carcinoma cells: possible implications for prevention and therapy. Cancer Res 60: 2304-2312.

Dilworth FJ, Fromental-Ramain C, Yamamoto K & Chambon P (2000) ATP-driven chromatin remodeling activity and histone acetyltransferases act sequentially during transactivation by RAR/RXR *in vitro*. Mol Cell 6: 1049-1058.

Dilworth F & Chambon P (2001) Nuclear receptors coordinate the activities of chromatin remodelling complexes and coativatos to facilitate initiation of transcription. Oncogene 20: 3047-3054.

DiRenzo J, Shang Y, Phelan M, Sif S, Myers M, Kingston R & Brown M (2000) BRG-1 is recruited to estrogen-responsive promoters and cooperates with factors involved in histone acetylation. Mol Cell Biol 20: 7541-7549.

Dong D & Noy N (1998) Heterodimer formation by retinoid X receptor: regulation by ligands and by the receptor's self-association properties. Biochemistry 37: 10691-10700.

Dressel U, Thormeyer D, Altincicek B, Paululat A, Eggert M, Schneider S, Tenbaum SP, Renkawitz R & Baniahmad A (1999) Alien, a highly conserved protein with characteristics of a corepressor for members of the nuclear hormone receptor superfamily. Mol Cell Biol 19: 3383-3394.

Duarte J, Perriere G, Laudet V & Robinson-Rechavi M (2002) NUREBASE: database of nuclear hormone receptors. Nucleic Acids Res 30: 364-368.

Dusso AS, Negrea L, Gunawardhana S, Lopez-Hilker S, Finch J, Mori T, Nishii Y, Slatopolsky E & Brown AJ (1991) On the mechanisms for the selective action of vitamin D analogs. Endocrinology 128: 1687-1692.

Egea PF, Klaholz PB & Moras D (2000a) Ligand protein interactions in nuclear receptors of hormones. FEBS Lett 476: 62-67.

Egea PF, Mitschler A, Rochel N, Ruff M, Chambon P & Moras D (2000b) Crystal structure of the human RXRα ligand-binding domain bound to its natural ligand: 9-cis retinoic acid. EMBO J 19: 2592-2601.

Egea PF, Rochel N, Birck C, Vachette P, Timmins P & Moras D (2001) Effects of ligand binding on the association properties and conformation in solution of retinoic acid receptors RXR and RAR. J Mol Biol 307: 557-576.

Erben RG (2001) Vitamin D analogs and bone. J Musculoskel Neuron Interact 2: 59-69.

Falsone SF, Kurkela R, Chiarandini G, Vihko P & Kungl AJ (2001) Ligand affinity, homodimerization, and ligand-induced secondary structural change of the human vitamin D receptor. Biochem Biophys Res Comm 285: 1180-1185.

Feldman D, Zhao X-Y & Krishnan AV (2000) Editorial/Mini-Review: Vitamin D and prostate cancer. Endocrinology 141: 5-9.

Fleet JC (1999) Vitamin D receptors: not just in the nucleus anymore. Nutr Rev 57: 60-62.

Fondell JD, Ge H & Roeder RG (1996) Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. Proc Natl Acad Sci USA 93: 8329-8333.

Fraydoon R, Trixie W, Qiang Z & Khorasanizadeh S (2000) Structure of the RXR-RAR DNA-binding complex on the retinoic acid response element DR1. EMBO J 19: 1045-1054.

Fry CJ & Peterson CL (2001) Chromatin remodeling enzymes: who's on first? Curr Biol 11: 185-197.

Gallagher JC & Riggs BL (1990) Action of 1,25-dihydroxyvitamin D_3 on calcium balance and bone turnover and its effect on vertebral fracture rate. Metabolism 39: 30-34.

Garcia-Villalba P, Jimenez-Lara AM & Aranda A (1996) Vitamin D interferes with transactivation of the growth hormone gene by thyroid hormone and retinoic acid. Mol Cell Biol 16: 318-327.

Gardezi SA, Nguyen C, Malloy PJ, Posner GH, Feldman D & Peleg S (2001) A rationale for treatment of hereditary vitamin D-resistant rickets with analogs of 1α,25-dihydroxyvitamin D_3. J Biol Chem 276: 29148-29156.

Garland CF, Garland FC & Gorham ED (1999) Calcium and vitamin D. Their potential roles in colon and breast cancer prevention. Ann N Y Acad Sci 889: 107-119.

Gerritsen MJ, Boezeman JB, van Vlijmen-Willems IM & Van de Kerkhof PC (1994) The effect of tacalcitol (1,24$(OH)_2D_3$) on cutaneous inflammation, epidermal proliferation and keratinization in psoriasis: a placebo-controlled, double-blind study. Br J Dermatol 131: 57-63.

Ghosh RE (1989) Institut Laue-Langevin Internal Publication No. 89GH02T, Institut Laue-Langevin, Grenoble, France.

Glass CK & Rosenfeld MG (2000) The coregulator exchange in transcriptional functions of nuclear receptors. Genes & Dev 14: 121-141.

Glatter & Kratky (1982) Small-angle X-ray scattering. Wiley, New York.

Glenville J, Hogan DB, Yendt E & Hanley DA (1996) Prevention and management of osteoporosis: Consensus statements from the Scientific Advisory Board of the Osteoporosis Society of Canada 8.Vitamin D metabolites and analogs in the treatment of osteoporosis. Canadian Medical Association J 155: 955-961.

Goltzman D, White J & Kremer R (2001) Studies of the effects of 1,25-dihydroxyvitamin D on skeletal and calcium homeostasis and on inhibition of tumor cell growth. J Steroid Biochem Mol Biol 76: 43-47.

Griffin MD, Lutz W, Phan VA, Bachman LA, McKean DJ, Kumar R (2001) Dendritic cell modulation by 1α,25 dihydroxyvitamin D_3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity *in vitro* and *in vivo*. Proc Natl Acad Sci U S A 98: 6800-6805.

Guinier A & Fournet G (1955) Small angle X-ray scattering. Wiley, New York

Gulliford T, Enlish J, Colston KW, Menday P, Moller S & Coombrs RC (1998) A phase I study of vitamin D analogue EB1089 in patients with advanced breast and colonrectal cancer. Br J Cancer 78: 6-13.

Haddad JG, Matsuoka LY, Hollis BW, Hu Y & Wortsman J (1993) Human plasma transport of vitamin D after its endogenous synthesis. J Clin Invest 91: 2552-2555.

Hanchette CL & Schwartz GG (1992) Geographic patterns of prostate cancer mortality: Evidence for a protective effect of ultraviolet radiation. Cancer 70: 2861-2689.

Hansen CM, Frandsen TL, Brunner N & Binderup L (1994) 1α,25-Dihydroxyvitamin D_3 inhibits the invasive potential of human breast cancer cells in vitro. Clin Exp Metastasis 12: 195-202.

Hansen JC, Tse C & Wolffe AP (1998) Structure and function of the core histone N-termini: more than meets the eye. Biochemistry 37: 17637-17641.

Hansen MC, Binderup L, Hamberg KJ & Carlberg C (2001) Vitamin D and cancer: Effects of 1,25$(OH)_2D_3$ and its analogs on growth control and tumorigenesis. Front in Biosci 6: 820-848.

Hard T, Kellenbach E, Boelens R, Maler BA, Dahlman K, Freedman LP, Carlstedt-Duke, J, Yamamoto KR, Gustafsson JA & Kaptein R (1990) Solution structure of glucocorticoid receptor DNA-binding domain. Science 249: 157-160.

Hassan AH, Neely KE & Workman JL (2000) Histone acetyltransferase complexes stabilize swi/snf binding to promoter nucleosomes. Cell 104: 817-827.

Haussler, MR, Whitfield GK, Haussler CA, Hsieh JC, Thompson PD, Selznick SH, Dominguez CE & Jurutka PW (1998) The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. J Bone Miner Res 13: 325-349.

Havas K, Flaus A, Phelan ML, Kingston RE, Wade PA, Lilley DMJ & Owen-Hughes T (2000) Generation of superhelical torsion by ATP-dependent chromatin remodeling activities. Cell 103: 1133-1142.

Hayes CE (2000) Vitamin D: a natural inhibitor of multiple sclerosis. Proc Nutr Soc 59: 531-535.

Heberden C, Denis I, Pointillart A & Mercier T (1998) TGF-beta and calcitriol. Gen Pharmacol 30: 145-151.

Heery DM, Kalkhoven E, Hoare S & Parker MG (1997) A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387: 733-736.

Heery DM, Hoare S, Hussain S, Parker MG & Sheppard H (2001) Core LXXLL motif sequences in CREB-binding protein, SRC1, and RIP140 define affinity and selectivity for steroid and retinoid receptors. J Biol Chem 276: 6695-6702.

Heidorn DB & Trewhella J (1988) Comparison of the crystal and solution structures of calmodulin and troponin C. Biochemistry 27: 909-915.

Henry HL (1992) Vitamin D hydroxylases. J Cell Biochem 49: 4-9.

Herdick M, Bury Y, Quack M, Uskokovic MR, Polly P & Carlberg C (2000) Response element and coactivator-mediated conformational change of the vitamin D_3 receptor permits sensitive interaction with agonists. Mol Pharmacol 57: 1206-1217.

Hewison M, Zehnder D, Bland R & Stewart PM (2000) 1α-Hydroxylase and the action of vitamin D. J Mol Endocrinol 25: 141-148.

Hittelman AB, Burakov D, Iniguez-Lluhi JA, Freedman LP & Garabedian MJ (1999) Differential regulation of glucocorticoid receptor transcriptional activation via AF-1 associated proteins. EMBO J 18: 5380-5388.

Hong L, Schroth GP, Matthews HR, Yay P & Brandbury EM (1993) Studies of the DNA binding properties of histone H4 amino terminus: thermal denaturation studies reveal that acetylation markedly reduces the binding constant of the H4 tail to DNA. J Biol Chem 268: 305-314.

Horlein AJ, Näär AM, Heinzel T, Torchia J, Gloss B, Kurokawa R, Ryan A, Kamei Y, Soderstrom M, Glass CK & Rosenfiel MG (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377: 397-404.

Hsieh JC, Whitfield GK, Oza AK, Dang HT, Price JN, Galligan MA, Jurutka PW, Thompson PD, Issa LL, Leong GM & Eisman JA (1998) Molecular mechanism of vitamin D receptor action. Inflamm Res 47: 451-475.

Hsieh JC, Dang HTL, Galligan MA, Whitfield GK, Jurutka PW, Thopson PD, Haussler CA & Haussler MR (2001) Phosphrylation of the human vitamin D receptor by protein kinase A downregulates 1,25$(OH)_2$ D_3-dependent transactivation by reducing retinoid X receptor β heterodimerization. J Bone Miner Res 16: (Suppl 1) 231.

Hu X & Lazar MA (2000) Transcriptional repression of nuclear hormone receptor. Trends Endocrinol Metab 11: 6-10.

Issa LL, Leong GM & Eisman JA (1998) Molecular mechanism of vitamin D receptor action. Inflamm Res 47: 451-475.

Issa LL, Leong GM, Sutherland PL & Eisman JA (2002) Vitamin D analogs-spesific recruiment of vitamin D receptor coactivators. J Bone Miner Res 17: 879-890.

Ito K, Barnes PJ & Adcock IM (2000) Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1β-induced histone H4 acetylation on lysines 8 and 12. Mol Cell Biol 20: 6891-6903.

Jacobson RH, Ladurner AG, King DS & Tjian R (2000) Structure and function of a human TAFII250 double bromodomain module. Science 288: 1422-1425.

Jensen SS, Madsen MW, Lukas J, Binderup L & Bartek J (2001) Inhibitory effects of 1α,25-dihydroxyvitamin D_3 on the G(1)-S phase-controlling machinery. Mol Endocrinol 15: 1370-1380.

Jimenez-Lara AM & Aranda A (1999) Vitamin D represses retinoic acid-dependent transactivation of the retinoic acid receptor-β2 promoter: the AF-2 domain of the vitamin D receptor is required for transrepression. Endocrinology 140: 2898-2907.

Johnson LE & DeLuca HF (2001) Vitamin D receptor null mutant mice fed high level of calcium are fertile. Nutr 131: 1787-1791.

Jones G, Strungnell SA & DeLuca HF (1998) Current understanding of the molecular actions of vitamin D. Physiol Rev 78: 1193-1231.

Jurutka PW, Hsieh JC, Nakajima S, Haussler CA, Whitfield GK & Haussler MR (1996) Human vitamin D receptor phosphorylation by casein kinase II at Ser-208 potentiates transcriptional activation. Proc Natl Acad Sci U S A 93: 3519-3524.

Jurutka PW, MacDonald PN, Nakajima S, Hshieh JC, Thompson PD, Whitfield GK, Galligan MA, Haussler CA & Haussler MR (2002) Isolation of baculovirus-expressed human vitamin D receptor: DNA responsive element interactions and phosphorylation of the purified receptor. J Cell Biochem 85: 435-457.

Jääskeläinen T, Ryhänen S, Mahonen A, DeLuca HF & Mäenpää PH (2000) Mechanism of action of superactive vitamin D analogs through regulated receptor degradation. J Cell Biochem 76: 548-558.

Kallay E, Pietschmann P, Toyokuni S, Bajna E, Hahn P, Mazzucco K, Bieglmayer C, Kato S & Cross HS (2001) Characterization of a vitamin D receptor knockout mouse as a model of colorectal hyperproliferation and DNA damage. Carcinogenesis 22: 1429-1435.

Kao HY, Downes M, Ordentlich P & Evans RM (2000) Isolation of a novel histone deacetylase reveals that class I and class II deacetylases promote SMRT-mediated repression. Genes Dev 14: 55-66.

Kato S, Takeyama K, Murayama A, Sekine K & Yoshizawa T (1999) In vivo function of VDR in gene expression- VDR knock-out mice. J Steroid Biochem Mol Biol 69: 247-251.

Keidel S, LeMotte P & Apfel C (1994) Different agonist- and antagonist-induced conformational changes in retinoic acid receptors analyzed by protease mapping. Mol Cell Biol 14: 287-298.

Khorasanizadeh S & Rastinejad F (2001) Nuclear-receptor interactions on DNA-response elements. Trends Biochem Sci 26: 384-390.

Kissmeyer & Binderup (1991) Calcipotriol (MC 903): pharmacokinetics in rats and biological activities of metabolites. A comparative study with $1,25(OH)_2D_3$. Biochem Pharmacol 41: 1601-1606.

Koli, K & Keski-Oja J (2000) 1α,25-Dihydroxyvitamin D_3 and its analogs down-regulate cell invasion-associated proteases in cultured malignant cells. Cell Growth Differ 11: 221-229.

Kong J, Li XJ, Gavin D, Jiang Y & Li YC (2002) Targeted expression of human vitamin D receptor in the skin promotes the initiation of the postnatal hair follicle cycle and rescues the alopecia in vitamin D receptor null mice. J Invest Dermatol 118: 631-638.

Kornberg RD & Lorch Y (1999) Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. Cell 98: 285-294.

Koszewski NJ, Reinhardt TA & Horst RL (1999) Differential effects of 20-epi vitamin D analogs on the vitamin D receptor homodimer. J Bone Miner Res 14: 509-517.

Krebs JE, Fry CJ, Samuels ML & Peterson CL (2000) Global role for chromatin remodeling enzymes in mitotic gene expression. Cell 102: 587-598.

Kristl S, Zhao S, Falsone SF, Somerville RL & Kungl AJ (2001) The influence of ATP on the association and unfolding of the tyrosine repressor ligand response domain of Haemophilus influenzae. Biochem Biophys Res Commun. 280: 81-84.

Kurokawa R, Söderström M, Hörlein A, Halachmi S, Brown M, Rosenfeld MG & Glass CK (1995) Polarity-specific activities of retinoic acid receptors determined by a co-repressor. Nature 377: 451-454.

Labuda M, Fujiwara TM, Ross MV, Morgan K, Garcia-Heras J, Ledbetter DH, Hughes MR & Glorieux FH (1992) Two hereditary defects related to vitamin D metabolism map to the same region of human chromosome 12q13-14. J Bone Miner Res 7: 1447-1453.

Laudet V (1997) Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. J Mol Endocrinol 19:207-226.

Lee E, Jeon SH, Yi JY, Jin YJ & Son YS (2001) Calcipotriol inhibits autocrine phosphorylation of EGF receptor in a calcium-dependent manner, a possible mechanism for its inhibition of cell proliferation and stimulation of cell differentiation. Biochem Biophys Res Commun 284: 419-425.

Lemire J (2000) 1,25-Dihydroxyvitamin D_3 a hormone with immunomodulatory properties. Rheumatol 59, Suppl 1: 24-27.

Leng X, Tsai SY, O'Malley BW & Tsai MJ (1993) Ligand-dependent conformational changes in thyroid hormone and retinoic acid receptors are potentially enhanced by heterodimerization with retinoic X receptor. J Steroid Biochem Mol Biol 46: 643-661.

Leo C & Chen JD (2000) The SRC family of nuclear receptor coactivators. Gene 245: 1-11.

Li Q & Wrange O (1995) The accessibility of a glucocorticoid response element dependent on its rotational positioning. Mol Cell Biol 15: 4375-4384.

Li YC, Pirro AE, Amling M, Delling G, Baron R, Bronson R & Demay MB (1997) Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia. Proc Natl Acad Sci USA 94: 9831-9835.

Li YC, Bolt MJ, Cao LP & Sitrin MD (2001) Effects of vitamin D receptor inactivation on the expression of calbindings and calcium metabolism. Am J Physiol Endocrinol Metab 281: 558-564.

Lin BC, Hong SH, Krig S, Yoh SM & Privalsky ML (1998) A conformational switch in nuclear hormone receptors is involved in coupling hormone binding to corepressor release. Mol Cell Biol 18: 7603-7608.

Liu M, Lee M-H, Cohen M, Bommakanti M & Freedman LP (1996) Transcriptional activation of the cdk inhibitor p21 by vitamin D_3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes & Dev 10: 142-153.

Liu YY, Nguyen C, Ali Gradezi SA, Schnirer I & Peleg S (2001) Differential regulation of heterodimerization by 1α,25-dihydroxyvitamin D_3 and its 20-epi analog. Steroids 66: 203-212.

Liu YY, Collins ED, Norman AW & Peleg S (1997) Differential interaction of 1α,25-dihydroxyvitamin D_3 analogs and their 20-epi homologues with the vitamin D receptor. J Biol Chem 272: 3336-3345.

Luo Z, Rouvinen J & Mäenpää PH (1994) A peptide C-terminal to the second Zn finger of human vitamin D receptor is able to specify nuclear localization. Eur J Biochem 223: 381-387.

Lusi BF, Xu WX, Otwinowski Z, Freedmann LP, Yamamoto KR & Sigler PB (1991) Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature 352: 497-505.

Lyakhovich A, Aksenov N, Miettinen S, Ahonen MH, Syvälä H, Ylikomi T & Tuohimaa P (2000) Vitamin D induced up-regulation of keratinocyte growth factor (FGF-7/KGF) in MCF-7 human breast cancer cells. Biochem Biophys Res Commun 273: 675-680.

MacDonald PN, Haussler CA, Terpening CM, Galligan MA, Reeder MC, Whitfied GK & Haussler MR (1991) Baculovirus-mediated expression of the human vitamin D receptor. Functional characterization, vitamin D response element interactions, and evidence for a receptor auxiliary factor. J Biol Chem 266: 18808-18813.

McDonnell DP, Mangelsdorf DJ, Pike JW, Haussler MR & O'Malley BW (1987) Molecular cloning of complementary DNA encoding the avian receptor for vitamin D. Science 235: 1214-1217.

Mackey SL, Heymont JL, Kronenberg HM & Demay MB (1996) Vitamin D receptor binding to the negative human parathyroid hormone vitamin D response element does not require the retinoid X receptor. Mol Endocrinol 10: 288-305.

Mak HY, Hoare S, Henttu PM & Parker MG (1999) Molecular determinants of the estrogen receptor-coactivator interface. Mol Cell Biol 19: 3895-3903.

Makin G, Lohnes D, Byford V, Ray R & Jones G (1989) Target cell metabolism of 1,25-dihydroxyvitamin D_3 to calcitroic acid. Evidence for a pathway in kidney and bone involving 24-oxidation. Biochem J 262: 173-180.

Malloy PJ, Pike JW & Feldman D (1999) The vitamin D receptor and the syndrome of hereditary 1,25-dihydroxyvitamin D-resistant rickets. Endocr Rev 20: 156-188.

Malloy PJ & Feldman D (1999) Vitamin D resistance. Am J Med 106: 355-370.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P & Evans RM (1995) The nuclear receptor superfamily: the second decade. Cell 83: 835-839.

Mangelsdorf DJ & Evans RM (1995) The RXR heterodimers and orphan receptors. Cell 83: 841-850.

Mantell, DJ, Owens PE, Bundred NJ, Mawer EB & Canfield AE (2000) 1α,25-Dihydroxyvitamin D_3 inhibits angiogenesis *in vitro* and *in vivo*. Circ Res 87: 214-220.

Martin KJ, Gonzalez EA, Gellens M, Hamm LL, Abboud H & Lindberg J (1998) 19-Nor-1-α-25-dihydroxyvitamin D_2 (Paricalcitol) safely and effectively reduces the levels of intact parathyroid hormone in patients on hemodialysis. J Am Soc Nephrol 9: 1427-1432.

Masuyama H & MacDonald PN (1997) Proteasome-mediated degradation of the vitamin D receptor (VDR) and a putative role for SUG1 interaction with the AF-2 domain of VDR. J Cell Biochem 71: 429-440.

Mawer EB, Walls J, Howell A, Davies M, Ratcliffe W & Bundred NJ (1997) Serum 1,25-Dihydroxyvitamin D may be related inversely to disease activity in breast cancer patients with bone metastases. J Clin Endocrinol Metab 82: 118-122.

McKenna NJ, Xu J, Nawaz Z, Tsai SY, Tsai MJ & O'Malley BW (1999) Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions. J Steroid Biochem Mol Biol 69: 3-12.

Miyamoto K, Kesterson RA, Yamamoto H, Taketani Y, Nishiwaki E, Tatsumi S, Inoue Y, Morita K, Takeda E & Pike JW (1997) Structural organization of the human vitamin D receptor chromosomal gene and its promoter. Mol Endocrinol 11: 1165-1179.

Moras D & Gronemeyer H (1998) The nuclear receptor lingand-binding domain: structure and function. Curr. Opin. Cell Biol 10: 384-391.

Mottershead DG, Polly P, Lyons RJ, Sutherland RL & Watts CK (1996) High activity, soluble, bacterially expressed human vitamin D receptor and its ligand binding domain. J Cell Biochem 61: 325-337.

Murayama A, Takeyama K, Kitanaka S, Kodera Y, Kawaguchi Y, Hosoya T & Kato S (1999) Positive and negative regulations of the renal 25-hydroxyvitamin D_3 1α-hydroxylase gene by parathyroid hormone, calcitonin, and $1\alpha,25(OH)_2D_3$ in intact animals. Endocrinology 140: 2224-2231.

Nagy L, Kao HY, Love JD, Li C, Banayo E, Gooch JT, Krishna V, Chatterjee K, Evans RM & Schwabe JW (1999) Mechanism of corepressor binding and release from nuclear hormone receptors. Genes Dev 13: 3209-3216.

Nakajima S, Hsieh J-C, MacDonal PN, Haussler CA, Galligan MA, Jurutka PW & Haussler MR (1993) Purified human vitamin D receptor overexpressed in E. coli and baculovirus systems does not bind 1,25-dihydroxyvitamin D_3 hormone efficiently unless supplemented with a rat liver nuclear extract. Biochem Biophys Res Comm 197: 478-485.

Narvaez CJ, Zinser G & Welsh J (2001) Functions of 1α,25-dihydroxyvitamin D_3 in mammary gland: from normal development to breast cancer. Steroids 66: 301-308.

Nayeri S & Carlberg C (1997) Functional conformations of the nuclear 1α,25-dihydroxyvitamin D_3 receptor. Biochem J 327: 561-568.

Nemer I, Schwartz Z, Pedrozo H, Sylvia VL, Dean DD & Boyan BD (1998) Identification of a membrane receptor for 1,25-dihydroxyvitamin D_3 which mediates rapid activation of protein kinase C. J Bone Miner Res 13: 1353- 1359.

Nickerson T & Huynh H (1999) Vitamin D analogue EB 1089-induced prostate regression is associated with increased gene expression of insulin-like growth factor binding proteins. J Endocrinol 160: 223-229.

Nishikawa J, Kitaura M, Imagawa M & Nishihara T (1995) Vitamin D receptor contains multiple dimerization interfaces that are functionally different. Nucleic Acids Res 23: 606-611.

Nolte RT, Wisely GB, Westin S, Cobb JE, Lambert MH, Kurokawa R, Rosenfeld MG, Willson TM, Glass CK & Milburn MV (1998) Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. Nature 395: 137-143.

Nomanbhoy TK, Leonard DA, Manor D & Cerione RA (1996) Investigation of the GTP-binding/GTPase cycle of Cdc42Hs using extrinsic reporter group fluorescence. Biochemistry 35: 4602-4608.

Norman AW (1995) The vitamin D Endocrine system: manipulation of structure-function relationships to provide opportunities for development of new cancer chemopreventive and immunosuppressive agents. J Cell Biochem Suppl 22: 218-225.

Norman AW, Song X-D, Zanello LP, Bula CM & Okamura WH (1999) Rapid and genomic biological responses are mediated by different shapes of the agonist steroid hormone 1α,25(OH)$_2$-vitamin D$_3$. Steroids 64: 120-128.

Norman AW, Ishizuka S & Okamura WH (2001) Ligands for the vitamin D endocrine system: different shapes function as agonists and antagonists for genomic and rapid response receptors or as a ligand for the plasma vitamin D binding protein. J Steroid Biochem Mol Biol 76: 49-59.

Näär AM, Lemon BD & Tjian R (2001) Transcriptional coactivator complexes. Annu Rev Biochem 70: 475-501.

Ogryzko VV, Schlitz RL, Russanova V, Howard BH & Nakatani Y (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 87: 953-959.

Ohyama Y, Ozono K, Uchida M, Shinki T, Kato S, Suda T, Yamamoto O, Noshiro M & Kato Y (1994) Identification of a vitamin D-responsive element in the 5'-flanking region of the rat 25-hydroxyvitamin D$_3$ 24-hydroxylase gene. J Biol Chem 269: 10545-10550.

O'Kelly J, Hisatake J, Hisatake Y, Bishop J, Norman A & Koeffer P (2002) Normal myelopoesis but abnormal T lymphocyte responses in vitamin D receptor knockout mice. J Clin Invest 109: 1091-1099.

Okuda KI (1994) Liver mitochondrial P450 involved in cholesterol catabolism and vitamin D activation. J Lipid Res 35: 361-372.

Okuda K, Usui E & Ohyama Y (1995) Recent progress in enzymology and molecular biology of enzymes involved in vitamin D metabolism. J Lipid Res 36: 1641-1652.

Park WH, Seol JG, Kim ES, Hyun JM, Jung CW, Lee CC, Binderup L, Koeffler HP, Kim BK & Lee YY (2000) Induction of apoptosis by vitamin D$_3$ analogue EB 1089 in NCI-H929 myeloma cells via activation of caspase and MAP kinase. Br J Haematology 109: 576-583.

Peleg S, Sastry M, Collins ED, Bishop JE & Norman AW (1995) Distinct conformational changes induced by 20-epi analogs of 1 α,25-dihydroxyvitamin D$_3$ are associated with enhanced activation of the vitamin D receptor. J Biol Chem 270: 10551-10558.

Peleg S, Qiu H, Reddy S, Harris D, Van Q, Estey EH, Talpaz M & Estrov Z (1997) 1,25-Dihydroxyvitamin D$_3$ and its analogs inhibit acute myelogenous leukemia progenitor proloferation by suppressing interleukin-1β production. J Cin Invest 100: 1716-1724.

Peleg S, Nguyen C, Woodard BT, Lee JK & Posner GH (1998) Differential use of transcription activation function 2 domain of the vitamin D receptor by 1,25-dihydroxyvitamin D3 and its A ring-modified analogs. Mol Endocrinol 12: 525-535.

Peleg S & Reddy GS (2001) Tissue specific metabolism of 1α,25-dihydroxy-20-epi-vitamin D$_3$ into new metabolites with significant biological activity: studies in rat osteosarcoma cells (UMR 106 and ROS 17/2.8). J Cell Biochem 82: 599-609.

Pike ACW, Brzozowski AM, Hubbard RE, Bonn T, Thorsell A-G, Engström O, Ljunggren J, Gustafsson J-A & Carlquist M (1999) Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. EMBO J 18: 4608-4618.

Pirianov G & Colston KW (2001) Interactions of vitamin D analogue CB1093, TNFα and ceramide on breast cancer cell apoptosis. Mol Cell Endocrinol 172: 69-78.

Polly P, Herdick M, Moehren U, Baniahmad A, Heinzel T & Carlberg C (2000) VDR-Alien: a novel, DNA-selective vitamin D$_3$ receptor-corepressor partnership. FASEB J 14: 1455-1463.

Quack M, Szafranski K, Rouvinen J & Carlberg C (1998) The role of the T-box for the function of vitamin D receptor on different types of response elements. Nuc Acids Res 26: 5372-5378.

Quack M & Carlberg C (1999) Selective recognition of vitamin D receptor conformations mediates promoter selectivity of vitamin D analogs. Mol Pharmacol 55: 1077-1087.

Rachez C, Suldan z, Ward J, Chang CP, Burakov D, Erdjument-Bromage H, Tempst P & Freedman LP (1998) A novel protein complex that interacts with the vitamin D$_3$ receptor in a ligand-dependent manner and enhances VDR transactivation in a cell-free system. Genes Dev 12: 1787-1800.

Rachez C & Freedman LP (2000) Mechanisms of gene regulation by vitamin D$_3$ receptor: a network of coactivator interactions. Gene 246: 9-21.

Rastinejad F, Perlmann T, Evans RM & Sigler P B (1995) Structural determinants of nuclear receptor assembly on DNA direct repeats. Nature 375: 203-211.

Rastinejad F, Wagner T, Zhao Q & Khorasanizadeh S (2000) Structure of the RXR-RAR DNA-binding complex on the retinoic acid response element DR1. EMBO J 19: 1045-1054.

Rastinejad F (2001) Retinoid X receptor and its partners in the nuclear receptor family. Curr Opin Struct Biol 11: 33-38.

Raval-Pandya M, Freedman LP, Li H & Christakos S (1998) Thyroid hormone receptor does not heterodimerize with the vitamin D receptor but represses vitamin D receptor-mediated transactivation. Mol Endocrinol 12: 1367-1379.

Reichrath J, Muller SM, Kerber A, Baum HP & Bahmer (1997) Biologic effects of topical calcipotriol (MC 903) treatment in psoriatic skin. J Am Acad Dermatol 36: 19-28.

Reichrath J (2001) Will analogs of 1,25-dihydroxyvitamin D_3 (calcitriol) open a new era in cancer therapy? Onkologie 24: 128-133.

Renaud JP, Rochel N, Ruff M, Vivat V, Chambon P, Gronemeyer H & Moras D (1995) Crystal structure of the RAR-γ ligand-binding domain bound to all-trans retinoic acid. Nature 378: 681-689.

Riachy R, Vandewalle B, Belaich S, Kerr-Conte J, Gmyr V, Zerimech F, d'Herbomez M, Lefebvre J & Pattou F (2001) Beneficial effect of 1,25 dihydroxyvitamin D_3 on cytokine-treated human pancreatic islets. J Endocrinol 169: 161-168.

Rhodes SJ, Chen R, DiMattia GE, Scully KM, Kalla KA, Lin S-C, Yu VC & Rosenfeld MG (1993) A tissue-specific enhancer confers Pit-1-dependent morphgen inducibility and autoregulation on the *pit-1* gene. Genes Dev 7: 913-932.

Rochel N, Wurtz JM, Mitschler A, Klaholz B & Moras D (2000) The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. Mol Cell 5: 173-179.

Rosenfeld MG & Glass CK (2001) The coregulator exchange in transcriptional functions of nuclear receptors. J Biol Chem 276: 36865–36868.

Rozen F & Pollak M (1999) Inhibition of insulin-like growth factor I receptor signaling by the vitamin D analogue EB1089 in MCF-7 breast cancer cells: A role for insulin-like growth factor binding proteins. Int J Oncol 15: 589-594.

Sack JS, Kish KF, Wang C, Attar RM, Kiefer SE, An Y, Wu GY, Scheffler JE, Salvati ME, Krystek SR Jr, Weinmann R & Einspahr HM (2001) Crystallographic structures of the ligand-binding domains of the androgen receptor and its T877A mutant complexed with the natural agonist dihydrotestosterone. Proc Natl Acad Sci USA 98: 4904-4909.

Sakai Y & Demay MB (2000) Evaluation of keratinocyte proliferation and differentation in vitamin D receptor knockout mice. Endocrinology 141: 2043-2049.

Sakai Y, Kishimoto J & Demay MB (2001) Metabolic and cellular analysis of alopecia in vitamin D receptor knockout mice. J Clin Invest 107: 961-966.

Schaefer-Klein J, Londowski JM & Kumar R (1993) Bacterial synthesis of truncated forms of the human vitamin D receptor and characterization of anti-receptor monoclonal antibodies. Biochem
Biophys Res Comm 196: 167-172.

Schioth HB, Kuusinen A, Muceniece R, Szardenings M, Keinänen K & Wikberg JE (1996) Expression of functional melanocortin 1 receptors in insect cells. Biochem Biophys Res Commun 221: 807-814.

Schräder M, Nayeri S, Kahlen J-P, Müller KM & Carlberg C (1995) Natural vitamin D_3 response elements formed by inverted palindromes: Polarity-directed ligand sensitivity of VDR-RXR heterodimer-mediated transactivation. Mol Cell Biol 15: 1154-1161.

Schwabe JW, Chapman L, Finch JT & Rhodes D (1993) The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. Cell 75: 567-578.

Shaffer PL & Gewirth DT (2002) Structural basis of VDR-DNA interactions on direct repeat response elements. EMBO J 21: 2242-2252.

Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA & Greene GL (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95: 927-937.

Shikama N, Lyon J & La Thangue BN (1998) The p300/CBP family: integrating signals with trancription factors and chromatin. Trends Cell Biol 7: 230-236.

Shinki T, Hin CH, Nishimura A, Nagai Y, Ohyama Y, Noshiro M, Okuda K & Suda T (1992) Parathyroid hormone inhibits 25-hydroxyvitamin D_3-24-hydroxylase mRNA expression stimulated by 1α,25-dihydroxyvitamin D_3 in rat kidney but not in intestine. J Biol Chem 276: 13757-13762.

Shinki T, Shimada H, Wakino S, Anazawa H, Hayashi M, Saruta T, DeLuca HF & Suda T (1997) Cloning and expression of rat 25-hydroxyvitamin D_3-1α-hydroxylase cDNA. Proc Natal Acad Sci USA 93: 8884-8888.

Sitrin MD, Bissonnette MJG, Bolt R, Wali S, Khare B, Scaglione-Sewell B, Sakrosi S & Brasitus TA (1999) Rapid effects of $1,25(OH)_2$ vitamin D_3 on signal transduction systems in colonic cells. Steroids 64: 137-142.

Siu-Caldera ML, Rao DS, Astecker N, Weiskopf A, Vouros P, Konno K, Fujishima T, Takayama H, Peleg S, Sastry M, Collins ED, Bishop JE & Norman AW (1995) Distinct conformational changes induced by 20-epi analogs of 1α,25-dihydroxyvitamin D_3 are associated with enhanced activation of the vitamin D receptor. J Biol Chem 270: 10551-10558.

Sone T, McDonnell DP, O'Malley BW & Pike JW (1990) Expression of human vitamin D receptor in *Saccharomyces cerevisiae*. Purification, properties, and generation of polyclonal antibodies. J Biol Chem 265: 21997-22003.

Steddon SJ, Schroeder NJ & Cunningham J (2001) Vitamin D analogs: how do they differ and what is their clinical role? Nephrol Dial Transplant 16: 1965-1967.

Sterner DE & Berger SL (2000) Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev 64: 435-459.

Sudarsanam P & Winston F (2000) The Swi/Snf family nucleosomae-remodelling complexes and transcriptional control. Trends in Gen 16: 345-350.

Svergun DI, Semenyuk AV & Feigin LA (1988) Small angle data scattering treatment by the regularization method. Acta Crystallogr A44: 244-250.

Svergun D, Barberato C & Koch MHJ (1995) CRYSOL a program to evaluate X-ray solution scattering of biological macromolecules from atomic coordinates. J Appl Cryst 28: 768-773.

Svergun DI, Richard S, Koch MHJ, Sayers Z, Kuprin S & Zaccai G (1998) Protein hydration in solution: experimental obsevation by X-ray and neutron scattering. Proc Natal Acad Sci USA 95: 2267-2272.

Syntichaki P, Topalidou I & Thireos G (2000) The Gcn5 bromodomain co-ordinates nucleosome remodelling. Nature 404: 414-417.

Tagami T, Lutz WH, Kumar R & Jameson JL (1998) The interaction of the vitamin D receptor with nuclear receptor corepressors and coactivators. Biochem Biophys Res Commun 253: 358-363.

Takeda S, Yoshizawa T, Nagai Y, Yamato H, Fukumoto S, Sekine K, Kato S, Matsumoto T & Fujita T (1999) Stimulation of osteoclast formation by 1,25-dihydroxyvitamin D requires its binding to vitamin D receptor (VDR) in osteoblastic cells: studies using VDR knockout mice. Endocrinology 140: 1005-1008.

Takeshita A, Ozawa Y & Chin WW (2000) Nuclear receptor coactivators facilitate vitamin D receptor homodimer action on direct repeat hormone response elements. Endocrinology 141: 1281-1284.

Takeyama K, Kitanaka S, Sato T, Kobori M, Yanagisawa J & Kato S (1997) 25-Hydroxyvitamin D_3 1α-hydroxylase and vitamin D synthesis. Science 277: 1827-1830.

Thompson PD, Jurutka PW, Haussler CA, Whitfield GK & Haussler MR (1998) Heterodimeric DNA binding by the vitamin D receptor and retinoid X receptors is enhanced by 1,25-dihydroxyvitamin D_3 and inhibited by 9-*cis*-retinoic acid. Evidence for allosteric receptor interactions. J Biol Chem 273: 8483-8491.

Tocchini-Valentini G, Rochel N, Wurtz JM, Mitschler A & Moras D (2001) Crystal structures of the vitamin D receptor complexed to superagonist 20-epi ligands. Proc Natl Acad Sci USA 98: 5491-5496.

Toell A, Gontzales MM, Ruf D, Steinmeyer A, Ishizuka S & Carlberg C (2001) Different molecular mechanisms of vitamin D_3 receptor antagonists. Mol Pharmacol 59: 1478-1485.

Towers TL, Luisi BF, Asianov A & Freedman LP (1993) DNA target selectivity by the vitamin D_3 receptor: mechanism of dimer binding to an asymmetric repeat element. Proc Natl Acad Sci USA 90: 6310-6314.

Towers TL & Freedman LP (1998) Granulocyte-macrophage colony-stimulating factor gene transcription is directly repressed by the vitamin D_3 receptor. Implications for allosteric influences on nuclear receptor structure and function by a DNA element. J Biol Chem 273: 10338-10348.

Towers TL, Staeva TP & Freedman LP (1999) A two-hit mechanism for vitamin D_3-mediated transcriptional repression of the granulocyte-macrophage colony-stimulating factor gene: vitamin D receptor competes for DNA binding with NFAT1 and stabilizes c-Jun. Mol Cell Biol 19: 4191-4199.

Tse C, Sera T, Wolffe AP & Hansen JC (1998) Disruption of higher order folding by core histone acetylation dramatically enhances transcription of nucleosomal arrays by RNA polymerase III. Mol Cell Biol 18: 4629-4638.

Umesono K, Murakami KK, Thompson CC & Evans RM (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D_3 receptors. Cell 65: 1255-1266.

Urnov FD & Wolffe AP (2001) Chromatin remodeling and transcriptional activation: the cast (in order of appearance). Oncogene 20: 2991-3006.

van De Kerkhof PC (2001) New developments in the treatment of psoriasis. Skin Pharmacol Appl Skin Physiol 14: 129-135.

Varga-Weisz P (2001) ATP-dependent chromatin remodeling factors: Nucleosome shufflers with many missions. Oncogene 20: 3076-3085.

Verlinden L, Verstuyf A, Convents R, Marcelis S, van Camp M & Bouillon R (1998) Action of $1,25(OH)_2D_3$ on the cell cycle genes, cyclin D1, p21 and p27 in MCF-7 cells. Mol Cell Endocrinol 142: 57-65.

Verstuyf A, Segaert S, Verlinden L, Bouillon R & Mathieu C (2000) Recent developments in the use of vitamin D analogs. Expert Opin Investig Drugs 9: 443-455.

Verstuyf A, Verlinden L, Segaert S, Van Etten E, Mathieu C & Bouillon R (1999) Nonclassical effects of $1\alpha,25$-dihydroxyvitamin D_3 and its analogs. Miner Electrolyte Metab 25: 345-348.

Vieth R (1990) The mechanisms of vitamin D toxicity. Bone Miner 11: 267-272.

Vihko P, Kurkela R, Porvari K, Herrala A, Lindfors A, Lindqvist Y & Schneider G (1993) Rat acid phosphatase: overexpression of active, secreted enzyme by recombinant baculovirusinfected insect cells, molecular properties, and crystallization. Proc Natal Acad Sci USA 90: 799-803.

Wagner RL, Apriletti JW, McGrath ME, West BL, Baxter JD & Fletterick RJ (1995) A structural role for hormone in the thyroid hormone receptor. Nature 378: 690-697.

Wallberg AE, Wright A & Gustafsson JA (2000) Chromatin-remodeling complexes involved in gene activation by the glucocorticoid receptor. Vitam Horm 60: 75-122.

Wasserman RH & Fullmer CS (1995) Vitamin D and intestinal calcium transport: facts, speculations and hypotheses. J Nutr 125: 1971-1979.

Weatherman RV, Fletterick RJ & Scanlan TS (1999) Nuclear-receptor ligands and ligand-binding domains. Annu Rev Biochem 68: 559-581.

Weisinger JR, Favus MJ, Langman CB & Bushinsky DA (1989) Regulation of 1,25-dihydroxyvitamin D_3 by calcium in the paratyroidectomized, parathyroid hormone-replete rat. J Bone Miner Res 4: 929-935.

Wen YD, Perissi V, Staszewski LM, Yang WM, Krones A, Glass CK, Rosenfeld MG & Seto E (2000) The histone deacetylase-3 complex contains nuclear receptor corepressors. Proc Natl Acad Sci U S A 97: 7202-7207.

Wikvall K (2001) Cytochrome P450 enzymes in the bioactivation of vitamin D to its hormonal form. Int J Mol Med 2: 201-209.

Williams SP & Sigler PB (1998) Atomic structure of progesterone complexed with its receptor. Nature 393: 392-396.

Wisely RT, Westin GB, Cobb S, Lambert JE, Kurokawa MH, Rosenfeld R, Willson MG, Glass CK & Milburn MV (1998). Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. Nature 395: 137-143.

Wolffe AP & Hayes JJ (1999) Chromatin disruption and modification. Nucleic Acids Res 27: 711-720.

Wong J, Li Q, Levi B-Z, Shi Y-B & Wolffe AP (1997) Structural and functional features of a specific nucleosome containing a recognition element for the thyroid hormone receptor. EMBO J 16: 7130-7145.

Wu J & Grunstein M (2000) 25 years after the nucleosome model: chromatin modifications. Trends Biochem Sci 5: 619-623.

Wu S, Finch M, Zhong E, Slatopolsky M, Grieff M & Brown AJ (1996) Expression of renal 25-hydroxyvitamin D-24-hydroxylase gene: regulation by dietary phosphate. AM J Physiol 271: 203-208.

Wu Y, Craig TA, Lutz WH & Kumar R (1999) Identification of 1α,25-dihydroxyvitamin D_3 response elements in the human transforming growth factor beta 2 gene. Biochemistry 38: 2654-2660.

Xiao JH, Davidson I, Matthes H, Garnier JM & Chambon P (1991) Cloning, expression, and transcriptional properties of the human enhancer factor TEF-1. Cell 65: 551-568.

Yang X-J, Ogryzko VV, Nishikawa J, Howard BH & Nakatani Y (1996) A p300/CBP-associated factor that competes with the adenoviral E1A oncoprotein. Nature 382: 319-324.

Yang W & Freedman LP (1999) 20-Epi analogs of 1,25-dihydroxyvitamin D_3 are highly potent inducers of DRIP coactivator complex binding to the vitamin D_3 receptor. J Biol Chem 274: 16838-16845.

Yao TP, Ku G, Zhou N, Scully R & Livingston DM (1996) The nuclear hormone receptor coactivator SRC-1 is a specific target of p300. Proc Natl Acad Sci USA 93: 10626-10631.

Yoh SM & Privalsky ML (2001) Transcriptional repression by thyroid hormone receptors. A role for receptor homodimers in the recruitment of SMRT corepressor. J Biol Chem 276: 16857-16867.

Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T & Kato S (1997) Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. Nat Genet 16: 391-396.

Yudoh K, Matsui H & Tsuji H (1997) Effects of 1,25-Dihydroxyvitamin D_3 on tumor cell invasion to the extracellular matrix in human fibrosarcoma HT1080 cells and its correlation with laminin. Tumour Biol 18: 69-79.

Zhao XY, Eccleshall TR, Krishanna AV, Gross C & Feldman D (1997) Analysis of vitamin D analog-induced heterodimeization of vitamin D receptor with retinoid X receptor using yeast two-hybrid system. Mol Endocrinol 11: 366-378.

Zhao Q, Chasse SA, Devarakonda S, Sierk ML, Ahvazi B & Rastinejad FJ (2000) Structural basis of RXR-DNA interactions. Mol Biol 296: 509-520.

Zhou XF, Shen XQ & Shemshedini L (1999) Ligand-activated retinoic acid receptor inhibits AP-1 transactivation by disrupting c-Jun/c-Fos dimerization. Mol Endocrinol 13: 276-285.

Zinser G, Packman K & Welsh J (2002) Vitamin D_3 receptor ablation alters mammary gland morphogenesis. Development 129: 3067-3076.