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INSIGHTS INTO THE BIOCHEMICAL LIFE CYCLE OF THE VITAMIN D RECEPTOR: PROTEIN AND DNA INTERACTIONS THAT TRANSDUCE THE SIGNAL FOR GENE EXPRESSION

by

Carlos Encinas Dominguez

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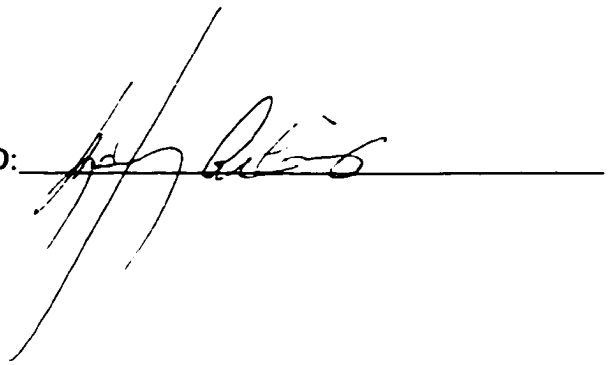
SIGNED: 

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DEDICATION

This dissertation is dedicated to my family and friends, whose love and support have helped make this dream possible. A special dedication is extended to my parents, Raul and Vicky for showing me to enjoy life to the fullest and helping me to follow my bliss.

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ABSTRACT

The biological actions of 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) are mediated by the nuclear vitamin D receptor (VDR), which functions as a ligand-dependent transcriptional regulator. We have developed a six-stage molecular model summarizing the VDR transcriptional activation-life cycle, and tested this model using a variety of experimental approaches, including pull-down assays with GST-fusion proteins, as well as assays of the functional activity of VDR and its putative coactivators in transiently transfected mammalian cells. The six stages of the VDR life cycle are: 1) unoccupied VDR binds to a transcriptional corepressor which serves as a chaperone, maintaining the main protein players in close contact in an inactive complex; 2) VDR becomes occupied by $1,25(OH)_2D_3$ ligand, enabling the receptor to heterodimerize strongly with a retinoid X receptor (RXR), leading to high affinity DNA binding and recruitment of coactivators with histone acetyl transferase (HAT) activity; 3) coactivator HAT activity promotes chromatin remodeling, rendering the gene promoter free to interact with the transcription preinitiation complex (PIC); 4) dissociation of VDR from the HAT coactivators, followed by association of a second set of coactivators that promote formation of the preinitiation complex (PIC); 5) transcriptional recycling of the liganded receptor and heteropartner to initiate additional rounds of transcription; and 6) ubiquitination and eventual degradation of VDR. Phosphorylation of VDR may influence all six stages. A testable conclusion from our model is that the role of the $1,25(OH)_2D_3$ hormonal ligand would be primarily in the transition from stage 1 to stage 2, but the continued presence of $1,25(OH)_2D_3$ appears to be necessary also for the

progression from stages 3 through 6 to VDR degradation, or alternatively for recycling via stage 5. This characterization of the macromolecular cofactors that transduce the signal of the $1,25(OH)_2D_3$ hormone to promote gene expression in vitamin D target tissues should add to our understanding of endocrine control of bone mineral remodeling and of epithelial cell differentiation. The present work also identifies new protein players that are candidates for mutation or dysregulation in the pathophysiology of vitamin D resistant bone disorders (osteoporosis), and in hyperproliferative diseases of vitamin D regulated epithelial tissues such as skin.

CHAPTER I. INTRODUCTION

The physiological and pathological roles of vitamin D

The traditional role of vitamin D, via its 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) hormonal metabolite, is to effect calcium and phosphate homeostasis in the body, ensuring that the concentration of these ions in the system is sufficient to promote normal bone mineralization; failure to achieve normal bone mineral accretion leads to rachitic syndromes. As illustrated in Fig. 1, this control of Ca^{+2} and PO_4^{-3} ion levels is executed by the $1,25(OH)_2D_3$ hormone, which acts by stimulating absorption of these ions from intestine, facilitating resorption from bone and increasing reabsorption at the kidney, thereby controlling their overall concentration in blood (Haussler *et al.* 1998).

The actions of the $1,25(OH)_2D_3$ hormone are not limited to calcium/phosphate translocating tissues, since the ligand elicits activities in several other tissues including skin, muscle and the immune system, indicating that the role of $1,25(OH)_2D_3$ and its receptor extend beyond the scope of calcemic and phosphatemic effects (MacDonald *et al.* 1995).

As shown in Fig. 1, there are two ways in which vitamin D can be acquired by the body. It can be absorbed from the diet or it can also be derived from sunlight-initiated photobiogenesis in skin (Holick 1996). From both of these sources, vitamin D_3 diffuses into the circulatory system and is then carried on the serum vitamin D binding protein (DBP) to the liver for 25-hydroxylation, and subsequently to the kidney to be hydroxylated at the number one carbon and converted to its known active form, 1α,25-dihydroxyvitamin D_3 (Haussler *et al.* 1998) (Fig. 1). Simultaneous deprivation of

dietary vitamin D and lack of sunlight exposure are therefore required to elicit nutritional rickets (Holick 1996). There also exist observed genetic mutations in the metabolic pathway for $1,25(OH)_2D_3$ production, as well as in genes that encode proteins which mediate vitamin D actions at the target tissue level. Each of these defects in vitamin D metabolism or function at the target cells cause the pathological phenotype of vitamin D-resistant rachitic syndromes (blue boxes in Fig. 1)

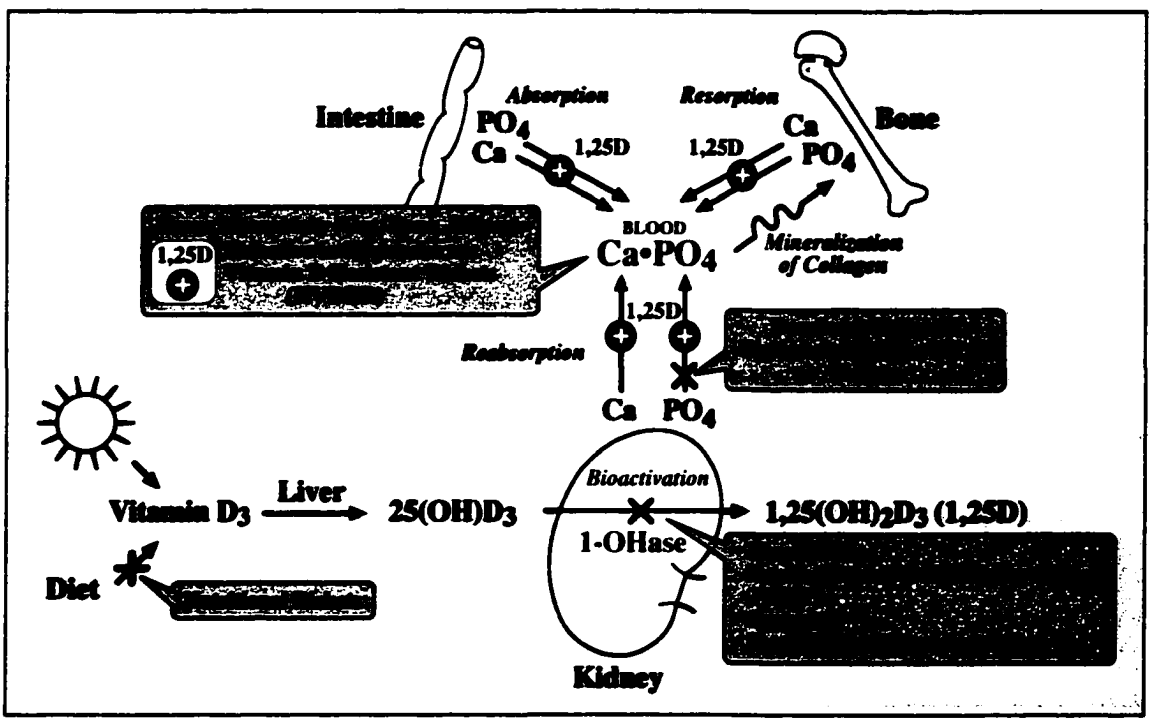


Fig. 1. Biosynthesis of $1,25(OH)_2D_3$ and its actions on intestine, bone and kidney. White plus signs on a red background signify the actions of $1,25(OH)_2D_3$ (1,25D) and blue boxes indicate nutritional, metabolic and signal transduction defects that interrupt the acquisition, bioactivation or tissue functions of vitamin D.

A classical example of vitamin D-resistance is insufficiency of the 1α-hydroxylase (1α-OHase) enzyme, either resulting from acquired chronic renal failure or hereditary defects (Haussler and McCain 1977). Chronic renal failure reduces renal

mass, compromising 1α-OHase activity and causing renal rickets and secondary hyperparathyroidism. Pseudo-vitamin D-deficiency rickets (PDDR) involves inactivating mutations in the gene coding for the 1α-OHase enzyme that prevent 25-hydroxyvitamin D_3, the intermediary metabolite, from being hydroxylated to $1,25(OH)_2D_3$ and becoming active (Haussler *et al.* 1998).

The biological actions of $1,25(OH)_2D_3$ are mediated at the molecular level by the nuclear vitamin D receptor (VDR), a member of a superfamily of zinc finger transcription factors that regulate gene expression in a ligand-dependent manner (Fig. 2) (Evans 1988; Haussler *et al.* 1998).

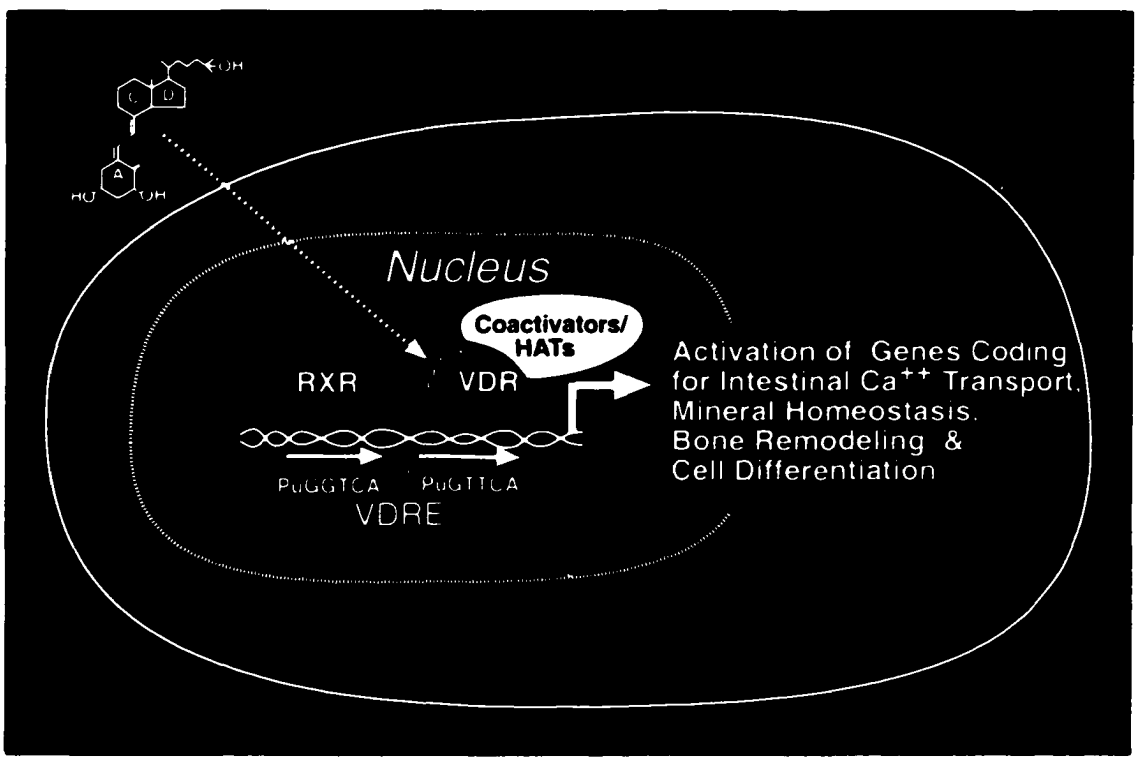


Fig. 2. Genomic activation by $1,25(OH)_2D_3$ in target tissues. The molecular actions of 1,25D hormone are mediated by its nuclear receptor, VDR. Once liganded, VDR controls transcription of target genes, either inducing or repressing (not shown) expression.

Genetic defects in VDR can confer $1,25(OH)_2D_3$ insensitivity, resulting in rachitic phenotypes characterized by severe bowing of lower extremities, short stature and alopecia. This autosomal recessive disorder is known as hereditary hypocalcemic vitamin D-resistant rickets (HVDRR; see also Fig. 1) (Rut *et al.* 1994; Whitfield *et al.* 1996; Malloy *et al.* 1999). The most intriguing aspect of HVDRR is the finding of alopecia in many patients, a novel disease phenotype not observed in vitamin D-deficiency or pseudo-vitamin D deficiency. This phenotypic distinction between VDR inactivation and $1,25(OH)_2D_3$ deficiency suggests a new and active role for VDR in skin and the hair cycle, independent of its endocrine ligand, $1,25(OH)_2D_3$ (Haussler *et al.* 2002).

The mechanism through which the $1,25(OH)_2D_3$-VDR complex influences calcium and phosphate homeostasis encompasses the induction of bone mineral ion translocating proteins in intestine and kidney (Taketani *et al.* 1997; Tenenhouse 1997). It has been observed in VDR null mice studies that these rodents display an HVDRR-like phenotype, and that rickets and secondary hyperparathyroidism can be prevented with a high calcium diet, suggesting a primary disorder in Ca transport from the intestine. Further analysis of these VDR knockout mice showed that the levels of transport proteins such as the calcium transport protein 1 (CaT1) and the epithelial calcium channel (ECaC) were considerably reduced, indicating that the expression of these transporters is highly dependent on $1,25(OH)_2D_3$ and its activation of the VDR protein (Van Cromphaut *et al.* 2001).

Hypophosphatemia induces transcription of the 1α-OHase enzyme (Fig. 3) to increase the levels of circulating $1,25(OH)_2D_3$ hormone, in turn, to promote PO_4 absorption. Oral phosphate plus $1,25(OH)_2D_3$ hormone administration can help reestablish PO_4 ion levels in patients with X-linked hypophosphatemic rickets (XHL), a dominant familial disorder of renal phosphate wasting (Drezner *et al.* 1980) (see Fig. 1). These findings support the role of $1,25(OH)_2D_3$-VDR complex in regulating PO_4 as well as Ca ion levels, both of which are ultimately required for bone mineralization.

Added to its role in controlling calcium and phosphate ion concentrations in the blood, the $1,25(OH)_2D_3$-VDR complex also controls the renal production of $1,25(OH)_2D_3$ by feedback regulatory loops (Fig. 3). The $1,25(OH)_2D_3$-VDR complex represses the transcription of 1α-OHase enzyme (Henry 1992), and also silences the synthesis of parathyroid hormone (PTH) (Brumbaugh *et al.* 1975), which is secreted under low blood calcium conditions and induces the production of 1α-OHase enzyme (Fig. 3).

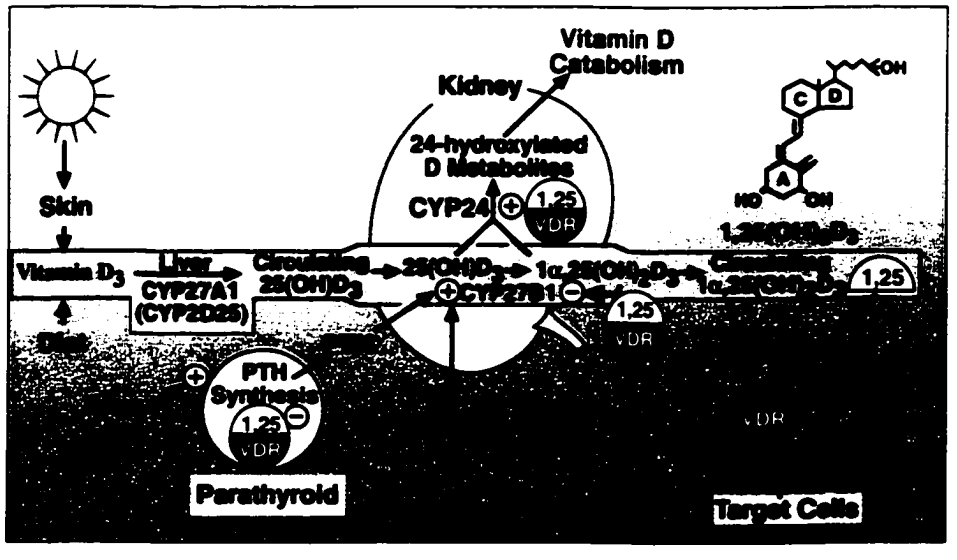


Fig. 3. Vitamin D acquisition, metabolism and regulation of synthesis/degradation of its active form.

Turnover of the active $1,25(OH)_2D_3$ metabolite is also a self-regulated process, involving the induction of the gene encoding the 24-hydroxylase (24-OHase) enzyme by the $1,25(OH)_2D_3$-VDR complex, an event characterized by 24-hydroxylation of either the $1,25(OH)_2D_3$ hormone or its $25(OH)D_3$ precursor (Fig. 3). This induction of the 24-OHase enzyme initiates the primary pathway for catabolic elimination of vitamin D metabolites (Reddy and Tserng 1989; Tomon *et al.* 1990). Failure to adequately catabolize $1,25(OH)_2D_3$ causes vitamin D intoxication, a condition characterized by high calcium levels, leading to calcified kidneys. An interesting observation is that 24-OHase null mice not only present with hypercalcemia and increased $1,25(OH)_2D_3$, but also defective intramembranous ossification, a bone developmental abnormality likely resulting from $1,25(OH)_2D_3$ excess (St-Arnaud *et al.* 1997; St-Arnaud *et al.* 2000).

Nuclear hormone receptors

The control of gene expression has long been a significant topic in molecular biology. Nuclear receptors are DNA binding proteins that, once they are bound to their cognate ligands, have crucial roles in cellular functions such as development, homeostasis and other biological processes in metazoic animals (Whitfield *et al.* 1999). Unlike hydrophilic hormones that bind to cell surface receptors, lipophilic hormones can diffuse through the cellular membrane and nuclear pores wherein hormone receptors transduce signals from glucocorticoids, mineralocorticoids, the sex steroids (estrogen, progesterone and androgen), thyroid hormones, and vitamins A and D (Olefsky 2001). Nuclear receptors, including the glucocorticoid receptor (GR), mineralocorticoid

receptor (MR), estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), thyroid hormone receptor (TR), all-*trans*-retinoic acid receptor (RAR), 9-*cis*-retinoic acid receptor (RXR) and vitamin D receptor (VDR), mediate hormonal responses by directly binding to DNA and regulating the transcription of target genes. These receptors have a similar structure, consisting primarily of a dual zinc finger-based DNA binding domain (DBD), nuclear localization signal(s), and a ligand or hormone binding domain (LBD) (Fig. 4). In addition, each receptor possesses multiple regions that participate directly in transcriptional activation (Beato *et al.* 1995). The LBD, localized in the protein's C-terminal half, selects the ligand or hormone for each specific receptor, leading to receptor activation and homodimerization or heterodimerization with other nuclear receptors, which increases the affinity and specificity for DNA binding (see below). The DBD, located toward the N terminus, mediates binding to DNA by direct interaction of the α-helical regions in the zinc finger domain with specific base pair sequences in the major groove of DNA. These sequences are localized in or near the promoter of controlled genes, and are organized as direct, inverted or everted repeats, as well as nonrepeat sequences, collectively known as hormone response elements (HREs). HREs constitute the target interaction sites for nuclear receptor heterodimers, homodimers, or monomers (Mangelsdorf and Evans 1995).

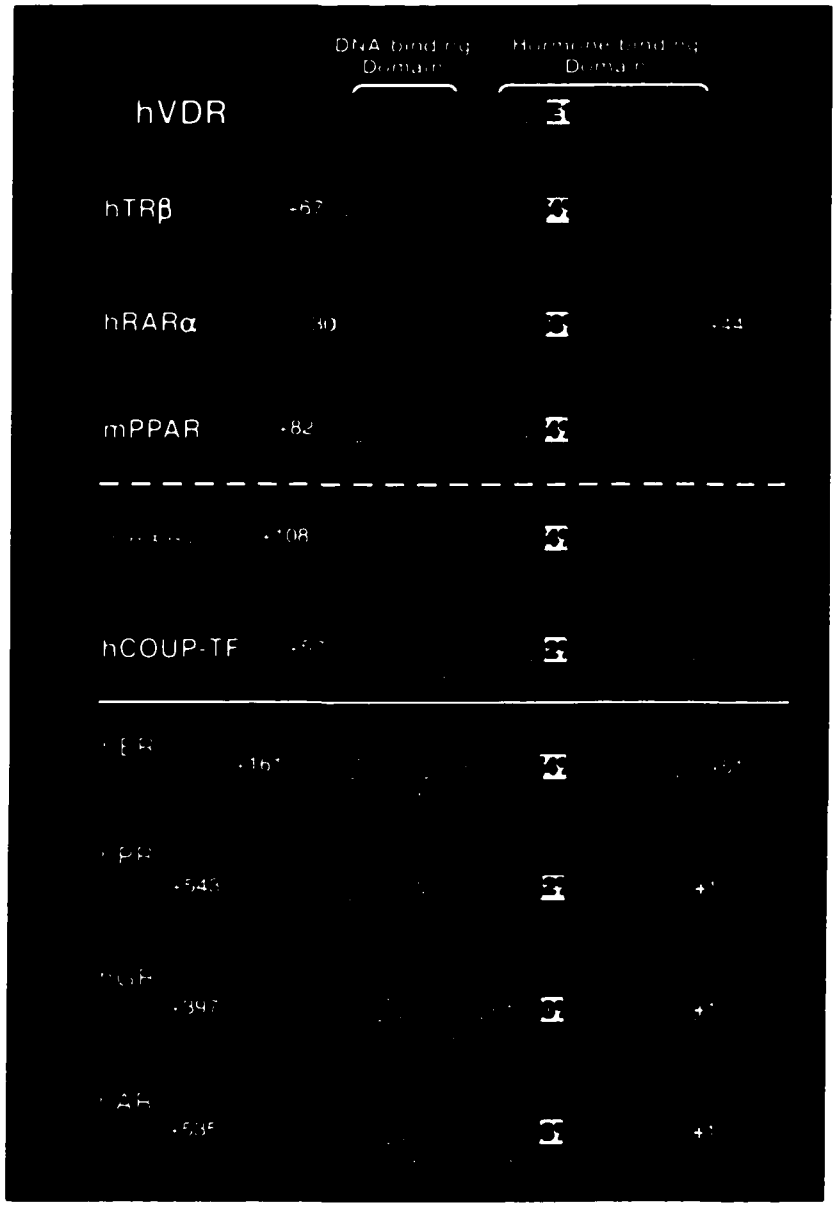


Fig. 4. Schematic model of the linear structure of several members of the nuclear receptor superfamily and homologies within key domains. The left most domain in the linear structures (dark gray) represents an N-terminal extension that some nuclear receptors possess, and the percentage numbers indicate the degree of homology to VDR. The blue band represents the Zn finger motif DNA binding domain. Following the DBD is the so called "hinge" region, including triangles in which the numbers depict a comparison with the number of residues found in VDR. The E1 region (yellow) is a conserved site that supports a dimerization interface located in the ligand binding domain (heptads, shown in red), followed by the activation function-2 (AF-2) at the C-terminus (not shown), and C-terminal extensions in dark gray, with the homology percentages imbedded in the diagram. mPPAR is mouse peroxisome proliferator activated receptor, and hCOUP-TF is the human homologue of the chicken ovalbumin upstream promoter-transcription factor.

Thus, as illustrated in Fig. 5, nuclear receptors function as homodimers or heterodimers, with each partner binding to specific HRE sequences that exist as half-sites separated by variable length nucleotide spacers between these direct or inverted half-site repeats (Olefsky 2001). Four categories of nuclear receptors have been proposed (Mangelsdorf *et al.* 1995), in which Class 1 consists of hormone receptors that bind as ligand-dependent homodimers on inverted repeats (Fig. 5, lower row). Class 2 receptors exist as heterodimers with RXR, and also function in a ligand-dependent manner to bind direct repeats (DRs) with various length bp spacers, i.e., DR1, DR2, DR3, DR4, DR5 (Fig. 5, upper row). Class 3 includes homodimeric receptors that bind to direct HRE repeats (i.e., RXR or COUP-TF shown in Fig. 5, middle row), and Class 4 receptors are monomeric species that bind to single site HREs (not shown in Fig. 5).

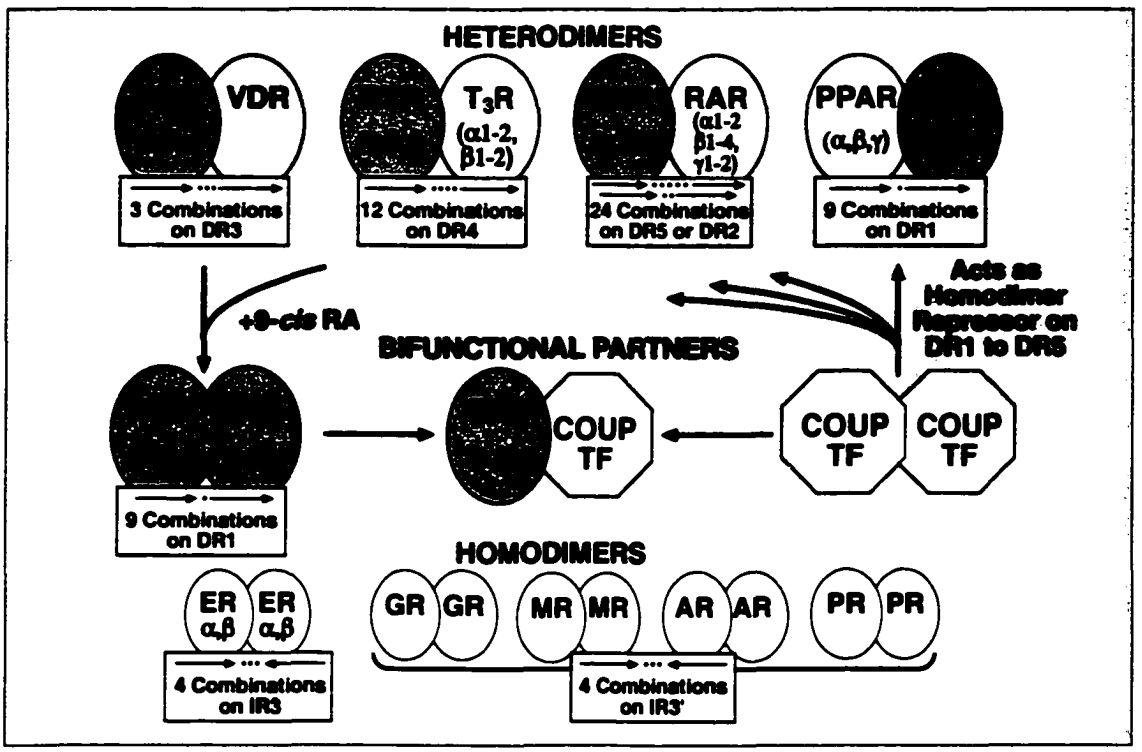


Fig. 5. Dimerization and DNA binding by the zinc finger nuclear receptors. The nuclear receptor superfamily is represented as heterodimers, homodimers or bifunctional receptors, and the type of response element to which they bind.

The structure and molecular role of VDR in mediating vitamin D hormone actions

As shown in Fig. 6, the hVDR molecule possesses various domains that contribute to the exertion of its actions at the molecular level. VDR consists of an N-terminal DNA-binding domain (DBD) comprised of a two-zinc finger motif of the C-4 type, highly conserved among the nuclear receptors, and a C-terminal ligand-binding domain (LBD) that includes conserved regions required for ligand-dependent RXR heterodimerization. These heterodimerization domains are observed in several subdomains within the VDR molecule, supporting the notion that the VDR-RXR association is required for specific interaction with DNA to control gene expression.

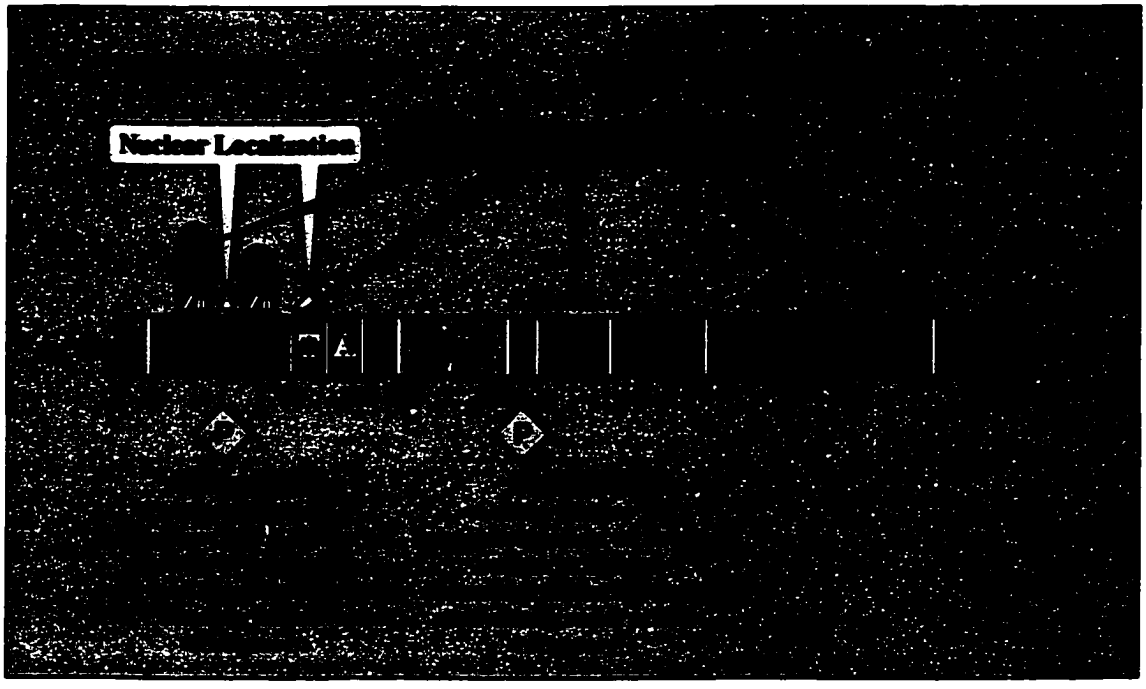


Fig. 6. Structure/function of the human vitamin D receptor represented in a linear form. The two major domains are depicted at the top of the diagram, the DBD and the LBD. Within these regions are embedded the nuclear localization signals and heterodimerization domains. Also shown are two major phosphorylation sites, and the effect their phosphorylation has on transcriptional activation by $1,25(OH)_2D_3$-VDR. The T and A regions (boxes) are elements mediating HRE selection that constitute the C-terminal extension of the Zn finger region of the DBD.

The DBD of VDR contains a cluster of 5 basic amino acids in the sequence ($_{49}$RRSMKRK$_{55}$) separating the zinc fingers. Four of these basic residues are believed to make direct contact with DNA, as well as constitute part of VDR's nuclear localization signal. In addition, this cluster includes Ser-51, a phosphorylation target site for protein kinase C, which when phosphorylated negatively influences the binding of the receptor to DNA (Fig. 6). There is a natural mutation found in this region, R50Q, which causes the HVDRR phenotype of $1,25(OH)_2D_3$ resistance in patients (Rut *et al.* 1994). There are other missense mutations in the DBD that elicit clinical vitamin D resistance, notably in his-35, lys-45, arg-73 and arg-80, all apparent contacts of VDR on DNA (Fig. 7).

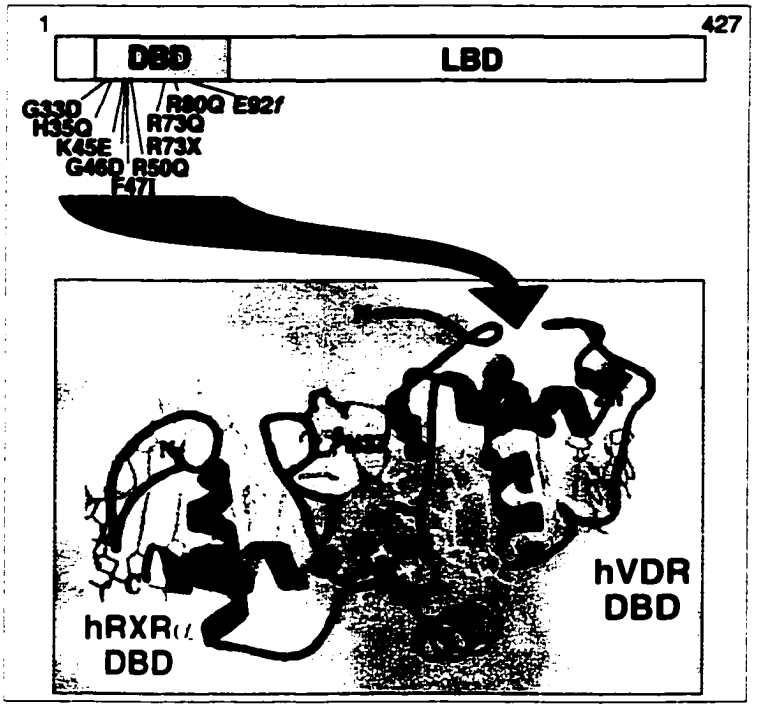


Fig. 7. Model of hVDR-hRXRα DNA-binding domains on a DR3 vitamin D responsive element (VDRE). Adapted froma model (Rastinejad *et al.* 1995), with location of natural mutations (•) causing vitamin D resistance (X signifies a stop codon mutation and *f* a frameshift).

The ligand binding domain of the receptor consists of a cluster of 12 α-helices and several β-strands that coalesce to form the lipophilic hormone-binding pocket (Fig. 8). There are three specific regions that form the bulk of this pocket, including amino acids 227-240, 268-316 and 396-422. Natural mutations found in this region, notably R274L (Kristjansson *et al.* 1993) and H305Q (Malloy *et al.* 1997), compromise ligand binding by collapsing the "pocket" site, making it difficult for the ligand to interact with the receptor. These mutations in ligand binding (Fig. 8) therefore cause the HVDRR phenotype.

Subregions of the DNA binding domain of VDR also support heterodimerization between VDR and RXR. This interaction involves specifically the zinc fingers, wherein

the RXR partner's second zinc finger interacts with both the first zinc finger (N37) and a region C-terminal of finger-2 (K91 and E92) in VDR (Rastinejad *et al.* 1995) (Fig. 7). This interaction not only supports heterodimerization, but also provides selectivity for the binding of both molecules, now integrated as a heterodimeric unit, to vitamin D responsive elements (VDREs). The VDREs are specific DNA sequences in the promoter of $1,25(OH)_2D_3$-regulated genes consisting of a hexanucleotide direct repeat separated by three base pairs (DR3), to which the receptor heterodimer binds and thereby controls transcription of target genes (Haussler *et al.* 1998).

A second surface of VDR-RXR interaction is found in the ligand-binding domain of the VDR. Here we can find two regions involved in ligand-dependent interaction. These domains correspond to helices 3-4 and 7-10, comprising residues 244-263 and amino acids 317-395, respectively (Bourguet *et al.* 1995). Natural mutations in this region that confer the vitamin D-resistant phenotype provide strong evidence for the structural interplay between hormone binding and heterodimerization that leads to selective DNA binding, and ultimately control of gene expression (Fig. 8). For example, hVDR I314S is primarily affected in hormone binding (Fig. 8) but also does not heterodimerize well with RXR, whereas hVDR R391C is predominantly an RXR heterodimerization mutant because it resides on the RXR contact surface of VDR (green shading in Fig. 8) (Whitfield *et al.* 1996).

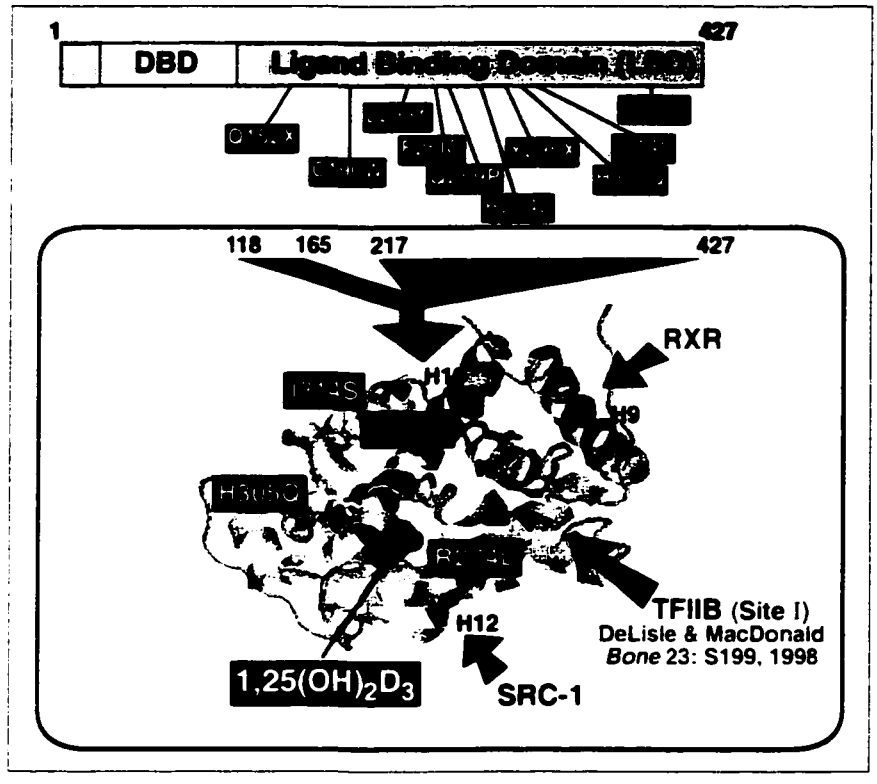


Fig. 8. LBD crystal structure of hVDR. From Rochel *et al.*, (Cell 5: 173, 2000) with location of selected natural mutations causing vitamin D resistance (mutations shown in yellow compromise ligand binding whereas those in green reduce RXR heterodimerization; X signifies a stop codon mutation and *f* a frameshift).

Other factors or comodulators interact with the VDR to transduce the signal for the control of expression of $1,25(OH)_2D_3$-regulated genes, and their mechanisms of action are just now becoming understood. There are at least two regions of contact between the receptor and these comodulators required for transcriptional activation. Both of these regions are found in the ligand-binding domain. The first one includes residues 244-263, a region that supports RXR-VDR interaction but does not provide for direct contact. In this region, lysine 246 is not involved in heterodimerization, but its alteration severely compromises transactivation (Whitfield *et al.* 1995a). It is believed

that this residue is involved in creating a binding interface with transcriptional coactivators. The second region of VDR involved in transcriptional activation is known as the ligand-dependent activation function-2 or AF-2 (Webster *et al.* 1988; Danielian *et al.* 1992; Saatcioglu *et al.* 1993; Leng *et al.* 1995). This region is found at the extreme C-terminus of the ligand-binding domain, and corresponds to helix-12 of the receptor (see Fig. 8). Site-directed mutagenesis studies of the AF-2 demonstrate this region to be a pure ligand-dependent transactivation domain, having little or no effect on the binding to hormone, RXR or DNA (Jurutka *et al.* 1997; Masuyama *et al.* 1997a). As will be discussed in detail later in this report, the helix-12/AF-2 domain of VDR comprises an interaction surface for transcriptional coactivators like steroid receptor coactivator-1 (SRC-1) (see Fig. 8). Finally, basal transcription factor II (TFIIB) also possesses a contact site in the LBD of VDR, denoted Site I and reported by DeLisle and MacDonald (DeLisle and MacDonald 1998) (Fig. 8), and TFIIB binding to VDR is obligatory for VDR-mediated transcriptional activation by $1,25(OH)_2D_3$ (Masuyama *et al.* 1997c).

Transcriptional activity of VDR also can be modulated by phosphorylation. In addition to VDR phosphorylation at Ser-51 by PKC which attenuates nuclear localization and DNA binding (Fig. 6), the VDR is phosphorylated by protein kinase A (PKA) in the hinge domain with a resultant decrease in $1,25(OH)_2D_3$-dependent activity (Jurutka *et al.* 1993a; Nakajima *et al.* 1996). The receptor can also be phosphorylated by casein kinase II (CK-II) at Ser-208 in the LBD, causing the potentiation of its transcriptional activity (Jurutka *et al.* 1993b; Hilliard *et al.* 1994) (Fig. 6). These

modulations of transcriptional VDR activity by phosphorylation provide cross-talk control between VDR and other signal transduction pathways.

The VDR natural partner, the retinoid X receptor (RXR)

Eukaryotic transcription systems commonly deploy dimeric complexes to generate cooperative binding to DNA and to amplify target sequence specificity (Glass 1994). This is the case for the steroid hormone receptors where dimerization enhances DNA recognition and high affinity binding. Once it is bound to the $1,25(OH)_2D_3$ ligand, the VDR has an enhanced affinity for interaction with the retinoid X receptor (RXR) (Haussler *et al.* 1997c). This interaction serves to align the DNA binding domains of both proteins in the heterodimer to optimally contact the specific response half-elements in the correct orientation, in particular where VDR occupies the 3' half-site of a positive DR3, whereas the 5' half-site is bound by RXR (Jin and Pike 1996). Once the DBD regions of both receptors are in place on the VDRE of a target gene promoter, the transactivation domains in the LBDs of both receptors are now able to interact with transcriptional coactivators and promote localized gene expression. There is significant evidence (Pathrose *et al.* 2001; Thompson *et al.* 2001) to demonstrate that RXR is not only a silent DNA-binding partner for VDR, but that it makes important contributions to increase the transcriptional potency of the heterodimer in response to the $1,25(OH)_2D_3$ signal. Thompson *et al.* (Thompson *et al.* 2001) showed via site directed mutagenesis, and Pathrose *et al.* (Pathrose *et al.* 2001) demonstrated using peptide antagonists, that the AF-2 terminal region of RXR is important for transcriptional signaling, primarily by

establishing intermolecular interactions between both its liganded primary receptor partner and with coactivators, anchoring these latter comodulator proteins to the promoter site and enhancing the $1,25(OH)_2D_3$ hormonal signal to effect transcription. Finally, heterodimerization of VDR and RXR will be expanded upon below, as it is the focus of Chapter 3.

Hormone responsive elements

Sequence and promoter analysis of $1,25(OH)_2D_3$-regulated genes has revealed specific DNA sequences that bind to the VDR-RXR heterodimer. These sequences are called vitamin D responsive elements or VDREs. For positive transcriptional regulation, the VDRE is defined as a DR3, usually an imperfect direct repeat sequence of hexanucleotides separated by a 3 base pair intervening sequence. Examples of these positive elements can be found in the promoter region of genes such as rat osteocalcin (MacDonald *et al.* 1991), human osteocalcin (Kerner *et al.* 1989), mouse osteopontin (Noda *et al.* 1990), rat 24-OHase (Jurutka *et al.* 1994; Zierold *et al.* 1994), etc. There is a great variability between VDREs, but a consensus VDRE determined by binding of the VDR-RXR heterodimers to randomly synthesized oligonucleotide sequences revealed a 5' half-site of purineGGTCA, a 3' half-site element consisting of a purineGTTCA, and a spacer in which a G at position 3 is important for VDR-RXR binding (Nishikawa *et al.* 1994; Colnot *et al.* 1995) (Fig. 9).

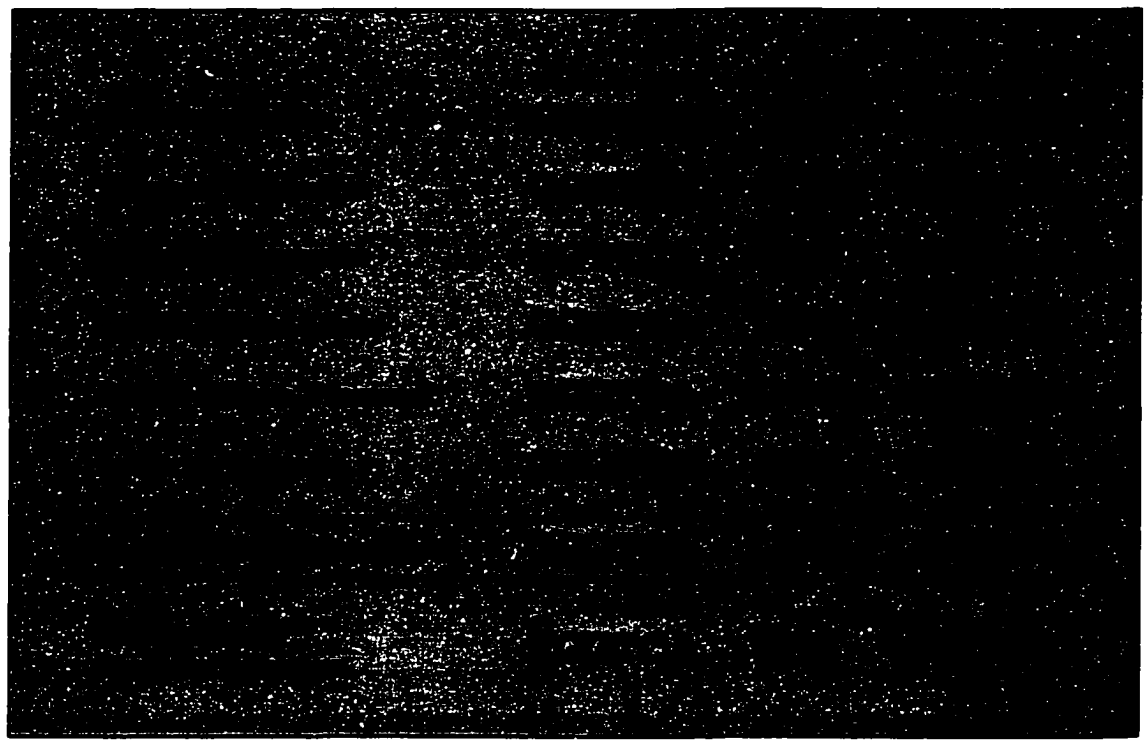


Fig. 9. A non-comprehensive summary of known positive VDREs. The red color represents bases that differ from the consensus (boxed) derived from the inspection of the six VDREs shown above. The green color in the randomly selected consensus (Nishikawa *et al.* 1994; Colnot *et al.* 1995) signifies bases not commonly found in VDREs but showing identity to those of the mouse osteopontin VDRE, which is known to have the highest affinity for VDR of any natural VDRE.

The great repertoire of VDRE sequences may be related to a need for graded potency of VDR action on regulated genes, likely allosterically generating distinct receptor conformational changes upon binding to DNA to diversify the $1,25(OH)_2D_3$-response. VDRE association of the heterodimer has been shown to modify the tertiary structure of the receptor, based on observations that alteration in the response element sequences influences VDR antibody accessibility and protease sensitivity (Staal *et al.* 1996). Such conformational changes, determined by the VDREs, could endow the receptor with the ability to recruit a variety of cell-specific coactivators resulting in differential modulation of the $1,25(OH)_2D_3$-response.

Control of $1,25(OH)_2D_3$-responsive gene transcription can also be negative. The expression of certain genes is repressed by the $1,25(OH)_2D_3$ hormone, through primary or secondary effects. The secondary effects may involve the induction of intermediary proteins that function as repressors, whereas the primary effects may involve the direct binding of the VDR in the promoter region of specific genes, effecting transrepression (Alroy *et al.* 1995). This effect could include the association of VDR with cell-specific corepressors, as in the case of other nuclear receptors like the thyroid hormone receptor and retinoic acid receptor (Chen and Evans 1995).

Cofactors and control of gene expression by $1,25(OH)_2D_3$

The mechanism of transcriptional control mediated by the $1,25(OH)_2D_3$ hormone and its receptor is not fully understood. A model for the molecular actions of VDR is portrayed in Fig. 10, and represents the hypothesis tested in the current dissertation research for actions mediated by VDR on a positively regulated gene. To test this hypothesis, we dissected the individual players pictured in Fig. 10 and investigated each as a potential VDR-interacting protein (VIP) in isolated biochemical and cellular systems. The ultimate goal was to elucidate the functional role of each participant in VDR signal transduction, and perhaps integrate this information into a sequential model of the activation of genes activated by the $1,25(OH)_2D_3$ hormone.

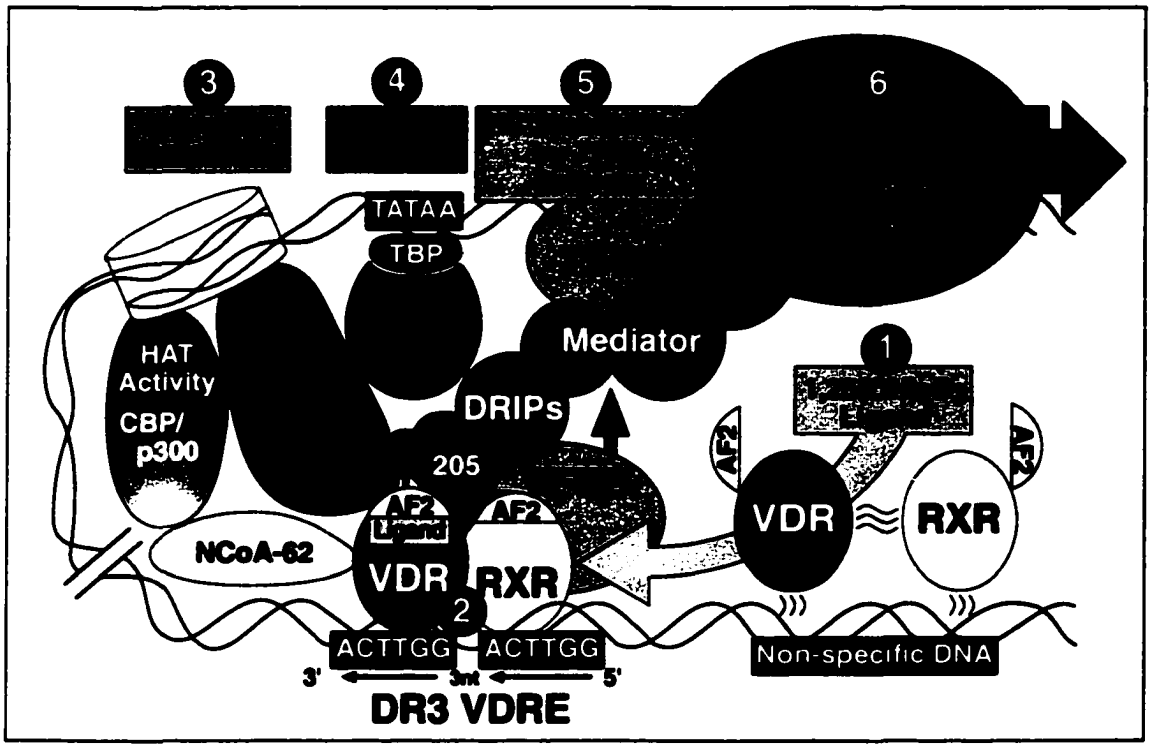


Fig. 10. A comprehensive model of the action of the $1,25(OH)_2D_3$ hormone mediated by the VDR on positively regulated genes. Once liganded by the hormone (1), the receptor dissociates any corepressor (not shown) and dimerizes with its partner RXR, changes its conformation, and is able to bind to specific sites on DNA (the mouse osteopontin VDRE is shown as an example) (2). Once bound to DNA, the heterodimer can attract modulator proteins that derepress chromatin structure via HAT activity (3), enabling the VDR-RXR dimer to interact with the transcription machinery to promote gene expression (4-6). Each step can be influenced by VDR phosphorylation. In negatively regulated genes, the assortment of cofactors can vary as well as the heterodimer associating with the response element.

By analogy with other steroid receptors, the unliganded VDR is presumably prevented from high affinity binding to the VDRE by direct interaction with a corepressor (not shown in Fig. 10), and unliganded VDR is envisioned to be loosely associated with RXR and nonspecifically "sliding" along the DNA phosphate backbone. Once bound to hormone, the ligand binding domain of VDR is thought to change its conformation, bringing the C-terminal activation function-2 (AF-2, see Fig. 10) into the proper orientation to promote corepressor release, RXR heterodimerization, specific

DNA element recognition and coactivator binding, all leading to transactivation. In addition to this conformational change, RXR interaction as well as DNA binding are believed to influence receptor conformation to uncover other sites for protein:protein interaction. VDR binding to coactivators or corepressors may depend upon the nature of the response element, facilitating either a positive or negative regulation of gene transcription. These latter interactions, when examined in analogous nuclear receptors, reside in many cases within the AF-2 domain at the C termini of the region involved in ligand binding (Le Douarin *et al.* 1995; MacDonald *et al.* 1995; Nawaz *et al.* 1995; Chakravarti *et al.* 1996; Schulman *et al.* 1996; Tong *et al.* 1996).

Despite the many factors hypothesized to be involved in transcriptional regulation depicted in Fig. 10, many of the molecular associations of VDR are still poorly defined and not rigorously demonstrated by direct protein:protein interaction experiments, as well as proven relevant by studies in intact cells. A systematic examination of these putative molecular interactions is required for an understanding of the mechanism by which the vitamin D receptor regulates transcription. Thus far, VDR's association with members of the transcriptional machinery, TFIIB and TAF$_{II}$28, has been evaluated (Blanco *et al.* 1995; MacDonald *et al.* 1995; May *et al.* 1996), and association with coactivators and corepressors has been reported for other members of the nuclear receptor superfamily, namely GR, AR, RXR, RAR and ER (reviewed in (Horwitz *et al.* 1996)). The present dissertation will describe the results of systematic research to identify and characterize VDR-interacting proteins (VIPs) that transduce the signal for VDR-mediated transcriptional activation or repression. By defining the

interactions of VDR with factors that directly or indirectly regulate the $1,25(OH)_2D_3$ hormonal response, it will be possible to comprehend the molecular mechanisms by which vitamin D mediates Ca^{+2} homeostasis, cell differentiation and modulation of the immune system.

CHAPTER II. VDR INTERACTION WITH COREPRESSORS

Hypothesis tested and findings

In this section, we tested the hypothesis that, like its close relative TR, unliganded VDR associates with SMRT and elicits repression of basal transcription. It was observed that VDR indeed interacts with SMRT in solution, but unlike the case of TR, hormonal ligand does not disrupt this association, and the complex does not repress basal transcription. Instead, SMRT functions with VDR in a novel fashion to facilitate coactivation once VDR is liganded.

Background

Nuclear receptor coregulators

Nuclear receptors exert their actions in the nucleus by controlling the expression of various genes involved in regulation of development, cell differentiation and organ physiology (Mangelsdorf *et al.* 1995). These receptors can act in *trans* as inducers or repressors via interactions with coregulators (coactivators and corepressors) to efficiently control gene expression. Coregulators are defined as rate limiting molecules that activate or repress the transcriptional potential of NRs, without affecting basal transcription. Coactivators are molecules that interact with NRs to enhance the transcriptional response, while corepressors are proteins that associate with NRs to decrease the expression of genes.

Recent research demonstrated that the mode of action of these proteins varies widely. Some coregulators directly interact with the transcription machinery, others

covalently modify histones and transcription-regulating proteins. Some of the coregulators are found in pre-assembled complexes, and are recruited individually to affect transcription (McKenna *et al.* 1999a). All these differences stem from the need to tightly regulate gene expression and enable the integration of several signal transduction pathways at the promoters governed by nuclear receptors.

The mechanism of NR control of gene expression is not well understood. Research supports the idea that once bound to cognate hormones, the NRs are translocated to the nucleus, bind to various partner proteins and eventually interact with specific DNA elements at the promoter region of target genes. The molecular events that follow are still uncharacterized, but it has become clear that the interaction of NRs with cofactors can enhance or repress the gene transcription response.

Transcriptional repression

Repression is considered as the ability of some nuclear receptors to lower the basal transcriptional activity of target genes in the absence of ligand. There are different ways in which the repression or silencing can take place. There is passive repression, also known as "squelching", in which the receptor unsuccessfully competes for dimerization partners because of overexpression of another transcription factor that uses common partners, thereby negatively affecting the interaction of the NR with DNA. Once DNA binding is abrogated, the NR cannot bind to the transcription machinery on the relevant promoter, thus shutting down gene expression (Hudson *et al.* 1990). Another form of repression, known as active repression, involves direct interaction of

the transcription factor with molecules that prevent the assembly of the transcription machinery or hinder the access of specific DNA sites in the promoter region of genes, a mechanism known as transrepression (Baniahmad *et al.* 1992). The complete mode of action of corepressors is not well understood, but they are commonly associated with histone deacetylases (HDACs), proteins known to modify the chromatin environment by making the DNA-histone interaction more dense and compact. This higher order of chromatin structure becomes a significant obstacle for gene transcription, limiting the access of transcription factors and the pre-initiation complex (PIC) to bind to DNA. The NR corepressors, nuclear corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT), by direct association with HDACs, can transduce the signal of repression via interacting with DNA bound, but unliganded, NRs.

Nuclear receptor corepressor (NCoR)

It has been demonstrated that the thyroid hormone receptor (TR) possesses specific silencing domains in its sequence, and when these domains are fused to other NR proteins, repression occurs (Baniahmad *et al.* 1992). O'Malley's laboratory demonstrated, in competition assays, the probable presence of a functional cofactor involved in this repression (Baniahmad *et al.* 1995). It was observed that the silencing activity of TR could be greatly reduced in cotransfection assays if these silencing domains were expressed concomitantly with TR, suggesting the presence of a limiting factor responsible for this phenotype (Baniahmad *et al.* 1995).

Biochemical assays with unliganded retinoid acid receptor (RAR) and TR have revealed the presence of corepressor proteins directly interacting with these NRs. One of these, a 270 kDa protein known as NCoR, was subsequently characterized by Hörlein *et al.* (Hörlein *et al.* 1995). It was observed that this interaction was specific for RARα and TRβ, but not with any other nuclear receptor. Mutational analysis of TR has elucidated a specific domain in its LBD involved in the interaction with NCoR. The domain was termed the NCoR box, and it was demonstrated that when this region is absent in a TR mutant, there is significant attenuation of the silencing capacity of unliganded TR, demonstrating that TR-NCoR association is required for silencing (Hörlein *et al.* 1995). Conversely, deletion mutants of NCoR C-terminus have identified two regions involved in NR interaction termed receptor interaction domains (RIDs). N-terminal deletions of NCoR also identified supportive domains indispensable for full repression. Competition assays have revealed that cotransfection of RIDs, alongside TR and RAR, abolishes the repressor action of NCoR with these unliganded NRs (Chen *et al.* 1996), providing evidence for the need of the NCoR-NR interaction for silencing purposes. It has also been shown that NCoR can interact with some members of the transcription machinery (TFIIB, TAFII32, TAFII70), suggesting that their actions may be to mediate interactions between the transcription apparatus and unliganded NRs (transrepression) (Muscat *et al.* 1998).

Silencing mediator for retinoid and thyroid hormone receptors (SMRT)

SMRT was isolated by the yeast two-hybrid system (YTHS) screen utilizing as bait RXR, RAR and TR (Chen and Evans 1995; Sande and Privalsky 1996). It is highly homologous to NCoR with subtle differences, specifically in the RID regions, with NCoR possessing more RIDs than SMRT (Chen *et al.* 1996). The YTH analysis has shown that the interaction between these receptors and SMRT can be abolished by the presence of ligand, and that for RAR and TR, the LBD possesses a ligand reversible interaction with SMRT. Receptor chimeras demonstrate that RAR and TR LBDs, fused to other transcription factors, can induce repression by binding to SMRT (Chen and Evans 1995).

Compared to NCoR, SMRT has different RIDs in the C-terminal region, and they are selectively used by distinct receptors. For example, RAR prefers binding to RID1 of NCoR, while TR does not display a preference (Wong and Privalsky 1998). An excess of SMRT protein in competitive assays between the Gal4DBD-RAR and RAR-403, a dominant negative mutant that retains interaction with SMRT despite the presence of hormone, relieves the lack of silencing effect created by the squelching that the RAR-403 promotes, and restores silencing (Tsai and Collins 1993). In fact, it has been demonstrated that mutations in the TRβ gene that convey resistance to thyroid hormone are the result of a stronger association between these receptor forms and corepressors like SMRT and NCoR (Yoh *et al.* 1997). Conversely, constitutively active TR isoforms lack the ability to interact with these repressor molecules (Chen and Evans 1995). Interestingly, it has been found that NCoR and SMRT can function as activators of

transcription when TR is bound to negative responsive elements. Moreover, an excess of repressor in a negative TRE transcription system enhanced the transcription activity of TR in a hormone-dependent manner. These results indicate that the actions of corepressors might be defined by the specific responsive element, ligand and possibly other factors involved in transcription.

VDR and nuclear corepressors

As discussed above, the interaction of TR and corepressors has been widely studied, and its function as a negative regulator of transcription is accepted as a mechanism of controlling gene expression and signal transduction. There have also been some additional studies that have evaluated the possible repressor properties of VDR, given the similarities between these receptors. Both VDR and TR belong to the same subfamily of nuclear receptors (Class 2), and share the same mode of action by functioning as heterodimers with RXR (Olefsky 2001).

Once identified as a nuclear receptor corepressor, NCoR was characterized and identified as a repressor that mediated ligand-independent inhibitor of gene transcription by nuclear receptors (Hörlein *et al.* 1995). It was shown in the yeast two-hybrid system it interacted with TRβ and RARα, but these interactions were abolished by the presence of ligand. For other receptors, specifically for the VDR, a significant interaction with NCoR was not detected in either the *in vivo* yeast system or *in vitro* GST-fusion protein pull down assay. Also, Chen *et al.* (Chen *et al.* 1996) did not find relevant interaction between SMRT and VDR in the mammalian two-hybrid system either in the presence or

absence of hormone, supporting the idea that VDR might not posses a repression domain. Conversely, Yen *et al.* (Yen *et al.* 1996), by examining different response elements and the "cross-talk" between the VDR and TR in co-transfection systems, observed that unliganded VDRs can repress basal transcription of a reporter gene when the osteopontin VDRE was fused to the promoter region. Employing the yeast two-hybrid system, Li *et al.* (Li *et al.* 1997) did demonstrate a very modest interaction between SMRT and VDR only in the absence of hormone, suggesting that SMRT might mediate transcriptional repression by VDR, although no transcriptional studies with VDR-SMRT were performed. However, in GST-SMRT fusion protein pull-downs, Wong and Privalsky (Wong and Privalsky 1998) did not detect interactions between SMRT and VDR in the presence or absence of hormone. Similarly, in the yeast two-hybrid system, a lack of association between these two proteins was observed (Wong and Privalsky 1998). As postulated previously, in order to control gene expression, nuclear receptors appear to first bind to their cognate ligands, dimerize with their partners and then bind to DNA, with some of them being able to interact weakly with partner proteins in the absence of hormone, as in the case of VDR, TR and RAR. Wong & Privalsky (Wong and Privalsky 1998) examined the effect of heterodimerization on SMRT-VDR interaction. In the yeast two-hybrid system, by introducing the SMRT protein as bait and the VDR and RXR as catch, a clear and robust interaction was revealed between these proteins, a significant difference from the response observed when the proteins were introduced individually. The addition of $1,25(OH)_2D_3$ or 9-*cis* retinoic acid destabilized the interaction between these receptors and SMRT. Therefore,

at least in the yeast two-hybrid system, weak RXR heterodimerization seems to enhance the ability of VDR to interact with SMRT in the absence of hormone. The mammalian two-hybrid system has also been used to test repressor interaction with VDR, specifically comparing NCoR-VDR and SMRT-VDR interaction. Tagami *et al.* (Tagami *et al.* 1998) found appreciable interaction between these proteins and VDR, but compared to that found between TR and corepressors, it appeared to be a weak, almost insignificant association. In order to evaluate this interaction in a natural setting, transactivation assays were carried out utilizing a natural VDRE (osteopontin) (Tagami *et al.* 1998). These studies revealed that in the absence of hormone, basal transcription was repressed, and that addition of the $1,25(OH)_2D_3$ ligand could relieve the repressive effect on transcription, a similar finding to that of Yen *et al.* (Yen *et al.* 1996). Therefore, after several studies that have evaluated the role of NCoR and SMRT in VDR signaling, repression in the $1,25(OH)_2D_3$ system remains controversial. This potential repressive property of unliganded VDR was examined in the present experiments, particularly in reference to SMRT as a possible VDR corepressor. SMRT was chosen because, unlike NCoR, which is accepted not to be a VDR interacting protein in virtually all reported studies, SMRT-VDR investigations are equally divided between positive and negative results in terms of biochemical and/or functional interactions of these two nuclear proteins.

Materials and methods

Transfection of mammalian cells

Nuclear receptors such as VDR, RXR and TR were cloned into the pSG5 expression vector (Green *et al.* 1988), a versatile eukaryotic expression vector possessing SV40 early and T7 promoters. COS-7 monkey kidney epithelial cells were transiently transfected by calcium phosphate-DNA coprecipitation (Kingston 1990). Each 60 mm plate containing 8.5 x 10^5 cells, received the empty expression vector pSG5 (500 ng), pSG5-hVDR (500 ng) or pSG5-hTRβ (500 ng), as well as a TKGH reporter vector containing either the rat osteocalcin VDRE or the rat myosin heavy chain TRE upstream of the human growth hormone (hGH) reporter gene (Terpening *et al.* 1991; Thompson *et al.* 1999). Cells were treated for 24 h following transfection with their cognate hormones or vehicle at physiological concentrations, and reporter gene expression was determined by radioimmunoassay (RIA) using a kit from Nichols Institute Diagnostics (San Juan Capistrano, CA).

INS-407 human embryonic intestinal cells were transiently transfected by calcium phosphate-DNA coprecipitation. Each plate received 1.5 μg of the pSG5 expression vector encoding for the specific cofactor (hRXRα, hSMRT, and hSRC-1), as well as with a VDRE-containing reporter construct ((CYP3A23-DR3)$_2$-TKGH, 10 μg/plate), and carrier pTZ18U DNA (6 μg/plate). The cells were incubated with 10^{-8} M 1,25(OH)$_2$D$_3$ (+) or vehicle (–) for 24 hours post-transfection and human growth hormone secretion into the medium was determined by RIA.

GST-pull-down assay

Initially, wild type (WT) hVDR was cloned into the pGEX vector (Promega, Madison WI) designed for expression of glutathione-S-transferase (GST) fusion proteins in *E. coli*. GST-hVDR fusion protein was overexpressed and linked to glutathione-Sepharose beads as described in the manufacturer's protocol. This immobilized hVDR matrix was utilized to recruit or pull down ^{35}S labeled proteins in solution to determine if they qualify as VIPs. The RXRα construct was described previously (MacDonald *et al.* 1993), and pCMX-mSMRTα-FL (Ordentlich *et al.* 1999) was kindly provided by M. Downes from the Salk Institute, San Diego CA. One μg of human RXRα and 1.2 μg of mouse SMRT cDNAs were *in vitro* transcribed and translated (IVTT) (Promega, Madison WI) utilizing a TNT rabbit reticulocyte lysate in the presence of ^{35}S labeled methionine. The mixture of the template DNA, lysate, amino acids and RNA polymerase was incubated for 2 hours at 30°C. The total contents of the reaction were then incubated with glutathione-Sepharose beads linked to glutathione-S-transferase (GST)-WThVDR for 30 minutes at 4°C in the presence of 10^{-7} M $1,25(OH)_2D_3$ hormone or vehicle. The GST-WThVDR beads were then washed 4X with 1 ml each of incubation buffer (0.15 M KCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.6, 0.3 mM $ZnCl_2$, 1 mM DTT (KETZD)) containing 0.1% Tween-20, 150 mM KCl, 1 mg/ml BSA, and the following protease inhibitors: aprotinin, leupeptin, pefabloc SC, and pepstatin) to release the non-specific interactions. The washes were carried out by adding the buffer directly to the beads with a repeat pipette, followed by a brief 1 min centrifugation and removal of the supernatant containing the non-specifically bound proteins. The remaining

associated proteins were solubilized with SDS and resolved by 5-15% Laemmli PAGE, followed by autoradiographic examination.

Results and conclusions

Analysis of potential corepression of VDR by SMRT

The functional effect, if any, of the putative interaction between VDR and SMRT was tested initially. The question of whether unliganded VDR represses transcription as does unliganded TR was first examined, utilizing a transfection system in which the effect of this interaction is measured by the behavior of reporter gene activation. In this system (Fig. 11A), the control expression vector by itself (without VDR insert, pSG5) establishes a basal activity of the reporter gene, as determined by radioimmunoassay (RIA), which is not influenced by the presence of either hormone. Once VDR or TR is expressed in the system (Fig. 11A; center or right panel), basal reporter gene expression is maintained when hormones are absent, although TRβ appears to repress it, whereas presence of either cognate ligand greatly enhanced the activity of each reporter gene. This indicates that each transfected receptor is sufficiently expressed to mediate a functional transcriptional activation response driven by its respective responsive element.

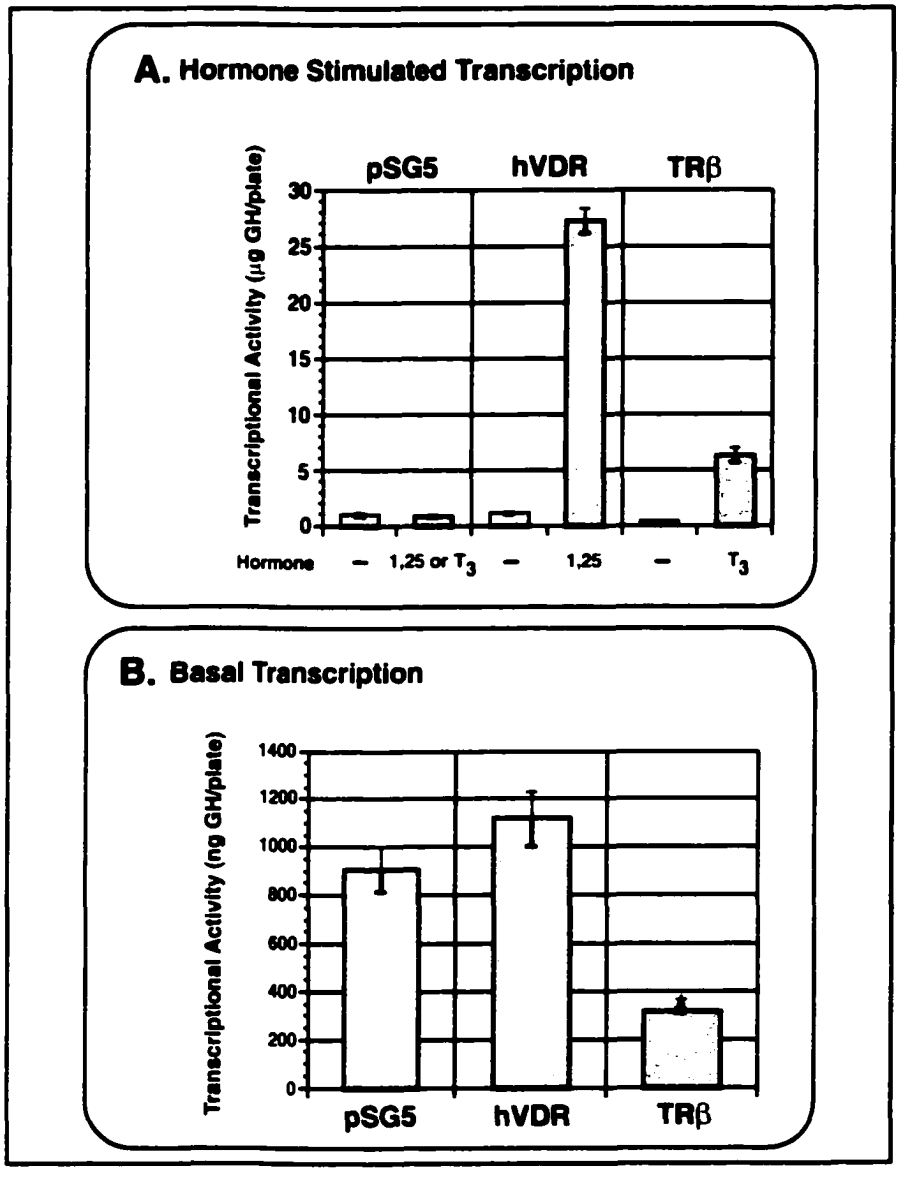


Fig. 11. Assessment of a potential functional interaction between SMRT and VDR. **A**. The effects of hormone on transcription for the VDR and TR systems. **B**. The basal (minus hormone) levels of reporter gene transcription in the presence of pSG5, hVDR and hTRβ expression vectors from panel A are depicted on an expanded scale to reveal differences. Results with VDR are not significantly different from those with pSG5 empty vector, whereas TRβ significantly (*) represses ($p<0.05$). Each value is the average of 3 determinations ± SEM.

A closer examination of the basal transcription in the absence of hormone via an expanded scale (Fig. 11B), reveals significant repression of basal transcription of the

TRE-linked reporter gene, whereas in the absence of ligand, VDR does not appreciably affect basal VDRE-mediated transcription. These results indicate that the TR is clearly capable of repression of target genes in the absence of ligand (Schulman *et al.* 1996), consistent with interaction with corepressors like SMRT. On the other hand, VDR unoccupied with $1,25(OH)_2D_3$ ligand does not act as repressor on the prototypical rat osteocalcin DR3 VDRE. Thus, unlike TR, VDR does not appear to constitute a transcriptional "off-on" switch, but instead may only exhibit ligand-dependent derepression and repression.

In vitro assessment of SMRT-VDR interaction

To further investigate the reason for the lack of repression by unliganded VDR observed in Fig. 11, we proceeded to study the interaction *in vitro* between a known nuclear receptor corepressor, SMRT, and VDR. As a positive control for protein:protein interactions, VDR and its natural RXR "partner" were employed. SMRT and the RXR alpha isoform each were cloned into the expression vector pSG5 and synthesized *in vitro* using the TNT rabbit reticulocyte lysate IVTT system (Promega, Madison WI) in the presence of ^{35}S-labeled methionine. Glutathione-Sepharose beads linked to GST-WT-hVDR were employed to study interactions between VDR and either of these two proteins.

With respect to VDR-RXR interactions, which is investigated in detail in the next chapter (Chapter 3) and is employed here as a positive control VIP for VDR-SMRT

association, it was observed via pull-down experiments (Fig. 12) that the interaction between VDR and RXR follows the nuclear receptor central dogma.

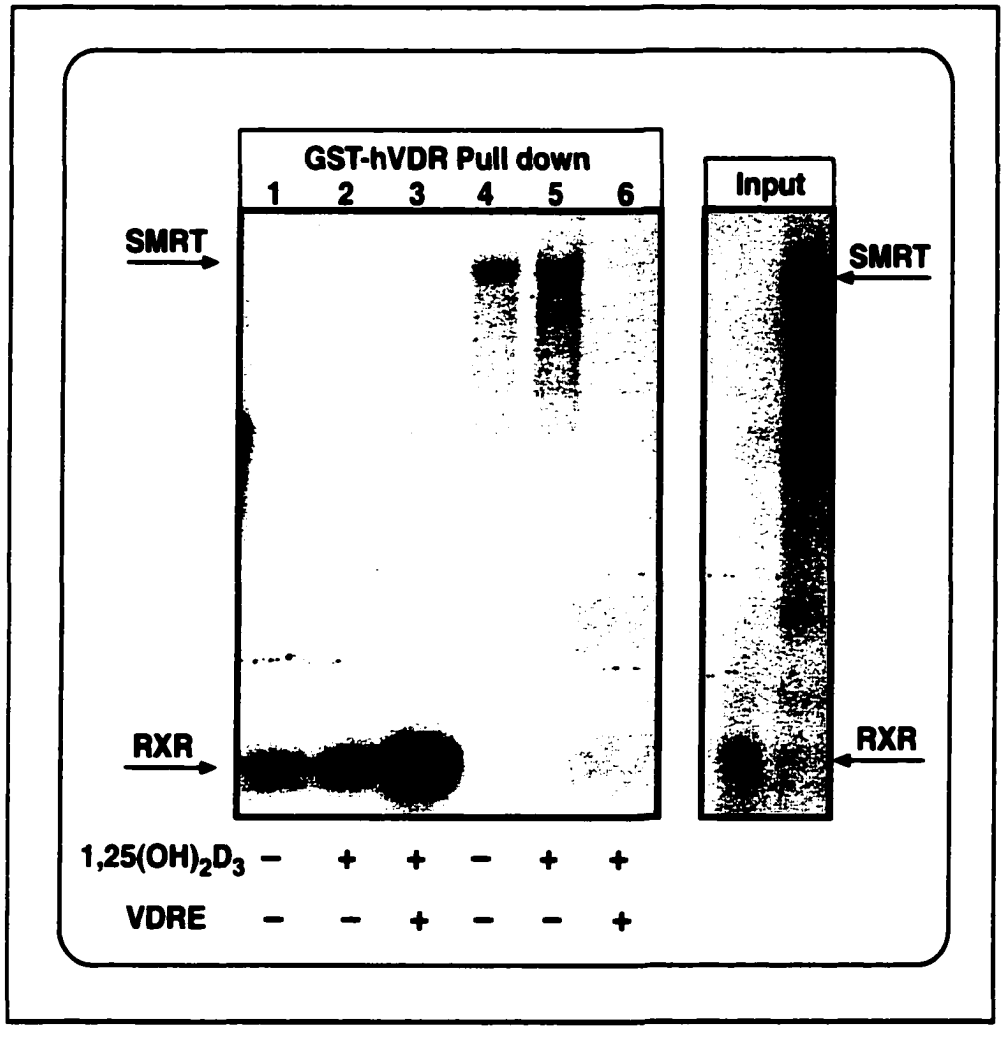


Fig. 12. VDR interacts with SMRT or RXR in solution: a VDRE in the presence of $1,25(OH)_2D_3$ ligand abolishes VDR-SMRT but enhances VDR-RXR association. One μg of RXRα or 1.2 μg of SMRT cDNA expression vectors were *in vitro* transcribed and translated into ^{35}S-labeled protein and used in pull down assays with VDR beads. In the absence of hormone (lane 1) a modest but significant interaction was found between VDR and RXR. $1,25(OH)_2D_3$ augmented this interaction (lane 2), which was then fully potentiated by the presence of the rat osteocalcin VDRE (lane 3). SMRT also interacts with VDR, independently of the presence of hormone (lanes 4 and 5). However, the VDRE completely abolishes the association of VDR with SMRT when $1,25(OH)_2D_3$ is present (lane 6). Input lanes represent 5% of that actually utilized in the GST pull down lanes.

The receptor somewhat weakly but significantly interacts with its RXR heteropartner in the absence of ligand (Fig. 12, lane 1). Once $1,25(OH)_2D_3$ is added and ligands VDR, VDR-RXR binding is markedly strengthened (Fig. 12, lane 2), making it possible for the heterodimeric complex to interact with DNA at defined recognition sites. Thus, the addition of the VDRE further stabilizes and greatly intensifies the VDR:RXR complex (Fig. 12, lane 3). As shown also in Fig. 12, lane 4, a modest but significant interaction is observed between VDR and SMRT in the absence of $1,25(OH)_2D_3$. This VDR-SMRT association is essentially unaffected when $1,25(OH)_2D_3$ hormone is added to the incubation (Fig. 12, lane 5). However, the binding of SMRT to VDR was totally abolished in the presence of both hormone and the VDRE DNA binding platform (Fig. 12, lane 6), revealing that when bound to its specific DNA element, VDR excludes the SMRT corepressor, likely in favor of coactivators and/or basal transcription factors. This sequence of events is in contrast to what is observed for TR and SMRT. In the case of TR, SMRT interacts strongly, but this association is obliterated when TR becomes liganded with T_3 (Chen and Evans 1995).

VDR does not exhibit appreciable repression of basal transcription in the absence of the $1,25(OH)_2D_3$ ligand, in stark contrast to hTRβ which is a significant repressor in its unliganded state. This repression may stem from the strong interaction between hTRβ and SMRT previously shown by other laboratories (Chen and Evans 1995; Sande and Privalsky 1996), whereas VDR may associate less avidly with this corepressor. By testing the VDR-SMRT interaction, *in vitro*, an association between VDR and SMRT was observed in solution (Fig. 12) that appears to be relatively weak, likely consistent

with the lack of significant basal repression by unliganded VDR in the *in vivo* transfection assays (Fig. 11). Thus, instead of being akin to TR, VDR appears to function molecularly more like RXR, which binds SMRT only marginally (Chen and Evans 1995), and does not exert significant repression when unliganded (Schulman *et al.* 1996). Nevertheless, SMRT-VDR association may still perform the function of preventing gene activation by VDR in the absence of $1,25(OH)_2D_3$, and more importantly, also may stabilize unoccupied VDR by analogy with the chaperone role played by heat shock proteins for unliganded GR (Howard and Distelhorst 1988; Dalman *et al.* 1991; Czar *et al.* 1995). Therefore, unlike TR, which is an "off-on" switch for transcription, depending on whether it is liganded, VDR would be a "neutral-on" switch or a "neutral-off" switch in the latter cases where VDR mediates $1,25(OH)_2D_3$-elicited repression of target gene transcription (e.g., 1α-OHase or PTH, see Fig. 3).

In this Chapter, we have shown that unliganded VDR interacts moderately but significantly with at least two nuclear proteins: SMRT and RXR. Previous work by several research groups (Blanco *et al.* 1995; MacDonald *et al.* 1995; Guo *et al.* 1997; Masuyama *et al.* 1997c; Jurutka *et al.* 2000b), indicates that TFIIB constitutes a third major VDR-interacting protein (VIP). VDR-TFIIB association occurs in the absence of $1,25(OH)_2D_3$, and is effected by two contact domains in VDR, Site I in the LBD (DeLisle and MacDonald 1998) (see Fig. 8), and Site II just N-terminal of the first zinc finger in the DBD (Jurutka *et al.* 2000b). Interestingly, $1,25(OH)_2D_3$ liganding of VDR conformationally alters Site I to partially dissociate TFIIB (Masuyama *et al.* 1997c;

DeLisle and MacDonald 1998), but this basal transcription factor apparently remains bound to Site II on VDR (Jurutka *et al.* 2000b).

We propose that there exists in intact cells a *quasi*-stable tetrameric complex of unoccupied VDR, RXR, SMRT and TFIIB that represents the unactivated form of VDR, perhaps sliding along the DNA-phosphate backbone (see Fig. 14, top). This multimeric complex of proteins is postulated to behave neither as a repressor, nor as an activator, at least until it is liganded with $1,25(OH)_2D_3$. Interestingly, the close SMRT corepressor relative, NCoR, was found in NCoR gene deletion experiments to be required for transcriptional activation of one class of retinoic acid response element (Jepsen *et al.* 2000). Therefore, unexpectedly, corepressors appear to be capable of activation under some circumstances, and a novel model to explain this has been put forth by McDonnell and coworkers (Li *et al.* 2002). They showed that the p160 coactivator ACTR (SRC-3) associates directly with NCoR, and this contact facilitates an interaction between unliganded TRβ and ACTR, perhaps raising the local concentration of ACTR at the target gene promoter. In support of this notion, they also observed that cotransfection of the NCoR corepressor actually enhanced TRβ-mediated transcriptional activation. Along these lines, in recent transfection assays using COS-7 cells, we have observed that overexpression of SMRT in a VDR-regulated transcription system enhances the expression of a VDRE-reporter gene construct in the absence of hormone (basal levels) (Fig. 13B). However, unlike the situation with TRβ and NCoR in the presence of excess ACTR coactivator, although SMRT cotransfection appears to boost hormone-stimulated transcription, it does not statistically significantly enhance VDR-mediated transcription

in the presence of $1,25(OH)_2D_3$ and endogenous coactivators in COS-7 cells (Fig. 13A). Nevertheless, it is clear from the data in Fig. 13 that SMRT overexpression does not in any way attenuate VDR mediated gene activation by $1,25(OH)_2D_3$, and instead seems to play a positive role in the initial steps of VDR function.

Although SMRT transfection of COS-7 cells did not statistically significantly potentiate $1,25(OH)_2D_3$ transcriptional responsiveness (Fig. 13A), we decided to re-examine this issue employing a human intestinal (embryonic jejunum) cell line (INS-407). As can be seen in Fig. 13C, again SMRT alone only slightly increased $1,25(OH)_2D_3$ stimulated transcription, and did so less than the p160 coactivator, SRC-1. However, when both SRC-1 and SMRT were over-expressed in INS-407 cells, there was a dramatic amplification of the transcriptional activation effect of $1,25(OH)_2D_3$ (Fig. 13C). These results are completely analogous to the observations of Li *et al.* (Li *et al.* 2002), that NCoR interacts directly with a p160 coactivator, ACTR, and facilitates transcriptional activation by the T_3-TR complex. Combined with our data on VDR, SMRT and SRC-1, this suggests a model for the SMRT "corepressor" in which its interaction with p160 coactivators actually prepares nuclear receptors such as VDR for hormonal activation of gene expression (see Fig. 14).

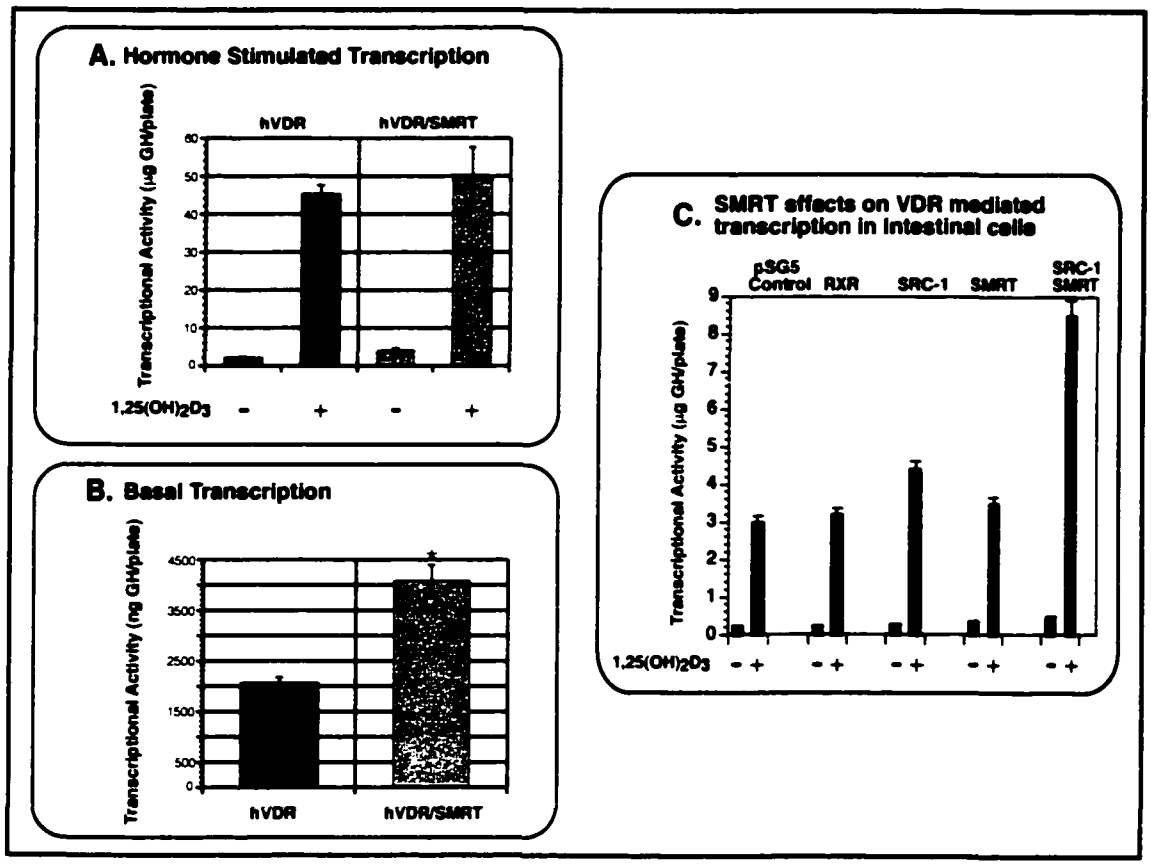


Fig. 13. The effect of SMRT cotransfection on VDR-mediated transcription. The basal (minus hormone) levels of reporter gene transcription in the presence of hVDR and hVDR/SMRT expression vectors from panel A are depicted in panel B on an expanded scale to reveal differences. Transactivation results for hVDR/SMRT overexpression differ significantly (*) from those to the hVDR basal transcription control ($p<0.05$). C. The effects of SMRT/SRC-1 cotransfection on $1,25(OH)_2D_3$ responsiveness in the INS-407 human embryonic intestinal cell line. Although as in A, SMRT cotransfection only slightly augmented the effect of $1,25(OH)_2D_3$, it greatly enhanced the coactivator effect of SRC-1, a VIP that will be examined in detail in Chapter 4. Each value is the average of 3 determinations ± SEM.

As described in detail above, in the case of unliganded VDR, there is evidence for association with TFIIB, and this interaction, like the VDR-RXR-SMRT triplex (Wong and Privalsky 1998), is increased by VDR-RXR heterodimerization in the absence of $1,25(OH)_2D_3$ (Jurutka *et al.* 2000b). These findings therefore support the model illustrated in Fig. 14, in which VDR and RXR are in loose contact in the absence of ligand as part of the tetrameric complex including SMRT and TFIIB (Fig. 14, top),

perhaps forming an apo-heterodimer that could improve the expression of genes at the basal level by delivering TFIIB at the promoter region of regulated genes in a fashion analogous to NCoR delivering ACTR during TR activation. This would explain the ability of SMRT overexpression to augment basal transcription (Fig. 13B).

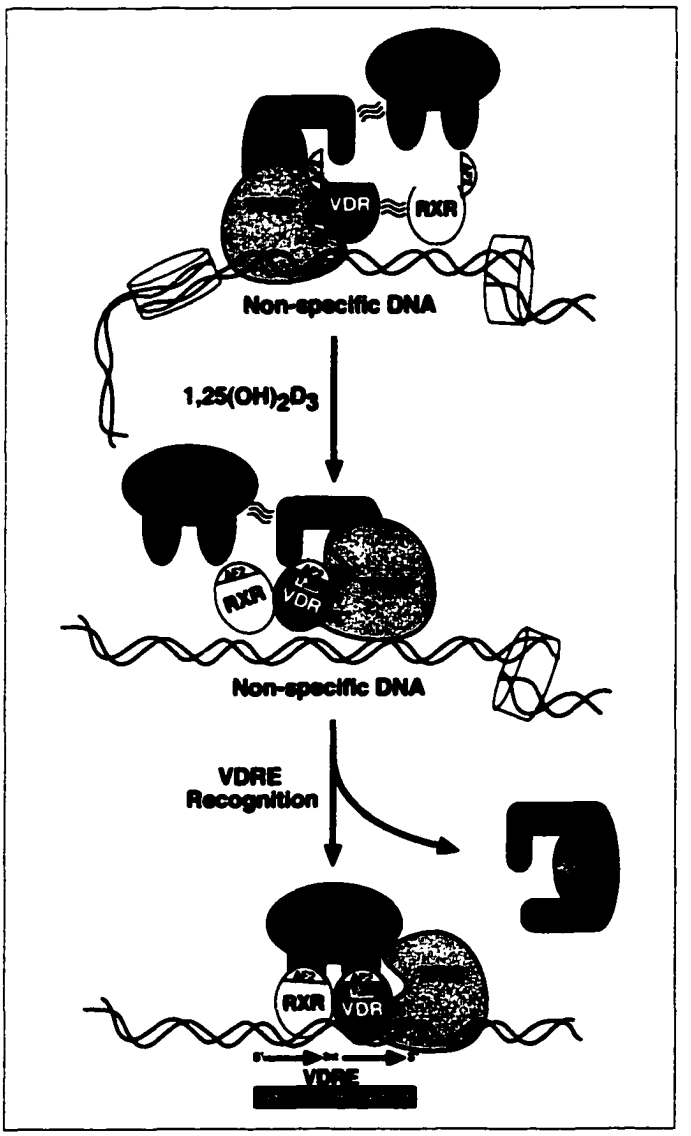


Fig. 14. DNA-sliding model of the unliganded VDR as a tetrameric complex: two step activation by $1,25(OH)_2D_3$ and VDRE ligands. In the absence of ligand, VDR modestly, but significantly interacts with RXR, SMRT and the PIC protein TFIIB, forming an inactive complex apparently attracted to DNA by electrostatic forces. From the work of McDonnell and colleagues (Li *et al.* 2002), and Fig. 13C, we propose that a p160 coactivator is also associated with the unliganded VDR protein complex, likely via binding directly to SMRT. Once liganded, VDR changes its conformation to heterodimerize strongly with RXR. This dimerization allows both receptors to position their DBDs in a manner that enables them to slide along and scan DNA for specific recognition sites (VDREs). Upon penetration of the major groove of DNA and VDRE recognition, the VDR-RXR heterodimer is proposed to undergo further conformational alteration, causing the release of the SMRT corepressor and creation of a unique platform for coactivator interaction that leads to expression of target genes. Because of its association with SMRT, coactivator is in the vicinity.

Upon binding to $1,25(OH)_2D_3$, VDR likely attracts the adjacent RXR into a high affinity heterodimer (Fig. 14, middle), and drives off SMRT when the complex docks on a VDRE (Fig. 14, bottom). Consistent with the last point and the lower portion of the model (Fig. 14) are the data in Fig. 12 revealing that in the presence of both the hormonal ligand and a VDRE, SMRT interaction with VDR becomes undetectable, presumably leaving the liganded $1,25(OH)_2D_3$ receptor free to activate transcription by recruiting coactivator as a DNA-enhanced RXR heterodimer. Additional results showing ligand-dependent VDR-RXR and VDR-coactivator associations will be presented in the next two chapters, but the conclusion at this point is that rather than functioning as a classic corepressor, SMRT instead plays a significant role in maintaining the integrity of the poised multimeric complex of proteins (including unliganded VDR, RXR, TFIIB, SMRT and a p160 coactivator) awaiting activation by sequential $1,25(OH)_2D_3$ and VDRE binding. It is interesting to note that such a maintenance or "chaperone" role which preserves GR in an inactive state until cortisol liganded, is played by Hsp90 and other proteins (Howard and Distelhorst 1988; Dalman *et al.* 1991). The concept that the "corepressor"/chaperone SMRT not only maintains the supercomplex but also directly recruits a p160 coactivator such as SRC-1 (Figs. 13 and 14), indicates that SMRT actually primes the poised unliganded VDR to efficiently bind the coactivator when liganded. Therefore, remarkably, the "corepressor" SMRT actually functions as both a chaperone and precoactivator.

CHAPTER III. VDR ACTIVATION BY 1,25(OH)$_2$D$_3$ AND INTERACTION WITH RXR

Hypothesis tested and findings

The questions asked in this section were: i) can the yeast two-hybrid system (YTHS) be utilized to probe VDR-RXR hormone-dependent interaction, ii) does the DNA platform influence VDR-RXR association in response to 1,25(OH)$_2$D$_3$ and iii) is RXR a functional participant in VDR-mediated stimulation of transcription by 1,25(OH)$_2$D$_3$? We found that the YTHS was a powerful tool in both fishing RXR isoforms from cDNA libraries and for quantitating this protein:protein interaction, which was observed to be dramatically ligand-dependent. Pull down and gel mobility shift technology revealed the strong positive effect of the VDRE on VDR-RXR heterodimerization. Finally, utilizing an RXR-poor myoblast cell line that expresses high levels of VDR, RXR cotransfection markedly potentiates 1,25(OH)$_2$D$_3$-stimulated transcription. Therefore RXR is the pivotal VIP that allows VDR to control gene expression.

Background

Nuclear receptors and RXR

The nuclear receptor superfamily involves 48 human proteins that control transcriptional responses to influence a wide array of cellular actions including differentiation and development (Maglich *et al.* 2001). Most of these proteins, once bound to a signaling lipophilic ligand, interact directly with DNA sequences known as

hormone response elements (HREs). HREs consist of minimal core hexad sequences that exist as half-sites separated by variable length nucleotide spacers between direct, inverted, or everted repeats (Olefsky 2001), and are found within the promoter region of target genes. In order to activate transcription, nuclear receptors bind to the HREs as homodimers or heterodimers, with each partner binding to a half-site of the element.

Although originally proposed to act as homodimers (Forman *et al.* 1989), in subsequent experiments the thyroid hormone (TR) and retinoic acid receptors (RAR) alone each failed to interact with DNA response elements of target genes in the presence of their cognate ligands in *in vitro* gel mobility shift assays (Wahlstrom *et al.* 1992). Thus, it was observed that TR, RAR (Kliewer *et al.* 1992) and VDR (MacDonald *et al.* 1991) required a factor present in nuclear extracts for HRE binding specificity, and for control of gene expression. The "missing link" for high affinity TR, RAR and VDR DNA binding, known as the retinoid X receptor (RXR) (Mangelsdorf and Evans 1995), was initially categorized as an orphan receptor. Further research demonstrated its role as a nuclear receptor that binds to a natural ligand, 9-*cis* retinoic acid, and functions as a homodimer when bound to its cognate ligand, or can function as an unliganded heterodimeric partner for other nuclear receptors including VDR (Mangelsdorf and Evans 1995). There are a number of receptors that heterodimerize with RXR to regulate gene expression (see Fig. 5), and with most of them, the RXR occupies the 5' half-site of the HRE and the partner receptor occupies the 3' site (Kurokawa *et al.* 1993). The exception is PPAR, which resides in the 5' half-element while RXR occupies the 3' half-element (Hsu *et al.* 1998). Interestingly, ligand-induced transcriptional activity for the

RXR homodimer is suppressed when it is complexed with a ligand-bound partner such as VDR and TR, and these heterodimers prevent the binding of RXR to its ligand, suggesting that TR and VDR are "nonpermissive" heteropartners for RXR, in which the primary receptor (TR or VDR) and its ligand play a dominant role over the subordinate RXR coreceptor (Thompson *et al.* 2001). However, in the case of RAR, which is a primary partner activated by all-*trans*-retinoic acid, the RXR heteropartner is still able to bind 9-*cis*-retinoic acid, and the two retinoids synergistically enhance transactivation from RAREs. Conversely, when 9-*cis*-retinoic acid is present in excess in the case of VDR-RXR or VDR-TR, this retinoid diverts RXR monomers away from forming heterodimers, instead facilitating RXR homodimers with a resulting attenuation of $1,25(OH)_2D_3$ or thyroid hormone responsiveness (see Fig. 5). Thompson *et al.* (Thompson *et al.* 1998), have proposed a general model for this nonpermissive behavior of VDR that is depicted in Figure 15, showing RXR as a subordinate receptor, acting to facilitate the response of the partner's cognate hormonal ligand unless diverted to form homodimers by 9-*cis* retinoic acid.

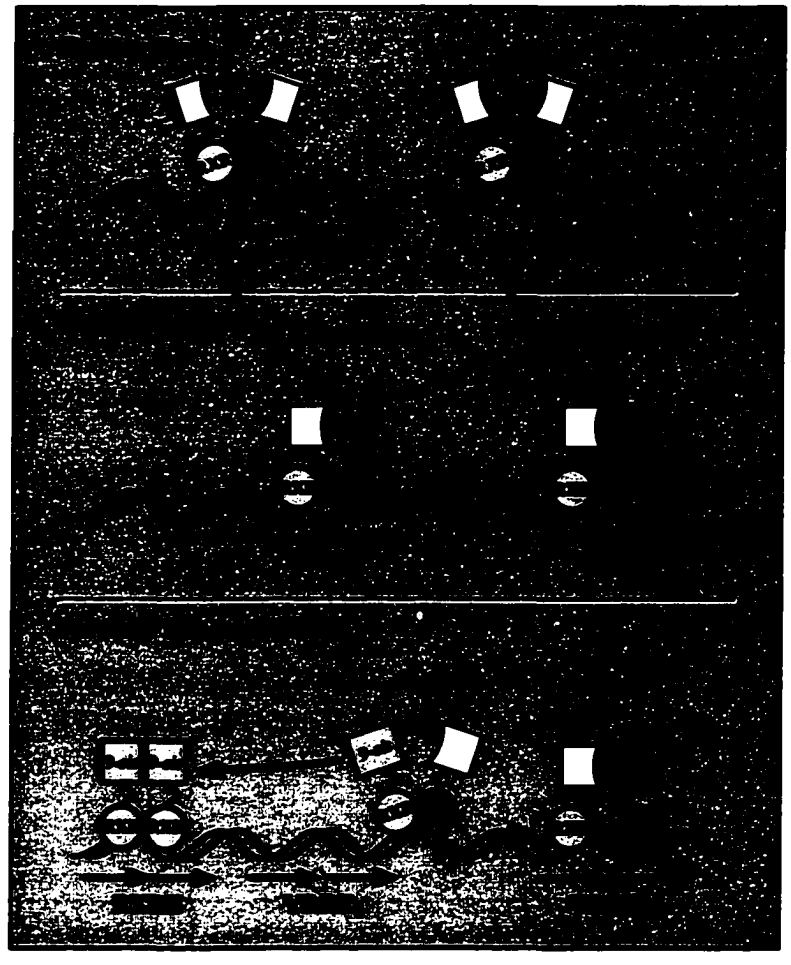


Fig. 15. DNA sliding model for 1,25(OH)$_2$D$_3$-VDR binding to the VDRE with RXR as a subordinate coreceptor, and diversion of the RXR heteropartner by 9-*cis* retinoic acid. In the resting state (top) the VDR-RXR apoheterodimer, bound through both receptor's DBDs, slides along DNA phosphate backbone, likely attracted by electrostatic forces. In the activated state (middle), the 1,25(OH)$_2$D$_3$ hormone binds to VDR, changing its conformation and enabling it to dimerize strongly with RXR and leading to DNA association at specific sites (VDREs). In the partially repressed state (bottom), the RXR partner can be diverted by its cognate ligand, 9-*cis* retinoic acid (9-*cis* RA), from the RXR-VDR apoheterodimer to instead mediate the action of 9-*cis* retinoic acid to control gene expression by binding to specific DNA response elements (RXREs) as an RXR homodimer. This diversion can be prevented if the RXR partner is efficiently heterodimerized with 1,25(OH)$_2$D$_3$-liganded VDR and acting on 1,25(OH)$_2$D$_3$ controlled genes.

1,25(OH)$_2$D$_3$ binding by VDR

As a member of Class 2 nuclear receptors (Mangelsdorf and Evans 1995), VDR interacts avidly with RXR to mediate the 1,25(OH)$_2$D$_3$-hormonal response, but does so only after ligand binding. Thus, the VDR-RXR heterodimer is considered the molecular switch in 1,25(OH)$_2$D$_3$ signaling. A critical step for efficient dimerization to occur is the ligand-triggered induction of the conformation change within the LBD of the VDR that allows full RXR partner recognition and binding (Bury *et al.* 2001). This conformational change has been previously observed in other receptors within the same subfamily as VDR, such as the retinoic acid receptor (RAR) (Driscoll *et al.* 1996) and the thyroid hormone receptor (TR) (Bendik and Pfahl 1995). In light of this evidence, a similar ligand induced allosteric mechanism was expected for the VDR (Thompson *et al.* 1998). The X-ray crystal structure of 1,25(OH)$_2$D$_3$-liganded VDR has been solved (Rochel *et al.* 2000), and the LBD fragment crystallized consists of a "sandwich" of 12 α-helices and 2 β-strands, several of which contact directly the hormone inside the LBD hydrophobic pocket. Liganding also stabilizes the tertiary structure of VDR, and this stabilization promotes changes in the receptor's AF-2 or helix-12 region by van der Waals contacts, hydrophobic interactions and salt bridges to reconfigure the AF-2 into an active platform, facilitating further binding of coactivators and ultimately leading to transcriptional activation (Rochel *et al.* 2000). Although it has not been demonstrated directly, it is presumed that VDR liganding with 1,25(OH)$_2$D$_3$ also stabilizes the helix-7 and helix-10 combined interface with RXR, thereby providing a physical basis for 1,25(OH)$_2$D$_3$-dependent enhancement of VDR-RXR heterodimerization. Recently, the

cocrystal structure of dual-liganded RAR-RXR was solved (Bourguet *et al.* 2000), and the exact RAR-RXR contacts elucidated. These are likely very similar to the VDR-RXR contacts, as the heterodimeric interfaces of all RXR heterodimerizing receptors are quite congruent (Dino Moras, personal communication via Mark Haussler). Finally, the allosteric modifications driven by the interaction between VDR and the $1,25(OH)_2D_3$ hormone also induce VDR to become hyperphosphorylated in order to fully heterodimerize with RXR and to interact with the specific DNA elements or VDREs (Thompson *et al.* 1998; Jurutka *et al.* 2002).

Without ligand binding, heterodimerization is compromised, rendering the receptor incapable of enhancing gene expression, at least with respect to its classical actions in bone target tissues. As discussed above in Chapter 1, natural mutations in the VDR gene can encode receptor proteins defective in their ability to bind ligand, resulting in hereditary vitamin D-resistant rickets (HVDRR). This condition, an autosomal recessive disease, prevents the body from responding to the $1,25(OH)_2D_3$ hormone, and creates a rachitic phenotype characterized by severe bowing of lower extremities and short stature. Several laboratories have identified such natural mutations and observed through *in vitro* analysis that the inability of the receptor to bind to $1,25(OH)_2D_3$ prevents it from subsequent RXR heterodimerization and DNA binding, impairing its ability to promote target gene expression (reviewed in (Haussler *et al.* 1998)).

Synthetic mutants have also been constructed to help demonstrate the relationship between VDR ligand binding, heterodimerization and transactivation (Whitfield *et al.* 1995b; Swamy *et al.* 2000; Solomon *et al.* 2001). Not only can

mutations in the LBD impair VDR's ability to bind to RXR (Whitfield *et al.* 1996), but it has also been suggested that the binding of different vitamin D analogs to the receptor can impart distinct receptor conformations that may enhance or diminish its interaction with RXR. These conformational changes often result in variable levels of target gene expression, depending on the strength of RXR interaction (Cheskis *et al.* 1995; Zhao *et al.* 1997; Carlberg *et al.* 2001; Liu *et al.* 2001). An alternative hypothesis is suggested by Tocchini-Valentini *et al.* (Tocchini-Valentini *et al.* 2001) who postulate that, according to the crystal structure of a VDR LBD fragment bound to 1,25(OH)2D3 and several analogs, the receptor has a longer half-life when occupied by certain drug analogs compared to the natural hormone, endowing VDR with higher stability and resistance to proteolysis. This extended half-life could promote additional rounds of expression on target genes, rendering it a more active receptor.

VDR-RXR heterodimerization

As mentioned above, the binding of VDR to RXR constitutes a pivotal step in which these nuclear receptors, as a heterodimeric unit, become a functional transcription factor. Natural and synthetic mutations that affect the ability of VDR to dimerize with RXR result in HVDRR-like phenotypes (Whitfield *et al.* 1996). Since its recognition as a "nuclear accessory factor" or NAF by Liao *et al.* (Liao *et al.* 1990), Sone *et al.* (Sone *et al.* 1991) and MacDonald *et al.* (MacDonald *et al.* 1991), RXR has been recognized as a necessary partner for VDR in specific DNA binding and control of gene expression (MacDonald *et al.* 1993; Nakajima *et al.* 1994; Jin and Pike 1996; Lemon *et al.* 1997;

Liu *et al.* 2000). When absent, certain RXR isoforms, particularly RXRα and RXRβ, produce a similar phenotype to VDR knockout mice, at least in terms of skin defects and lack of hair cycling (Li *et al.* 2000b), whereas the RXRγ counterpart appears to have a dispensable role in this system (Krezel *et al.* 1996). Because heterodimerization with RXR plays a pivotal role in VDR action, it was of interest to identify the regions on the VDR involved in contacting RXR, as well as the regions that support this interaction. Because of the lack of an available co-crystal structure for the RXR-VDR complex, the mapping of the interaction has been mainly achieved through the use of deletion and point mutations; so far four regions in hVDR have been implicated (see Fig. 6, Chapter 1). Human VDR deletion mutant interaction experiments by Nakajima *et al.* (Nakajima *et al.* 1992) revealed that a C-terminal region between Lys-382 and Arg-402 is required for RXR association. Point mutations validated this finding and recognized a second element between Lys-325 and Leu-332 that supported the interaction. Further research recognized a highly conserved region in VDR (residues 244-263, known as E1) essential for RXR binding. Point mutations in this domain revealed several residues that were required for RXR contact. Jin *et al.*, (Jin *et al.* 1996) further supported this finding by mapping VDR-RXR interactions in a yeast two-hybrid system, and identified an area localized between residues 320-397. The fourth interaction domain was identified by Hsieh *et al.*, (Hsieh *et al.* 1995) who discovered residues found in the DBD of VDR as critical for heterodimerization. Asn-37 in the first zinc finger as well as Lys-91 and Glu-92 have been proposed to contact the second zinc finger of RXR, and to facilitate the

recognition of the responsive elements to which the VDR-RXR heterodimer binds on DNA (Haussler *et al.* 1997a).

Thus, VDR-RXR heterodimerization involves both the LBD and the DBD of VDR, leading to a two-step hypothesis for binding of heterodimers to DNA. In the first step, the receptors associate weakly through their DBDs, enabling the VDR-RXR apoheterodimeric unit to bind electrostatically (nonspecifically) and slide along DNA. The second step involves high affinity association of the receptors through their LBDs, an event caused by the conformational change of VDR driven by hormone binding, thereby allowing the heterodimer to penetrate the major groove of DNA and recognize specific DR3 VDREs.

VDRE binding

As depicted in Fig. 15, in the current model that explains the actions of the $1,25(OH)_2D_3$ hormone, the vitamin D receptor is an inactive protein in the absence of ligand, weakly interacting with its natural partner RXR, and in a similar manner, the apo-heterodimer binds non-specifically to DNA in chromatin. The opposite effect is observed once ligand is present: heterodimerization is a strong response to ligand binding by VDR, and DNA-specific site docking by the heterodimer to drive control of target genes is the ultimate effect.

As already discussed in Chapter 2, there have been several studies attempting to implicate unliganded VDR in gene repression, but none of the studies has been conclusive (Hörlein *et al.* 1995; Chen *et al.* 1996; Li *et al.* 1997; Tagami *et al.* 1998;

Wong and Privalsky 1998). As concluded above in Chapter 2 from the present results, unoccupied VDR neither binds with sufficient affinity to recognize VDREs, nor recruits the necessary quantity of corepressor (SMRT or NCoR) required to function as a gene silencer as do TR and RAR. Therefore, the central dogma for VDR as a nuclear receptor, at least in its traditional target tissues of intestine, bone and kidney, presents the receptor as an active molecule able to stimulate transcription only in the presence of hormone and bound to its RXR natural partner (Haussler *et al.* 1998). Ligand binding to VDR changes the conformation of the receptor, resulting in strong heterodimerization with RXR via their LBDs. This heterodimerization event provides the receptor dimeric complex with the high affinity and specificity required to bind to VDREs in the promoter regions of target genes as described earlier.

In order for the heterodimeric receptor to interact with DNA, specifically with the VDRE, it is necessary for the genomic chromatin and its nucleosome architecture to undergo a structural change that releases histones, rendering the DNA in that region accessible to transcription factors. Some of these physical changes, from the repressive to the derepressed state of chromatin, are driven by the SWI/SNF complex (Cairns *et al.* 1994). This complex, isolated initially from yeast, and with several mammalian homologues, has been shown to alter nucleosome conformation in an ATP-dependent manner, leading to an increased accessibility of nucleosomal DNA to transcription factors (Imbalzano *et al.* 1994). SWI/SNF complexes previously were found to potentiate hormonal responsiveness of GR in yeast (Yoshinaga *et al.* 1992), and it has been demonstrated that this effect is mediated in part by direct interaction between the

GR and the SWI/SNF complex in both yeast and mammalian cells (Wallberg *et al.* 2000). Finally, Lemon *et al.* (Lemon *et al.* 2001), recently observed that a SWI/SNF homologue in humans known as PBAF (polybromo BRG-1-associated factor (BAF)), enhances activation mediated by several nuclear receptors, including VDR, in an integrated in vitro transcription reaction with chromatin templates. Importantly, relevant experiments by Dilworth *et al.* (Dilworth *et al.* 2000), utilizing RXR-RAR heterodimer activated by retinoid in a transcription system with "purified" chromatin templates, established a temporal order for required chromatin modifying activities. They showed that ATP-driven chromatin remodeling is the first step that leads "tight" binding of RXR-RAR to its cognate DR5 RARE, associated with a selective disruption of nucleosomal structure in the RARE region. Thus, although the RXR-RAR complex can bind to DNA, responsive element recognition/tight association requires nucleosomal structure rearrangement by SWI/SNF-like factors but not histone acetylation (Dilworth *et al.* 2000).

As discussed above, VDREs are imperfect direct repeats of hexanucleotides with a spacer of three nucleotides (DR+3; see Chapter 1, Fig. 9). Based on the analysis of the crystal structure of the TR-RXR DBD heterodimer bound to DNA, it has been possible to extrapolate this information and predict the behavior of the VDR-RXR moiety, bound to the VDRE. In the crystal structure model (Rastinejad *et al.* 1995) (see Fig. 7), plausible positions and functions of both VDR zinc fingers have been determined, with each one of them anchoring the protein on DNA and stabilizing its interactions with RXR. The N-terminal finger of VDR presents its α-helix to directly contact the bases of

the major groove of DNA. In addition, both fingers make phosphate backbone contacts. The aforementioned model structure has shown the heterodimers arranged on a direct repeat, and suggests binding of the proteins in an asymmetrical form, stabilized by protein-protein interactions. This deduced structure displays an extensive head to tail subunit arrangement involving nonreciprocal regions of each protein forming an interface (Freedman 1997). Residues of the second zinc finger of RXR directly interact with residues of the first VDR finger and C-terminal of the second. The DBDs of both RXR and VDR possess select contact residues that bind to VDRE half-element bases, and this HRE specificity is likely determined by the length of the VDRE spacer (Umesono *et al.* 1991; Hsieh *et al.* 1999). The binding of the heterodimer to the element presumably effects a change in the conformation of both receptors that may expose different interaction surfaces, possibly enabling distinct cofactors to bind to the dimer to ultimately modulate transcription in a VDRE-specific manner (Staal *et al.* 1996).

The goals of the present chapter were to examine the strength and ligand dependence of the VDR-RXR interaction utilizing the yeast two-hybrid system (YTHS), and to determine the influence of a specific DNA element platform on this protein:protein association. The YTHS was utilized to study RXR herein, but its use was also a prelude to employing this technique to select potentially novel VIPs (see Chapter 6). Finally, the functional significance of the RXR partner in supporting VDR stimulated transcription was evaluated in an RXR-poor cell line.

Material and Methods

Yeast two-hybrid system (YTHS)

The YTHS was employed to test the interaction between hVDR and mRXRβ utilizing the yeast strains and expression vectors provided by the Clontech Matchmaker™ 1997 (Clontech; Palo Alto, CA). Yeast cells were transformed with the appropriate vectors, following the protocol provided by either Bio 101 (Carlsbad, CA) or Stratagene (La Jolla, CA), and screened for growth in selective media by the use of their auxotrophic markers.

In both protocols, the yeast strains are grown in complete media at 30°C to mid-log phase. The cells are then extracted by centrifugation and treated with a mildly acidic solution (cesium acetate) that permeabilizes the cell wall. The cells are subsequently exposed to expression plasmids as well as carrier DNA, and incubated at permissive temperature for 15 minutes. Finally, the cells are treated with a high-density alkaline solution, heat shocked and plated in selective media at 30°C for 48-72 hours until colonies start to appear.

The Gal4 DBD vector contains the TRIP1 gene which allows transformants to grow on Trp^- media. The Gal4 activation domain (AD) vector contains the LEU2 gene permitting yeast growth on a Leu^- media. Once transformed with both plasmids, the yeast cells are plated in control (Leu^-/Trp^-) media, where growth indicates a successful transformation. Growth in $Leu^-/Trp^-/His^-$ medium indicates the presence of protein:protein interactions by activation of the HIS3 gene. A second reporter gene (lacZ) reduces the amount of false positives when its activation is tested in an X-gal

assay, where positive interactions are visualized by the presence of blue colonies in an X-gal substrate. The testing of these associations was performed in the presence and absence of $1,25(OH)_2D_3$ (10^{-6} M). In the present study, we utilized the strains SFY526 and HF7c. For controls, we employed positive interactions between the proteins encoded by the pP53 (Trp^+) and pSV40 T antigen (Leu^+) vectors and, as negative controls, we used the absence of interaction between the proteins encoded by the pP53 (Trp^+) and the pLAM5' lamminin C (Leu^+) vectors.

X-gal assay

The plates containing candidate transformants received 7 ml of chloroform and were incubated for 2 minutes with rocking movement. After incubation, the chloroform was drained and the plates were dried for about 6 minutes. Approximately 10 ml of 0.3% low-melt containing 100 mM KH_2PO_4, pH 7.0 and 1.5 mg/ml of X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) from a 20 mg/ml stock solution in N,N-dimethylformamide (DMF) are then added to the plates, and the media allowed to harden. The plates were then incubated at 30°C for 1-3 hours, an optimal time for the color reagent to develop.

β-gal assay

The yeast cells containing vectors with ORFs for putative interacting proteins were grown overnight in selective media at 30°C until saturation. Two ml of the saturated culture were centrifuged and resuspend in 1 ml of HLT^- media, and the cell

suspension added to a 9 ml lactose-selective media. $1,25(OH)_2D_3$ hormone was then added to a final concentration of 10^{-9} M. The cell suspension was incubated with shaking at 30°C for approximately 22 hrs. After that time, 1 ml of media is taken to determine the cell concentration at 600 nm (1 X 10^6 cells ~ OD_{600}= 0.0334). The remainder of the cells were extracted by centrifugation for 3 min at 3000 rpm in a Beckman AccuSpin centrifuge (Volume = V in ml = 9 ml), and resuspended in 1 ml of Z buffer (60 mM $Na_2HPO_4•7H_2O$, 40 mM $NaH_2PO_4•H_2O$, 10 mM KCl, 1 mM $MgSO_4•7H_2O$, pH 7.0) with 2.7 µl/ml of β-mercaptoethanol. The cells were then freeze-thawed in liquid N_2 3X and the extract preincubated for 5 minutes at 28 °C. The reaction starts by the addition of 0.2 ml of o-nitrophenyl β-D-galactopyranoside (β-ONPG), and time is recorded. The reaction was stopped once color was visible by the addition of 0.5 ml of Na_2CO_3, and time elapsed (t) recorded. Cell debris was removed by centrifugation at 14 krpm for 10 minutes in an Eppendorf centrifuge 5415C. Absorbance was read at 420 nm and the reading used to calculate the β-gal units produced per yeast cell. $U = 1000 \times [(A_{420}) / (OD_{600} \times V \times t)]$.

GST-pull-down assay

Sepharose beads containing immobilized hVDR were prepared as described in Chapter II and used in pull down assays for labeled RXR. One µg of hRXRα cDNA was employed in an *in vitro* transcription/translation (IVTT) system (Promega, Madison WI) utilizing a TNT rabbit reticulocyte lysate in the presence of ^{35}S-labeled methionine to generate full length WT hRXRα protein. The mixture of the template DNA, lysate,

amino acids and RNA polymerase was incubated for 2 hours at 30°C. The total contents of the reaction were then incubated with glutathione-Sepharose beads linked to GST-WThVDR for 30 minutes at 4° C in the presence of 10^{-7} M $1,25(OH)_2D_3$ hormone or vehicle in the presence or absence of VDREs. The GST-WThVDR beads were then wash 4X (as described in Chapter II) with incubation buffer containing 0.1% of Tween-20 detergent to eliminate non-specific interactions. The remaining interacting proteins were resolved by a 5-15% Laemmli PAGE gel and examined by autoradiography. This assay was used to study the interactions between the VDR and putative interacting proteins.

Ligand-dependent gel mobility shift assays

Human VDR (10-100 ng) generated via the baculovirus expression system (MacDonald *et al.* 1991) was added to a mixture containing 10 mM Tris-HCl, pH 7.6, 150 mM KCl, 1.0 mM dithiothreitol, 15% glycerol, 1 mg/ml acetylated bovine serum albumin and 50 μg/ml poly(dI•dC), followed immediately by an amount of mRXRβ (also expressed in the baculovirus system) equivalent to that of hVDR. Prior to the addition of the receptor(s), $1,25(OH)_2D_3$ was added to the reaction mixture in ethanol vehicle to give a final concentration of 10^{-12} to 10^{-5} M and ethanol vehicle controls were included. The binding portion of the experiment included incubation for 45 minutes at 22°C. One μl of radioactively labeled DNA probe (containing 0.5 ng of DNA, or approximately 0.8 nM; 50,000-100,000 cpm) was added to yield a final volume of 40 μl, then incubated for an additional 30 minutes. Thereafter, the entire reaction was

subjected to nondenaturing electrophoresis on a 4% polyacrylamide gel. The dried gels were and examined by autoradiography.

Cellular transfections

C2C12 mouse myoblast cells were cotransfected with hRXRα pSG5 expression vector (500 ng/plate) as well as with a VDRE-containing reporter construct ((CYP3A23-DR3)$_2$-TKGH, 1 μg/plate), and carrier pTZ18U DNA (9 μg/plate). The cells were incubated with 10^{-8} M 1,25(OH)$_2$D$_3$ (+) or vehicle (–) for 24 hours post-transfection and human growth hormone secretion into the medium was determined by RIA.

Results

Because the active form of vitamin D, 1,25(OH)$_2$D$_3$, plays a vital role in regulating calcium and phosphate homeostasis via the vitamin D receptor (Haussler *et al.* 1988; Haussler *et al.* 1997b; Haussler *et al.* 1998), we characterized the effect that its partner RXR plays in the control of gene expression. The approaches were molecular, biochemical and biological in trying to determine the influence of this co-receptor on transcription.

The YTHS proved to be a valuable tool in identifying the molecular/biological role of RXR as a VDR natural partner. Utilizing VDR fused to the Gal4-DBD as "bait" and mRXRβ fused to the Gal4-AD as "catch", we were able to demonstrate the hormone dependency of this interaction as well as quantify it. Once the interaction occurs, the yeast are capable of turning on expression of the HIS3 and LacZ reporter genes. HIS3

allows the yeast to grow on media lacking the amino acid histidine, while the LacZ allows a visible confirmation of this interaction by production of the LacZ enzyme which can then metabolize a galactose colorimetric reagent (X-gal). The reporter gene product, β-galactosidase, allows us to quantify this association by measurement of the activity of the β-galactosidase enzyme.

As shown in Fig. 16, the yeast growth and colorimetric response to heterodimerization of VDR and mRXRβ is strongly dependent on the presence of hormone. Plates without the ligand did not demonstrate appreciable growth or the characteristic blue color exhibited by the X-gal assay. A similar result was obtained for VDR-hRXRα interaction in this assay (data not shown). The YTHS and $1,25(OH)_2D_3$-enhancement of protein-VDR interaction depicted here is also a useful method to screen for other VDR-interacting proteins, and will be employed accordingly in later chapters (especially Chapter 6).

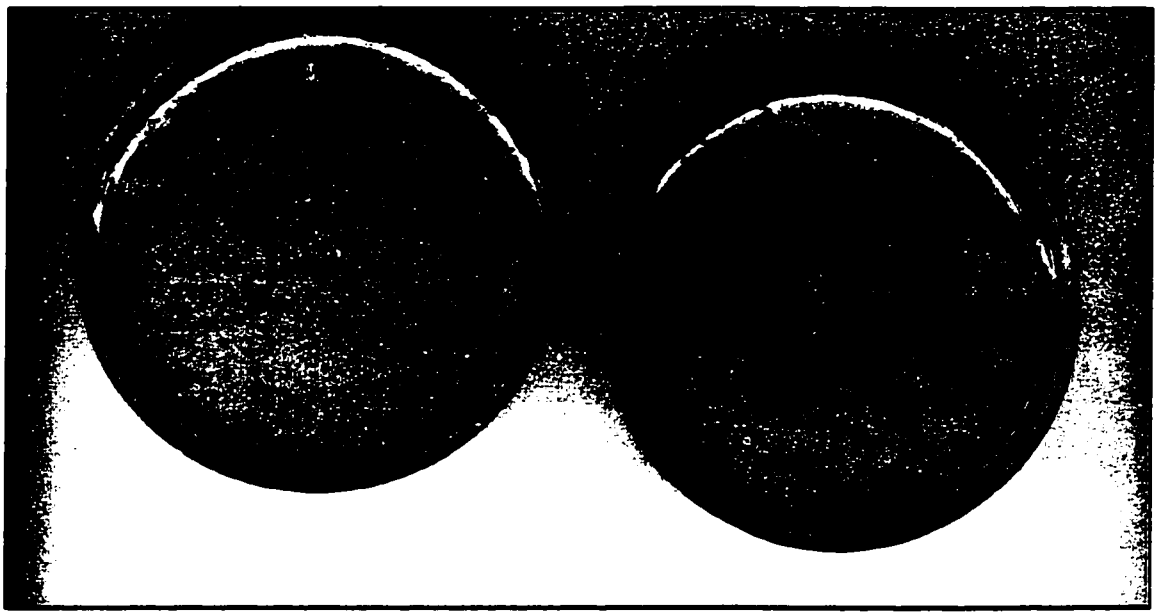


Fig. 16. The colorimetric X-gal assay, illustrating the hormone-dependent interaction between VDR and RXRβ. To the left, the VDR-RXRβ interaction in the absence of hormone is weak and does not significantly express the Lac-Z reporter gene. To the right, the presence of hormone stimulates HIS3 expression, hence growth on His⁻ media and expression of Lac-Z with a positive response in the X-gal assay.

Quantifying the interaction revealed that the strongest association tested for VDR was with the mRXRβ protein in the presence of ligand. This was observed by performing the β-gal assay on cells carrying expression vectors for both receptors (Fig. 17). The P53/SV40 antigen positive interaction control exhibits a strong expression of the LacZ reporter gene when compared to the lack of interaction observed for the P53 and lamminin C protein. In a similar fashion, the VDR/RXR interaction proved to be weak in the absence of hormone. Once the ligand is added, this association becomes stronger and results in activation of the reporter gene (5-fold).

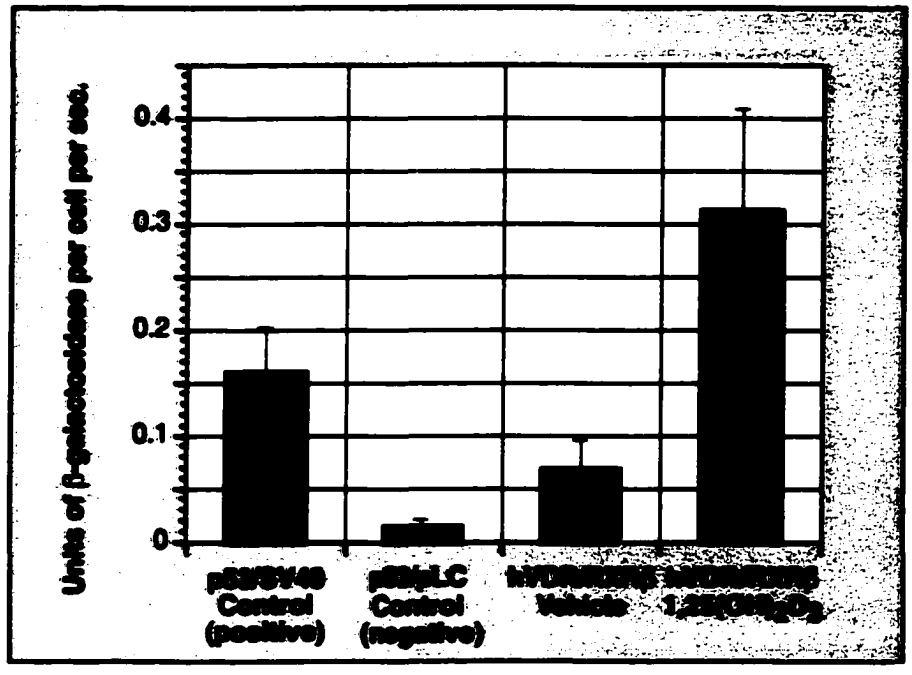


Fig. 17. The effect on Lac-Z induction of the VDR-RXR interaction in yeast, driven by the presence of $1,25(OH)_2D_3$. The p53-SV40 T-antigen interaction served as a positive control. Both proteins, when in contact, enhance the transcription of the Lac-Z gene, an interaction that can be quantified by the number of units of β-galactosidase they produce. The absence of interaction between p53 and lamminin C reveals basal levels of reporter activity. VDR and RXRβ interact weakly with each other in the absence of hormone. When $1,25(OH)_2D_3$ is present, the interaction is enhanced approximately 5-fold, supporting previous findings that hormone binding drives this interaction. Error represents SEM, with n=3.

The interaction of VDR and RXR, seen in the yeast system, was independently evaluated in a defined *in vitro* system by pull-down assay (Fig. 18). Once *in vitro* transcribed and translated, hRXRα was tested for its association with hVDR fused to glutathione S-transferase and immobilized on glutathione-Sepharose beads. The results (Fig. 18) support the findings observed in the yeast system in that RXRα displayed dependency on the $1,25(OH)_2D_3$ ligand for strong interaction with VDR in solution. In addition, we also found that the presence of DNA, in this case specifically the rat osteocalcin VDRE, markedly amplified the association between these two proteins. A control experiment (data not shown) revealed only a slight enhancement of unliganded

VDR-RXR association in the presence of the VDRE. The results from two independent approaches (Figs. 17 and 18) lead us to the conclusion that, in order to interact in a high affinity manner with DNA, VDR must be liganded by its cognate hormone, and be dimerized to its natural RXR partner. Furthermore, liganded VDR-RXR heterodimerization is stabilized and dramatically accentuated when the VDRE DNA platform is available.

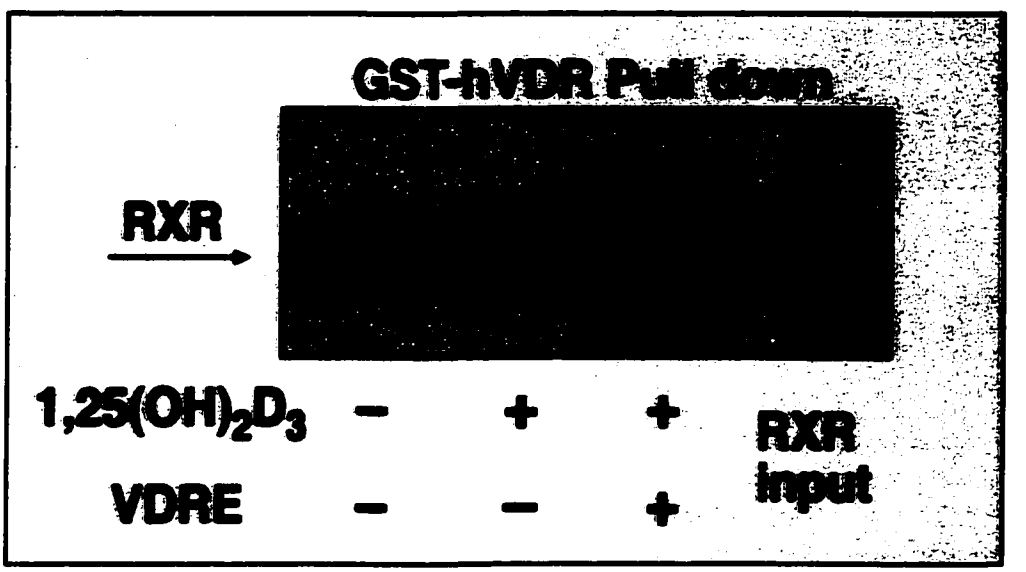


Fig. 18. Hormone and VDRE enhance VDR-RXRα heterodimer formation. A pull-down assay was used to evaluate the interaction between these receptors in a defined *in vitro* system. In the absence of hormone, VDR-RXRα interaction is weak, but can be stimulated (2.5-fold) when the $1,25(OH)_2D_3$-hormone is present, although ultimately the highest level of association (5-fold) occurs when the hormone and a VDRE (rat osteocalcin) are present. Input lane represents 5% of the RXRα added to other lanes.

The next step in studying the process by which VDR mediates the molecular actions of $1,25(OH)_2D_3$ was to further analyze the influence that the responsive element exerts on ligand-dependent VDR-RXR heterodimerization. Therefore, we utilized the Electrophoretic Mobility Shift Assay (EMSA), in which partially purified hVDR and

hRXRβ were incubated with distinct VDREs in the presence and absence of ligand, and the interaction of the RXR-VDR heterodimer with the DNA elements were established.

By employing both the rat osteocalcin and mouse osteopontin DR3 VDREs (see Fig. 9 and Fig. 19 for DNA sequences), we were able to observe that the interaction between VDR and RXR on DNA was greatly enhanced in the presence of hormone, in the range of 10^{-9} M to 10^{-5} M $1,25(OH)_2D_3$. Concentrations of $\leq 10^{-10}$ M $1,25(OH)_2D_3$ only slightly enhanced VDR-RXR-VDRE formation, and the dose-dependency for the hormone is most apparent for the mouse osteopontin VDRE. Therefore, by performing these *in vitro* experiments, we were able to demonstrate the significant hormonal dependency that the receptor displays in order to interact with DNA, as well as the need for the RXR partner to accomplish this function (Figs. 18 and 19).

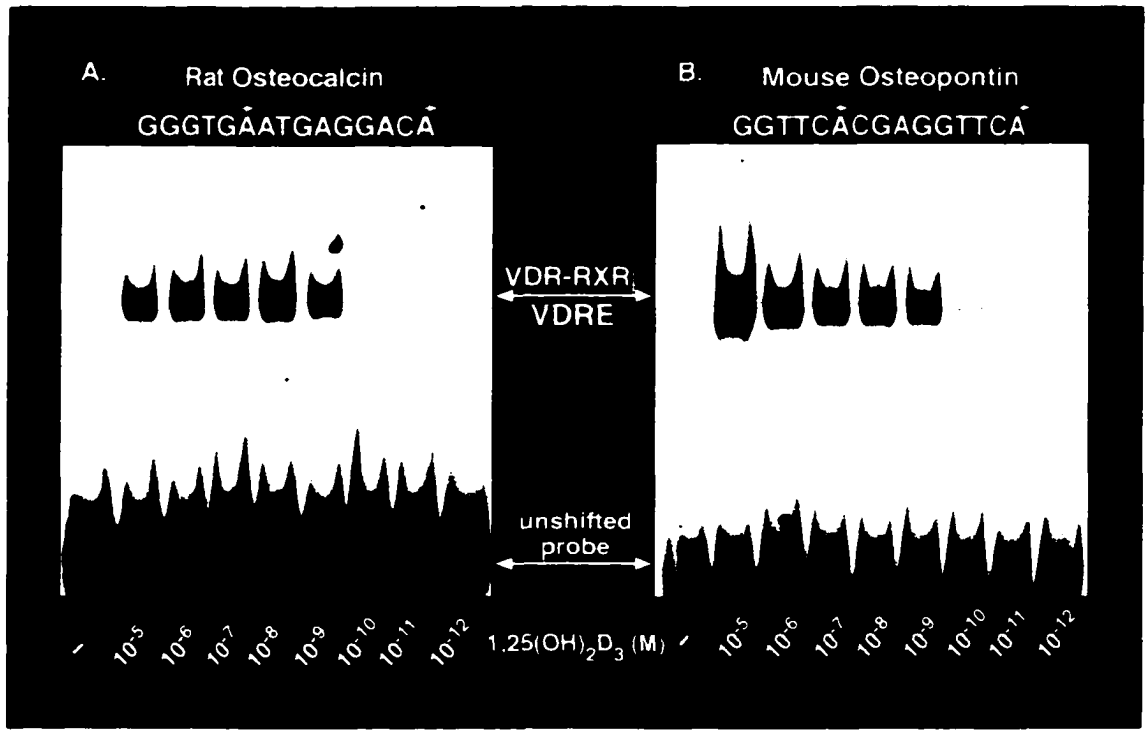


Fig. 19. VDR-RXR interaction with DNA is hormone dependent. Rat osteocalcin (rOC) and mouse osteopontin (mOP) VDREs were used to determine the interaction between the VDR-RXR heterodimer and DNA by EMSA. Under these conditions, in the absence of hormone, no interaction between the heterodimer and the VDREs is detected. When physiological to high concentrations of $1,25(OH)_2D_3$ are added to the system, a gel shift is observed, representing the VDR-RXR dimer retarding the mobility of the labeled-DNA probe.

Finally, an *in vivo* (intact mammalian cell) assay was employed to complement the observations made in the yeast two-hybrid and *in vitro* (pull-down and gel shift) systems, regarding the molecular mechanism by which the $1,25(OH)_2D_3$ hormone stimulates transcription of target genes through the actions of the VDR-RXR heterodimer. The C2C12 mammalian myoblast cell line, containing high levels of endogenously expressed VDR, comparable to those of classic vitamin D target cells such as osteoblasts, was transiently transfected with expression plasmids encoding hRXRα, a VDRE-containing reporter construct ($(CYP3A23\text{-}DR3)_2$-TKGH (Makishima *et al.* 2002), 1 μg/plate) carrying two copies of a synthetic perfect DR3 VDRE

(AGTTCAtgaAGTTCA), and carrier DNA. As illustrated in Fig. 20, in the presence of hormone and the VDRE-reporter (left panel), the induction levels of the GH reporter gene were 33% higher than that of the basal (no hormone treatment). This is statistically significant, but very modest effect considering that the C2C12 cells, based on other transfection assays in which various coactivator levels are boosted by transfection, respond similarly to ROS 17/2.8 cells that contain on average 10,000 copies of VDR per cell. This indicated that a cofactor(s) may be limiting in these cells, and we tested the hypothesis that RXR isoforms may not be well expressed in the C2C12 cell line. The cotransfection of C2C12 cells with hRXRα increased induction levels by $1,25(OH)_2D_3$ to 2.5-fold (Fig. 20, right panel), indicating that the RXR partner enhanced the response. These results are consistent with the findings in the *in vitro* and yeast systems, which demonstrate that in order for VDR to act on transcription, it requires the cooperation of the RXR partner in addition to the $1,25(OH)_2D_3$ ligand and a DNA responsive element.

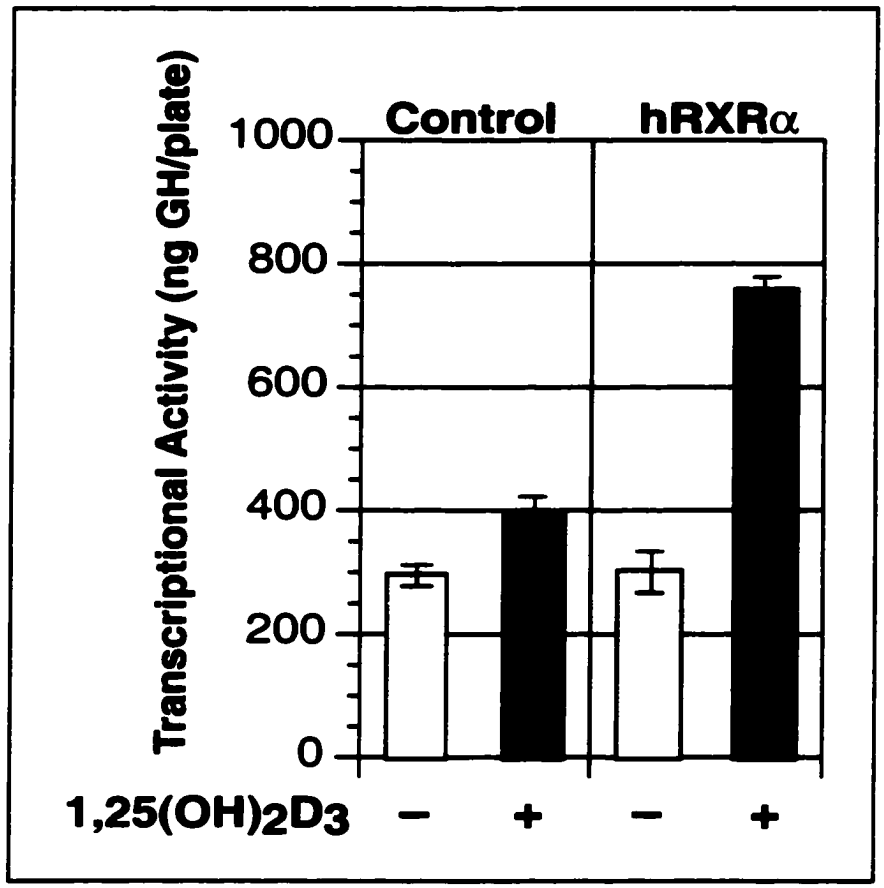


Fig. 20. The $1,25(OH)_2D_3$ transcriptional response mediated by VDR is dramatically boosted by RXR in myoblasts. C2C12 mouse myoblasts, containing significant levels of endogenous VDR, displayed only a minor increase in GH reporter gene induction in the presence of hormone (left panel). This activity was significantly augmented by the cotransfection of hRXRα, supporting the concept that in order to control gene expression, VDR requires cooperation with RXR, which is apparently poorly expressed in C2C12 cells.

Conclusions

In order to control the expression of $1,25(OH)_2D_3$ target genes, VDR requires the direct interaction with ligand to promote conformational changes that will allow the receptor to associate with RXR, an event that then supports high affinity DNA binding and creates an interaction surface platform for cofactors to help control gene

transcription. This concept of VDR activation by $1,25(OH)_2D_3$ ligand, high affinity association with RXR and docking on a VDRE platform is illustrated in Fig. 21. As discussed above, PBAF (Lemon *et al.* 2001), and other SWI/SNF homologues, are likely required for chromatin remodeling that facilitates the access of VDR-RXR to the VDRE for high affinity association. Therefore, as depicted in Fig. 21, for the $1,25(OH)_2D_3$-liganded VDR-RXR heterocomplex to assume tight VDRE binding (states II to III and II' to III), chromatin restructuring, but not histone acetylation, is required first.

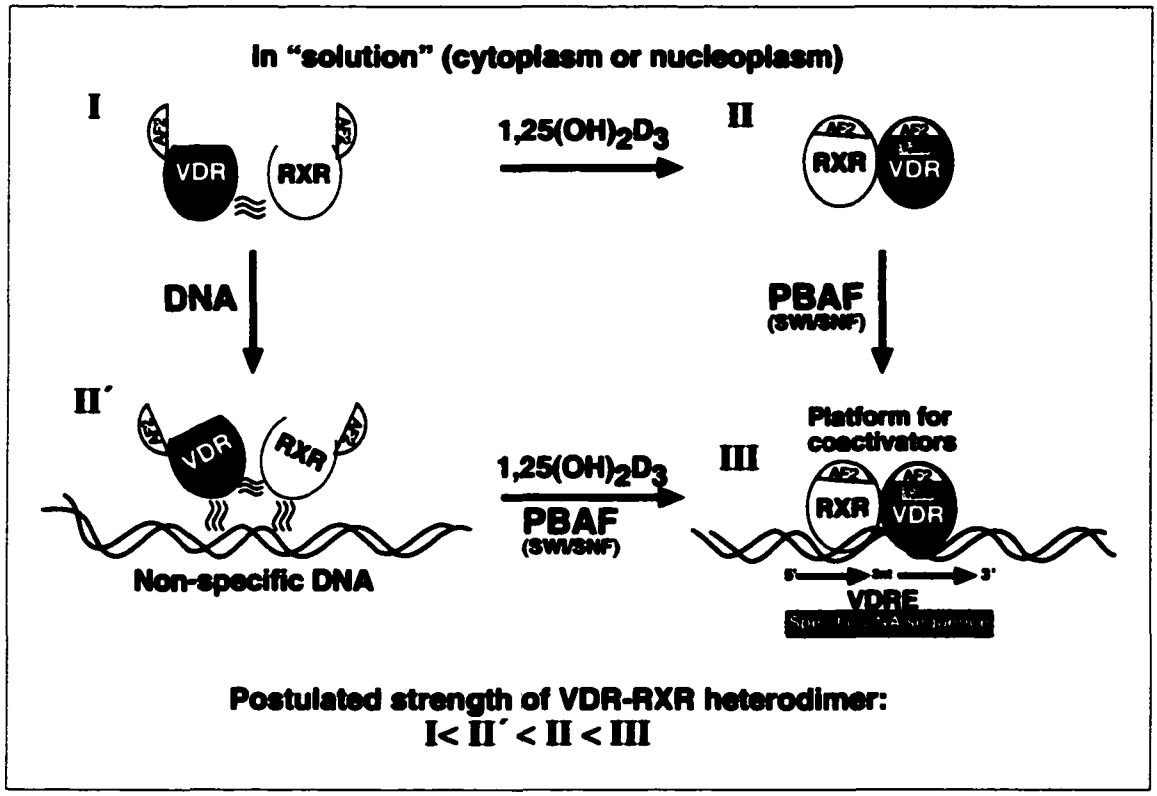


Fig. 21. VDR activation by $1,25(OH)_2D_3$ promotes heterodimerization with RXR and DNA binding. In state I, unliganded VDR interacts weakly with RXR in solution and most of the interaction occurs between the DBDs. Once DNA is present, in state II', dual DBD association with nonspecific DNA is postulated to strengthen the intermolecular interaction within the receptor apoheterodimer. Alternatively, if the free apoheterodimer becomes liganded with $1,25(OH)_2D_3$ in solution, strong VDR-RXR heterodimerization occurs, as depicted in state II. The $1,25(OH)_2D_3$-liganded heterodimer is capable of high affinity DNA binding and recognizes the specific VDRE (state III) to provide a coactivator platform in the promoter region of target genes. It has been suggested (Lemon *et al.* 2001) that the ongoing activity of the chromatin remodeler protein, PBAF, provides nuclear receptors with histone-free DNA, enabling them to locate the specific DNA sequences to which they bind and promote gene expression.

After ligand binding, the receptor undergoes apparent conformational changes, enabling it to interact with RXR with higher affinity. We have been able to demonstrate this effect in the yeast two-hybrid system, in which the absence of hormone precludes detectable interaction between VDR and RXR, and hence the transcriptional regulation of target genes. As discussed above, mutations that prevent hormone binding can also influence the rate at which VDR dimerizes with RXR. Whitfield et al. (Whitfield *et al.*

1996) identified two natural mutations, I314S and R391C in the hormone binding domain of hVDR that hindered heterodimerization with RXR, affecting the overall response to the $1,25(OH)_2D_3$ hormone and thereby resulting in the clinical syndrome of hypocalcemic vitamin D resistant rickets in patients that harbor these mutations. The molecular pathology in these patients, as well as the demonstration that conditional knockout of RXRs in skin renders mice alopecic (Li *et al.* 2000b), just as does VDR knockout (Li *et al.* 1998), provides strong support for the obligatory role of the RXR in VDR signal transduction.

The β-galactosidase quantitative assay helped us measure the effect that hormone binding exerts on dimerization. An increase in reporter gene expression of 5-fold was observed when hormone is present, thus supporting previous findings by Liao *et al.* (Liao *et al.* 1990), and more recently by Jurutka *et al.* (Jurutka *et al.* 2001b), that VDR-RXR heterodimerization and subsequent DNA-binding are driven primarily by hormone binding. Additional experimental support for this molecular mechanism was obtained from the hVDR-GST fusion protein pull-down assay. The presence of $1,25(OH)_2D_3$ hormone enhanced VDR-RXR heterodimerization that was further augmented by the presence of a VDRE. In this assay however, we were able to observe a weak interaction between these receptors when hormone was absent, supporting the findings of Thompson *et al.* (Thompson *et al.* 1998), that the vitamin D receptor can exist weakly associated with its RXR partner prior to ligand binding, with hormone association causing a significantly enhanced interaction.

Another *in vitro* test, the gel mobility shift assay, also enabled us to observe the effect that hormone imposed on DNA binding. In the absence of hormone, very little shift in the mobility of the labeled VDRE probe is observed. Once again, these findings support the general notion that hormone binding and heterodimerization enable this heterodimeric transcription factor, working as a unit, to interact with DNA and support gene expression. As proposed by Staal *et al.* (Staal *et al.* 1996), the binding of VDR-RXR to DNA positively affects the heterodimer conformation making it possible for the receptors to expose domains that will serve as docking sites for coregulators to interact and further enhance the transcriptional response.

A final *in vivo* assay further supported the role of RXR on the actions of VDR in transactivation. In transfection assays, where VDR is endogenously abundant, the addition of ligand promoted the expression of a transiently transfected GH reporter gene only at a minimal level. However, the overexpression of hRXRα dramatically enhanced this response, indicating a need for the RXR partner in the system to transduce the transcriptional response.

Thus far we have studied the initial steps of gene control by $1,25(OH)_2D_3$ and VDR: liganded-VDR heterodimerization with RXR, stabilization of the VDR-RXR heterodimer on a DNA binding platform, and VDR-RXR-VDRE-driven activation of transcription. In order to understand the molecular mechanisms by which VDR promotes gene expression, it is important to dissect the phases of the signal transduction pathway into isolated phenomena, being able to analyze each step that is part of an entire series of molecular events that occur almost simultaneously, or perhaps as part of a

cycle. In the next chapter, we will evaluate the role of potential VDR coactivators in the mechanism of $1,25(OH)_2D_3$-mediated transactivation, and define the contribution of these coregulator molecules to the transcriptional life cycle of VDR.

CHAPTER IV. FUNCTIONAL AND PHYSICAL INTERACTION OF VDR/RXR WITH P160, P300 AND P62 COACTIVATORS

Hypothesis tested and findings

The questions asked in this section were: i) which HAT coactivators interact directly with VDR through its AF-2, ii) are there domains in VDR besides the AF-2 that influence VDR-coactivator interaction, iii) does the non-HAT NCoA-62 coactivator associate with VDR in a hormone-dependent manner and iv) what is the functional effect of p160, p300 and p62 coactivators on VDR-mediated transcription? It was observed that only SRC-1 HAT coactivator directly binds to VDR and this interaction bridges to the CBP HAT cointegrator, forming a ternary complex that likely remodels chromatin nucleosome architecture. Other regions of VDR in addition to the AF-2 are required for full SRC-1 binding, including helix-3 and the N-terminal domain. Both SRC-1 and CBP HAT coactivators amplify $1,25(OH)_2D_3$-stimulated transcription in cotransfected mammalian cells, a functional effect potentiated by NCoA-62. Thus, hormone-dependent binding of NCoA-62 to VDR helps recruit HAT coactivators for chromatin remodeling.

Background

Role of coactivators in nuclear receptor control of gene expression

The mechanism of control of gene expression by nuclear receptors has remained elusive for more than a decade (Fry and Peterson 2002). By analogy with prokaryotic transcriptional activation systems such as CAP protein directly contacting and recruiting

RNA polymerase (Benoff *et al.* 2002), it was originally thought that when nuclear receptors are bound to the promoter regions of target genes, they initially associated directly with key components of the transcription machinery. However, the most recent hypothesis states that nuclear receptors must first recruit several different proteins, including chromatin-modifying enzymes, in order to promote gene transcription. The manner in which these different proteins and enzymes interact, and the order in which they appear within the active transcriptional enhancing complex, remains a subject of intense study. Chromatin-remodeling enzymes appear to be present in every step of the transcriptional process, altering the folding, fluidity and structure of chromatin to make DNA accessible to activators (Fry and Peterson 2002). It will be important to understand the role that nuclear receptors play to coordinate chromatin-remodeling, and how they are then able to attract the transcription machinery leading to activation of target genes mediated by VDR.

It is well established that after binding to their specific ligand, the nuclear receptor undergoes conformational changes leading to sequential receptor-coactivator interactions that culminate in transcription initiation (McKenna and O'Malley 2000). In order to comprehend how this process is achieved, it will be necessary to understand the order in which these interactions occur, as well as the functions that this variety of enzymes serve. The enzymatic activities include ATPases (Fryer and Archer 1998), acetyltransferases (Chen *et al.* 1997; Spencer *et al.* 1997), methyltransferases (Chen *et al.* 1999), ubiquitin ligases (Nawaz *et al.* 1999), etc., and a number of signals orchestrate these catalytic events (McKenna and O'Malley 2000). Binding of hormone to the

receptor promotes the association of the NR with chromatin-modifying complexes such as SWI/SNF and/or PCAF, as well as the SRC (p160) and CBP (p300) families of coactivators, which possess histone acetyltransferase (HAT) activity that promotes chromatin disruption. Once the DNA is accessible, the complex next brings to the promoter region, via DRIP/ARC and Mediator, the general transcription machinery, resulting in gene expression. As mentioned above, the mechanism of these events is not well understood, but recent evidence suggests that this cascade may be partially defined by post-translational modifications of a variety of substrates mediated by these coactivators (McKenna and O'Malley 2000).

Coactivators can be defined as proteins that potentiate the activity of specific transcription factors (Rachez and Freedman 2000). Their actions appear to be effective once they bind through protein:protein interactions with the DNA-bound receptors. An early indication of the need for coactivators was the observed phenomenon of squelching in transient transfection assays, that is vast overexpression of one NR can squelch the ability of other endogenous NRs to activate transcription in response to cognate ligand. (Meyer *et al.* 1989). The explanation for this effect is that a limited concentration of each coactivator was available, and that these common cofactors crucial for activating gene expression were sequestered by the overexpressed NR.

Steroid receptor coactivator-1 (SRC-1)

The steroid receptor coactivator-1 (SRC-1), a p160 class of coactivator, was first identified by Oñate *et al.*, (Oñate *et al.* 1995) in the yeast two-hybrid system, utilizing the progesterone receptor (PR) LBD as a bait, screening a human β-lymphocyte cDNA library. Later, it was demonstrated that a broader range of liganded receptors such as the glucocorticoid receptor (GR), estrogen receptor (ER), thyroid hormone receptor (TR) and retinoid X receptor (RXR) could also interact with this protein. It was also observed that the squelching effect observed in PR/ER coupled transfections could be relieved by overexpression of p160s. The results of these experiments indicated that SRC-1 might be the limiting factor recruited by the receptors to activate transcription. Furthermore, competitive assays utilizing the SRC-1-receptor interaction domain (RID) suppressed PR activation by SRC-1 in a dominant-negative fashion, both *in vivo* and *in vitro* (Jenster *et al.* 1997), suggesting a direct effect of SRC-1-receptor interaction in gene expression activation. Interactions between SRC-1 and nuclear receptors appear to be dependent largely on the integrity of the receptor AF-2 domain (Gill *et al.* 1998). SRC-1 possesses two transferable autonomous transactivation domains, that when fused to heterologous DBDs of transcription factors, are capable of enhancing gene expression (Onate *et al.* 1998). In addition to nuclear receptors, SRC-1 can also enhance the response of other transcription factors such as AP-1 (Lee *et al.* 1998), serum response factor (Kim *et al.* 1998) and NFκB (Na *et al.* 1998).

The SRC-1/p160 family, named after the 160 kDa steroid receptor coactivator-1, is comprised of three types of factors, depending on their homology: SRC-1/NCoA-1,

SRC-2/GRIP1/TIF2/NCoA-2 and SRC-3/pCIP/RAC3/ACTR/AIB1/TRAM-1 (McKenna *et al.* 1999a). The originally described activity intrinsic to these coactivators was the HAT activity that catalyzes the acetylation of lysine residues at the N-terminus of histones, making them negatively charged and releasing them from interaction with the DNA-phosphate backbone (Chen *et al.* 1997; Spencer *et al.* 1997), resulting in chromatin destabilization and DNA accessibility for transcription factors.

Specific inactivation of SRC-1 shows that there may be redundant activity, since SRC-1 null mice display only limited resistance to nuclear hormones such as T_3, and the mRNA levels of homologous coactivators like SRC-2/TIF2 are high, perhaps compensating for the SRC-1 lack of function (Xu *et al.* 1998). The effects of SRC-1 in potentiating ligand-dependent nuclear receptor activity have not been limited to *in vivo* assays. *In vitro* assays utilizing templates with and without chromatin have shown that SRC-1 potentiates gene expression in both systems, suggesting a further effect of the coactivator in gene expression besides chromatin remodeling (Liu *et al.* 1999). This potentiation in the absence of chromatin may come from the interaction between SRC-1 and the p300 family CREB binding protein (CBP), shown to enhance gene activation mediated by ER (Smith *et al.* 1996).

Thus, the p160 family of proteins are coactivators found at limiting amounts *in vivo* that directly interact with NR superfamily members to enhance the transcriptional response to ligand by remodeling chromatin architecture through HAT activity, rendering DNA accessible for the transcription factors and transcription machinery to promote gene expression.

LXXLL motifs

In addition to their HAT activity regions, receptor interacting domains (RIDs) have been identified in the SRC-1/p160 family of coactivators. The RIDs, NR boxes or nuclear receptor interaction domains (NIDs), as they are called, are α-helices composed of leucine stretches (LXXLL, where X is any amino acid) (Heery *et al.* 1997; Torchia *et al.* 1997), necessary and sufficient to mediate the binding of the coactivators to liganded NRs. Three RID motifs are conserved in the SRC-1 family, separated by 50-60 amino acid each (McKenna *et al.* 1999b). Mutations in key residues of the RID boxes of SRC-1 have been shown to abolish interaction with the AF-2 of ER, but these alterations do not affect its interaction with the CBP cointegrator (Heery *et al.* 1997), suggesting that these domains are specific for NR association. Multiple RID boxes involved in the receptor-coactivator interaction suggest distinct RID boxes for different classes of nuclear receptors (Ding *et al.* 1998). Also, select combinations of RID motifs direct the interaction of the coactivator with distinct combinations of nuclear receptors (Darimont *et al.* 1998; Ding *et al.* 1998; McInerney *et al.* 1998; Voegel *et al.* 1998). For example, RXR-TR and RXR-RAR heterodimers require the second and third RID motifs of SRC-2/GRIP1 for interaction, while RXR-PPAR requires the first and second motif for the same association, and the ER homodimer relies solely on the second RID box to bind to SRC-1 (McInerney *et al.* 1998; Mak *et al.* 1999). The NR domain thought to direct the interaction between receptors and p160s is the AF-2/helix-12. Jurutka *et al.* (1997), Masuyama *et al.* (1997), Liu *et al.* (1998) and Gill *et al.* (1998) (Jurutka *et al.* 1997; Masuyama *et al.* 1997a; Gill *et al.* 1998; Liu *et al.* 1998), amongst others, described the

importance of this highly conserved AF-2 region (Fig. 22) in facilitating the interaction of NRs with coactivators.

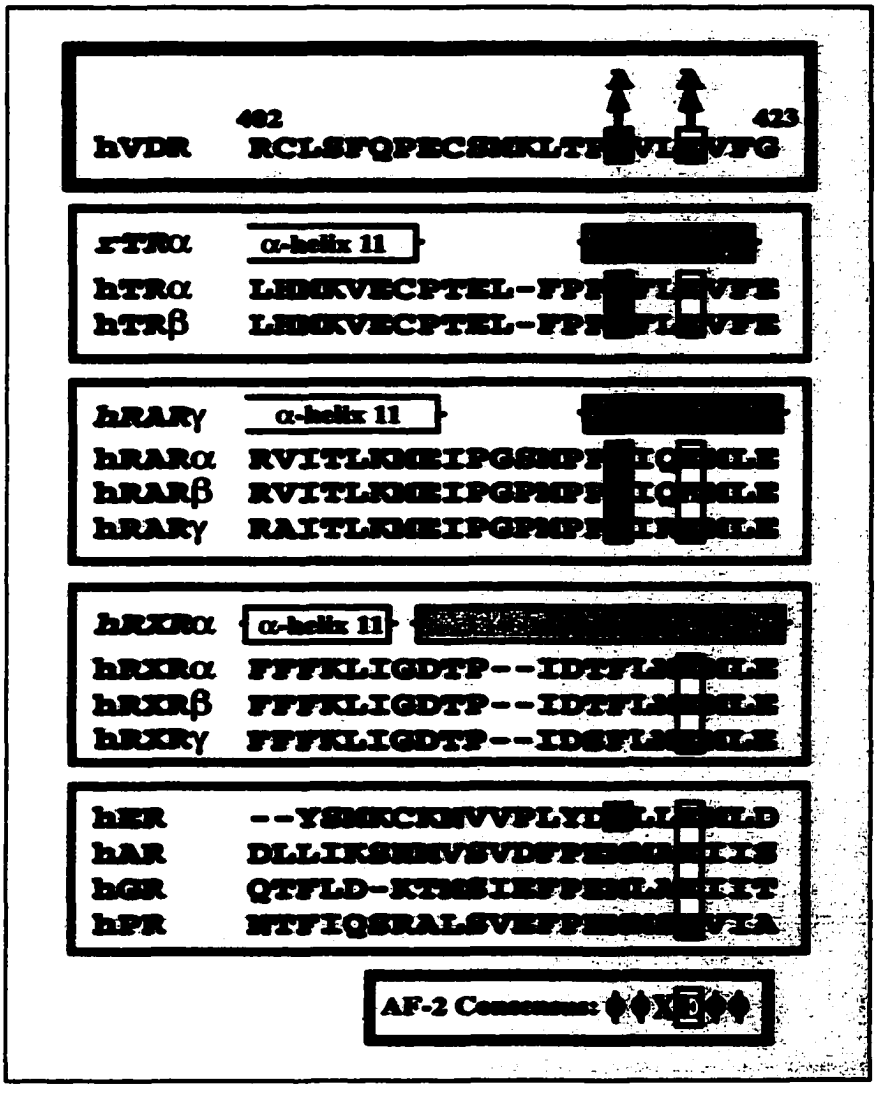


Fig. 22. Comparison of the C-terminal amino acid sequence of the AF-2 region in the nuclear receptor superfamily. The conservation of a glutamic acid residue throughout the superfamily is shown along with a consensus sequence for AF-2 activity (φ= hydrophobic amino acid). Also depicted are the point mutations generated for hVDR at residues 417 and 420, where changes to alanines result in a transcriptionally defective proteins (Jurutka *et al.* 1997).

Mutations of any of the key residues in helix-12 abrogate NR-coactivator association, hence, preventing the expression of target genes. The association between NRs and coactivators does not rely solely on the helix-12 domain, rather it has been demonstrated that the presence of hormone redirects positioning of the helix-12/AF-2 from the solution towards the receptor itself, near the location of helices 3, 4 and 5, creating a hydrophobic groove to which these cofactors can bind and make stable contacts with the receptor (Feng *et al.* 1998). Crystallographic studies of the liganded NRs have shed some light on this interaction. In the case of TR (Liu *et al.* 1998), the RID interacts with the hydrophobic platform that is created when the receptor binds hormone, positioning the AF-2 near helices H3, H4 and H5 (Feng *et al.* 1998). The liganded PPARγ-LBD crystal structure in the presence of two RID motifs of SRC-1 has suggested a model in which the second and third RID box are each binding one receptor of the heterodimer, providing a stoichiometry of one SRC-1 molecule per heterodimer (Nolte *et al.* 1998; Gampe *et al.* 2000). Therefore, recent observations made for the interactions between coactivators and NRs have found that the RID or NIDs are indispensable for their association with nuclear receptors, and that these interactions occur primarily at the hydrophobic cleft generated by the AF-2/helix-12 interfacing with helices 3-5 on the surface of the LBD. This interaction can be abrogated by mutations found in either the RID site in the cofactor or in the receptor's AF-2 or helix-3.

Cointegrator CBP/p300

The CREB binding protein (CBP) was initially identified as a coactivator required for the activation of cAMP-regulated genes controlled by the cAMP response element-binding protein (CREB) (Kwok *et al.* 1994). Because of its HAT activity, CBP has been implicated as a coactivator for multiple transcription factors such as p53 (Avantaggiati *et al.* 1997), NFκB (Perkins *et al.* 1997), and NRs (Chakravarti *et al.* 1996; Kamei *et al.* 1996), where interactions with TR, RXR and ER have been found to be mediated by a RID box located in the N-terminal portion of CBP (Kamei *et al.* 1996). CBP also interacts with members of the SRC family, forming ternary complexes with SRC members and NRs, thereby enhancing gene expression (Kamei *et al.* 1996; Torchia *et al.* 1997; Voegel *et al.* 1998). These interactions have been observed for the ER and PR systems (Smith *et al.* 1996), where CBP synergizes with SRC-1, leading to a positive effect on gene expression. CBP forms a stable complex with the SRC-1 homologue, ACTR (Demarest *et al.* 2002), and its interactions with liganded ER and PR are weaker than those observed with p160s like ACTR and SRC-1 (McKenna *et al.* 1998). O'Malley's group has proposed that liganded receptors interact initially with SRC-1, and that this coactivator associates with CBP to further promote gene activation, as observed in SRC-1 immunodepletion assays (Torchia *et al.* 1997) where CBP alone could not restore the activity of a RARE-linked reporter gene in the presence of liganded RXR-RAR heterodimer. Because of its involvement with several transcription factors, it has been postulated (Kamei *et al.* 1996) that CBP may be a limiting factor controlling and

integrating multiple and divergent signals initiating from different pathways that converge at a unique promoter to regulate the expression of a particular gene.

p300 (Eckner *et al.* 1994) shares many similarities with CBP, including an ability to interact with several transcription factors like MyoD (Yuan *et al.* 1996), p53 (Avantaggiati *et al.* 1997) and NRs (Chakravarti *et al.* 1996), but the activities of p300 and CBP are not redundant, as demonstrated by the p300 knockout mouse (Yao *et al.* 1998). In this system, the effects of CBP alone were not sufficient to rescue the p300 lack of function in certain tissues, and at specific embryonic stages. Recent reports have shown functions for the HAT activity of CBP/p300 in addition to chromatin remodeling. For example, this coactivator can acetylate proteins other than histones, such as p53, promoting DNA-binding by this transcription factor (Gu and Roeder 1997). Components of the transcription machinery are also acetylated by p300 and pCAF, but the relevance of this modification is still not well understood (Imhof *et al.* 1997).

The current view of the actions of p160 coactivators is that they recruit CBP/p300 to the nuclear receptors, with CBP/p300 representing the predominant, but not exclusive, source of HAT activity in the ternary complex. A "fire/reload" model for the actions of p300 has been postulated (McKenna *et al.* 1999a). This model invokes a mechanism in which the effects of ER/p300 are essential to enhancing transcription (fire), but only the ER is required for reassembly of the transcription complex (reload). This model illustrates the complexity that exists at the promoter region of active genes, and the role that these factors may play in stimulating gene transcription.

SRC-1 and VDR

After its discovery as a PR, TR, GR and RXR coactivator (Oñate *et al.* 1995; Takeshita *et al.* 1996), SRC-1 was independently cloned through the use of the yeast two-hybrid screening of a human kidney cDNA library, utilizing VDR as a bait (Gill *et al.* 1998). It was found that this interaction was $1,25(OH)_2D_3$-dependent and that the hormone concentrations needed to promote the association were similar to those levels found physiologically. With the previous knowledge that coactivators bind to the highly conserved AF-2 region in NRs, the VDR AF-2 was the subject of analysis (Jurutka *et al.* 1997; Gill *et al.* 1998; Kraichely *et al.* 1999) in order to dissect the role that this domain plays in coactivator binding, and hence in transcription activation. Deletion and point mutation analysis revealed that this region is required for both functions. Gill *et al.* (Gill *et al.* 1998) demonstrated that the C-terminal amino acids 419-423 of hVDR are required for interaction with SRC-1. The integrity of this domain is also required for ligand binding (Danielian *et al.* 1992; Barettino *et al.* 1994; Durand *et al.* 1994), but point mutagenesis of two conserved residues in this region that did not disrupt ligand binding (Leu-417 and Glu-420) still abrogated transactivation (Jurutka *et al.* 1997). Thus, the AF-2 domain of hVDR contains amino acids that form an amphipathic α-helix that promotes interaction with SRC-1. Based upon X-ray crystallographic data from other NRs, in the unoccupied receptor, the AF-2 or helix-12 projects into the solvent (Bourguet *et al.* 1995), and in the presence of ligand, the helix folds back to helices 3-5 of the receptor (Renaud *et al.* 1995; Wagner *et al.* 1995) to create a platform for SRC-1 association (Fig. 23). The repositioning of helix-12 also "traps" the ligand in the

hydrophobic pocket, increasing the half-life of the $1,25(OH)_2D_3$-VDR noncovalent association and potentiating the action of the liganded receptor on transcription.

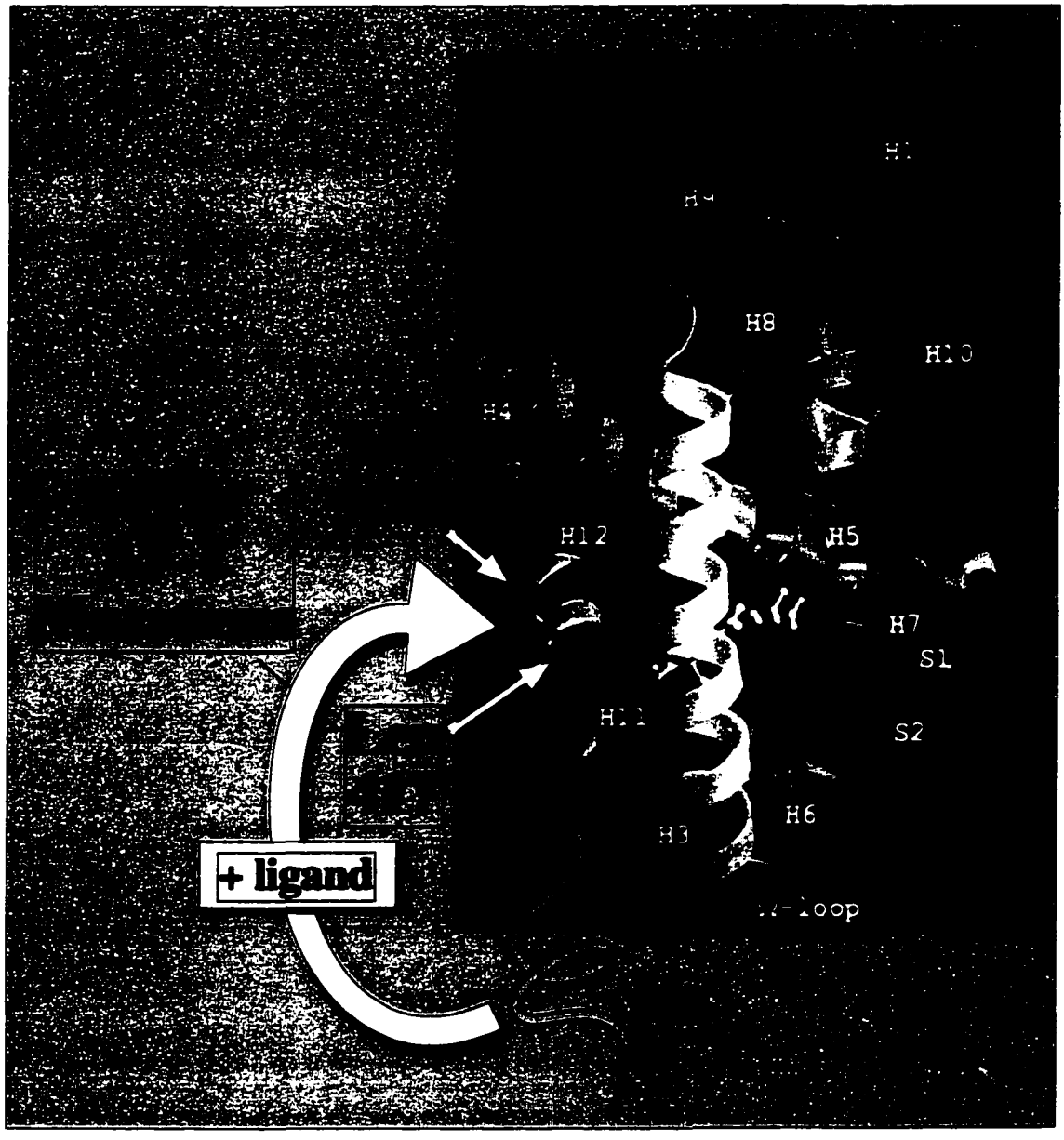


Fig. 23. The crystal structure of RARγ bound to its all-*trans* retinoic acid ligand. From Renaud *et al.* (1995), showing the proposed H12/AF-2 realignment, once ligand is present. The unliganded position of H12 in RARγ is inferred from the crystal structure of apo RXRα (Bourguet *et al.* 1995), wherein H12 protrudes outward in to the solvent. Also illustrated are the corresponding and positionally conserved VDR AF-2 residues in which point mutations were generated (L417A and E420A; see Fig. 22).

In addition to the role of the VDR AF-2 in contacting SRC-1, the RXR partner, once bound tightly to the liganded VDR, is also proposed to conform its AF-2 in a similar way to that of VDR, so that the SRC-1 can also contact the RXR AF-2 (Thompson *et al.* 2001), providing the aforementioned stoichiometry proposed by Nolte (Nolte *et al.* 1998), i.e., one molecule of SRC-1 coactivator per heterodimer. Crystallographic analysis and scanning surface mutagenesis of the hTRβ by Feng *et al.* (Feng *et al.* 1998) identified a hydrophobic cleft formed by helices H3, H4, H5 and H12, crucial for receptor binding to coactivators SRC-1 and GRIP1. MacDonald's group (Kraichely *et al.* 1999), through mutagenesis analysis utilizing *in vivo* and *in vitro* systems, identified residues within helix 3 (H3) required for ligand-dependent interaction of VDR and SRC-1 leading to transcriptional activation. They proposed that H3 and H12 in VDR fold in a way that creates a single transactivation surface in which both helices are required for optimal interaction with the p160 coactivators. It has been suggested that the mechanism of VDR action is similar to that of TR, where liganded receptor induces AF-2 reconfiguration and RXR heterodimerization, enabling this newly formed transcription factor heterodimer to bind DNA and coactivators to promote expression of target genes (Jurutka *et al.* 1997). Furthermore, Takeyama *et al.* (Takeyama *et al.* 1999) reported that in *in vivo* and *in vitro* assays, different analogs of the $1,25(OH)_2D_3$ hormone bound to the VDR and preferentially activated the receptor in its binding to coactivators, suggesting a more integral role of the ligand in control of gene expression. Taken together, these observations suggest that the role of hormone binding is to reconfigure the receptor's structure, specifically the AF-2 domain to create

a docking site to which coactivators can bind and help promote and control the expression of target genes.

Nuclear Receptor Coactivator-62 kDa (NCoA-62)

As stated above, nuclear receptor coactivators are proteins that directly interact with the receptors and function as mediators of transcriptional activation in response to ligand. MacDonald's group (Baudino *et al.* 1998) recently identified a 62 kDa protein that interacts with VDR and other nuclear receptors in a hormone-enhanced manner to mediate transcriptional activation. Initially identified as a human homologue of *Drosophila melanogaster* BX42, a nuclear protein involved in ecdysone-stimulated transcription (Wieland *et al.* 1992), NCoA-62 is recruited to the promoter of target genes by liganded VDR and promotes gene expression. This coactivator is not a member of any previously identified family of VDR coactivators and does not posses any LXXLL RID motifs (Baudino *et al.* 1998), indicating that its association with the receptor is AF-2/helix-12-independent (MacDonald *et al.* 2001). This suggests that NCoA-62 might function concomitantly with other known coactivators, specifically the p160 family (SRC-1, GRIP1, ACTR) that require an intact AF-2/helix-12 domain and ligand for VDR association. It was observed, in recent *in vitro* experiments by Zhang *et al.* (Zhang *et al.* 2001), that the vitamin D receptor may simultaneously interact with NCoA-62 and p160 coactivators in the presence of ligand, thus forming a ternary complex. This complex was capable of synergistically enhancing the transcriptional response in cotransfection assays of p160s, NCoA-62 and VDR in the presence of ligand compared

to that of liganded VDR and p160 alone. By sequence analysis it was observed that the NCoA-62 coactivator does not possess any intrinsic HAT activity, thus suggesting that its actions on transcription are at a different level than the chromatin remodeling process (MacDonald *et al.* 2001). It has been suggested that NCoA-62 may act as a bridging protein between the HATs and VDR to anchor these proteins at the promoter region of target genes, amplifying the function of HATs to completely release histones from the DNA, making it possible for the PIC to start transcription. However, further studies are required to better understand the nature and functions of the ternary complex (NCoA-62-VDR-p160) in the signaling pathways with which they may be involved (Zhang *et al.* 2001).

Material and Methods

GST-pull-down assays

Sepharose beads containing immobilized hVDR or an AF-2 mutant hVDR (E420A) were prepared as described in Chapter II and used in pull down assays. The human SRC-1 expression plasmid (pCR3.1-hSRC-1A) was kindly provided by Dr. Ming Tsai (Spencer *et al.* 1997), Baylor College of Medicine. One μg of hRXRα (MacDonald *et al.* 1993) and 1.2 μg of SRC-1 expression plasmids were used as templates in an *in vitro* transcription and translation protocol, and the synthesized proteins were labeled with ^{35}S-methionine (Promega, Madison WI). GST-hVDR fusion proteins linked to glutathione-Sepharose, as well as the GST-E420A VDR mutant were used to test the interaction between the receptor and the SRC-1 coactivator in the presence of 10^{-7} M

$1,25(OH)_2D_3$ or ethanol vehicle, employing RXR, the natural VDR partner, as a positive control for VDR association. The counterpart experiment was also conducted, in which GST-SRC-1-Sepharose beads were used to map the interaction domains between VDR and the coactivator. For this experiment, several VDR point and deletion mutants were employed (see below).

The mouse CBP expression vector (pRc/RSV-mCBP) was kindly provided by Dr. Roland Kwok (Chrivia *et al.* 1993), Vollum Institute, Oregon Health Sciences University, Portland. This construct was used to prepare GST-mCBP fusion proteins linked to glutathione-Sepharose that tested the interaction of the cointegrator with full length VDR, RXR and the p160 coactivator, SRC-1, in the presence and absence of 10^{-7} M $1,25(OH)_2D_3$.

The human NCoA-62 expression plasmid (pSG5-hNCoA-62) was kindly provided by Dr. Troy Baudino (Baudino *et al.* 1998), St. Louis University Health Sciences Center, St. Louis MO.

Site-directed mutagenesis

The expression vector pSG5-hVDR was utilized in synthesizing deletion and point mutants by *in vitro* site directed mutagenesis (Stratagene, La Jolla CA). Alterations of specific residues/regions were created using previously described double stranded DNA protocols to generate changes in the hVDR sequence (Jurutka *et al.* 1997). The hVDR deletion mutants created were: Δ5-13, Δ14-23, Δ1-88, Δ304, Δ403, Δ424-427.

Transfections

ROS 17/2.8 cells were cotransfected with pSG5-hSRC-1 and/or pRc/RSV-mCBP expression plasmids (1 μg/plate), a single copy VDRE-containing reporter construct possessing approximately 1000 bp of 5' upstream promoter of the rat osteocalcin gene (rBGP-GH; 2 μg/plate), and carrier pTZ18U DNA (6 μg/plate). The cells were incubated with 10^{-8} M $1,25(OH)_2D_3$ (+) or vehicle (–) for 24 hours post-transfection and human growth hormone secretion into the medium was determined by RIA.

COS-7 cells were cotransfected with pSG5-hVDR and pSG5-hRXRα expression vectors (500 ng/plate) as well as with pSG5-hSRC-1 and/or pRc/RSV-mCBP (100 ng/plate), a four copy rat osteocalcin VDRE-containing reporter construct ($(CT4)_4$-TKGH, 10 μg/plate), and carrier pTZ18U DNA (9 μg/plate). The cells were incubated with 10^{-8} M $1,25(OH)_2D_3$ (+) or vehicle (–) for 24 hours post-transfection and human growth hormone secretion into the medium was determined by RIA.

C2C12 mouse myoblast cells were cotransfected with pSG5-hRXRα expression vector (500 ng/plate) and with 1 μg of pSG5-hSRC-1 and/or pSG5-hNCoA-62 in different combinations, as well as with 1 μg/plate of VDRE-containing reporter construct ($(CYP3A23\text{-}DR3)_2$-TKGH, detailed in Chapter 3), and carrier pTZ18U DNA (9 μg/plate). The cells were incubated with 10^{-8} M $1,25(OH)_2D_3$ (+) or vehicle (–) for 24 hours post-transfection and human growth hormone secretion into the medium was determined by RIA.

Results

In an effort to define the role of SRC-1, CBP/p300 and NCoA-62 coactivators in $1,25(OH)_2D_3$ signaling, the effect of overexpression of these cofactors on VDR mediated gene expression was investigated. We also tested the ability of these coactivator proteins to interact directly with VDR in biochemical pulldown experiments.

Which putative VDR coactivators interact directly with VDR?

First, we attempted to demonstrate direct, hormone-dependent interaction between VDR and SRC-1. By utilizing the *in vitro* GST-fusion protein pull down system, we were able to transcribe and translate *in vitro* (IVTT) the hSRC-1 coactivator, and show its association with the hVDR to be almost completely hormone-dependent (Fig. 24). In order to demonstrate the efficacy of the system, we employed VDR's natural partner (RXR) as a control, and demonstrated again that RXR heterodimerization with VDR is also significantly hormone dependent (Fig. 24A, lanes 1-2). Only in the presence of hormone was the SRC-1 coactivator capable of binding to VDR (Fig. 24A, lanes 3-4). It is also important to note that this interaction between SRC-1 and VDR is solely dependent on the $1,25(OH)_2D_3$ hormone because other ligands, such as less biologically active $1,25(OH)_2D_3$ precursors, and other steroid hormones were less effective, and ineffective, respectively, in recruiting SRC-1 to VDR (data not shown).

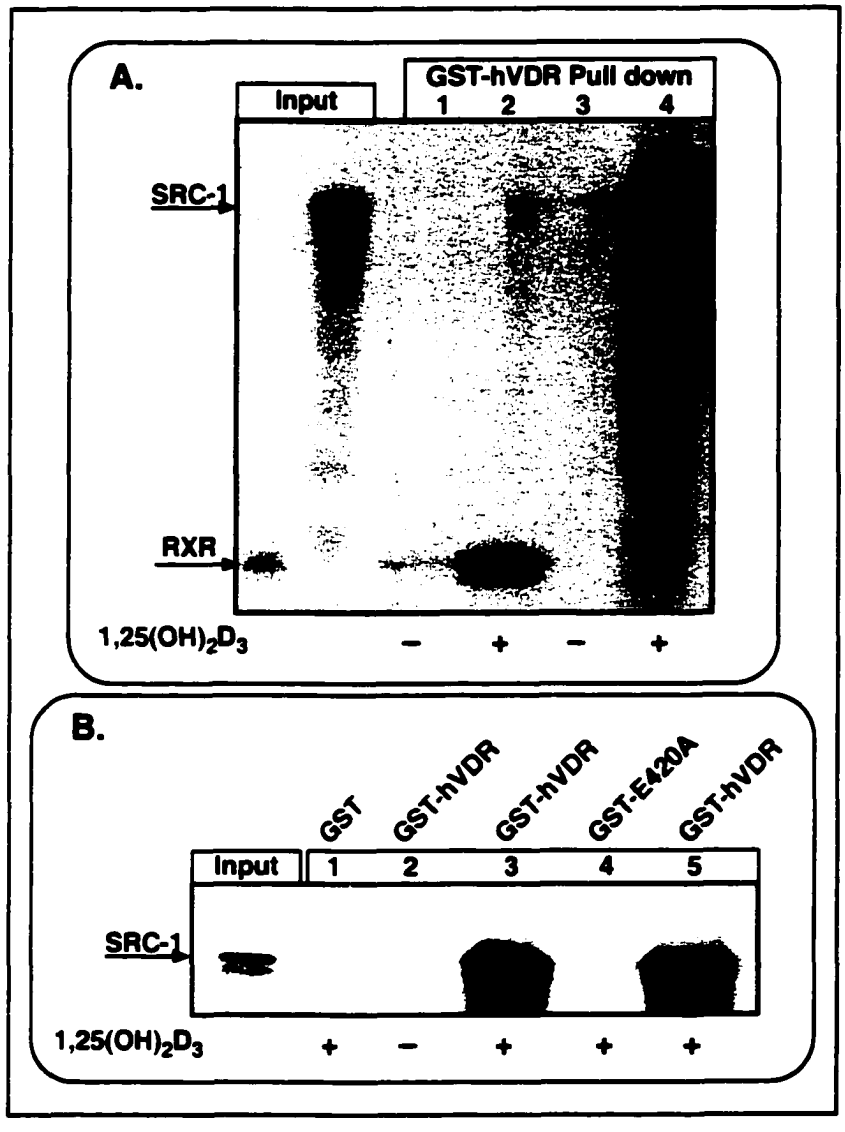


Fig. 24. GST-VDR pulldown assay of the interaction of VDR with SRC-1. **A**. The interaction between VDR and the SRC-1 coactivator is highly dependent on the presence of the $1,25(OH)_2D_3$ hormone (10^{-7} M), as is the association between VDR and its RXR partner, here shown as a positive control (lanes 1 and 2). **B**. The VDR-SRC-1 interaction is hormone and AF-2 dependent, because disruption of helix-12 integrity of the receptor compromises the interaction between these two molecules (lane 4), and the association between VDR and SRC-1 occurs only in the presence of ligand (lanes 3 and 5). Input represents 5% of the amount used in GST pulldown lanes.

We also evaluated the significance of the AF-2 domain in sustaining this interaction (Fig. 24B). The VDR point mutant E420A alters helix-12, in which an

otherwise negatively charged glutamic acid is replaced by an aliphatic residue (alanine), disrupting the charged character of this domain and its interaction with the SRC-1 (Fig. 24B). In the case of PPARγ, Nolte *et al.* (Nolte *et al.* 1998) have shown by x-ray crystallography that this highly conserved glutamic residue corresponding to E420 in hVDR is an actual contact site with a p160 coactivator RID peptide. Thus, the SRC-1 interaction with VDR is $1,25(OH)_2D_3$ hormone-dependent, and requires an intact AF-2/helix-12 to support the association.

A potential mCBP association with VDR was also evaluated through the GST-pull down assay, utilizing GST-CBP-Sepharose as a fixed matrix. In this assay, it was observed that the vitamin D receptor does not interact directly with the p300 family protein, regardless of the presence of $1,25(OH)_2D_3$ hormone (Fig. 25). This observation was consistent with other results investigating nuclear receptor-CBP interactions, specifically Heery *et al.* (Heery *et al.* 1997) studying RXR and RAR, and Li, *et al.* (Li *et al.* 2000a), probing TR. Both of these groups found that the CBP cointegrator interacts only weakly with certain members of the nuclear receptor superfamily in *in vivo* assays, especially compared to the interactions found between these receptors and the p160 coactivator, SRC-1. Interestingly, we have found that a ternary complex is formed between VDR, SRC-1 and CBP, with SRC-1 apparently performing a bridging function between VDR and CBP. As shown in Fig. 25, VDR exhibits very little interaction with the CBP cointegrator, even when liganded with $1,25(OH)_2D_3$ (Fig. 25, lanes 1-2). In contrast, the SRC-1 coactivator, displays a robust interaction with CBP (Fig. 25, lanes 5-6). The further addition of the RXR partner to the VDR-CBP reaction neither facilitates

(via allosteric influences) direct interaction between VDR with CBP, nor provides a bridge between VDR and CBP (Fig. 25, lanes 7-8), as the trace amount of direct contact between CBP and either of these two nuclear receptors is no greater when combined (Fig. 25, lanes 7, 8) as when they are present individually (Fig. 25, lanes 1-4). Interestingly, the concomitant addition of SRC-1 and VDR to the CBP bead system shows not only the association of SRC-1 with CBP beads, but also establishes an indirect interaction between VDR and CBP (Fig. 25, lanes 9-10), providing evidence for the existence of a ternary complex. This complex, generated by SRC-1 coactivator bridging from VDR to CBP, may be important in the enhancement of transcription mediated by the liganded vitamin D receptor.

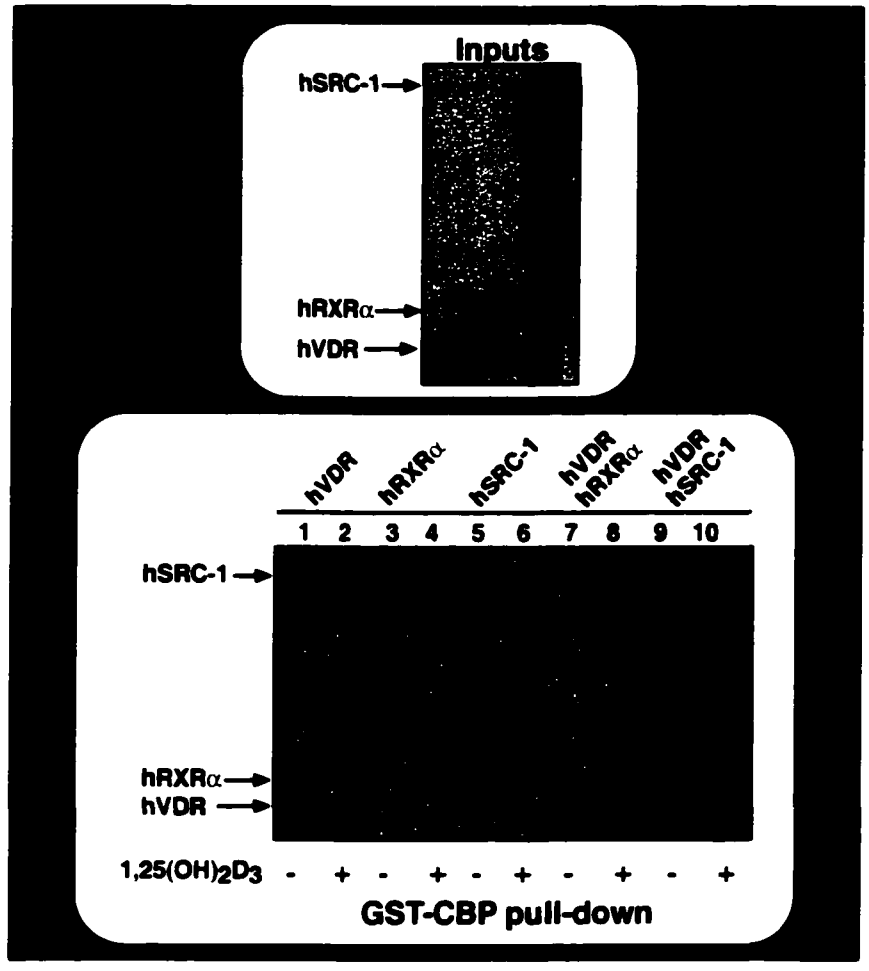


Fig. 25. GST-CBP pulldown assay testing the interaction of the cointegrator CBP with VDR and SRC-1. There is only a trace interaction between CBP and VDR, regardless of the presence of the $1,25(OH)_2D_3$ hormone (10^{-7} M) (lanes 1-2), as well as for its natural partner, hRXRα (lanes 3-4), or the VDR-RXR heterodimer (lanes 7-8). However, the SRC-1 coactivator interacts strongly with the CBP cointegrator (lanes 5-6). The concomitant addition of the p160 coactivator, SRC-1, and VDR to the reaction causes VDR to be pulled down indirectly by CBP, through a ternary complex between the CBP beads, SRC-1 and VDR (lanes 9 and 10).

The association of the NCoA-62 coactivator and VDR was also tested in the GST-pull down assay. In this case, we utilized the wild type VDR fused to glutathione-S-transferase and linked to glutathione-Sepharose beads. Similar to that observed by Baudino *et al.* (Baudino *et al.* 1998), the NCoA-62 coactivator binds to the GST-VDR fusion protein more avidly in the presence of the $1,25(OH)_2D_3$ hormone (Fig. 26, right

panel). However, unlike coactivators of the p160 family, NCoA-62 binding to VDR is not dependent on a functional AF-2 domain in the receptor, as shown by MacDonald and colleagues (MacDonald *et al.* 2001). Thus, NCoA-62 represents a different class of coactivator which, as discussed below, contacts a domain(s) in VDR other than the AF-2 and may function to facilitate the recruitment of SRC-1/CBP to the receptor.

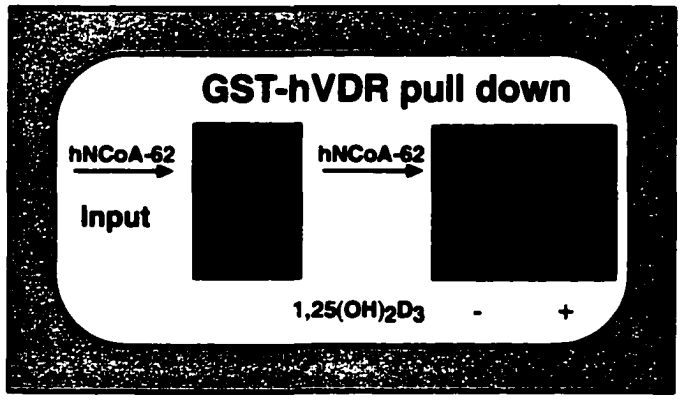


Fig. 26. GST-VDR pull down assay to probe the interaction of VDR with hNCoA-62. The VDR-NCoA-62 interaction is hormone dependent. Input, left, represents 5% of the amount used in GST pull down lanes.

What is the functional effect of putative VDR coactivators?

A second set of experiments was performed *in vivo*, utilizing transfection of cell lines that represent classical VDR target tissues such as bone osteoblasts (ROS 17/2.8) and kidney (COS-7). We first determined whether there was an enhancement in the $1,25(OH)_2D_3$-VDR mediated gene transcription response in the presence of overexpressed SRC-1 (Fig. 27). The overexpression of SRC-1 increased the transcriptional activity of $1,25(OH)_2D_3$ by 30 to 40 percent in either ROS 17/2.8 or COS-7 cell lines, in which the endogenous and exogenous VDR response was evaluated,

respectively (compare first and second panels in Fig. 27A and 27B). We next assessed the effect of overexpression of the CBP cointegrator in these systems. Similarly to SRC-1, exogenous CBP significantly augmented VDR-mediated transcriptional activation above that observed with hVDR transfection alone (compare first and third panels in Fig. 27A and 27B).

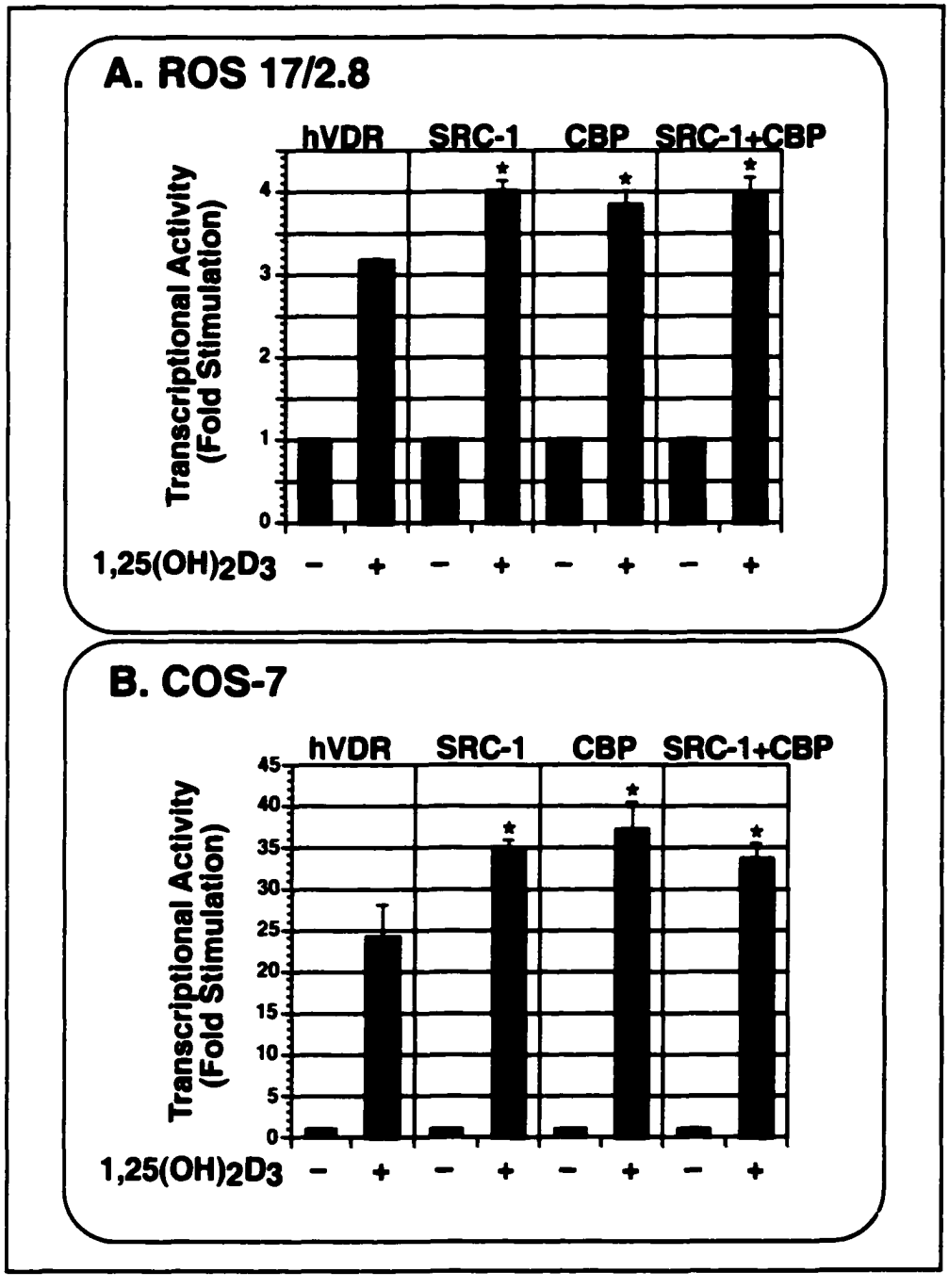


Fig. 27. The effect of overexpression of coactivator SRC-1 and/or CBP on the $1,25(OH)_2D_3$-VDR gene response system in intact bone (A, endogenous VDR) **and kidney** (B, exogenous VDR) **cells.** (*) Significantly different from VDR alone hormone-dependent activation ($p<0.05$). Each value is the average of 3 determinants ± SEM.

In cotransfections with both coactivator (SRC-1) and cointegrator (CBP), no further stimulation of $1,25(OH)_2D_3$-VDR-mediated transcription was observed beyond that found with either coactivator alone (compare second and third panels with the fourth panel in Fig. 27A and 27B). The fact that SRC-1 and CBP cotransfection effects are not additive may be explained by a "topping out" of transcriptional activation in the assay when either one alone is overexpressed. These observations in *in vivo* assays indicate that both the p160 coactivator (SRC-1) and the p300 cointegrator (CBP) enhance VDR-mediated transcription, and are therefore likely molecular players in VDR transcriptional signal transduction.

Next the effects of the NCoA-62 coactivator on VDR-mediated transactivation were evaluated in transfection assays, utilizing the mouse myoblast cell line C_2C_{12} (Fig. 28). In these cells with limiting concentrations of endogenous RXR, the addition of the RXRα partner amplified the $1,25(OH)_2D_3$ transcriptional response, confirming that the levels of RXR expressed in this cell line might not be sufficient to fully support VDR-mediated gene expression (Fig. 28, compare first and second panels). The concomitant transfection of an SRC-1 coactivator expression plasmid, along with RXRα, did not statistically significantly increase the transcriptional response to $1,25(OH)_2D_3$ (Fig. 28, third panel vs. second). However, cotransfection of NCoA-62, in addition to RXRα, increased reporter gene expression by 20%, displaying a statistically significant difference from that of RXRα alone (Fig. 28, compare fourth panel to second panel). The cotransfection of both SRC-1 and NCoA-62 in this system resulted in an additional 20% enhancement of that produced by NCoA-62 alone, and an overall 40%

augmentation of the VDR-RXR response (Fig. 28, fifth panel). These results are consistent with the independent findings of Zhang *et al.* (Zhang *et al.* 2001), in which cotransfection of NCoA-62 and p160 coactivators elicited a VDR transcriptional response exceeding that of either cofactor alone. The fact that SRC-1 cotransfection does not boost VDR-mediated transcription, but NCoA-62 does, suggests that the former is expressed sufficiently in C_2C_{12} cells, but the later coactivator is limiting. Interestingly, in this VDR-rich muscle cell line transfected with RXRα, once NCoA-62 is overexpressed, exogenous SRC-1 further augments the VDR response (Fig. 28). These findings (Fig. 28 and Zhang *et al.* 2001) suggest a model in which NCoA-62 facilitates the recruitment of SRC-1 by VDR, probably via contact with both nuclear receptor and HAT coactivator.

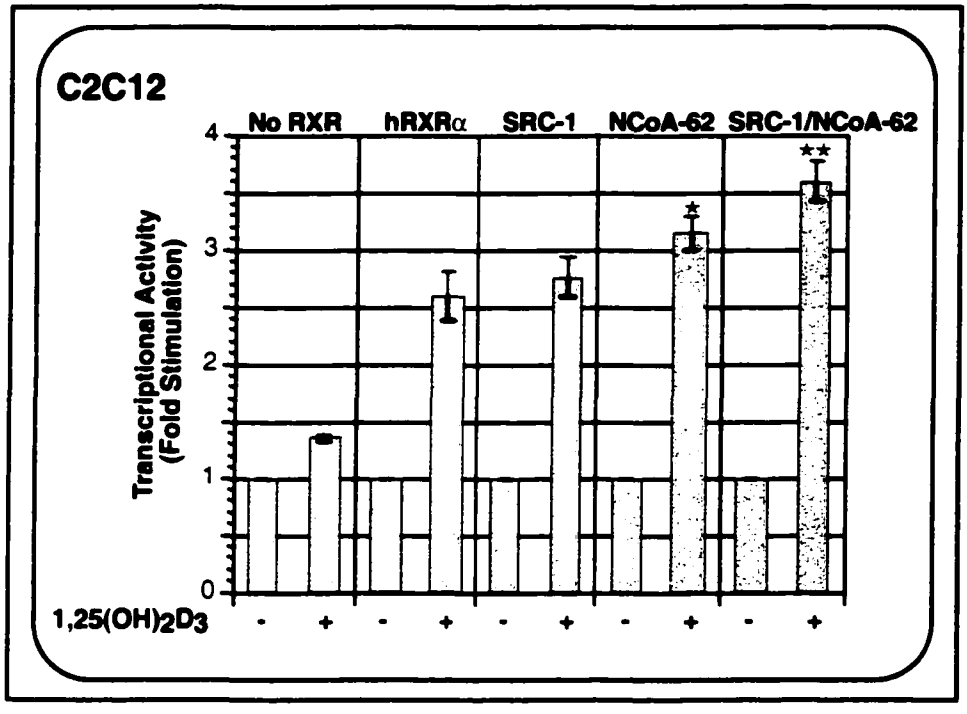


Fig. 28. Effect of the coexpression of coactivators SRC-1 and/or NCoA-62 on the $1,25(OH)_2D_3$-VDR gene response in myoblasts. Experiments in panels 3, 4 and 5 to the right include hRXRα cotransfection. (*) Statistically significantly different from RXR expression alone ($p<0.05$). (**) Statistically significantly different from NCoA-62 plus RXRα coexpression system ($p<0.05$). Each value is the average of 3 determinants ± SEM.

To extend the conclusion that SRC-1 is a positive player in VDR-mediated transcriptional signal transduction, we next investigated if additional regions of the VDR beyond the AF-2/helix 12 are involved in the association of the receptor with SRC-1. As shown in Fig. 29, several deletion mutants were utilized to characterize the interaction via an *in vitro* pull-down assay, utilizing a GST-SRC-1 fusion protein as a fixed matrix. As demonstrated previously using GST-VDR beads and pulling down SRC-1 (see Fig. 24), SRC-1 and WT hVDR interaction is enhanced by the presence of $1,25(OH)_2D_3$ ligand (Fig. 29, lanes 1 and 2), although the hormone dependence is not nearly as dramatic when SRC-1 is immobilized and VDR is free in solution. Perhaps when the N-

terminus of VDR is untethered it is able to influence ligand-dependent p160 coactivator recruitment, as in the case of other nuclear receptors such as ER (Sathya *et al.* 2002) and AR (Alen *et al.* 1999). Accordingly, we evaluated two amino-terminal deletion mutants of VDR for their ability to bind SRC-1 in the presence and absence of hormone. As shown in Fig. 29, lanes 2 and 3, the two N-terminal deletions displayed different behavior in their association with the SRC-1 coactivator. The Δ5-13 hVDR mutant exhibited (lanes 3 and 4) hormone-enhanced interaction of the receptor with SRC-1 that was intensified over that seen with the WT receptor. This enhanced association was diminished in the more central N-terminal deletion mutant, Δ14-23 hVDR (lanes 5 and 6), which was more like WT hVDR in character. Thus, the Δ14-23 region of VDR may contain an N-terminal, activation function-1 (AF-1), as was proposed previously (Jurutka *et al.* 2000b), but more importantly the Δ5-13 domain of hVDR appears to contain a repressive motif, that when either deleted (Fig. 29) or "neutralized" by adjacent covalent immobilization in GST-Sepharose, allows hVDR to recruit SRC-1 efficiently, and in a hormonal ligand-dependent manner. Presumably, in the intact cell, the DBD of unliganded VDR is associated with the DNA phosphate backbone and the N-terminus is occupied with TFIIB (see Fig. 14), leaving the C-terminal LBD free to interact with $1,25(OH)_2D_3$, much like the situation with hVDR-GST-beads.

Complementarily, we analyzed two C-terminal deletion mutants of hVDR for interaction with SRC-1 beads. The C-terminal hVDR deletion mutant Δ403, which lacks the AF-2/helix-12, displayed a significant loss in interaction with SRC-1, especially in the presence of hormone (Fig. 29, lane 8). The Δ403 hVDR still binds to $1,25(OH)_2D_3$,

but with a 10-fold higher K_d than WT hVDR (Nakajima *et al.* 1994), explaining the enhancement of Δ403 hVDR-SRC-1 interaction observed at the relatively high ligand concentration of 10^{-7} M (compare lanes 8 and 7 in Fig. 29). Nevertheless, the relatively weak association the Δ403 mutant showed with SRC-1 provided further evidence for the importance of the AF-2 region in the interaction of VDR with SRC-1, but at the same time suggested that this region is not the only one involved in VDR/SRC-1 attraction. This mechanism is supported by results with the Δ304 hVDR, a mutant lacking a large portion of the VDR LBD that consequently does not bind $1,25(OH)_2D_3$, but still displayed a strong association with SRC-1 independent of the presence of hormone (lanes 9 and 10). This finding with Δ304 hVDR indicates that there is a second region of the receptor involved in the interaction between VDR and SRC-1.

Thus, it was observed that the interaction between VDR and SRC-1 involves regions of the receptor N-terminal of the AF-2/helix-12. From Fig. 29, lanes 9 and 10, and as previously reported by MacDonald *et al.* (MacDonald *et al.* 2001), the helix-3 of VDR constitutes a second site in VDR for the interaction with coactivators, and we concluded that the Δ304 deletion mutant "exposes" this region in the tertiary structure of the protein to increase the overall association between hVDR and SRC-1, despite the absence of an AF-2 in hVDR. Finally, although the primary platform for SRC-1 interaction with VDR appears to be composed of helices 3-5 plus 12, the short N-terminal domain of hVDR also modulates receptor-coactivator association, in the case of hVDR in a suppressive fashion.

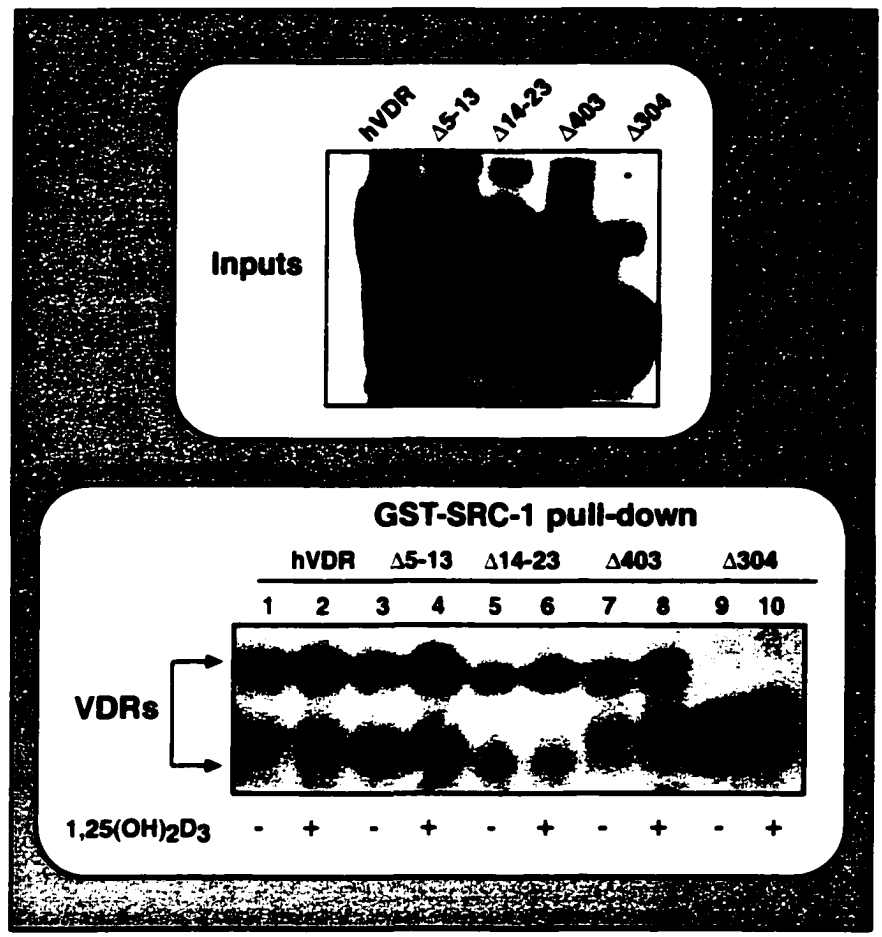


Fig. 29. Pull-down assay to map the domains of VDR involved in SRC-1 interaction. WT VDR and several deletion mutants were evaluated for their capacity to interact with SRC-1. WT VDR as well as the Δ5-13 and Δ14-23 deletion mutants display significant hormone-augmented interaction with SRC-1 beads (lanes 3-4 and 5-6 respectively). C-terminal deletion mutants (Δ403 and Δ304) do not display the same degree of hormone dependence in their association with SRC-1, but Δ304 exhibits a marked level of binding to this protein (lanes 9-10). Inputs (top panel) are 5% of that used in the pull down lane.

Conclusions

The experiments carried out above have helped to refine the model for VDR transcriptional activation by supporting the hypothesis that SRC-1 is a hormone-dependent coactivator for VDR, with SRC-1 binding directly to the receptor AF-2

domain. For activation to occur, the integrity of the AF-2 is necessary so that the helix-12/AF-2 region is able to "close the pocket" to which the ligand is bound and thus form the "docking" platform for coactivator interaction (Jurutka *et al.* 1997; Gill *et al.* 1998; Kraichely *et al.* 1999). In contrast NCoA-62 constitutes a second VDR coactivator that requires only ligand but not the AF-2 to interact with the receptor, but appears to facilitate VDR association with SRC-1, perhaps forming a ternary complex of VDR, NCoA-62 and SRC-1. This triplex has been observed recently for VDR by MacDonald's group (Zhang *et al.* 2001), in which the NCoA-62 coactivator interacts with certain members of the p160 family and VDR *in vitro*, helping mediate $1,25(OH)_2D_3$ driven control of gene expression (see data in Fig. 28).

We have also observed that VDR coregulators with HAT activity, such as SRC-1 and CBP, serve to potentiate gene expression induced by $1,25(OH)_2D_3$, and their actions are likely tied to their ability to remodel chromatin. In our observations, the modest level of transcriptional potentiation achieved with SRC-1 and/or CBP (Fig. 27) is probably due to the existence of nearly sufficient endogenous coactivator and cointegrator concentrations in ROS 17/2.8 and COS-7 cells, or the existence of additional redundant coactivator molecules present in these cells. Furthermore, the association between VDR and CBP is not through direct interaction. The association is mediated by the p160 coactivator, SRC-1, as part of a ternary complex as seen from the data in Fig. 25. Recent experiments have provided further evidence for this observation. In nuclear magnetic resonance spectrometric studies (Demarest *et al.* 2002), a mechanism called synergistic folding was characterized by which p160 coactivators

recruit the p300 family of cointegrators. In this mechanism, although isolated domains are intrinsically disordered, they combine with high affinity to form a cooperatively folded heterodimer. This suggests that it is through the p160s that the nuclear receptors such as TR and RAR, and in this case, VDR, can indirectly recruit and interact with p300s to activate gene expression. The actions of p300/CBP in nuclear receptor mediated transcription, at least for the homodimer subgroup, is suggested to be acetylation of the receptor (as in the case of ERα), leading to a higher response of the receptor to ligand, resulting in an overall enhancement of the transcriptional response

The regions of VDR involved in the association with SRC-1 include more than just the AF-2, as demonstrated by the VDR deletion mutants in pull-down assays (Fig. 29). These results suggest that even when a significant portion of the LBD is not present the receptor can interact with the coactivator, but does so in a ligand-independent manner. The Δ304 VDR, a mutant with a partial LBD deletion interacts significantly with SRC-1, perhaps because this mutant exposes additional internal regions of VDR (helices 3-5) involved in the association with the coactivator independent of the helix-12/AF-2 portion of the docking platform.

The overall actions of coactivators may be to create a high level of acetylation in the promoter region of hormone-dependent genes leading to chromatin remodeling. Further acetylation may promote autoacetylation of these enzymes once substrate (histone) is not available, serving as an exiting signal and enabling the next set of coregulators to deliver the transcription machinery and promote gene expression (McKenna *et al.* 1999a; Leo and Chen 2000).

The idea of coactivators serving as acetylation factors to make DNA accessible has been illustrated in Fig. 30. In this model, once VDR is liganded, heterodimerized and bound to specific VDREs (Fig. 30, step I) it is capable of interacting with HAT-coactivators (SRC-1, CBP) that in turn can be stabilized on the receptor by non-HAT proteins (NCoA-62) and promote the process of the chromatin remodeling. Once the coactivator is bound to the promoter-assembled VDR, the cofactors exert their actions on chromatin (Fig. 30, step II). Histone acetylation promotes chromatin reorganization leading to the release of histone from DNA contacts (Fig. 30, step III).

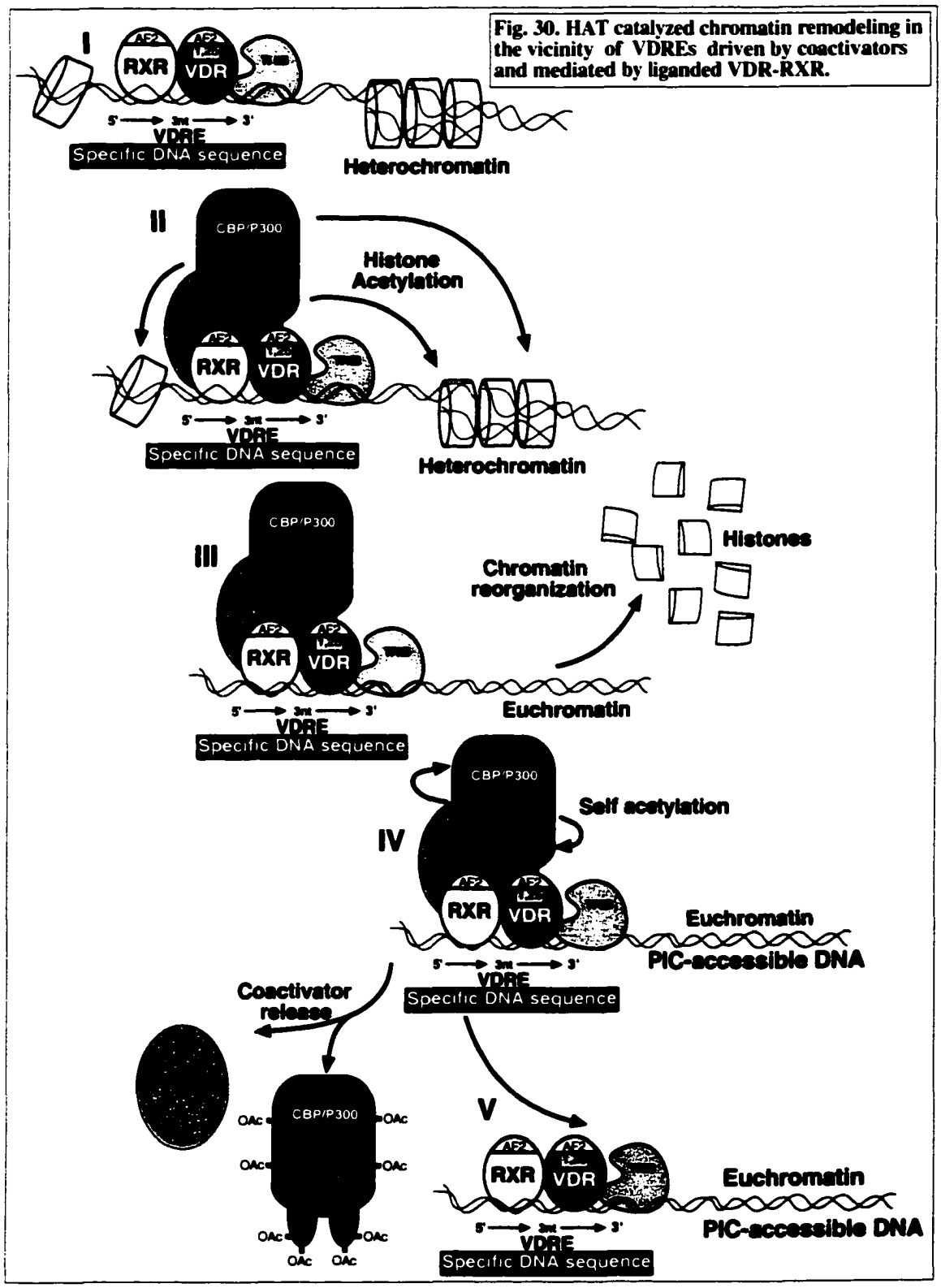


Fig. 30. HAT catalyzed chromatin remodeling in the vicinity of VDREs driven by coactivators and mediated by liganded VDR-RXR.

Acetylation continues until coactivators undergo self-acetylation (Shang *et al.* 2000), disrupting the electrostatic forces that promote protein:protein interactions (Fig. 30, step IV), leaving the VDR target gene promoter ready to attract the PIC and RNA polymerase II (Fig. 30, step V) to initiate transcription.

CHAPTER V. PARTICIPATION OF THE MEDIATOR COMPLEX IN VDR-STIMULATED TRANSCRIPTION

Hypothesis tested and findings

The hypothesis tested in this section is that the RNA polymerase II/Mediator associated protein, $DRIP_{205}$, functions as a VIP via contact with the same AF-2 region that interacts with SRC-1, lending credence to the sequential model of coactivator action. We also determined if $DRIP_{205}$ contacted other surfaces on VDR, and assessed its functional impact on $1,25(OH)_2D_3$-stimulated transcription. It was found that $DRIP_{205}$ binds to VDR in a hormone- and AF-2-dependent manner, but $DRIP_{205}$ also requires the N-terminus of VDR for full association with the receptor. It was additionally observed that $DRIP_{205}$ cotransfection significantly augmented $1,25(OH)_2D_3$-stimulated transcription, rather than squelching VDR activity, indicating that $DRIP_{205}$ is a loosely bound subunit of the Mediator complex that links the receptor to the transcriptional preinitiation complex.

Background

The role of Mediator complexes

The regulation of gene expression by nuclear receptors requires the interaction of a series of complexes known as coregulators, serving as a basic mechanism for switching from the inactive to the active state (Rosenfeld and Glass 2001). In addition to the p160 family of coactivators, the CBP integrator and NCoA-62, VDR-mediated transcription requires the recruitment of a complex that has no known nucleosome

remodeling activity, i.e., the DRIP complex (vitamin D receptor interacting proteins) (Rachez and Freedman 2000). This complex, alternatively known as TRAP, ARC, NAT or Mediator, enhances the transcriptional activity of nuclear receptors, *in vitro*, in assays involving purified components of the transcription machinery (Rachez *et al.* 1998; Fondell *et al.* 1999). The cloning of these complexes by independent groups in several organisms has revealed a high level of homology among them, suggesting that they constitute a single, universal complex (Rachez and Freedman 2000). To date, the proposed model for the actions of mediators states that they provide a functional bridge between RNA polymerase II and activators such as the members of the nuclear receptor family. This bridging function facilitates the recruitment of the polymerase directly to the promoter, supporting the role of nuclear receptors to facilitate gene transcription.

It has been demonstrated that the RNA polymerase II is not a component of the mediator complex, but that it is recruited by it to transcribe genes. This association between the RNA polymerase II and the Mediator complex driven by the nuclear receptor family requires that the receptors exist in the liganded form, as in the case of the vitamin D receptor (Chiba *et al.* 2000). It has also been suggested that Mediator complexes may play a role in reinitiation. Yudkovsky *et al.* (Yudkovsky *et al.* 2000), using an *in vitro* immobilized promoter template assay, observed that some of the transcription machinery factors in yeast, e.g., TFIID, TFIIA, TFIIH and TFIIE, as well as the Mediator complex, remained at the promoter site. Thus, this preformed complex creates a template for RNA polymerase II and several of its rate-limiting-step subunits (TFIIB) to reassemble and reinitiate transcription. Still, it is not clear how the Mediator

complex operates in the context of a chromatin-bound promoter. It has been suggested that the recruitment of RNA polymerase II to the promoter requires previous rounds of remodeling to make it accessible to the transcription machinery (Cosma *et al.* 1999; Agalioti *et al.* 2000). It is evident that different complexes carry out different functions to activate transcription efficiently. Are the actions of each individual complex (for example, p160 and Mediator) occurring in a sequential or simultaneous manner? This issue is still a subject of ongoing debate and controversy.

The structural domains that support interactions between nuclear receptors and p160, and between nuclear receptors and Mediator are very similar, and both sets of interactions are known to be hormone/ligand-dependent (Treuter *et al.* 1999; Rachez *et al.* 2000; Ren *et al.* 2000). Chromatin immunoprecipitation on three estradiol controlled promoters indicates that p160 coactivators, Mediator proteins, p300 and RNA polymerase II are bound to the promoter region within 30 minutes of the addition of ligand, hinting that these interactions occur rapidly and likely overlap (Shang *et al.* 2000). Indeed, ChIP assays reveal that DRIP/Mediator and RNA polymerase II are recruited by estradiol to the ER-ERE complex at about the same time as are p160 and p300 HAT coactivators. These experiments suggest a model in which the p160 and Mediator complexes may act simultaneously and contribute to fully activate gene expression initiated by the liganded receptor. One hypothesis that could help explain the simultaneous interaction of these complexes is that a dimeric receptor could bind to both complexes, one through each receptor monomer. This model is supported by the findings of Yang *et al.* (Yang *et al.* 2000), in which DNA-bound PPARγ-RXR heterodimers were

observed to selectively interact with a Mediator and a p160 complex in the presence of their respective specific ligands. However, data from immunoprecipitation studies in HeLa cells of recruitment of p160/HAT coactivators and Mediator by TR (Sharma and Fondell 2000), indicate that TR interaction with p160s occurs rapidly (~10 min), whereas the association and the function of the Mediator-TR complex occurs significantly later (~3 hr) post T_3 ligand treatment of the cells.

Thus far, the mechanism of action of the Mediator complex and its many components remains unclear. It will be important to understand the precise molecular effects of the mediator complex to begin to elucidate the manner in which this group of proteins enhances gene transcription. As stated above, it is still unresolved whether mediator must wait until histone acetylation by recruited p160/p300 coactivators has rendered chromatin more accessible (Sharma and Fondell 2000), or whether mediator acts in combination with the aforementioned complexes on the same nuclear receptor responsive promoter (Shang *et al.* 2000).

DRIP complex actions on VDR-mediated transcription

The DRIP complex consists of more than a dozen polypeptides that have no known enzymatic function (Rachez *et al.* 1998). It is believed that this complex functions to recruit the RNA polymerase II holoenzyme to ligand-bound nuclear receptors. The RNA polymerase II, not being part of the DRIP complex, can be coimmunoprecipitated with liganded VDR through DRIP mediator, suggesting a bridging role of the complex in helping activate gene expression (Rachez *et al.* 1999).

This complex strongly potentiates ligand-dependent VDR activation of transcription, independently of the actions of the p160 family of coactivators, where none of its members have been found to be part of this complex (Rachez and Freedman 2000).

The DRIP mediator proteins lack HAT activity, suggesting that their role in enhancing gene response may be either novel, distinct from that of chromatin remodeling, or to serve as a recruiting agent to bring HAT activity enzymes to the promoter site. The activities that the DRIP and the p160 protein provide to the system may have a synergistic effect on $1,25(OH)_2D_3$-mediated activation, or they may well provide specificity to its regulation. VDR-mediated transcriptional regulation may require chromatin remodeling activities and the efficient recruitment of RNA polymerase II via its basal factors (Rachez and Freedman 2000). Currently, the model that explains VDR regulation of gene expression suggests that the p160 family of chromatin remodeling enzymes is required to expose DNA, rendering the promoter more accessible to the transcription machinery, with the DRIP/Mediator complex then making it possible for the RNA polymerase II to localize at the relevant promoter and initiate transcription. This line of reasoning supports the sequential model of action for coactivator complex recruitment to elicit $1,25(OH)_2D_3$-mediated responses.

$DRIP_{205}$ interactions with VDR

$DRIP_{205}$ is a key subunit of the DRIP complex that interacts directly with nuclear receptors in a ligand dependent-manner, and anchors the other DRIP subunits to form the complex (Rachez *et al.* 2000). $DRIP_{205}$ contains two LXXLL motifs that serve to

contact nuclear receptors and have been named NR1 and NR2. As with other coactivator-nuclear receptor interaction domains, these motifs provide specificity and selectivity for the receptor interaction.

As a member of the nuclear receptor superfamily, VDR interacts with $DRIP_{205}$ in a hormone-dependent manner, and does so presumably through the activation function-2 or AF-2 (Rachez *et al.* 2000). $DRIP_{205}$ utilizes only its NR2 motif to contact VDR as demonstrated by peptide competition assays (McInerney *et al.* 1998). In addition to the NR2 motif, the NR1 also plays a part in the interaction with the RXR heteropartner, the latter motif being the one postulated to interact with the AF-2 of RXR. These results suggest that each NR domain is designed to bind selectively to a different nuclear receptor, and support the findings of McInerney *et al.* (McInerney *et al.* 1998), which state that in order to achieve nuclear receptor-binding specificity, the coactivator must bind to the heterodimer with one of the NR motifs bound to each heterodimer monomeric unit.

To date, association studies between $DRIP_{205}$ and VDR have revealed that only the AF-2/helix-12 region of VDR is required for recruiting the Mediator complex (Rachez *et al.* 1998), and this is the same domain of the receptor that is required for association with the p160 family of proteins. Therefore, we further evaluated the regions in VDR that contact $DRIP_{205}$ mediator, attempting to further define the degree of overlap between VDR surfaces that contact p160s and $DRIP_{205}$. We anticipated that such data might provide insight into whether simultaneous action of DRIP/mediator and p160s with VDR is feasible, or whether a sequential model is more likely. Also, we

investigated the importance of $DRIP_{205}$ on VDR mediated transcriptional activation in response to $1,25(OH)_2D_3$ in order to evaluate the relative functional significance of this comodulator with respect to VDR action.

Material and Methods

GST-pull-down assay to study protein:protein interactions

Sepharose beads containing immobilized hVDR or an AF-2 mutant hVDR (E420A) were prepared as described in Chapter II and used in pull down assays. A human $DRIP_{205}$ expression construct (pcDNA3-$hDRIP_{205}$) was kindly provided by Dr. Leonard Freedman (Rachez *et al.* 1998), from the Memorial Sloan-Kettering Cancer Center.

Approximately 1.2 μg of pcDNA3-$hDRIP_{205}$ and pSG5-hSRC-1 expression plasmids were used as templates in an *in vitro* transcription and translation protocol, and the synthesized proteins were labeled with ^{35}S-methionine (Promega, Madison WI). GST-hVDR fusion protein Sepharose was used to test the interaction between the receptor and $DRIP_{205}$ Mediator, employing SRC-1 coactivator as a positive control (see also Chapter 4). The counterpart experiment was also conducted, in which GST-$DRIP_{205}$ Sepharose beads were prepared by inserting the $hDRIP_{205}$ into pGEX-4T-2 vector (Promega, Madison WI), and employing the beads to map the interaction domains between VDR and the Mediator protein. For this experiment, several VDR point mutants and deletions mutants were evaluated, *in vitro*.

Site-directed mutagenesis

The expression vector pSG5-hVDR was employed in synthesizing deletion and point mutants by *in vitro* site directed mutagenesis (Stratagene, La Jolla, CA). Alterations of specific residues/regions were created using previously described double stranded DNA protocols to generate changes in the hVDR sequence (Jurutka *et al.* 1997). The hVDR deletion mutants created were: Δ5-13, Δ14-23, Δ 1-88, Δ304, Δ403, Δ424. The hVDR point mutants created were: I242D, K246R, L417A E420A, and V421D.

Cotransfection/Transcriptional activation assays

ROS 17/2.8 cells were cotransfected with pcDNA3-hDRIP$_{205}$ expression plasmid (1 μg/plate), a TKGH reporter vector containing two copies of the rat 24-hydroxylase proximal VDRE upstream of the hGH reporter gene (4 μg/plate), and carrier pTZ18U DNA (6 μg/plate). The cells were incubated with 10^{-8} M 1,25(OH)$_2$D$_3$ (+) or vehicle (–) for 24 hours post-transfection, and human growth hormone secretion into the medium was determined by RIA.

Results

In order to determine the interaction between VDR and hDRIP$_{205}$, we initially employed the pull down assay to evaluate the nature of their association, and then utilized transfection of culture cells to observe the effect exerted on VDR-mediated transactivation by expression of the DRIP$_{205}$ protein.

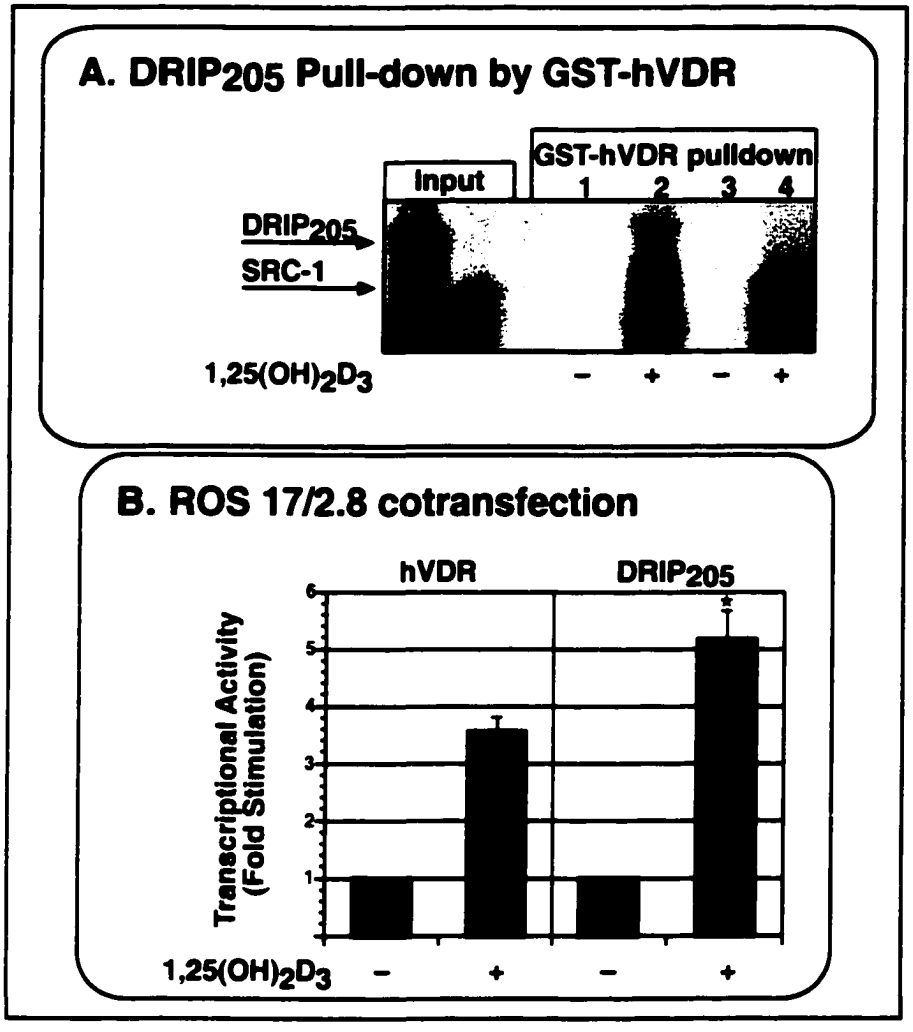


Fig. 31. $DRIP_{205}$ interaction with, and effect on, VDR function are $1,25(OH)_2D_3$ hormone-dependent. A). $DRIP_{205}$ pull-down by GST-hVDR demonstrates that the interaction between this subunit of Mediator and the receptor is ligand-dependent (compare lanes 1 vs. 2), as also observed for the coactivator SRC-1 (lanes 3 vs. 4). B). In transactivation assays, the hormone-dependent interaction of VDR and $DRIP_{205}$ results in a functional response. In the left panel, the presence of hormone induces the expression of the reporter gene by 3.5-fold, an induction that is amplified to 5-fold when $DRIP_{205}$ is co-expressed (right panel). This further stimulation is statistically significant (*) $p<0.05$.

The *in vitro* pull-down assay helped us observe that, as depicted in Figure 31A, the interaction between $DRIP_{205}$ and VDR is absolutely dependent on the present of hormone (compare lanes 1 vs. 2), as is also the case for the coactivator SRC-1 (lanes 3

and 4), used here as a positive control. The interaction between $DRIP_{205}$ and VDR was also reported previously by Freedman's group (Rachez *et al.* 1998), who showed a similar hormone dependency for this association. We also studied the effect that this Mediator subunit exerts on $1,25(OH)_2D_3$-mediated transcriptional activity. Utilizing endogenous VDR present in the rat osteosarcoma cell line, ROS 17/2.8, we observed that the presence of hormone stimulates (3.5-fold; left panel of Fig. 31B) the transcription of a VDRE-reporter gene containing approximately 1100 bp of natural promoter region from the rat osteocalcin gene. This response is further enhanced 40% (right panel Fig. 31B) to yield a 5-fold transactivation effect of $1,25(OH)_2D_3$ when the Mediator component, $DRIP_{205}$, is overexpressed in this system. These results implicate the $DRIP_{205}$ protein in a mechanism that contributes to the stimulation of transcriptional activity by VDR in the presence of $1,25(OH)_2D_3$, likely involving the recruitment of RNA polymerase II.

Because the nuclear receptor interaction domains of $DRIP_{205}$ have already been characterized (Rachez *et al.* 2000), and consist mainly of the LXXLL motifs in NR1 and NR2, we set out to identify the complementary regions of VDR involved in this association. We constructed several deletion mutants within each domain of the receptor (DBD and LBD), and tested them for their ability to interact with a GST-$DRIP_{205}$ fusion protein in the presence and absence of $1,25(OH)_2D_3$ hormone.

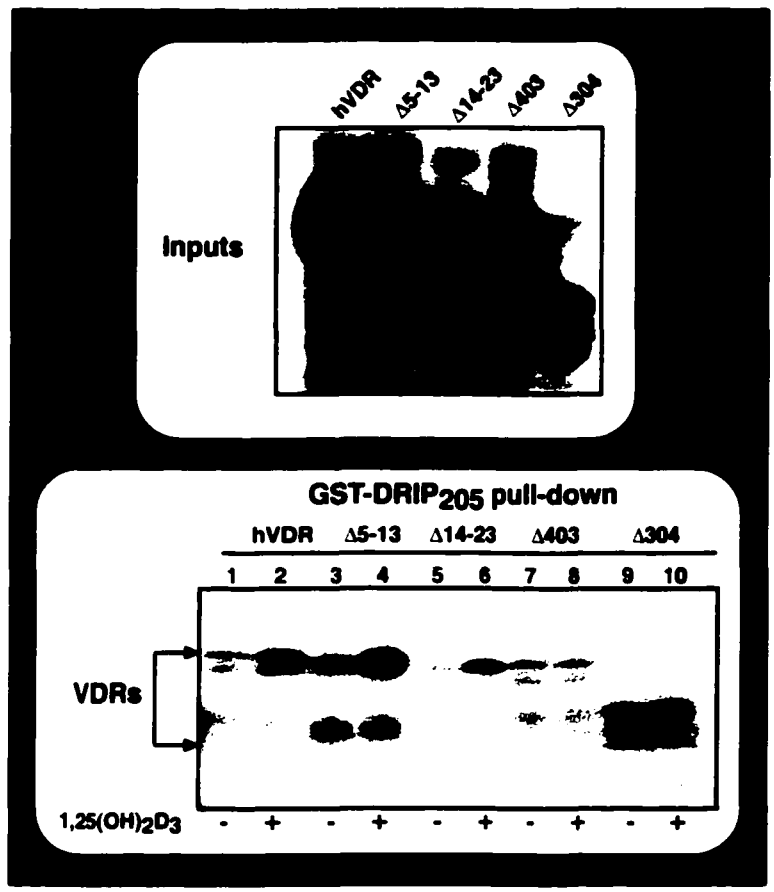


Fig. 32. Pull-down assay to determine the domains of VDR involved in $DRIP_{205}$ interaction. WT VDR and several deletion mutants were evaluated for their capacity to interact with $DRIP_{205}$. The upper panel depicts ^{35}S-labeled inputs, and represents 5% of that used in each pull down lane. In the lower panel, WT VDR (lanes 1-2), as well as the Δ5-13 deletion mutant (lanes 3-4), display a strong hormone-enhanced interaction with $DRIP_{205}$, but this association is blunted in the Δ14-23 hVDR deletant (lanes 5-6). C-terminal truncation mutants (Δ403 and Δ304) appear to have no hormone dependency in their association with $DRIP_{205}$, but Δ304 seems to acquire $DRIP_{205}$ binding capacity mostly absent in the Δ403 truncation lacking the AF-2.

Figure 32 illustrates the results of pull down experiments with $DRIP_{205}$ beads, and several hVDR deletion mutants. Wild type hVDR displays a significant $1,25(OH)_2D_3$ ligand-enhanced interaction with immobilized $DRIP_{205}$ (Fig. 32, lanes 1 and 2), demonstrating that $DRIP_{205}$ and hVDR interact not only functionally (Fig. 31B), but also physically (Figs. 31A and 32). The transcriptionally inactive C-terminal Δ403

truncation of hVDR (Nakajima *et al.* 1994), which removes the AF-2/helix-12 p160 interaction site, also causes the receptor to lose virtually all of its ability to interact with $DRIP_{205}$ beads, and the remaining weak association is not increased by $1,25(OH)_2D_3$ (Fig. 32, lanes 7-8). The $1,25(OH)_2D_3$-dependent association of VDR with $DRIP_{205}$ beads was enhanced in the Δ5-13 small deletant, while the transcriptionally inactive Δ14-23 hVDR exhibits attenuated $DRIP_{205}$ contact, apparently identifying the N-terminal domain of hVDR that either directly contacts or supports association with $DRIP_{205}$ as a charged region (including arginines 18 and 22) that also has been characterized as a TFIIB interaction site (Jurutka *et al.* 2000b). Therefore, at this point we conclude that VDR possesses at least two platforms for $DRIP_{205}$ interaction, with the AF-2/helix-12 being the major contact, and N-terminal residues 14-23 (GDFDRNVPRI) playing a supportive role. There is evidence from results on the estrogen receptor (Sathya *et al.* 2002), the androgen receptor (Alen *et al.* 1999) and RXRα (Bommer *et al.* 2002), that the AB region N-terminal of the DBD of these receptors interacts functionally with the LBD AF-2 to facilitate coactivator binding and ligand-dependent transcriptional activation.

Also illustrated in Fig. 32 is the observation that although AF-2 deficient Δ403 hVDR truncation binds $DRIP_{205}$ beads very weekly, this association is markedly enhanced by further C-terminal truncation of hVDR to Δ304. The Δ304 hVDR truncation does not bind $1,25(OH)_2D_3$ and is transcriptionally inactive (Nakajima *et al.* 1994), but associates strongly with $DRIP_{205}$ in a hormone-independent fashion (Fig. 32). Notably, the Δ304 truncation still includes helices 3-6 of hVDR, which now constitute an unencumbered C-terminal region that appears to be more available to protein:protein

surface interactions than occur in the context of full-length hVDR. A similar phenomenon was observed for enhanced Δ304 hVDR interaction with SRC-1 (see Chapter IV). As discussed earlier in this dissertation, helices 3-6 create a hydrophobic cleft, which, in combination with helix-12, comprises a key surface for nuclear receptor-coactivator interaction. We therefore conclude that the helix 3-6 domain of hVDR represents yet a third significant site that supports $DRIP_{205}$ association with hVDR. As reported later in this Chapter, experiments involving point mutagenesis of hVDR within helix-3 provide further evidence for this conclusion.

Next, additional deletion/truncation mutants in hVDR were created in order to investigate further the extreme N- and C-termini of the receptor in terms of $DRIP_{205}$ interaction. A short Δ424 C-terminal truncation of hVDR that leaves the AF-2 intact does not significantly impact $DRIP_{205}$ binding (Fig. 33B), consistent with the observation that this mutant hVDR retains full $1,25(OH)_2D_3$-stimulated transcriptional activity (data not shown). Similarly, the Δ424 hVDR C-terminal truncation also associates with a p160 coactivator (SRC-1) and the RXR heteropartner to an identical degree as that of the wild type receptor (Fig. 33B). In contrast, the Δ1-88 N-terminal hVDR truncation, which removes the N-terminal "AB" domain of 23 amino acids, plus the entire DBD zinc finger region, does exhibit a significant reduction in $DRIP_{205}$ interaction, while associating with RXRα and SRC-1 relatively normally (Fig. 33A). Because this hVDR truncation, which consists essentially of the LBD, is compromised in $DRIP_{205}$ binding, a third $DRIP_{205}$ contact site, besides the AF-2 and helices 3-6, is likely present in the N-terminal region of VDR. As discussed above, this third $DRIP_{205}$ interaction site likely

occurs between residues 14 and 23 of hVDR, since deletion of this region attenuates $DRIP_{205}$ binding (Fig. 32). This conclusion is also supported by the observation that a partial deletion of the N-terminal region in Δ5-13 hVDR creates a superinteracting receptor with respect to $DRIP_{205}$ association (Fig. 32). As with VDR-TFIIB interaction (Jurutka *et al.* 2000b), deletion of the repressive extreme N-terminal domain apparently allows hVDR to contact $DRIP_{205}$ more avidly (Fig. 32), and transactivate more efficiently (Jurutka *et al.* 2000b).

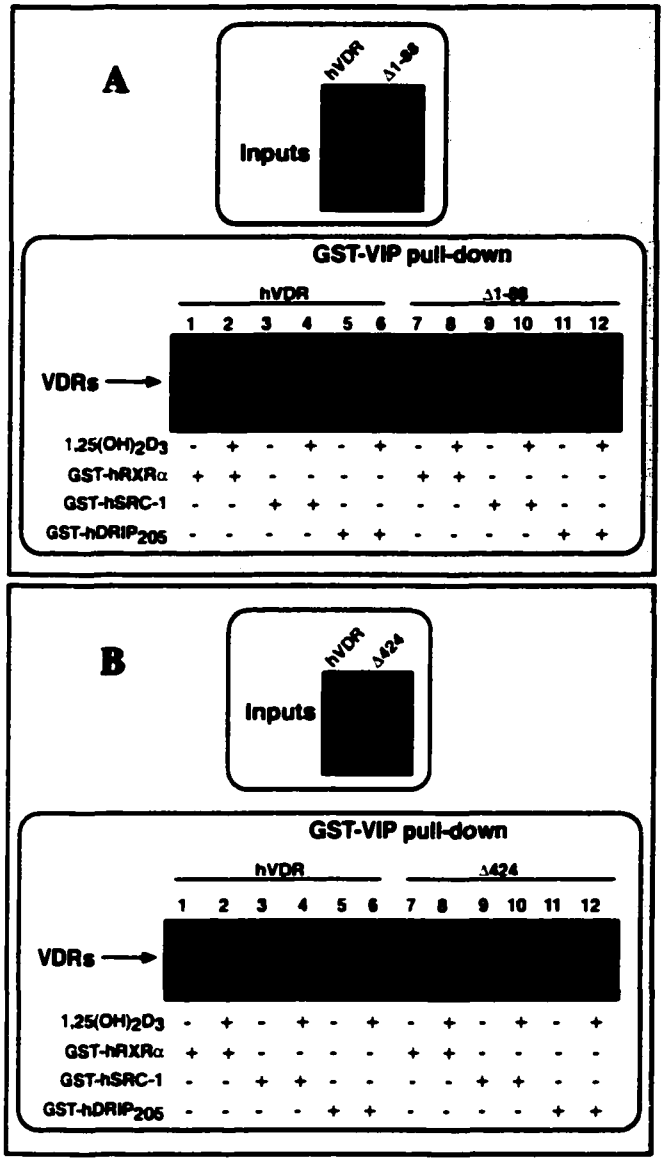


Fig. 33. Pull-down assay to evaluate the LBD domain of VDR (minus the N-terminal/DBD region), as well as its extreme C-terminus, for their interactions with DRIP$_{205}$ and other VIPs. WT hVDR, a DBD deletion mutant (Δ1-88) and the Δ424 hVDR, were evaluated for their capacity to interact with hDRIP$_{205}$ and positive control VIPs. A). WT VDR displays a strong hormone-dependent interaction with GST-hRXRα beads (lanes 1-2), as well as with the GST-hSRC-1 (lanes 3-4) and GST-hDRIP$_{205}$ beads (lanes 5-6). The hVDR-hRXRα association remains strong and hormone dependent for the Δ1-88 hVDR deletant (lanes 7-8) , but somewhat blunted for hSRC-1 (lanes 9-10) and extremely attenuated for hDRIP$_{205}$ (lanes 11-12). B). The hVDR extreme C-terminal truncation Δ424 displays a WT-like, hormone-dependent response in its ability to associate with hRXRα, hSRC-1 and hDRIP$_{205}$ in a pull down assay.

To evaluate in more depth the relative importance of the other two putative $DRIP_{205}$ interacting regions of hVDR, namely the helix-12/AF-2 (see Fig. 22 for sequence), and helix-3, the major player in the helices 3-6 LBD group, we next engineered point mutations into each of these domains. The helix-12 (L417A and V421D) and helix-3 (I242D and K246R) point mutant hVDRs were examined in an *in vitro* pull down assay, utilizing $DRIP_{205}$ beads in the presence or absence of $1,25(OH)_2D_3$ hormone. As presented in Fig. 34, the strong $1,25(OH)_2D_3$-enhanced interaction of the wild type hVDR with $DRIP_{205}$ beads was essentially abolished in the helix-12 mutants, and significantly attenuated, but still present in the helix-3 mutants. Thus, these point mutants confirm the involvement of both helix-3 and helix-12 in $DRIP_{205}$ recruitment by hVDR, and establish helix-12 as being relatively more important in this protein:protein association.

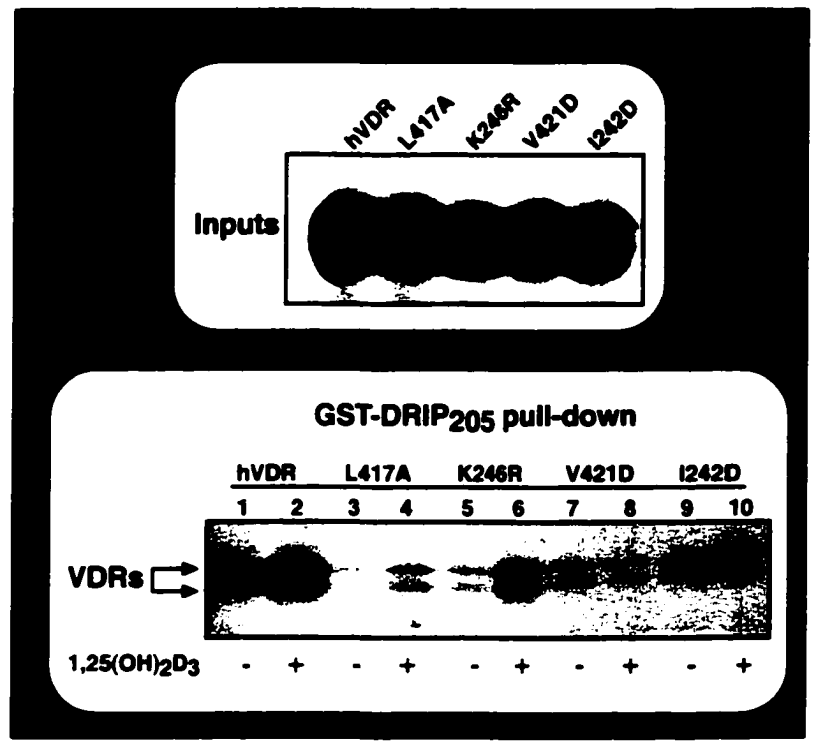


Fig. 34. In vitro interaction between $DRIP_{205}$ and VDR point mutants. The involvement of specific regions of VDR in $DRIP_{205}$ interaction was evaluated. Synthetic point mutations found in helix-12 (L417A and V421D) and in Helix 3 (I242D and K246R) were analyzed for their ability to associate with $DRIP_{205}$, utilizing WT VDR as a control.

To verify the dominant role played by the AF-2/helix-12 of hVDR in $DRIP_{205}$ recruitment, we next performed the reverse pull down experiment, attempting to attract $DRIP_{205}$ in solution to either wild type or E420A hVDR beads. As can be seen from the results in Fig. 35, transcriptionally inactive (Jurutka *et al.* 1997) E420A hVDR is unable to bind $DRIP_{205}$ in response to $1,25(OH)_2D_3$ as opposed to wild type hVDR, whereas RXRα is recruited equally well by wild type and E420A hVDR in response to $1,25(OH)_2D_3$.

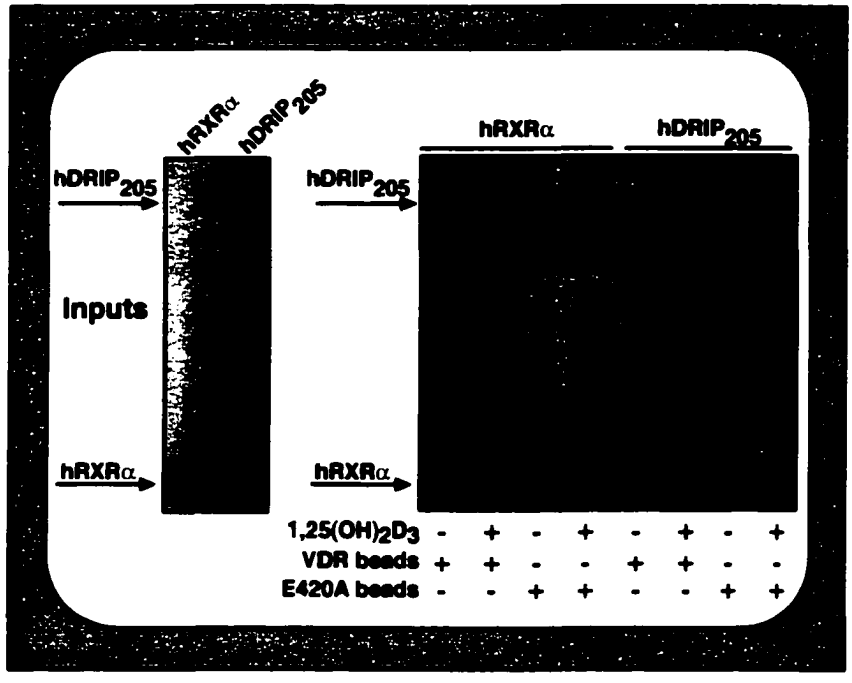


Fig. 35. GST-VDR pull down assay of the interaction of VDR with DRIP$_{205}$. The VDR-DRIP$_{205}$ interaction is hormone and AF-2 dependent, because disruption of helix-12 integrity of the receptor compromises the interaction between these two molecules (compare lanes 6 vs. 8, right panel), and the association between VDR and DRIP$_{205}$ occurs only in the presence of ligand (lanes 5 and 6). Input, left panel, represents 5% of the amount used in GST pull down lanes.

The results described above further support the idea that these two sets of modulators (coactivators and mediators) bind to VDR in the same region(s) of the receptor, supporting a previous hypothesis (Sharma and Fondell 2000), which states that the interactions between nuclear receptors like TR and coactivators or mediators likely occur in a sequential manner.

Conclusions

With the *in vitro* (biochemical) and *in vivo* (intact cultured cells) assays performed to establish the effects of $DRIP_{205}$ on $1,25(OH)_2D_3$-mediated gene expression, we have been able to conclude that VDR interacts directly with the $DRIP_{205}$ mediator subunit, an association that is further enhanced in the presence of the $1,25(OH)_2D_3$ ligand, and that this interaction serves to potentiate hormone-stimulated gene activation.

The characterization of $DRIP_{205}$-VDR interaction demonstrated that the AF-2 region is indispensable for the association to take place, and that other regions in VDR also appear to influence this interaction, i.e., the N-terminus and helix-3. In pull-down assays, it was observed that some hVDR deletion mutants (e.g. Δ5-13 and Δ304) displayed a stronger association than that seen for the WT VDR. It is reasoned that these deletion mutants expose regions within the receptor that normally participate weakly or indirectly in the interaction between VDR and $DRIP_{205}$, but once they are liberated from the native tertiary structure in the deletion/truncation mutant, their influence on the association or contact between the two proteins becomes more avid.

To probe further the AF-2 and the helix-3 domains in VDR and their influence on $DRIP_{205}$ binding, several point mutations in hVDR were evaluated. Three AF-2 mutants (L417A, E420A and V421D) and two helix-3 mutants (I242D and K246R) were tested for their ability to retain $DRIP_{205}$ binding capacity. It was observed that the AF-2 mutations exert a profound negative influence on the interaction between $DRIP_{205}$ and VDR, demonstrating the need for an intact AF-2 domain in the receptor to associate productively with the mediator protein. These results suggest that the full $1,25(OH)_2D_3$

transcriptional response mediated by VDR is driven by direct protein:protein interactions between the VDR and coactivators as well as between VDR and Mediator, each through the receptor's AF-2/helix-12 region.

It was also found that another region of the receptor, namely helix-3, once exposed in deletion mutants (e.g., Δ304), enhances interaction between VDR and $DRIP_{205}$, a finding further supported by the observation that helix-3 mutations (I242D and K246R) blunt the association of hVDR and $DRIP_{205}$. Interestingly, this attenuation of $DRIP_{205}$ binding is less than the reduction in SRC-1 binding that occurs in the case of these two helix 3 mutants (Jurutka *et al.* 2001a), suggesting that helix-3 is relatively more important in establishing the SRC-1/p160 platform than it is in supporting $DRIP_{205}$ association. Fig. 36 illustrates in schematic summary fashion, the domains in hVDR that are significant in $DRIP_{205}$ interaction.

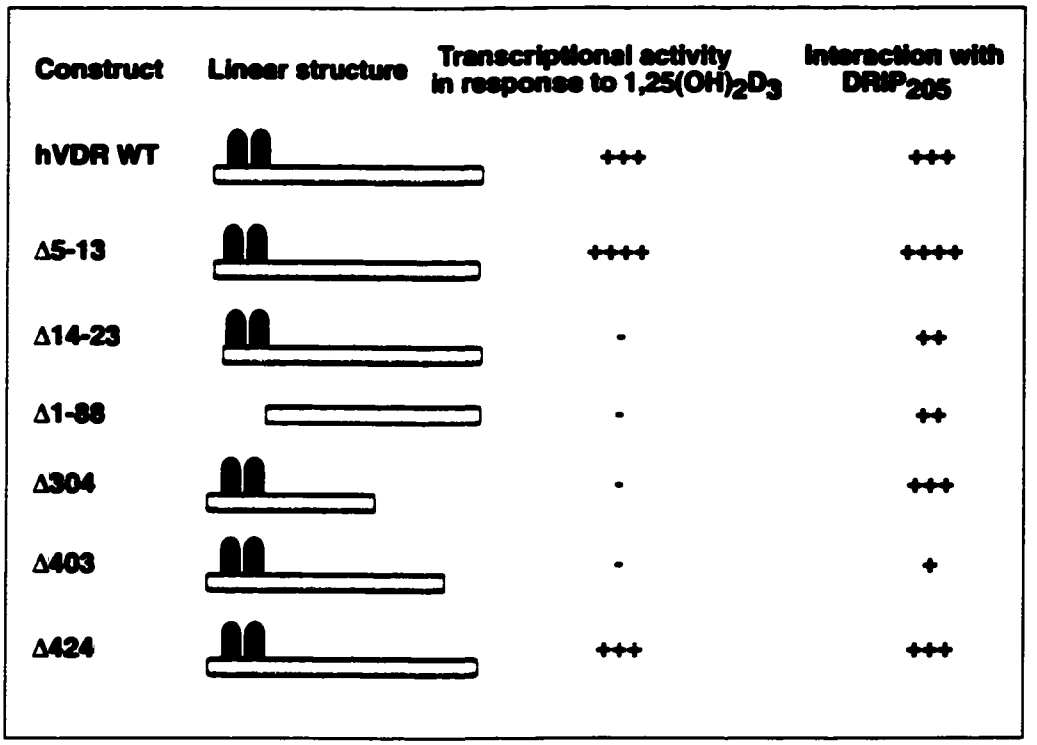


Fig. 36. Summary of interactions between GST-DRIP$_{205}$ and hVDR mutant constructs in the presence of 1,25(OH)$_2$D$_3$. This figure depicts a series of hVDR deletion mutants in their linear form and the level of interaction with the mediator protein in the presence of hormone, and compares this to the transactivation response of each hVDR construct.

In conclusion, as summarized in Fig. 36, and supported by point mutagenesis of specific hVDR residues in helices-3 and -12 (Figs. 34 and 35), the major site of DRIP$_{205}$ contact with hVDR consists of helix-12 (missing in Δ403 truncation), and secondary regions in hVDR that influence or support this protein:protein interaction lie in the helix-3 and the N-terminal domains of the receptor. Our data also support the theory that these three regions of VDR are required for interactions with more than one family of transcription factors. For example, the p160 family of coactivators and the Mediator family (DRIP$_{205}$), physically overlap in contacting VDR on the helix-12/helix-3 cleft surface, arguing that they likely function as sequential rather than simultaneous

coactivators (Sharma and Fondell 2000). Similarly, the observed $DRIP_{205}$ association with the hVDR N-terminal domain (residues 14-23 in hVDR), constituting a third contact site besides helix-12 and helix-3 in the receptor, coincides with an important TFIIB site (Jurutka *et al.* 2000b), suggesting that when $DRIP_{205}$ replaces a p160 coactivator at helix-12 of hVDR, it also displaces TFIIB from the N-terminus of the receptor, perhaps releasing TFIIB to complete the PIC by associating with RNA polymerase II. Accordingly, we have developed the model illustrated in Fig. 37, to account for the apparent sequential actions of SRC-1, $DRIP_{205}$ and TFIIB in transducing transcription of vitamin D regulated genes from the $1,25(OH)_2D_3$-liganded VDR.

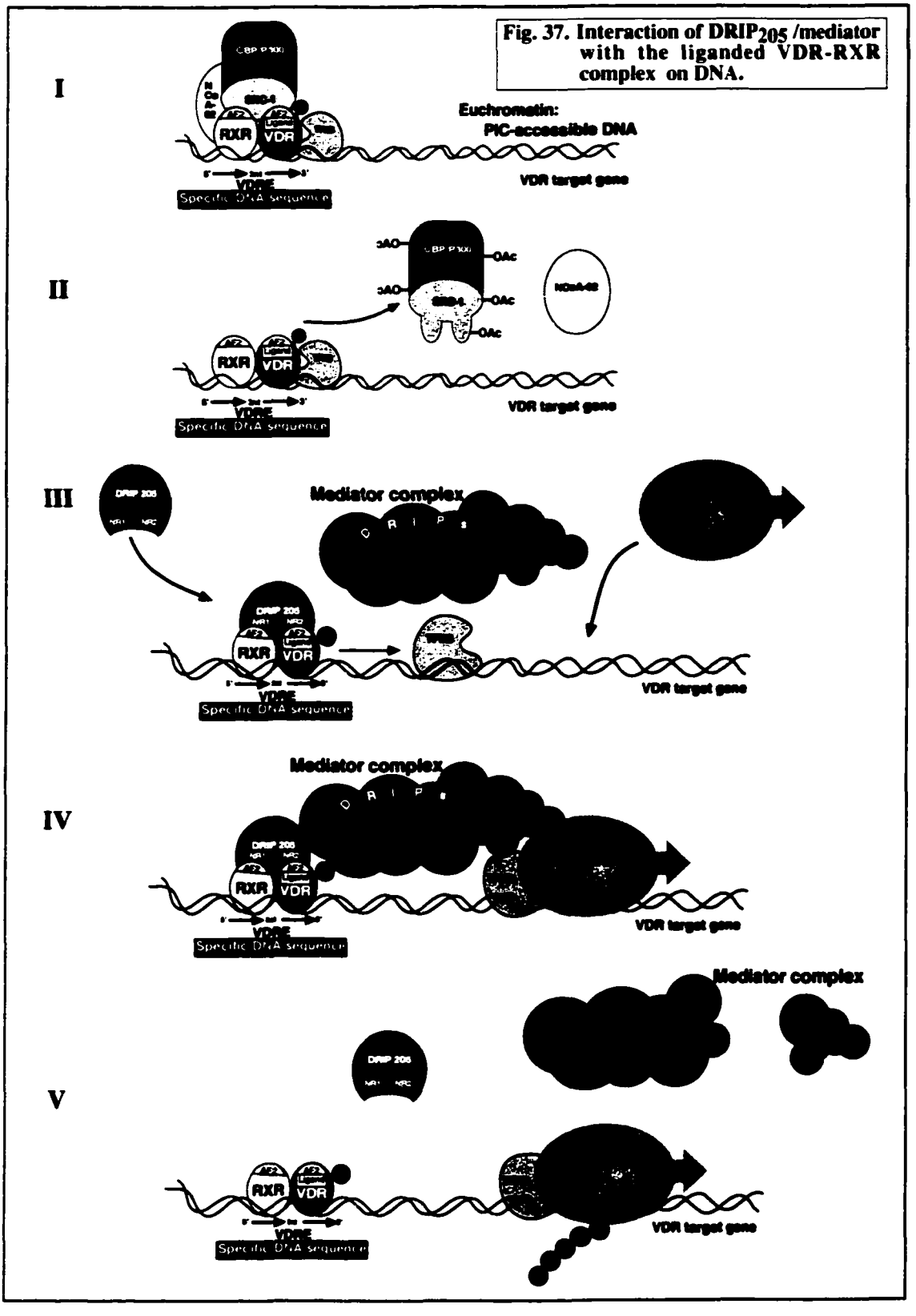


Fig. 37. Interaction of DRIP$_{205}$ /mediator with the liganded VDR-RXR complex on DNA.

In this model (Fig. 37), the activated VDR bound to the p160 and p300 family of coactivators and cointegrators has been able to remodel chromatin to render it PIC accessible through the actions of HAT enzyme activity that is found in this class of cofactors (I). Once the chromatin has been released from its repressed state, autoacetylation of these cofactors (Shang *et al.* 2000) is postulated to inhibit their association with VDR, promoting their release (II). NCoA-62 could dissociate from VDR because it is also acetylated, or it may leave simply because of the departure of the acetylated p160 that formerly participated in a VDR-NCoA-62-SRC-1 ternary complex. The liberation of p160/p300/NCoA-62 from VDR facilitates sequential binding of $DRIP_{205}$ to the VDR-RXR heterodimer (III). With the $DRIP_{205}$ protein in place, the balance of the mediator complex can also be recruited the promoter (IV). The completed $DRIP_{205}$/Mediator complex then attracts RNA polymerase II, which is activated by the delivery of TFIIB from VDR, an event that occurs when $DRIP_{205}$ displaces TFIIB from the N-terminal domain of VDR (III). With the mediator complex and the RNA polymerase II bridging from the VDR in the promoter to the transcription start site, and the VDR-RXR activated heterodimer having released TFIIB (the rate limiting step in the assembly of the preinitiation complex (PIC)), transcriptional machinery assembly is now complete (IV), leading to the expression of the VDR target genes after phosphorylation of the C-terminal domain (CTD) of RNA polymerase II (V).

CHAPTER VI. REGULATION OF VDR TRANSCRIPTIONAL ACTIVATION CAPACITY BY TRIP1

Hypothesis tested and findings

The questions asked in this section were: i) can a novel VIP be identified via the YTHS, ii) what is the functional effect of this VIP on VDR action and iii) does this VIP interact with VDR following the SRC-1/DRIP$_{205}$ paradigm of hormone-dependent AF-2 binding? A controversial VIP, namely Trip1, was isolated through the YTHS, and found to be a significant attenuator of VDR action. Trip1 was found to interact with both the AF-2 and the N-terminus/DBD of VDR in a 1,25(OH)$_2$D$_3$-stimulated fashion. Based upon experiments utilizing a proteasome inhibitor, it was concluded that Trip1 operates as a terminator of VDR mediated transcription by triggering ubiquitination and eventual proteolysis of the receptor.

Background

Control of gene expression via the proteasome

The regulation of eukaryotic gene expression requires the assembly of the preinitiation complex (PIC) at the promoter region of target genes, an action that is directed and coordinated by multiple transcription factors and other regulatory complexes from different signal transduction pathways that converge at the enhancer region (Desterro *et al.* 2000). Some of the proteins influencing the role of PIC assembly are the nuclear receptors, which are activated by liganding with lipophilic hormones, but whose activity as transcription factors can also be regulated in many additional fashions

by: changing their rate of synthesis, regulating the degradation of the receptor protein, post-translational modifications, or altering receptor subcellular localization. Proteolysis is an important form of regulation for a large number of factors. One molecular strategy for targeting proteins for degradation is carried out by the ubiquitin-proteasome system (Ottosen *et al.* 2002).

Cellular proteins exist in a dynamic equilibrium, their levels being maintained by a tightly controlled balance of synthesis and degradation. The degradation of proteins via ubiquitination is a well-coordinated mechanism and is one of the processes that the cell utilizes to control the concentration of transcription factors. This system targets many cellular proteins besides transcription factors, such as cell growth modulators, signal transducers, cell cycle regulators, tumor suppressors, oncoproteins, enzymes, viral gene products, membrane receptors, as well as damaged, misfolded or misassembled proteins (Hershko and Ciechanover 1998). Considering the great number of proteins that are regulated by ubiquitination, it is clear that this system is involved in the management of many basic cellular processes. Degradation of a protein via the ubiquitin pathway involves two major steps: attachment of the ubiquitin molecule(s) to the substrate and degradation of the tagged protein by the 26S proteasome complex. Ubiquitin is a 76 amino acid polypeptide that is highly conserved in evolution, showing only 3 amino acid differences between the human and the yeast homologue (Yeh *et al.* 2000). Ubiquitin conjugation initiates with the ATP-dependent activation of the C-terminus of this moiety by the ubiquitin activation enzyme (E1). After the E1 step, the ubiquitin is transferred to a conserved cysteine residue found in the ubiquitin-conjugating enzyme (E2). Finally,

ubiquitin is transferred from E2, and covalently linked to a lysine residue in the substrate protein targeted for degradation. This final step requires the participation of the ubiquitin protein ligase (E3) that also acts as the substrate recognition enzyme (Hershko and Ciechanover 1998). Target protein specificity is obtained by a combination of activities between 2 groups of proteins. In the ubiquitin system, protein substrates must be recognized and bound to the E3 enzyme prior to the modification. The recognition is modulated by specific structural motifs within the targeted proteins. The stability of these proteins depends on their state of oligomerization, post-translational modifications, phosphorylation or association with ancillary proteins (such as chaperones) that act as recognition elements. Some transcription factors must dissociate from specific DNA elements to which they bind in order to be identified and tagged. Thus, recognition of the target protein will depend on the E3 specificity, modifying enzymes, ancillary proteins and specific DNA sites (Ciechanover *et al.* 2000).

Once the substrate protein has been recognized and the ubiquitin moiety(ies) added, it is directed to the 26S proteasome where the targeted protein is degraded. The proteasome is a major cytosolic and nuclear protease complex, responsible for the ATP-dependent extralysosomal proteolytic pathway, and degrades most of the cellular proteins (Rock *et al.* 1994; Tanaka 1995; Coux *et al.* 1996). As a highly conserved structure, it is found as a two subunit complex: the 20S proteasome with multiple peptidase activities, and the smaller regulatory subunit, the 19S, composed of multiple ATPases that are necessary for protein binding and recognition (Tanaka 1995; Coux *et al.* 1996).

The structure of the 20S complex from *S. cereviseae* has been determined. It is composed of 14 pairs of protein subunits assembled into a single structure arranged into four seven-membered rings with α-type subunits forming an outer ring guarding the inner part consisting of catalytic β-type subunit rings (Groll *et al.* 1997). The 19S complex is composed of a minimum of 15 subunits, 6 of which are ATPases and members of the AAA ATPase family of proteins (ATPase associated proteins with a variety of cellular activities) (Baumeister *et al.* 1998). The roles that the ATPases play in these complexes are still unknown, but they may involve the recognition and unfolding of the target proteins, helping in their translocation to the 20S proteolytic complex.

Specific inhibitors for the proteasome complex have been developed. Most of these act as competitive inhibitors that become covalently linked to the active sites of the β-subunits; inactivating chymotryptic and tryptic-like activities. The most widely used inhibitors are aldehydes such as MG-132, MG-115, ALLN, and some natural products such as lactacystein (Lee and Goldberg 1998).

Proper regulation of gene expression is essential to mediate accurate and normal eukaryotic development and cellular homeostasis. Organisms have developed several mechanisms to tightly control the activities of *trans*-acting factors that regulate gene expression at the transcriptional level. The nuclear receptor superfamily illustrates quite well this tightly regulated system (Mangelsdorf *et al.* 1995). In contrast to what is known about the induction and transcriptional control by nuclear receptors, little is known about the mechanism that cell utilize to turn off these transcription factors once activated (Osburn *et al.* 2001). Recent work suggests that protein concentrations of many

transcription factors, including nuclear receptors, are controlled by proteolysis driven by ubiquitination. Ligand binding seems to decrease the receptor's half-life (Adam-Stitah *et al.* 1999; Nawaz *et al.* 1999; Zhu *et al.* 1999; Boudjelal *et al.* 2000; Dace *et al.* 2000; Hauser *et al.* 2000; Lonard *et al.* 2000), indicating that the mechanism to turn the system off or attenuate receptor-mediated transcription is via degradation of the transcriptionally active receptor, a finding that correlates protein stability and gene expression (Kopf *et al.* 2000; Salghetti *et al.* 2000). These observations suggest that interactions between nuclear receptors and coactivators and components of the transcription machinery may serve as a signal that targets the receptors for proteolysis, ultimately regulating the expression of target genes.

Ubiquitin (Ub) mediated proteolysis and nuclear receptors

Studies targeted to elucidate the control of gene expression by nuclear receptors reveal that hormone treatment of cells leads to a decrease in the ability of the receptor to bind hormone, a poorly understood phenomenon (McIntyre and Samuels 1985). One such example is the case of the glucocorticoid receptor.

Glucocorticoids are steroid hormones produced by the adrenal glands and are necessary for normal growth and development. Their actions are mediated by binding to the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily, which, in turn, regulates transcription of target genes. Analysis of the GR utilizing antibodies showed that ligand binding leads to a reduction of receptor protein levels (Dong *et al.* 1988; Hoeck *et al.* 1989), decreasing its half-life from 18 to 9 hours. GR

phosphorylation-deficient mice exhibited a reduced ligand-dependent GR down-regulation, suggesting that phosphorylation also influences receptor stability (Webster *et al.* 1997). Furthermore, Wallace *et al.* (Wallace and Cidlowski 2001) showed by immunoprecipitation that this receptor, in the presence of ligand, was ubiquitinated and that pretreatment of cells with the MG-132 proteasome inhibitor effectively blocked the GR down-regulation driven by ligand, suggesting that the control of gene expression by glucocorticoids and mediated by GR was affected by ubiquitin-directed proteolysis (Wallace and Cidlowski 2001).

The progesterone receptor (PR), another member of the nuclear receptor superfamily, also undergoes ubiquitin-mediated proteolysis to control its effects on gene expression. Syvälä *et al.* (Syvala *et al.* 1998), showed that PR levels, as well as its half-life, decrease once progesterone is included with cultured human breast cancer cells (Mullick and Katzenellenbogen 1986; Alexander *et al.* 1989). Progesterone treatment decreases the amount of receptor detected without affecting the total mRNA found in the system. Immunoprecipitation of PR demonstrated that the presence of hormone increased the levels of ubiquitin linked to the receptor, indicating that the lower levels of PR were due to ubiquitin-driven proteolysis (Syvala *et al.* 1998).

It has been reported that ERα is also subject to ligand mediated proteolysis (Horigome *et al.* 1988), and that this receptor is also ubiquitinated preferentially in the presence of estradiol (Nirmala and Thampan 1995). Recent studies on ERα involving the ubiquitin proteasome pathway showed that ligand-mediated degradation of ERα could be blocked in the presence of inhibitors (Alarid *et al.* 1999; El Khissiin and

Leclercq 1999; Nawaz *et al.* 1999). However, ligand-mediated ubiquitination of nuclear hormone receptors is not solely linked to their down-regulation, for example Lonard *et al.* (Lonard *et al.* 2000), have shown that the ubiquitin proteasome function is required for ERα to serve as a transcriptional activator.

Interestingly, a novel group of steroid receptor-interacting proteins that influence transcriptional response have been reported and identified as components of the ubiquitin proteasome degradation system, as in the case of SUG1/Trip1 (Lee *et al.* 1995b; vom Baur *et al.* 1996).

Trip1 (thyroid hormone receptor interacting protein 1) has been shown to associate with TR and RXR baits in the yeast two-hybrid system (YTHS) in a ligand dependent manner (Lee *et al.* 1995b). Identified as a member of the CAD (conserved ATPase domains) family of proteins, Trip1 exhibits significant sequence identity with the yeast transcriptional regulator SUG1, originally identified as a suppressor of a gene expression-deficiency mutation for the transcription factor Gal 4. Yeast SUG1 was also found in purified RNA polymerase II holoenzyme complexes *in vitro* (Kim *et al.* 1994), but according to Rubin *et al.* (Rubin *et al.* 1996), it does not appear to be a subunit of the holoenzyme. Instead, SUG1 is part of the 26S proteasome complex, specifically one member of ATPase proteins in the 19S regulatory subunit of the proteasome (Tanaka 1995). Fraser *et al.* (Fraser *et al.* 1997) suggest that SUG1 exhibits intrinsic 3'-5' DNA helicase activity dependent on its ATPase domain, and Makino *et al.* (Makino *et al.* 1997) assert that this activity is stimulated and controlled by specific mRNA molecules. All these results suggest that the proteasome itself might be involved in transcription

(Ottosen *et al.* 2002), and that SUG1/Trip1 may regulate more than one cellular function (Fraser *et al.* 1997).

VDR interactions with SUG1/Trip1

Using the Yeast two-hybrid system, it was demonstrated that SUG1, the component of the 26S proteasome complex, interacts with VDR in a ligand-dependent manner (vom Baur *et al.* 1996; Masuyama *et al.* 1997a; Encinas Dominguez *et al.* 2001). It was found that this interaction with VDR, as well as for other nuclear receptors, was AF-2 and ligand-dependent. Masuyama and MacDonald (Masuyama and MacDonald 1998) showed that overexpression of SUG1 selectively altered VDR proteolysis, suggesting that the mechanism for VDR degradation may involve the proteasome complex via SUG1 interaction with the AF-2, but so far, no evidence showing VDR ubiquitination has been published.

The difference in VDR conformation driven by the binding of the receptor to $1,25(OH)_2D_3$ analogs affects the interaction of the receptor to co-modulators and ultimately influences the rate of transcription (Peleg *et al.* 1995; Liu *et al.* 1997). Recently it has been shown that the binding of 20-epi analogs to VDR facilitates its association with the DRIP complex in a more efficient fashion than with calcitriol (Yang and Freedman 1999). Jääskeläinen *et al.* (Jaaskelainen *et al.* 2000), studying the effects of 20-epi analogs on VDR degradation in human osteosarcoma cells, observed that the presence of analogs increased the half-life of the receptor and protected the receptor from degradation over longer periods of time than with $1,25(OH)_2D_3$. This increase in

the receptor half-life may result from conformation differences of the analog-VDR complexes and differential binding of proteins involved in the degradation of VDR, with SUG1/Trip1 being a strong candidate for this differential binding.

This Chapter reports first our successful retrieval of Trip1 from a cDNA library using the YTHS and hVDR as bait. Then, we evaluated the effects that SUG1/Trip1 exerts on VDR as either a transcriptional modulator or a proteasome degradation complex protein utilizing several experimental approaches *in vitro*, and in intact cells.

Materials and Methods

Yeast two-hybrid system (YTHS)

The YTHS has been described in Chapter 3, and was employed to identify novel VIPs from several cDNA libraries, from which the Trip1 cofactor was isolated.

X-gal assay

The plate(s) containing the candidate transformants received 7 ml of chloroform and was incubated for 2 minutes with rocking movement. After incubation, the chloroform is drained and the plates are dried for about 6 minutes. Approximately 10 ml of 0.3% low-melt containing 100 mM KPO_4, pH 7.0 and 1.5 mg/ml of X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) from a 20 mg/ml stock solution in N,N-dimethylformamide (DMF) are then added to the plates, and the media allowed to harden. The plates were then incubated at 30°C for 1-3 hours, an optimal time for the color reagent to develop.

Pull-down assays

Sepharose beads containing immobilized hVDR was prepared as described in Chapter II and used in pull down assays. The human thyroid receptor interacting protein (Lee *et al.* 1995a) expression vector pCDM8-Flag-hTrip1, was kindly provided by Dr. David Moore from the Department of Molecular and Cellular Biology of Baylor College of Medicine, Houston Texas.

Approximately 1 μg of pSG5-Trip1 and pSG5-hRXRα expression plasmids were used as templates in an *in vitro* transcription and translation protocol, and the synthesized proteins were labeled with ^{35}S-methionine (Promega, Madison WI). GST-hVDR fusion protein Sepharose was used to test the interaction between the receptor and Trip1, employing RXRα, as a positive control. The counterpart experiment was also conducted, in which GST-Trip1 Sepharose beads were used to map the interaction domains between VDR and the Mediator protein. For this experiment several VDR point mutants and deletion mutants were developed *in vitro*.

The effect of the rat osteocalcin VDRE was also tested in a similar pull-down assay. In the presence of the $1,25(OH)_2D_3$ hormone, the interaction of both RXR and Trip1 with VDR was compared and evaluated. The effect of the proteasome inhibitor MG-132 (1 mM) was also tested in the pull-down assay to observe the influence this aldehyde had on the amount of intact R49K mutant hVDR remaining in the IVTT system as well as its ability to interact with Trip1.

Site-directed mutagenesis

The expression vector pSG5-hVDR was employed in synthesizing deletion and point mutants by *in vitro* site directed mutagenesis. Alterations of specific residues/regions were created using previously described double stranded DNA protocols to generate changes in the hVDR sequence (Jurutka *et al.* 1997). The deletion mutants created were: Δ5-13, Δ1-88, Δ123-130, Δ304, Δ403, Δ424. The point mutants created were: R49K, R49G and E420A.

A Trip1 point mutant K196H was also generated as described by Masuyama *et al.* (Masuyama *et al.* 1997b), following the *in vitro* site directed mutagenesis protocol presented by Jurutka *et al.* (Jurutka *et al.* 1997).

Transfections

ROS 17/2.8 cells were cotransfected with pSG5-hTrip1 expression plasmid or a non-functional mutant pSG5-Trip1, K196H (1 μg/plate), a VDRE-containing reporter construct (rBGP-GH, 2 μg/plate), and carrier pTZ18U DNA (6 μg/plate).

COS-7 cells were also cotransfected with pSG5-hTrip1 or a non-functional Trip1 mutant, K196H, and hVDR expression plasmids (1 μg/plate), as well as with a VDRE-containing reporter construct ($(CT4)_4$-TKGH; 10 μg/plate), and carrier pTZ18U DNA (9 μg/plate).

All the cells (ROS 17/2.8 as well as COS-7) were incubated with 10^{-8} M $1,25(OH)_2D_3$ (+) or vehicle (–) for 24 hours post-transfection and human growth hormone secretion into the medium was determined by RIA.

Preparation of Cellular Extracts and Immunoblotting

Transfected COS-7 cells (as described above) were lysed directly in loading buffer (2% SDS, 5% ß-mercaptoethanol, 125 mM Tris-HCl, pH 6.8, and 20% glycerol), and 40 μg of cellular protein were run on 5–15% gradient SDS/polyacrylamide gels. After electrophoretic fractionation, proteins were electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a Transblot apparatus (Bio-Rad Laboratories, Inc. Richmond, CA) in 25 mM Tris-HCl, pH 7.4, 192 mM glycine, 0.01% SDS, and 20% methanol. The membrane was then blocked by incubation for 3 h with 3% blotto. Immunodetection of bound hTrip1 protein was then performed using the monoclonal anti-Flag antibody, (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After the first antibody treatment, the Immobilon-P membrane was washed and treated at room temperature for 1 h with goat antimouse IgG conjugated to biotin followed by four 15-min washes. A 5-ml mixture of biotinylated alkaline phosphatase and neutravidin (Pierce Chemical Co., Rockford, IL; in a ratio of 1:4) was preincubated for 45 min at 22° C in 1% blotto. The mixture was diluted to 30 ml with 1% blotto and added to the membrane for a 2-h incubation with rocking at room temperature and then washed four more times, followed by a fifth wash with biotin blot buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100). Finally, the blot was exposed to color reagent containing 50 μg/ml of 5-bromo-4-chloro-3-indolyl-phosphate and 100 μg/ml of 4-nitroblue tetrazolium chloride. The color reaction was stopped by washing with distilled water.

VDR expression analysis was also monitored by immunoblotting by a procedure analogous to the one described for Trip1, but using the 9A7 VDR specific monoclonal antibody as described in detailed elsewhere (Jurutka *et al.* 2000b).

Results

As described above in Chapters 3 and 4, driven by the original interest of this dissertation research in cloning novel VIPs via YTHS technology, we were successful to this point only in isolating RXRα and SRC-1, two of the most well recognized VIPs and general nuclear receptor partners that interacted in a ligand-dependent fashion. This chapter describes the isolation and cloning by the YTHS of a less well understood VIP, Trip1. This VIP was originally considered to be a coactivator (Lee *et al.* 1995a), but had fallen into temporary disfavor when it was learned that it was a component of the 26S proteasome proteolysis system. Consequently, when we screened out Trip1 as a VIP, we decided to characterize its influence on VDR-mediated transcription.

With the screening of a human keratinocyte cDNA library in the yeast two-hybrid system, we were able to isolate a clone that exhibited interaction with hVDR *in vivo* in a hormone-dependent manner (Fig. 38).

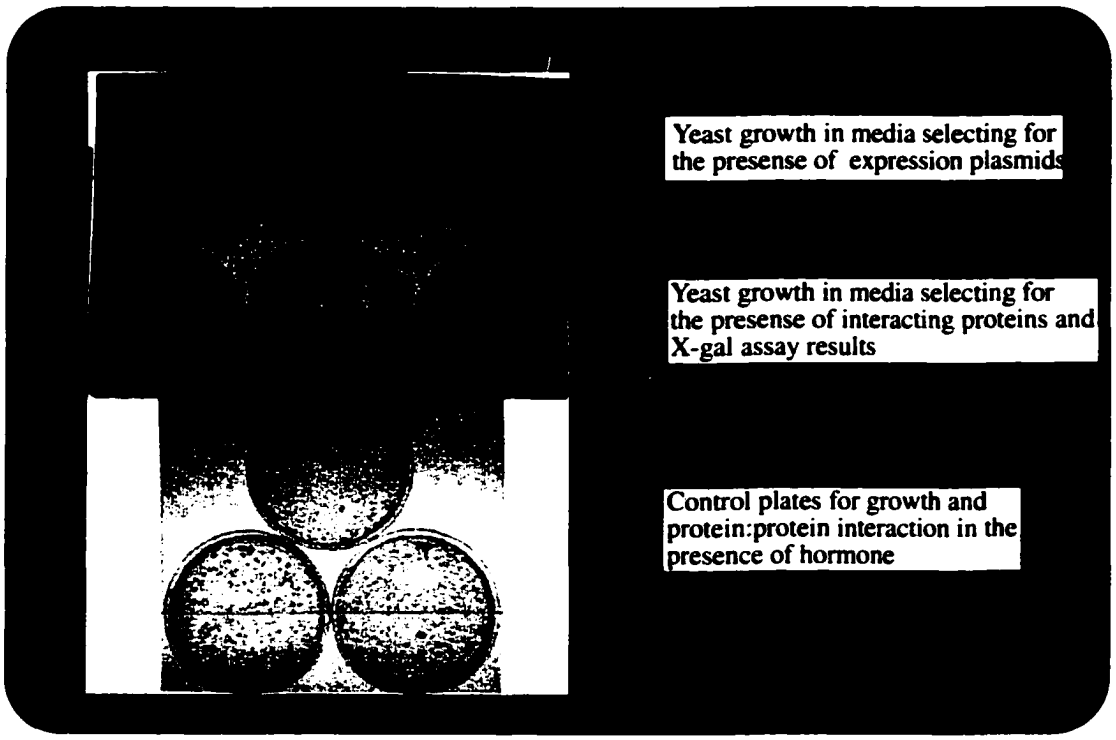
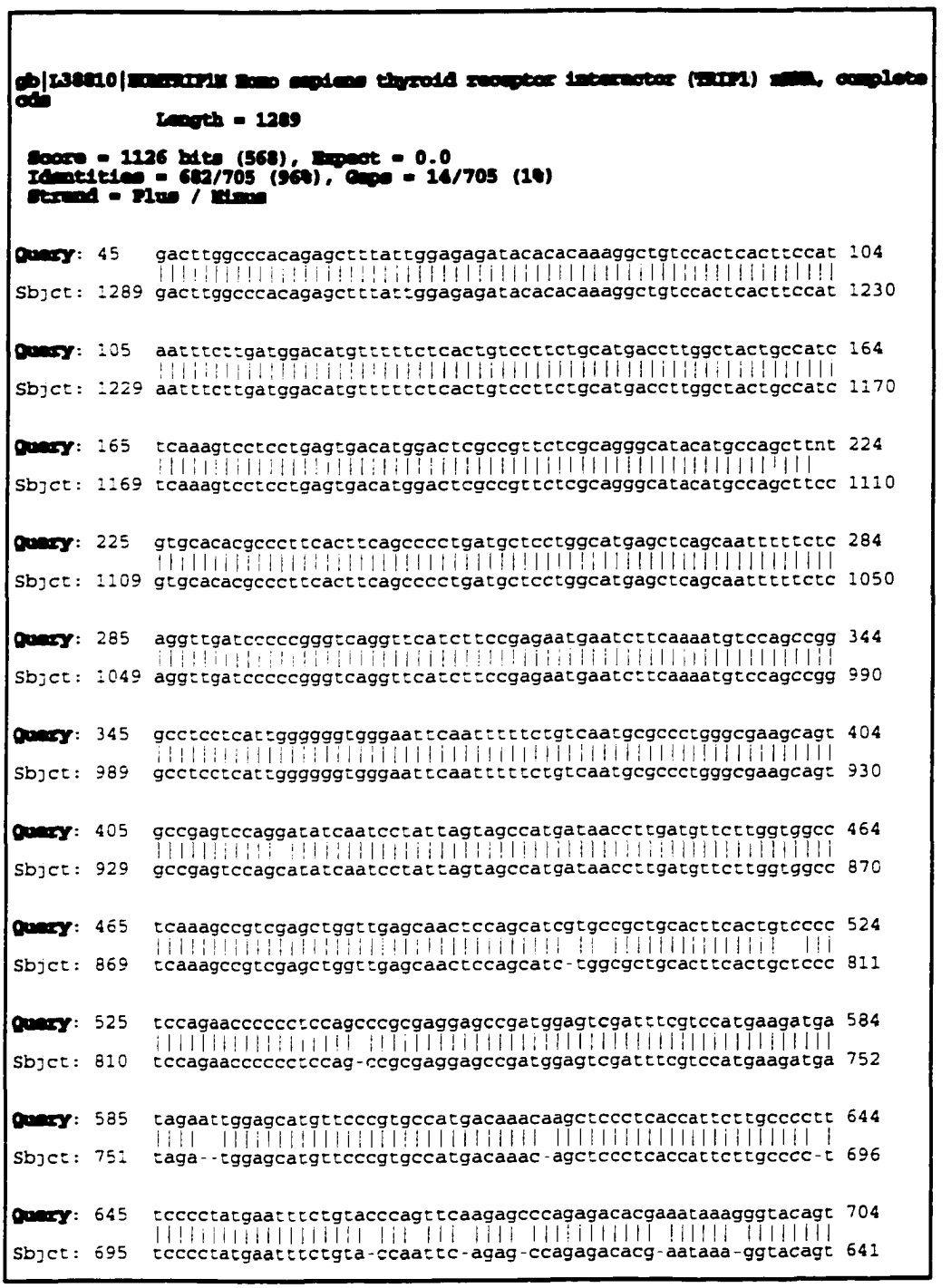


Fig. 38. Yeast two-hybrid system cDNA library screen. Petri dishes display yeast growth in selective media. A) Yeast growth in plates lacking the amino acids leucine and tryptophan selects for clones containing the expression plasmids evaluated. B) Yeast growth in plates lacking the amino acid histidine selects for protein:protein interaction. Furthermore, this interaction can be visualized and quantified by controlling the expression of the reporter gene LacZ encoding for the β-galactosidase enzyme in the X-gal assay. C) Control transformation of yeast with known protein:protein interacters also help to select candidate clones.

After its isolation, DNA sequence and database analysis showed that the isolated VIP was the human homologue of the yeast SUG1, also known as Trip1 (see Fig. 39 for clone sequence and DNA database alignment analysis).



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gb|L38810|HSTRIP1X Homo sapiens thyroid receptor interactor (TRIP1) mRNA, complete
cds
            Length = 1289

 Score = 1126 bits (568), Expect = 0.0
 Identities = 682/705 (96%), Gaps = 14/705 (1%)
 Strand = Plus / Minus

Query: 45   gacttggcccacagagctttattggagagatacacacaaaggctgtccactcacttccat 104
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query: 105  aatttcttgatggacatgtttttctcactgtccttctgcatgaccttggctactgccatc 164
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
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Query: 225  gtgcacacgcccttcacttcagcccctgatgctcctggcatgagctcagcaatttttctc 284
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Query: 645  tcccctatgaatttctgtacccagttcaagagcccagagacacgaaataaagggtacagt 704
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Fig. 39. DNA sequence alignment of the selected VIP clone, proved to be Trip1.

Being one of the most controversial VIPs since its detection as a VDR interacting protein (Lee *et al.* 1995b; vom Baur *et al.* 1996; Masuyama *et al.* 1997a; Encinas Dominguez *et al.* 2001), Trip1 has been identified as possessing several putative domains with very diverse activities, e.g., ATPase (Tanaka 1995), 3'-5' helicase (Fraser *et al.* 1997), mRNA binding (Makino *et al.* 1997), etc. Trip1 has also been observed to act as a transcriptional enhancer (Russell and Johnston 2001), as well as a negative regulator of transcription through its involvement with the proteasome complex (Rubin *et al.* 1996; Masuyama *et al.* 1997b). With all its potential functions and the controversy surrounding its biological significance, after independently identifying Trip1 as a VIP via the yeast two-hybrid screen, we set out to study the effects of Trip1 on the transcriptional activities mediated by VDR.

The initial step was to define the effect that this protein exerts on $1,25(OH)_2D_3$–stimulated transcription (Fig. 40). In transiently transfected cells, specifically ROS 17/2.8 osteoblast-like and COS-7 renal cell lines, we observed that VDR-mediated transcriptional activation by $1,25(OH)_2D_3$ was suppressed by approximately 20 and 30%, respectively, upon cotransfection of Trip1 (Figs. 40A and B). Trip1 attenuation of VDR action was statistically significant, but not complete, probably because of the expression of endogenous Trip1 in COS-7, and especially in ROS 17/2.8 cells. In order to ensure that this effect of Trip1 in VDR-driven transactivation was a functional consequence of the presence of the Trip1 cofactor, a Trip1 inactivation mutant was employed as a control. As shown by Masuyama *et al.* (Masuyama *et al.* 1997b), the K196H Trip1 mutant is ATPase-defective and exhibits a

compromised protein degradation-inducing effect. We created this mutation in our Trip1 expression construct and tested its influence on $1,25(OH)_2D_3$-stimulated transcription compared to the effect of wild type Trip1. As shown in Fig. 40, we observed that in the ROS 17/2.8 cell line, the addition of the inactivated mutant not only restores the VDR transcriptional response, but actually amplifies it, increasing VDR activity by about 20% (Fig. 40A, far right panel). Presumably, K196H Trip1 competes with endogenously expressed Trip1 in ROS 17/2.8 cells to reverse the negative effect of this VIP cofactor. In the COS-7 cell line (Fig. 40B, far right panel), the effects of the K196H Trip1 mutant on VDR-mediated transactivation were qualitatively similar to those observed in the ROS 17/2.8 cell line in that the ATPase inactive mutant was less effective than native Trip1 in blunting $1,25(OH)_2D_3$-stimulated transactivation. However, the K196H Trip1 mutant failed to fully restore the transcriptional response to that observed for VDR in the presence of hormone (compare Fig. 40B lanes 3 and lane 1). Western blot analysis of the expression in COS-7 cells of Trip1 and its mutant K196H showed that the reduced recovery effect of K196H was not due to the presence of low levels of this mutant, as it was expressed equally to wild type Trip1 (Fig. 40C). Therefore, in general we can conclude that Trip1 exerts a negative influence on VDR-mediated transactivation, likely via its ATPase function. We postulate that the interaction of Trip1 with VDR leads to the eventual proteolytic degradation of VDR, and consequent downregulation of its actions. Indeed, as shown directly in Fig. 40D, overexpression of wild type Trip1 in COS-7 cells leads to a significant diminution of immunoreactive hVDR protein levels, correlating with reduced transactivation by VDR

under these conditions (Fig. 40A and B). On the other hand, results in Fig. 40D illustrate that hVDR protein levels are sharply elevated when the ATPase defective Trip1 mutant (K196H) is cotransfected with hVDR in COS-7 cells. This finding is in concert with less attenuation of VDR-mediated transactivation (COS-7 cells) or even enhanced transactivation (ROS 17/2.8 cells), and suggests that blunting effects of Trip1 on VDR stimulated gene expression are caused by ligand-dependent proteolysis of the receptor.

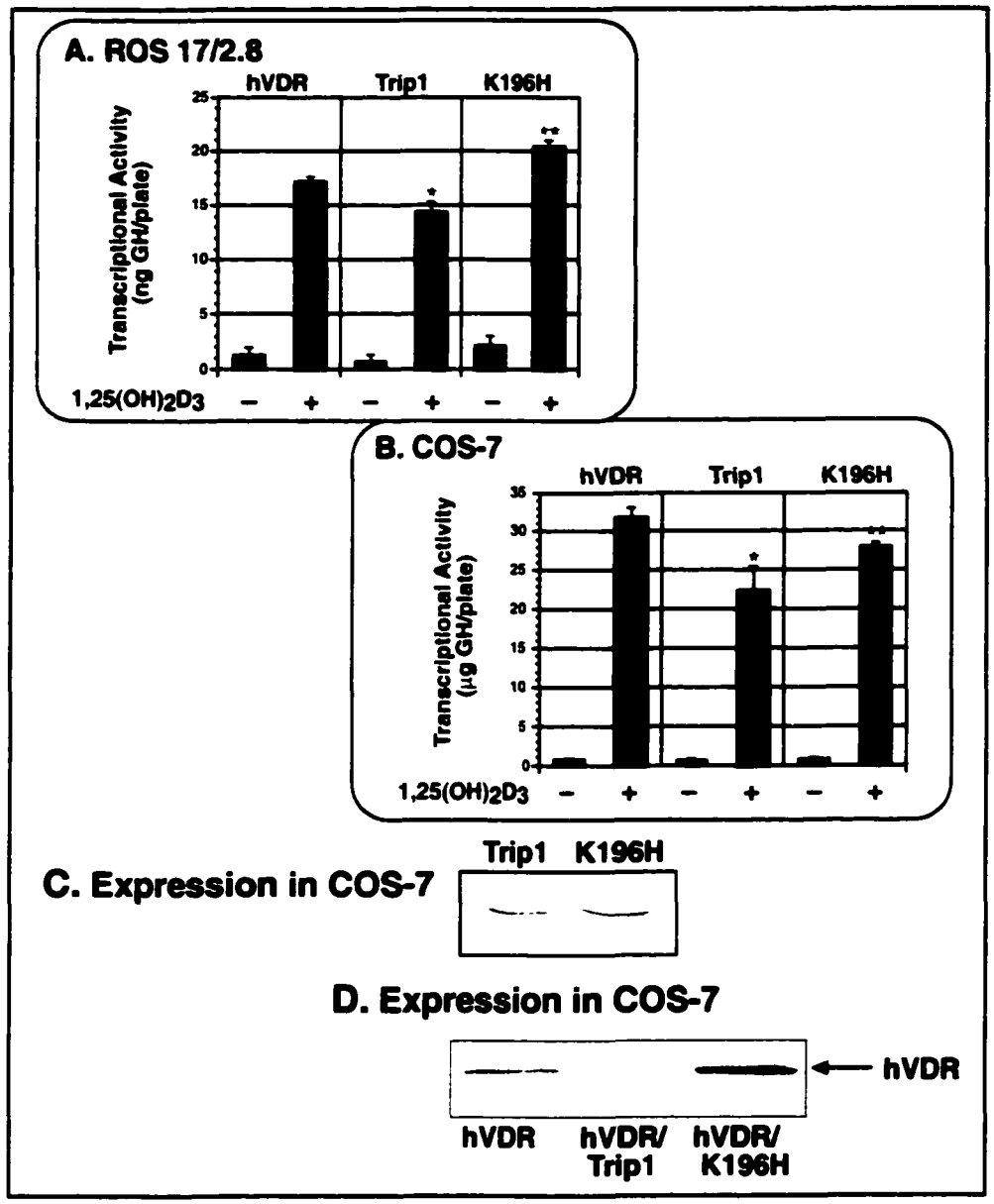


Fig. 40. Effect of the expression of Trip1 and its ATPase defective mutant, K196H (Masuyama *et al.* 1997b), **on the $1,25(OH)_2D_3$-VDR gene response in A) rat osteosarcoma and B) monkey kidney cell lines.** In both systems it can be observed that the addition of the Trip1 cofactor inhibits the transcriptional response driven by hVDR. The addition of the Trip1 mutant, K196H somewhat restores the transcriptional response, suggesting that the ATPase catalytic domain of this protein is important for degradation of VDR and reduction of its signaling. (•) Statistically significantly different from hVDR alone ($p<0.05$). (••) Statistically significantly different from Trip1 coexpression system ($p<0.05$). Each value is the average of 3 determinants ± SEM. **C**). Expression levels of the Trip1 and its K196H mutant via Western blotting demonstrate that the quantitative difference in transcriptional blunting effectiveness is derived from the actions of the cofactor and not differences in expression levels. **D**). Trip1 overexpression reduced hVDR levels as monitored by Western blotting, but cotransfection of the K196H mutant potentiates the concentration of hVDR, presumably by diminishing its proteolytic degradation.

With negative Trip1 effects on VDR-mediated transactivation established, we then proceeded to determine if the interaction between VDR and Trip1 revealed in the YTH system could be recapitulated utilizing the more biochemical GST-pull down assay. The results (Fig. 41) indicate that a weak direct interaction was found between these two proteins in the absence of hormone, whereas the addition of $1,25(OH)_2D_3$-ligand significantly enhanced Trip1 binding to VDR, *in vitro* (Fig. 41A, compare lanes 1 and 2). The potential effect of the rat osteocalcin VDRE as a DNA platform was also tested in the GST pull-down assay (Fig. 41B), employing as a control, the RXR natural heteropartner of VDR. Again, the presence of the $1,25(OH)_2D_3$ hormone enhanced the interaction of both RXR (Fig. 41B lane 2) and Trip1 (Fig. 41B lane 5) with VDR. However, whereas inclusion of the VDRE greatly amplified the association of RXR and VDR (Fig. 41B, compare lanes 2 and 3), this DNA element blunted the interaction of Trip1 and VDR (Fig. 41B, compare lanes 5 and 6). These results indicate that the VDR-Trip1 interaction is hormone dependent and that it may occur primarily in solution. They also suggest that the VDR-Trip1 interaction is somewhat reduced in the presence of DNA, specifically a VDRE, revealing that the DBD of VDR might be involved in supporting the association of the receptor with Trip1. Conversely, when VDR has dissociated from DNA in the cell, it may be more vulnerable to Trip1-triggered proteolysis via the 26 S proteasome.

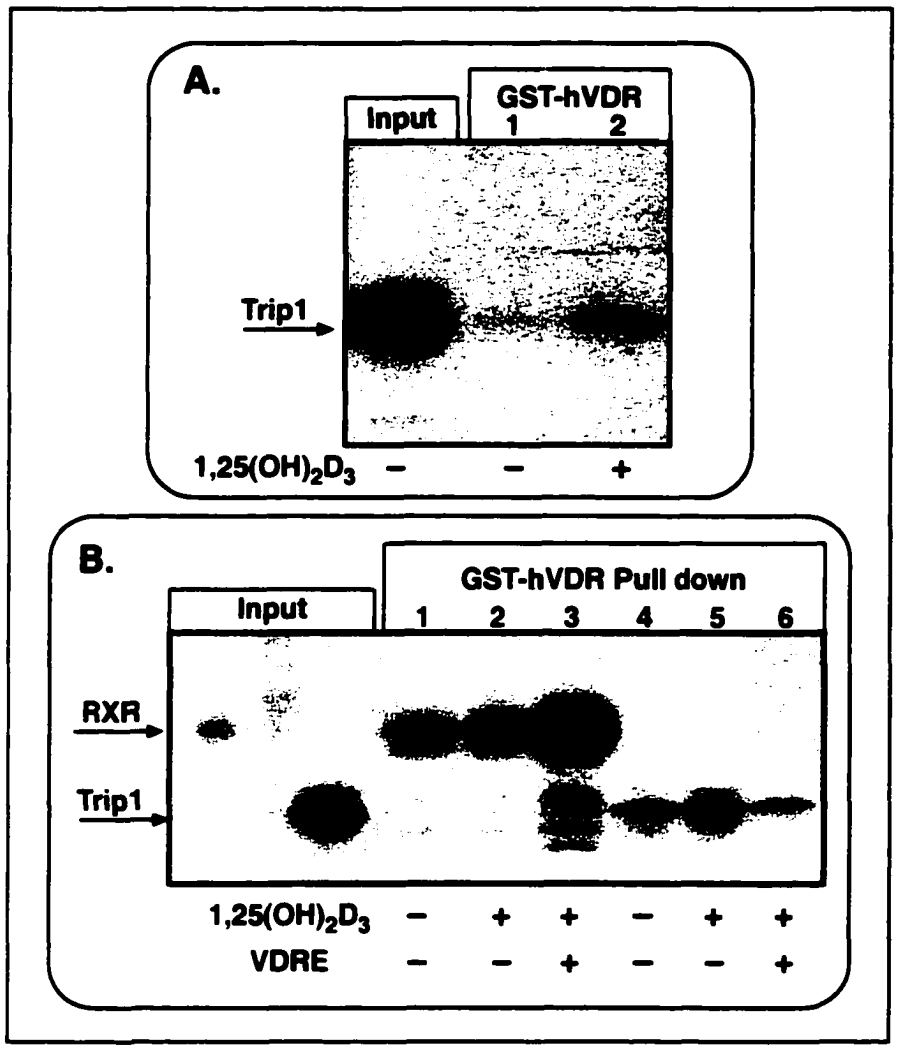


Fig. 41. Trip1 interacts with hVDR in a ligand-enhanced fashion in solution: a VDRE in the presence of $1,25(OH)_2D_3$ ligand diminishes VDR-Trip1 interaction, but enhances VDR-RXR association. One μg of RXRα or 1 μg of hTrip1 cDNA expression vectors were *in vitro* transcribed and translated into ^{35}S-labeled protein and used in pull down assays with VDR beads. **A**). In the absence of hormone (lane 1), little or no interaction was found between hTrip1 and hVDR. $1,25(OH)_2D_3$ promoted this interaction (lane 2). **B**). In the absence of hormone (lane 1) a modest but significant interaction was found between VDR and RXR. $1,25(OH)_2D_3$ augmented this interaction (lane 2), which was then fully potentiated by the presence of the rat osteocalcin VDRE (lane 3). Trip1 interacts weakly with VDR, independently of the presence of hormone (lane 4). However, the presence of $1,25(OH)_2D_3$ enhanced this association (lane 5), whereas the addition of the VDRE interferes with the interaction of hTrip1 with hVDR when $1,25(OH)_2D_3$ is present (lane 6). Input lanes represent 5% of that actually utilized in the GST pull down lanes.

Because Trip1 appears to interact directly with VDR in solution, the next set of experiments were designed to probe the various domains of hVDR for Trip1 association

sites. Masuyama *et al.* (Masuyama *et al.* 1997b) have previously established that the association between VDR and Trip1 is AF-2/helix-12-dependent, as well as ligand-dependent. However, with other VDR cofactors, protein:protein interactions had been shown to require (or be modulated by) additional regions of the receptor, likely necessary for VDR to fully exert its actions, or be downregulated in the case of Trip1. With the finding (Fig. 41) that the presence of a VDRE inhibited the association between VDR and Trip1, we hypothesized that perhaps the DBD or N-terminal domain of hVDR might be involved in contacting Trip1.

Utilizing the GST-pull down assay with Trip1 as a fixed matrix, we tested several VDR deletion mutants for their interaction with this cofactor (Fig. 42). Similar to previous results, the wild type hVDR displayed a hormone-enhanced interaction with Trip1 (Fig. 42, lanes 1 and 2). The hVDR N-terminal deletion mutant Δ1-88 (LBD) displayed a very weak, but ligand-dependent association with Trip1 (Fig. 42, lanes 3 and 4). In contrast, the Δ123-130-deletion mutant exhibited a stronger but hormone-independent interaction (Fig. 42, lanes 5 and 6). The Δ403 AF-2-deletion mutant did not display any detectable interaction with Trip1 (Fig. 42, lanes 7 and 8). The results in Fig. 42, indicate clearly that both the AF-2 region and the DBD/N-terminal domain are critical for the association of hVDR and Trip1. With respect to the hVDR AF-2, the data in Fig. 42 (lanes 7 and 8) are consistent with the independent work of Masuyama *et al.* (Masuyama *et al.* 1997a), who showed that two point mutations that compromise the AF-2, namely L417S and E420Q, eliminate SUG1 binding to hVDR. Based upon the data in Fig. 42, the AF-2 is relatively more important for Trip1 contact than is the DBD,

but both domains are significant. The stronger association of Trip1 with Δ123-130 VDR suggests that just as the p160 coactivator and the $DRIP_{205}$ mediator may interact more avidly with Δ304 hVDR because of the conformational exposure of contacts when internal deletions are utilized (Chapters 4 and 5), the Δ123-130 internal deletant seemingly alters the positioning of the N-terminal/DBD domain and the AF-2 to potentiate hVDR-Trip1 interaction. Interestingly, the Δ123-130 hVDR deletant shows a 50% reduction in concentration of expressed receptor compared to wild type and attenuated transcriptional activity (Peter Jurutka, personal communication), suggesting that the enhanced interaction of this deletant with Trip1 (Fig. 42) leads to increased proteolytic degradation.

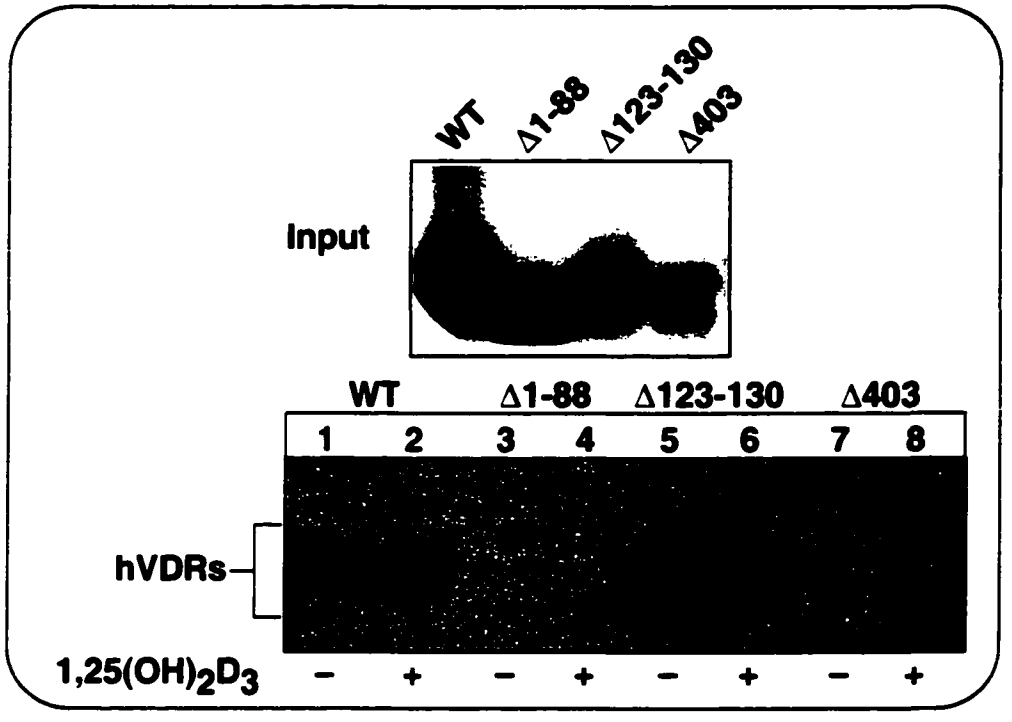


Fig. 42. Pull-down assay to identify the general domains of VDR involved in Trip1 interaction. WT VDR and several deletion mutants were evaluated for their capacity to interact with Trip1. WT VDR as well as the Δ123-130 deletion mutant display significant interaction with Trip1. N and C-terminal hVDR truncation mutants (Δ1-88 and Δ403) exhibit weak and non-existent association with Trip1, respectively.

Hsieh *et al.* (Hsieh *et al.* 2000), investigating residues in the DBD of VDR that provide specificity for DNA binding observed that a synthetic hVDR mutant, R49K is inactive, displaying neither VDRE binding, nor transactivation potential. It was also observed that this VDR mutant, when *in vitro* transcribed and translated, was degraded at a rapid rate, suggesting that the artificial introduction of a lysine at position 49, between the zinc fingers of hVDR, seems to generate a recognition site for proteolysis. We therefore tested the R49K synthetic mutant for interactions with Trip1, in an attempt to determine if the observed degradation is proteasome dependent, and perhaps driven by

hyperassociation of Trip1 with R49K hVDR. WT hVDR was used as a positive control, with N-terminally deleted hVDR (Δ1-88), and a point mutant (R49G) that is not rapidly degraded, serving as a negative controls. As shown in Fig. 43, the 36kDa fragment of the rapidly degraded hVDR point mutant, R49K, exhibited a markedly enhanced interaction with Trip1, independent of the presence of hormonal ligand (Fig. 43, lanes 3 and 4). In fact, assuming approximately 12kDa of C-terminal sequence is removed from R49K hVDR, the 36kDa fragment likely is hormone-binding negative. The control R49G hVDR point mutant displayed a profile of Trip1 interaction similar to that of WT (Fig. 43, lanes 5 and 6) and, once again, the VDR LBD (Δ1-88) displayed diminished, but hormone-dependent association with Trip1 (Fig. 43 lanes 7 and 8). These results suggest that the R49K hVDR point mutant might generate either an amino acid recognition sequence that targets the protein for degradation or introduce a ubiquitination site, either of which renders this form of VDR susceptible to proteolysis.

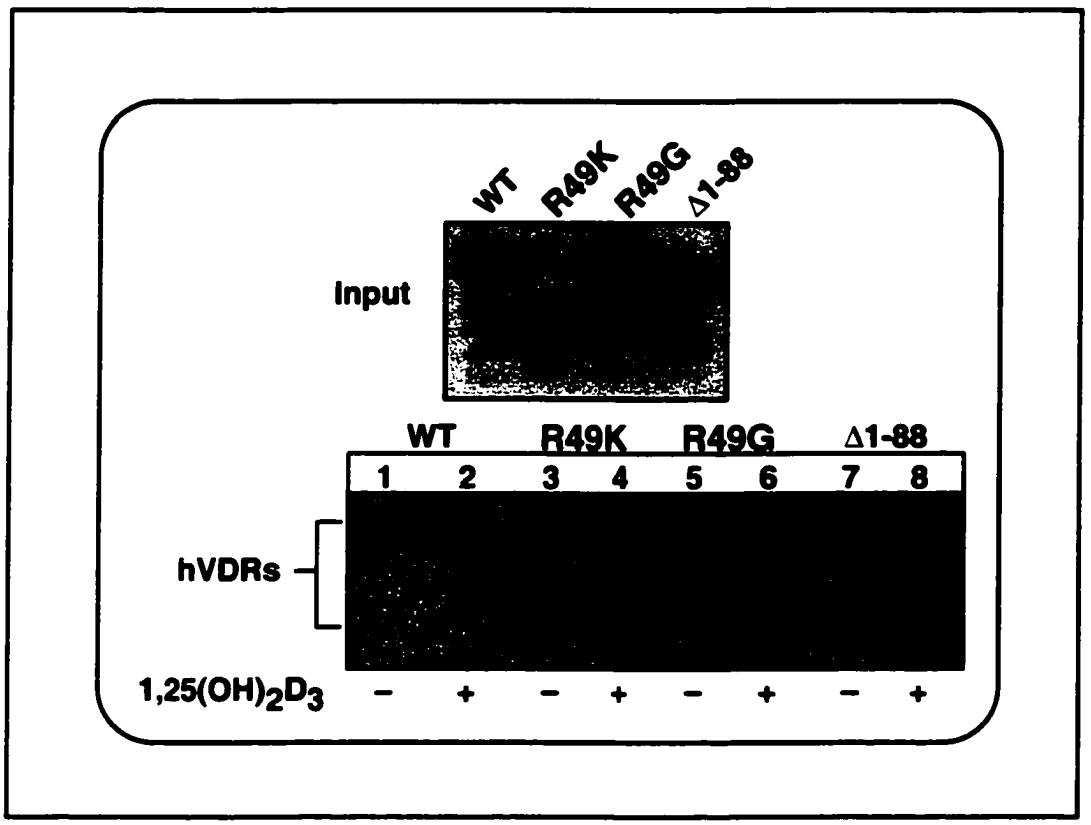


Fig. 43. In vitro interaction between Trip1 and VDR point and deletion mutants. Synthetic point mutations found in the DBD of hVDR (R49K and R49G) were observed in their ability for Trip1 interaction, utilizing WT VDR as control. The proteolytic fragment of hVDR mutant R49K exhibits a strong interaction with Trip1 (lanes 3-4), suggesting a correlation between interaction and degradation. R49G, a non-proteolyzed mutant, (lanes 5-6) shows WT-like behavior (lanes 1-2). The VDR DBD deletion mutant exhibited somewhat impaired interaction with Trip1, but still associated with this VIP in a hormone dependent manner (lanes 7-8). Inputs (top panel) are 5% of that used in the pull down lane.

In order to test the role of Trip1 and the proteasome complex in the degradation of the R49K hVDR point mutant, we examined the effect of an inhibitor of the proteasome complex on the behavior of the aforementioned synthetic point mutant (Fig. 44). The proteasome inhibitor, MG-132, has been utilized previously to prevent VDR degradation driven by the proteasome complex (Masuyama and MacDonald 1998). In our experiments, we were able to protect the R49K proteolyzed mutant hVDR from degradation, in part, by the addition of the MG-132 compound to the reaction (Fig. 44,

top panel). Partial prevention of degradation also allowed the full length R49K point mutant to interact with the Trip1 cofactor in the pull down assay (Fig. 44, lane 5), providing further evidence that in this mutant the change from arginine to lysine might mimic recognition sequences that drive receptor proteins to ubiquitination and further degradation via the proteasome complex.

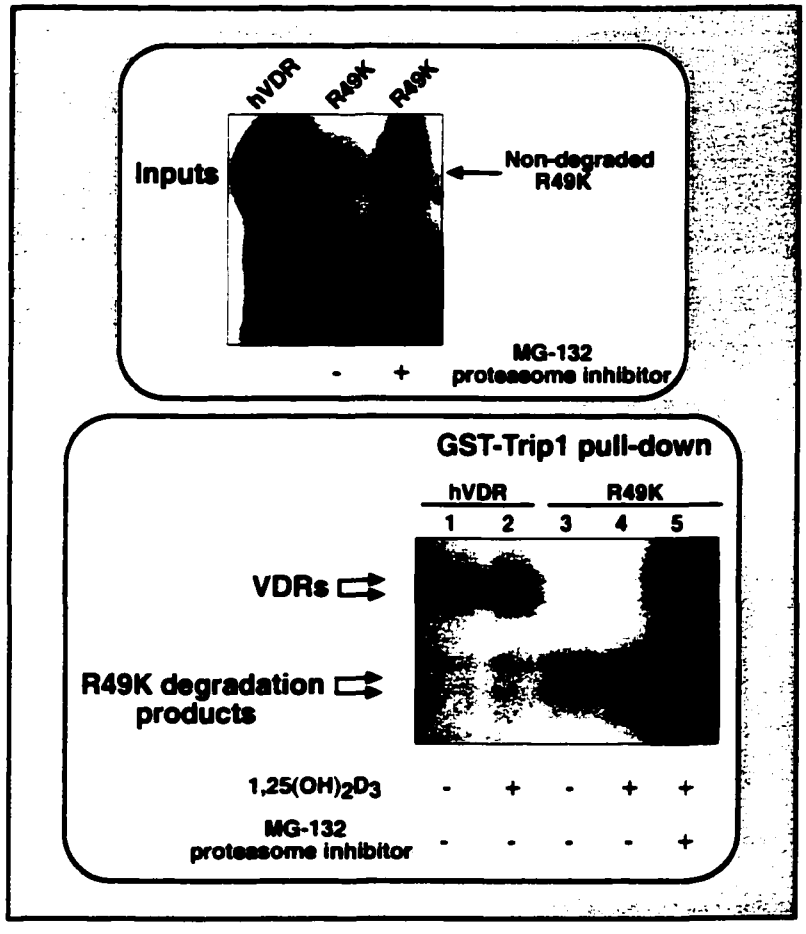


Fig. 44. A proteasome inhibitor, MG-132, retards the degradation of R49K hVDR. The synthetic point mutant R49K was investigated for its ability to interact with Trip1 when a proteasome inhibitor (MG-132) is added to the *in vitro* transcription-translation reaction. As previously observed, the proteolytic fragment of mutant R49K exhibits a strong interaction with Trip1 (lanes 3-4). The addition of the MG-132 inhibitor reduced R49K degradation, as seen in the input box above, and promoted the ability of full length R49K mutant to interact with the Trip1 cofactor. This suggests that R49K degradation is driven by the proteasome complex, probably following ubiquitination of K49 in the hVDR mutant. Inputs (top panel) are 5% of that used in the pull down lane.

Fig. 45 depicts a summary of the evaluation performed to determine the hVDR domains involved in Trip1 interaction. Three deductions can be made from this summary: i) the major Trip1 interacting site in hVDR is the AF-2/helix-12 domain (based upon the inactivity of Δ403 hVDR), ii) a second Trip1 contact site lies in the N-terminal/DBD region of hVDR (based upon the reduced binding activity of Δ1-88), and iii) hVDR is subject to probable ubiquitination and proteasomal catalyzed degradation, phenomena that either can be stimulated by introducing a ubiquitinatable lysine at position 49, or partially blocked by MG-132. Thus, we conclude from the evidence that Trip1 binding to hVDR signals ubiquitination and degradation to cause downregulation of VDR-mediated transactivation.

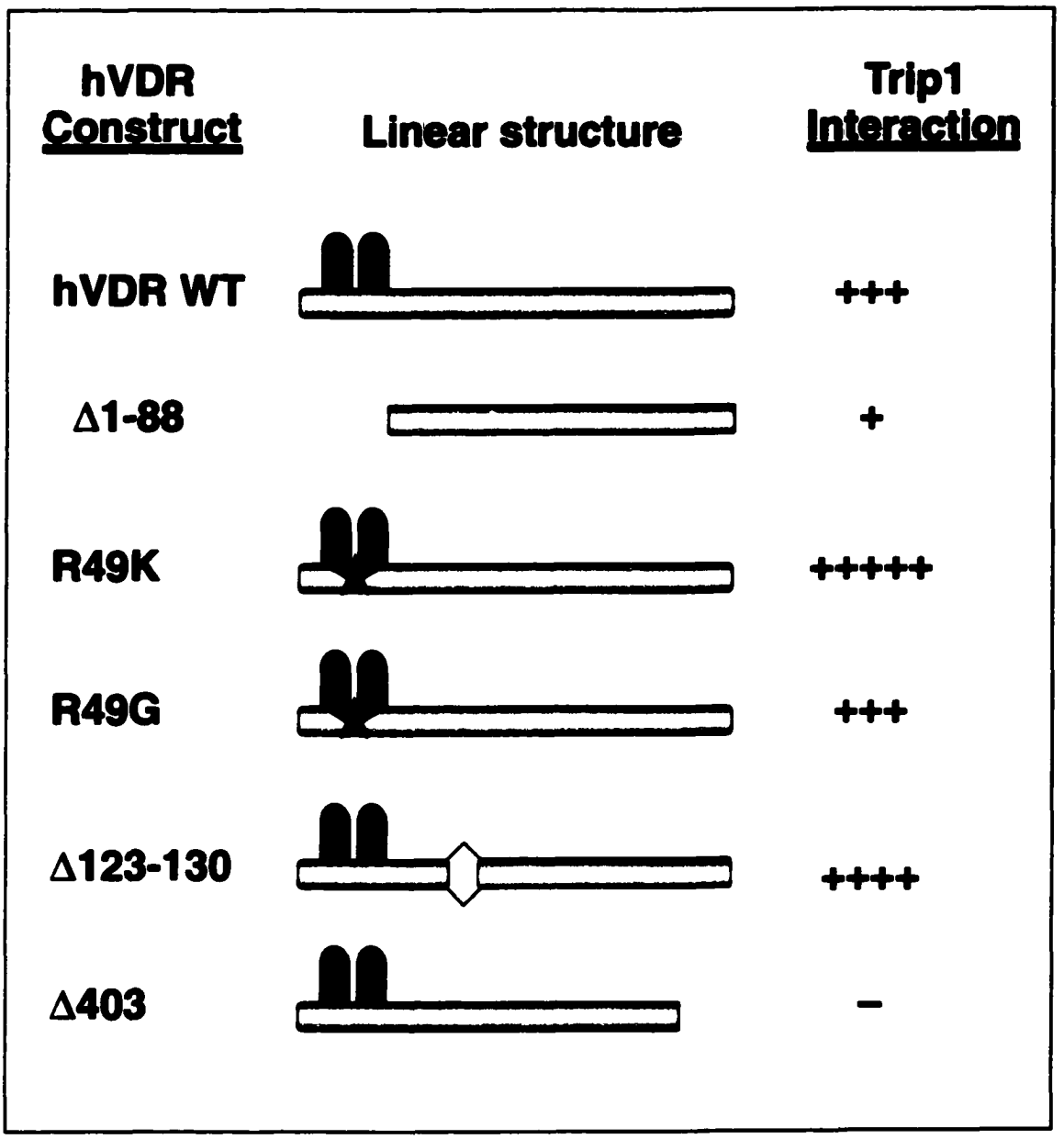


Fig. 45. Summary of interactions between GST-Trip1 and hVDR mutant constructs in the presence of $1,25(OH)_2D_3$. This figure depicts a series of hVDR deletion mutants in their linear form, and the level of interaction with the Trip1 protein in the presence of hormone. The red star signifies introduction of a lysine proteolysis signal, whereas the green star indicates that an arginine to glycine mutation is silent with respect to proteolysis.

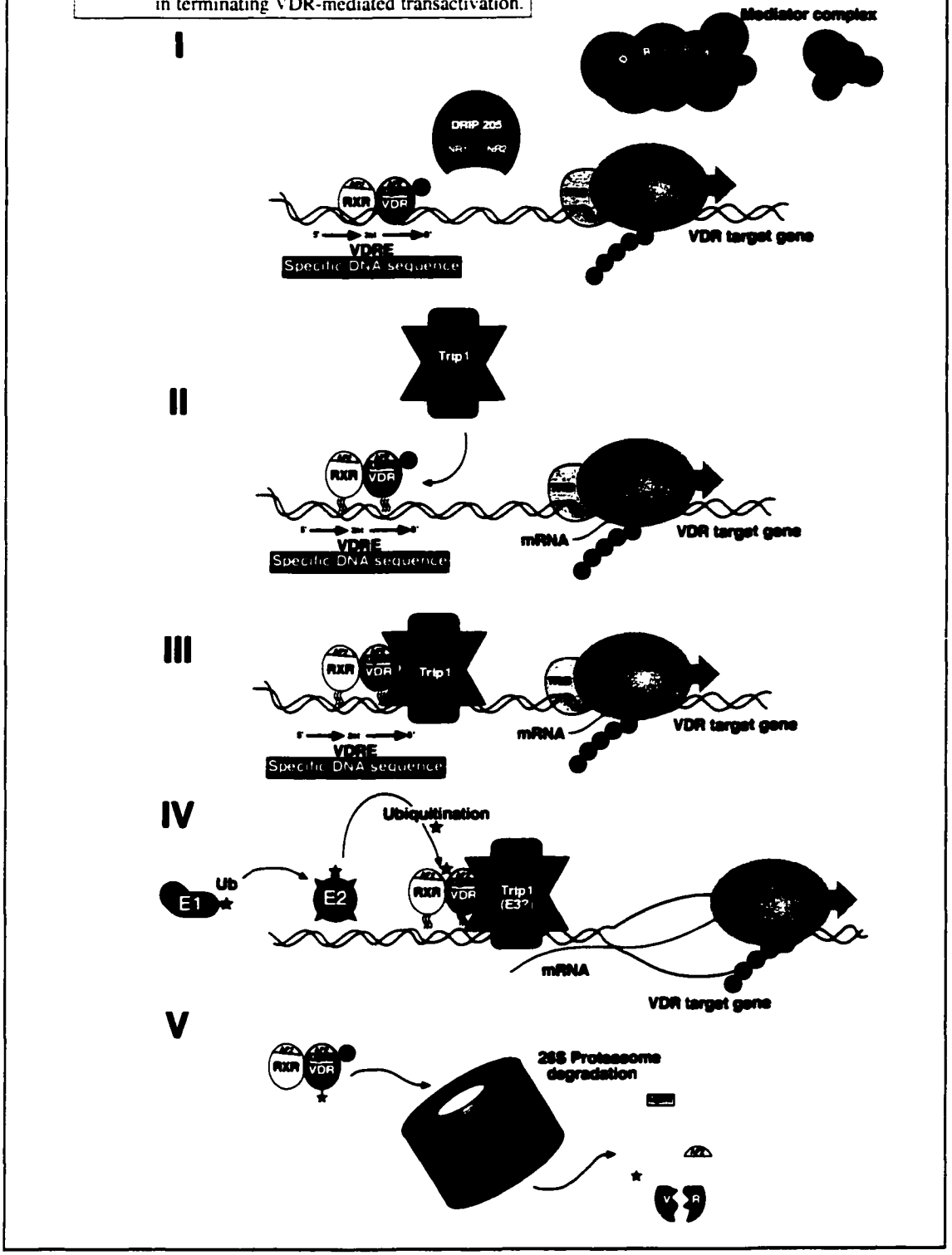
Conclusions

Trip1 attenuates the transcriptional activity of hVDR. This cofactor also exhibits a hormone-dependent interaction with the receptor in solution. With the use of a VDR mutant susceptible to degradation that intensely interacts with Trip1, we were able to correlate Trip1 association with protein degradation. These results revealed that one of the mechanisms through which VDR-mediated gene expression could be down regulated is via the proteolytic inactivation of the receptor. The interaction between hVDR and Trip1 can be diminished by the presence of a VDRE, suggesting that VDR is less susceptible to Trip1-triggered degradation when bound to DNA. Thus, one Trip1 contact in hVDR may be occluded by DNA binding to the zinc finger N-terminal domain of the receptor. Accordingly, via VDR deletion mutant analysis we were able to establish that the VDR DBD/N-terminus is required but not sufficient for full Trip1 interaction, and that the VDR LBD, specifically the C-terminal AF-2 region, is required but not sufficient for full Trip1 interaction. Thus, the involvement of the DBD in VDR association with Trip1 might explain the negative influence that DNA, specifically the VDRE, has on this interaction, and indicates that proteolysis or VDR inactivation is prevented while VDR is bound to DNA and acting as a transcription factor regulating target gene expression. Similarly, while activating transcription via SRC-1 and $DRIP_{205}$ associating sequentially with the hVDR AF-2, the other Trip1 binding site on VDR is occupied. The bottom line is that Trip1 triggered proteolysis of hVDR likely doesn't come into play in the transcriptional life cycle of the receptor until after $DRIP_{205}$ and

RNA polymerase II have left VDR/RXR at the VDRE when transcriptional initiation occurs.

Therefore, we have proposed a two-site interaction model (Fig. 46), to explain the manner in which Trip1 interacts with VDR to complete the VDR transcriptional control life cycle, and to down regulate the expression of target genes.

Fig. 46. Model of the proposed mode of action of Trip1 in terminating VDR-mediated transactivation.



As shown in panel I, Fig. 46, once the CTD of RNA polymerase II is phosphorylated and transcription of the vitamin D target gene begins, the DRIP/Mediator dissociates from the VDR/RXR heteropartner. This dissociation is proposed to partially destabilize heteropartner binding to the DNA/VDRE, leading perhaps to an equilibrium between DNA bound and unbound forms of the heterodimer. This equilibrium is postulated to allow the association of the Trip1 cofactor with VDR in solution (Fig. 41 and Fig. 46 panels II and III). The competition found between DNA and Trip1 for VDR (Fig. 41) suggests that this VIP associates with the receptor in part through its DBD (see Fig. 42), likely preventing it from high affinity re-interaction with the VDRE (see panel III, Fig. 46). The binding of Trip1 to VDR is further driven by Trip1's ability to contact the receptor's AF-2, which is now vacant of $DRIP_{205}$ (or p160 coactivators) that would otherwise stabilize the forces that maintain receptor-DNA interaction. Following ubiquitin activation by E1, and transfer to E2 (Fig. 46, panel IV), the ubiquitin-charged E2 is poised to interact with the VDR substrate, a process that is proposed to require Trip1 functioning as a ubiquitin protein ligase, or E3. Next, as depicted in Fig. 46, panels IV and V, Trip1 triggers ubiquitination of the receptor, a modification that tags the protein for degradation by the 26S proteasome complex. VDR proteolysis effectively ends the transcriptional life cycle of VDR (panel V, Fig. 46).

CHAPTER VII. SUMMARY AND FINAL REMARKS

Research project objectives

The goal of this dissertation research project was the cloning and characterization of novel and known VIP(s) that serve to regulate the transcription of genes mediated by the vitamin D receptor. Several laboratories have isolated a wide variety of cofactors that control the activity of members of the nuclear receptor superfamily, including VDR, with the assistance of the yeast two-hybrid system (MacDonald *et al.* 1995; Jin and Pike 1996; vom Baur *et al.* 1996; Tagami *et al.* 1998; MacDonald *et al.* 2001). By utilizing this technique, we have been able to identify three previously recognized VIPs, two of them extensively studied (RXR and SRC-1), and one of them initially disregarded as a false positive (Trip1), but proven by others (Masuyama *et al.* 1997b), and in the present dissertation, to be a significant player in downregulating the action of VDR on transcription.

The present research results were presented, in part, at the American Society for Bone and Mineral Research (ASBMR) meeting held in the fall of 2001 in the form of a poster (Encinas Dominguez *et al.* 2001), and these results have been the backbone of the data and schematic models contained in this dissertation.

Accomplishment of objectives and integrated model

Although mildly disappointed that we did not fish out any absolutely novel VIPs with the YTHS, we built on the success of retrieving RXRα, SRC-1 and Trip1 by extensively characterizing the interaction of these VIPs with VDR, both physically and

functionally. Moreover, we obtained from other investigators four additional VIPs (or putative VIPs), namely SMRT, NCoA-62, CBP and $DRIP_{205}$, and set out to elucidate the complete transcriptional life cycle of the VDR protein. Thus, the molecular function of each of a total of seven potential VIPs was probed with respect to their domain contacts in hVDR, and their role in transducing signal for gene transcription from the ligand-activated VDR. Table 1 lists the VIPs investigated in the current dissertation, along with the balance of VIPs that have been studied by other investigators.

	VIPs studied in this dissertation		Work from other investigators	
VDR interacting proteins (VIPs)	Yeast two-hybrid system (YTHS)	Biochemical assays	In vivo assays (YTH/MTH)	Biochemical assays
SMRT		+		+
RXRα,β	+*	+		+
SRC-1 (p160s)	+*	+	+	+
VIP170 (i)				+
NCoA-62		+	+	+
CBP (ii)		+		+
DRIP205		+		+
TFIIB (iii)			+	+
Trip1	+*	+	+	+

Table 1. Vitamin D interacting proteins (VIPs) and assays used to identify them. The yeast two-hybrid system was used to isolate VIPs from cDNA libraries made from classic vitamin D target tissues such as bone, kidney and skin. The VIPs identified in this dissertation through this technique are marked with an asterisk (*). RXRβ was not isolated by this method, and was used as a positive control for interactions with VDR in the presence of hormone. Biochemical assays were used to characterize VDR-VIP interaction as isolated events, mainly through the use of the GST pull down assay. Cotransfection of mammalian cells was employed to investigate the functional significance of each VIP. Other investigators also studying VDR cofactors have isolated VIPs through *in vivo* assays such as the yeast two-hybrid (YTH) and mammalian two-hybrid (MTH) systems and *in vitro* assays such as GST pull down and affinity chromatography column separation. i. (Jurutka *et al.* 2000a), ii. CBP is not a true VIP, interacting indirectly with VDR via SRC-1 bridging, iii. (Jurutka *et al.* 2000b)

The yeast two-hybrid system allowed us isolate three distinct VIPs (shown in Table 1 with an asterisk), and with the use of biochemical techniques such as the GST pull down assay, we were able to define the nature of the interaction as well as the domains in the hVDR protein involved in the association. All of the VIPs identified and characterized were evaluated for their ability to influence VDR's transcriptional response in intact cells by transient transfection of a limited number of different cultured mammalian cell lines. We expect that the data obtained from this research will help to define the mechanism by which nuclear receptors, specifically VDR, control and coordinate the expression of target genes. Further, probing the role that each of these VDR support players execute in the signaling of gene expression by $1,25(OH)_2D_3$ may allow us to predict effects that the disruption of any member of this tightly controlled machinery may have on the physiology of $1,25(OH)_2D_3$ and VDR regulated systems.

The complete proposed life cycle of VDR as a transcription factor is illustrated in Fig. 47, and is based on information obtained from this dissertation (Chapters 2-6), additional results from our research laboratory, and data gathered by other groups investigating the control of gene expression mediated by members of the nuclear receptor superfamily. Step 1 (Fig. 47) depicts the unactivated or unliganded state of VDR, as discussed previously (Chapter 2), functioning molecularly in a similar fashion to RXR, binding to SMRT marginally (Chen and Evans 1995), and not exerting significant repression in this state (Schulman *et al.* 1996). The function of SMRT with respect to VDR at this point may be analogous to that of the chaperone-role played by heat shock proteins for unliganded GR (Howard and Distelhorst 1988; Dalman *et al.*

1991; Czar *et al.* 1995). Thus, unliganded VDR interacts in solution with at least two nuclear proteins: SMRT and RXR, and as demonstrated by several research groups, TFIIB is the third major VDR-interacting protein (VIP) for unliganded VDR (Blanco *et al.* 1995; MacDonald *et al.* 1995; Guo *et al.* 1997; Masuyama *et al.* 1997c; Jurutka *et al.* 2000b).

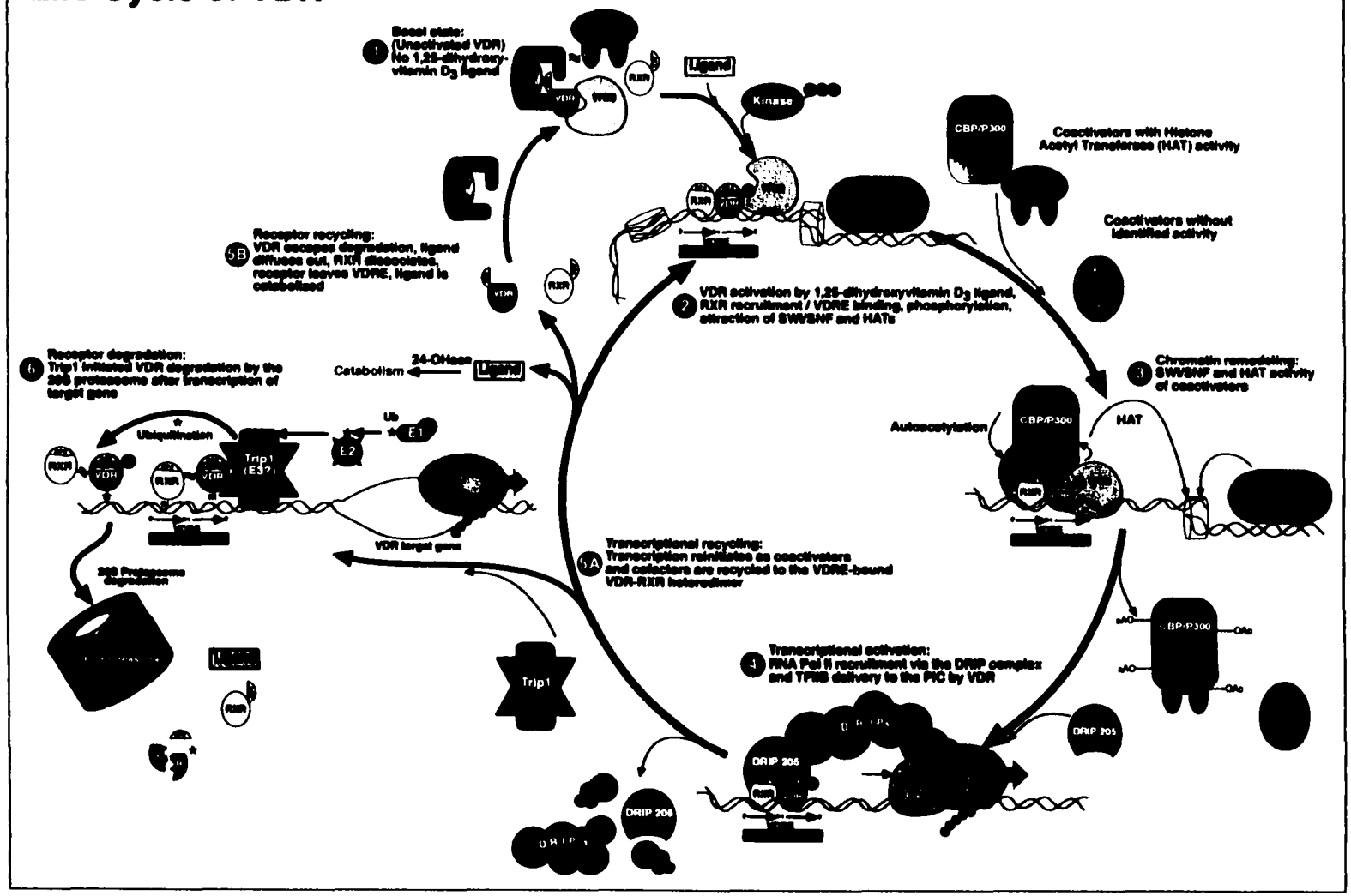
With these players involved with the "inactive" receptor, we propose that there exists in intact cells a *quasi*-stable tetrameric complex of unoccupied VDR, RXR, SMRT and TFIIB sliding along the DNA-phosphate backbone. This complex also prepares VDR to activate transcription once bound to hormone by bringing coactivator close to the promoter site at target genes via direct SMRT-coactivator contact as proposed by McDonnell group (Li *et al.* 2002). Independent work from ours' and other's laboratories, have helped us place SMRT in a significant role maintaining the poised pentameric complex of proteins including unliganded VDR, RXR, TFIIB, SMRT and p160 coactivator, awaiting activation by sequential $1,25(OH)_2D_3$ and VDRE binding. Also depicted in Fig. 47, phosphorylation of liganded VDR is an early step in VDR signal transduction, being required for VDRE binding and transactivation (Jurutka *et al.* 2002).

Step 2 (Fig. 47) illustrates that in order for VDR to control the expression of target genes, the receptor requires direct interaction with the $1,25(OH)_2D_3$-ligand to promote conformational changes. This change allows the receptor to strongly associate with RXR, leading the heterodimer to bind the VDRE with high affinity and specificity, events that both decrease the levels of interaction with the SMRT "corepressor" and

create an interaction surface platform for cofactors to help control gene transcription. For the heterodimeric receptor to interact with DNA, specifically with the VDRE, it is necessary for the genomic chromatin first to undergo a structural change that releases histones and relaxes the nucleosome network, rendering the DNA in that region accessible to transcription factors. These physical changes in chromatin, from the repressive to the derepressed state, are driven by the members of SWI/SNF complex (Cairns *et al.* 1994), specifically a PBAF containing multimer in the case of hVDR (Lemon *et al.* 2001).

We have also observed that VDR coregulators with HAT activity, such as SRC-1 and CBP, serve to potentiate gene expression induced by $1,25(OH)_2D_3$, and their actions are likely tied to their ability also to remodel chromatin (step 3, Fig. 47). The association between VDR and CBP is not through direct interaction, because the p160 coactivator, SRC-1, acts to bridge VDR and CBP as part of a ternary complex. The overall actions of p160 coactivators and p300 cointegrators may be to create a high level of protein acetylation (e.g., histone, etc.) in the promoter region of hormone-dependent genes, leading to chromatin remodeling. Once the coactivator is bound to the promoter-assembled VDR, the cofactors exert their acetylation actions on histones in nucleosomes to promote reorganization, leading to the release of histone from DNA contacts. Acetylation is proposed to continue until coactivators undergo self-acetylation (Shang *et al.* 2000), disrupting the electrostatic forces that promote protein:protein interactions, leaving the VDR target gene promoter poised to attract the PIC and RNA polymerase II and initiate transcription.

Fig. 47. The Transcription Factor Life Cycle of VDR



VDR interacts directly with the $DRIP_{205}$ Mediator subunit, an association that is further enhanced in the presence of the $1,25(OH)_2D_3$ ligand, serving to potentiate hormone-stimulated gene activation. Full $1,25(OH)_2D_3$ transcriptional response mediated by VDR is driven by direct protein:protein interactions between the VDR and coactivators, as well as between VDR and Mediator. Our data suggest that the regions of VDR required for interactions with the p160 family of coactivators and with the mediator family (i.e., $DRIP_{205}$), physically overlap in VDR, arguing that p160 HATs and DRIPs/Mediator likely function as sequential rather than simultaneous coactivators (steps 3 and 4, Fig. 47) (Sharma and Fondell 2000). Once the chromatin has been released from its repressed state, autoacetylation apparently promotes the liberation of p160/p300/NCoA-62 from VDR contact, facilitating sequential binding of $DRIP_{205}$ to the VDR-RXR heterodimer. The subsequently assembled $DRIP_{205}$/Mediator complex attracts RNA polymerase II, which is activated by the delivery of TFIIB from VDR, an event that occurs when $DRIP_{205}$ displaces TFIIB from the N-terminus of VDR. With the release of TFIIB, transcriptional machinery assembly is now complete, leading to the expression of the VDR target gene. After phosphorylation of the C-terminal domain (CTD) of RNA polymerase II, the DRIP/Mediator is postulated to dissociate from the VDR/RXR heteropartner, causing the latter to be loosely bound to the DNA/VDRE, leading to an equilibrium between the bound and unbound forms. This equilibrium permits the association of the Trip1 cofactor with VDR in solution. The competition found between DNA and Trip1 for VDR (see Fig. 41) prevents the receptor from further interaction with the VDRE. As depicted in step 6, Fig. 47, Trip1 association with the

receptor not only inhibits its binding to DNA, but also to coactivators such as the p160s and Mediator that might help stabilize the forces that drive receptor-DNA interaction. Trip1 association with VDR is proposed to trigger and drive the ubiquitination of the receptor, a modification that tags VDR for degradation by the 26S proteasome complex, ending the transcriptional life cycle of VDR (step 6, Fig. 47).

There is an alternative route that the receptor may follow in order to avoid degradation and continue the process of gene activation, perhaps permitting the cell to optimize resources and energy by sustaining multiple rounds of transcriptional initiation by VDR. Once the RNA polymerase II has initiated the transcriptional process and the coactivator complex has disassembled, the receptor, in the absence of Trip1 interaction may remain bound to the promoter site to continue another round of transcription, saving the cell the need of further VDR biosynthesis, a time and energy-consuming process. In this case (step 5A, Fig. 47), the VDR-RXR heterodimer remains loosely bound to the VDRE, ready to attract another round of cofactors and initiate the process of target gene expression. Such repeated rounds of transcriptional initiation could be required to sustain bioresponses such as vitamin D-induced calcium absorption from the intestine, but may not occur for transient effects of $1,25(OH)_2D_3$ such as the induction of p21 to elicit cessation of the cell cycle. There may be an additional scenario wherein VDR recycles and avoids the requirement for resynthesis. We propose that in this case (step 5B, Fig. 47), the receptor dissociates its hormonal ligand from the binding pocket, an event that leads to disruption of the heterodimer and VDRE binding. Thus, the resulting apo-receptor is now receptive to stabilization in its unliganded form by binding to the

SMRT cofactor, RXR and TFIIB. This recreates the tetrameric complex shown in panel 1, awaiting ligand binding and a further round of transcriptional control of target genes.

An integrated mechanism by which VDR controls the expression of target genes has been postulated in the model illustrated in Fig. 47. We consider the life of VDR as a transcription factor to be a turning cycle, with an exit mechanism that leads to an "end game". Because in endocrine control systems, hormonal ligands and receptors operate at very low concentrations in the cell (pM-nM), the small amount of receptor available likely must be recycled in order to amplify the signal generated by a burst of hormone from an endocrine organ. Therefore, the existence of a regenerating cycle of VDR action we propose in Fig. 47 makes teleological sense. Conversely, because hormonal signals are molecular expressions of adaptive responses to a changing environment, they must also be terminated to prevent VDR-induced proteins from spiraling out of control in a target cell. There are two main mechanisms for signal termination (Fig. 47): i) $1,25(OH)_2D_3$ ligand catabolism via the 24-OHase CYP enzyme, which is induced by VDR, and ii) VDR degradation via proteolysis (step 6, Fig. 47), a process also potentiated by liganding of VDR. Once the transcription of target genes has initiated, the process is proposed to gain momentum and continue through several rounds of transcription, until the need for a specific protein levels are satisfied and this operation must be shut down, both to maintain the integrity and energy of the cell. For this latter event to occur, the system likely must be repressed or silenced, being deactivated by a process that includes ligand and protein degradation, as well as disruption of the assembled coactivator complexes. This system also possesses a feedback loop for ligand

autoregulation, as in the case of controlling the expression of the 24-hydroxylase, the enzyme that initiates the primary pathway for catabolic elimination of vitamin D metabolites (Reddy and Tserng 1989; Tomon *et al.* 1990). As mentioned above, this is a highly regulated system that has been developed throughout the course of evolution, with the main goal of keeping the balance of calcium and phosphate minerals in blood and bone, assuring the formation of efficient calcified structures that constitute the physical support for animals walking on land.

The effects of the ubiquitin proteasome system on ligand-induced expression of genes via nuclear receptors varies among these transcription factors. For example PPARα is downregulated by ubiquitination, but this modification mainly occurs with the unliganded receptor, and dynamically controls its cellular level (Blanquart *et al.* 2002). For TR and RAR, each complexed with RXR, agonist ligand binding leads to degradation of the transcriptionally active TR or RAR subunits, as well as the RXR subordinate partner (Osburn *et al.* 2001). GR is subject to hormone-dependent downregulation that is linked to its ubiquitination and engagement of the proteasome degradation machinery (Wang *et al.* 2002), however, if fewer than four ubiquitin moieties are linked to the receptor, GR appears capable to re-engaging HAT/DRIP/PIC complexes and completing additional rounds of transcription (Gonzalez *et al.* 2002). Interestingly, select subunits of Mediator contain components that seems to be involved in recruiting ubiquitin adding enzymes to the transcriptional machinery. For instance, the srb10 kinase Mediator subunit signals ubiquitination and destruction of the transcriptional activator, Gcn4, and the MED8 subunit assembles into Elongin BC-based

E3 (Conaway *et al.* 2002). These latter concepts suggest that in our cyclic model of VDR action (Fig. 47), $DRIP_{205}$/Mediator recruitment of the PIC may be coupled to the subsequent recruitment of Trip1, which we propose as a candidate E3 for VDR ubiquitination. Yet if the VDR follows the GR-pattern of mono-, di-, tri-, tetra-ubiquitination, then mono-ubiquitinated VDR may recycle via step 5 (Fig. 47) instead of being degraded by the proteasome. Thus sequential addition of single ubiquitin units provides a conceptual framework for the turning of the cycle in Fig. 47 until four moieties are added, at which time VDR exits the cycle via proteasomal degradation.

Indeed, the fact that substitution of R49 in hVDR with a lysine (R49K), which is the residue present at the corresponding position in GR, sensitizes VDR to efficient ubiquitination/proteolysis (Fig. 44) argues that VDR is only minimally subject to degradation compared to GR. However, this proteolysis can be accelerated by artificially converting the heterodimerizing VDR into a homodimerizing steroid hormone-like receptor (Fig. 44), or simply overexpressing Trip1 in the presence of VDR (Fig. 40). The functional effect of Trip1 overexpression on VDR action is modest, but it is negative. This links Trip1 to an eventual degradative role in the VDR life-cycle instead of playing a positive, non-proteolytic part in transcriptional elongation as has been shown for its SUG1 homologue in yeast (Ottosen *et al.* 2002).

One of the conundrums associated with constructing a cyclic model of transcriptional factor action as in Fig. 47 is the order in which each coregulator participates in the cycle. In fact, one recent study of the human α_1-antitrypsin promoter during enterocyte differentiation revealed that a complete PIC, including phosphorylated

RNA polymerase II, was assembled on the promoter prior to transcriptional activation (Soutoglou and Talianidis 2002). HATs such as CBP and PCAF were recruited subsequently and local histone hyperacetylation was delayed. In this case, transcriptional initiation occurred when the human Brahma homologue hBrm was attracted and local nucleosome remodeling transpired. Therefore, chromatin reconfiguration can initiate transcription after assembly of the RNA polymerase II machinery, and the order of events pictured in Fig. 47 will not be absolutely defined until real time chromatin immunoprecipitation (ChIP) assays are completed for VDR on several of its target gene promoters.

In our model of VDR action in Fig. 47, we postulate that the "turning of the cycle" is initiated by $1,25(OH)_2D_3$ ligand and progresses via successive recruitment of coregulators. Because these coregulators bind to overlapping regions on the multifaceted surface of VDR, there must by definition occur a succession of disassembles and reassembles. One mechanism for coactivator release discussed in Chapter 4 is that of HAT self-acetylation, but recently Yamamoto and colleagues (Freeman and Yamamoto 2002), observed that a chaperone protein, p23, disrupts nuclear receptor-coregulator complexes at genomic response elements, *in vivo*, and in a hormone-dependent manner. Thus, they conclude that p23, and Hsp 90 in a weaker fashion, acts as chaperones in the disassembly of transcriptional regulatory complexes, and such a mechanism could facilitate the "turning of the cycle" in Fig. 47. On the other hand, others laboratories agree with the mechanism but argue that instead of p23/Hsp 90 chaperones, the SWI/SNF family of proteins execute this disassembly role. SWI/SNF-like enzymes

apparently function primarily to alter individual nucleosomes, in contrast to HATs, which disrupt nucleosome-nucleosome interactions (Horn and Peterson 2002). Whether SWI/SNF enzymes also act as nuclear receptor or complex disruptors is unproven, but is clear that these enzymes interact with gene-specific activators to ensure that chromatin remodeling is targeted to the correct gene, in the proper cell, and at the right time (Fry and Peterson 2002). In the detailed scheme for VDR-RXR-VDRE association (Fig. 21), and in the hypothetical cyclic model (Fig. 47), we propose that SWI/SNF, specifically the PBAF tethered complex (Lemon *et al.* 2001), acts both to render the VDRE accessible to the VDR-RXR heterocomplex (Dilworth *et al.* 2000), and facilitates and controls the recruitment of HAT coregulators. The effect of SWI/SNF is analogous to its role in regulating the yeast *HO* gene promoter, in which the SWI/SNF complex actively recruits a HAT complex and together they destabilize chromatin condensation to in turn attract a second cofactor and eventually the PIC (Fry and Peterson 2002). However, this order is different for the α_1-antitrypsin gene mentioned above, wherein HAT and SWI/SNF complexes are recruited after PIC assembly, and the human IFN-β gene promoter, in which HATs are recruited during PIC assembly, and subsequent histone acetylation promotes SWI/SNF attraction to disrupt the architecture of a promoter bound nucleosome (Fry and Peterson 2002). Basically, the requirement for chromatin remodeling before, during and after PIC formation seems to vary from gene to gene, depending on where the particular promoter region is encased in nucleosomes. Apparently nuclear receptors are endowed with the flexibility to stimulate transcription of multiple target genes, each in different chromatin contexts.

Interestingly, the concept of comodulators like SWI/SNF functioning at multiple steps to facilitate gene expression also extents to a new class of steroid receptor coregulators involved in RNA processing, such as heterogeneous nuclear ribonucleoprotein-like proteins TLS and CoAA (Auboeuf *et al.* 2002). Thus, when recruited, CoAA both stimulates transcription and regulates differential RNA splicing, determining whether CGRP or calcitonin mRNA is processed alternatively from the calcitonin gene transcript. In fact, in spite of the dissection of VDR controlled transcription provided herein, it is likely that eukaryotic gene expression involve several multi-component cellular machines, with each machine carrying out a distinct step in the pathway. Rather than a simple assembly line, there probably exists a coupled network for transcription, pre-mRNA processing, and export of mature mRNA to the cytoplasm (Maniatis and Reed 2002). Coregulators could conceivably participate in more than one machine, and the individual machines, i.e., chromatin remodeling, Mediator/PIC, ubiquitin ligase/proteasome, hnRNA elongation and processing, etc., could be tethered to one another to form a "gene expression factory". Amazingly, the signal to activate such factories appears to be the liganding by hormones of nuclear receptors, their dimerization and recognition of the specific gene promoters to be stimulated.

Pathological uncoupling of the above described series of cellular machines could have a dramatic effect on nuclear hormone responsiveness, and in this dissertation several new molecular locations for inactivating point mutations, or reduced activity gene polymorphisms, have been identified, e.g., RXRs, SRCs, DRIPs and Trip1. Disruption or dysregulation of these VIPs may cause vitamin D hypo- or hyper-

responsiveness, for example producing osteoporosis and calcium urolithiasis, respectively. It is already known that patients with breast cancer overexpress AIB1 (SRC-3) (Reiter *et al.* 2001). Also, one common VDR gene polymorphism is associated with low bone mineral density in several populations, with the mechanism being a suboptimal interaction of its N-terminally extended hVDR isoform with the VIP, TFIIB (Jurutka *et al.* 2000b). Perhaps most relevant to the present dissertation is the fact that inactivating mutations in hVDR that confer the vitamin D-resistant rachitic phenotype exist not only in the DBD that block VDRE binding, and in the LBD that preclude $1,25(OH)_2D_3$ binding, but also in the VIP contact surface for RXR (Whitfield *et al.* 1996), and in the helix-12/AF-2 contact for HATs, DRIPs and Trip1 (Malloy *et al.* 2002). Intriguingly, DNA and RXR contact mutations in VDR elicit rickets and alopecia, whereas $1,25(OH)_2D_3$ and AF-2-VIP interacting alterations generate rickets without alopecia. This suggests that unlike the case of bone mineral metabolism, for hair cycling control VDR employs both a novel, non-vitamin D ligand and interacts with non-HAT and non-DRIP comodulators/corepressors. In conclusion, in governing calcium and bone metabolism, VDR follows the pattern of other nuclear receptors in recruiting commonly used VIPs that serve many transcription factors, such as RXR, SRC-1, CBP, $DRIP_{205}$, TFIIB, Trip1, etc., but for unique VDR actions like skin maintenance and hair cycling, novel undiscovered VIP/corepressors may transduce the signal that positively or negatively influence target cell gene expression. This latter story is reminiscent of the recent elucidation of the molecular coregulator determinants for the tissue specificity of the selective estrogen receptor modulators (SERMs) (Shang

and Brown 2002). That is, some ER antagonist ligands that recruit corepressors in one cell type (e.g., breast, bone) can attract coactivators to ER when it is bound to a nonclassical ERE in a different cell type (e.g., endometrium). By analogy, there may exist not only a novel VDR ligand in skin, but cell-specific- and promoter-selective-differences in coregulator recruitment that could determine distinct cellular responses to liganded VDR. It will be of great interest to continue the present study of VIPs using novel, non-vitamin D VDR ligands (Makishima *et al.* 2002), as well as target cells such as those in the hair follicle, perhaps uncovering a new set of VIPs transducing the VDR signal from DNA platforms modulating the expression of genes controlling the hair cycle.

In summary, we have developed a six-stage molecular model that summarizes the VDR transcriptional activation-life cycle, and have tested this model using a variety of experimental approaches, including pull-down assays with GST-fusion proteins, as well as assays of the functional activity of VDR and its putative coactivators in transiently transfected mammalian cells. Stage 1: pull-down experiments with GST-fusion proteins indicate that unliganded VDR associates with a corepressor such as the silencing mediator of retinoid and thyroid receptors (SMRT), and a basal transcription factor (TFIIB). Stage 2: yeast two-hybrid system and gel mobility shift experiments show that liganded VDR acquires a strong attraction for RXR, and once heterodimerized is capable of binding specifically to a vitamin D responsive element (VDRE), concomitantly dissociating SMRT. Stage 3: based upon data from GST pulldown experiments, the liganded VDR-RXR complex on the VDRE recruits coactivators such

as steroid receptor coactivator-1 (SRC-1) and CREB-binding protein (CBP), both of which possess histone acetyltransferase (HAT) activity that derepresses chromatin via nucleosome reorganization. Transactivation assays reveal that both SRC-1 and CBP enhance VDR activity, but only SRC-1 directly contacts VDR. Thus, we conclude that the CBP-VDR interaction is indirect through a SRC-1 bridge forming a ternary complex that is a prerequisite for transactivation by liganded VDR. Stage 4: From GST pulldown experiments, VDR interacts with the Mediator protein, $DRIP_{205}$, which is in turn proposed to attract the PIC containing RNA polymerase II. Indeed, $DRIP_{205}$ cotransfection amplifies VDR-mediated transcription. We postulate that VDR interaction with $DRIP_{205}$ follows dissociation of SRC-1 and CBP, which likely lose their affinity for VDR after self-acetylation. Moreover, $DRIP_{205}$ association with VDR is postulated to promote the release of TFIIB and its delivery to the PIC. Stage 5: derived from data with other nuclear receptors, we propose that the VDRE-bound VDR-RXR heterodimer, free of interactions with the DRIP complex once transcription is initiated, can be recycled to initiate additional rounds of transcription by repeated sequential rebinding of SRC-1/CBP and $DRIP_{205}$/PIC. Stage 6: by cotransfection experiments, we observe that Trip1 attenuates VDR activity, likely due to the ability of Trip1 to promote ubiquitination, followed by proteolysis of VDR. Thus, the VDR life cycle can be summarized by the progressive action of four sets of coregulators: SMRT, SRC-1/CBP, $DRIP_{205}$/PIC, and Trip1.

Caveats

It is important to mention that there are a great number of factors that influence the transcriptional response mediated by VDR, and that the conclusions reached in the present study relate only to very specific conditions. For example, we utilized only the single most prevalent isoform of hVDR in the research for this dissertation, but, as summarized in Fig. 48, there exist several common hVDR polymorphisms that affect the expression and activity level of the receptor (Whitfield *et al.* 2001). They provide a great deal of genetic variability that may influence the level of calcium and phosphate minerals that individual humans acquire for efficient bone mineralization. Similarly, we employed only the single most active VDR ligand, $1,25(OH)_2D_3$, but many drug analogs have been synthesized. Ligand binding has a profound influence on receptor conformation and enables VDR to interact with its natural partners such as several RXR isoforms, primarily RXRα or RXRβ (Thompson *et al.* 1998). The conformation of VDR attained following ligand binding is dictated by the nature of the ligand, varying depending on whether $1,25(OH)_2D_3$, or a synthetic analog, or the recently discovered novel natural ligand, lithocholic acid (Makishima *et al.* 2002), is bound to the receptor. All of these lipophilic signalling molecules can influence the receptor affinity for its RXR isoform partner, which in turn, can affect the composition of coactivator supercomplexes. Phosphorylation is another important process involved in the regulation of the VDR transcriptional response, as summarized in Chapter I (Jurutka 1993; Hsieh *et al.* 2001). This event may have a positive or negative effect in the overall transcriptional response mediated by VDR. PKC catalyzed phosphorylation attenuates VDR nuclear

localization and DNA binding, whereas PKA catalyzed phosphorylation decreases receptor activity, and CK-II exerts the opposite effect by potentiating VDR transcriptional activity via phosphorylation. Protein kinase expression and resulting protein phosphorylation patterns likely are cell-specific (Fig. 48), as is the occurrence of VDR-RXR transmodulators. We utilized only a limited array of kidney (COS-7), bone (ROS 17/2.8), intestine (INS-407) and muscle (C2C12) cells, and therefore the conclusions reached may apply only to these specific cell types.

With the VDR ligand, the RXR heteropartner and post-translational modifications in place directing the conformation of the receptor, VDR is now able to bind to DNA at specific sites (VDREs) in the promoter regions of target genes and initiate transcription. Again, we studied primarily one VDRE, that from the rat osteocalcin gene, with several experiments using mouse osteopontin or CYP (24 or 3A23) VDRE platforms. The binding to different DNA sequences of several VDREs has also been shown to influence VDR conformation and its overall activity (Staal *et al.* 1996). Other factors linked to DNA binding affecting the activity of the receptor are transmodulators, i.e., *trans*-acting factors binding to *cis*-elements in the proximal regions of the particular VDRE in the context of the various promoters of vitamin D-regulated genes, that also impart allosteric effects on the conformation of VDR (Ozono *et al.* 1990; Pike 1990).

In summary, as pictured in Fig. 48, the VDR-RXR "nucleus" that recruits coactivators for $1,25(OH)_2D_3$ signal transduction is a multifaceted entity that is differentially influenced in an allosteric fashion by hVDR gene polymorphisms, variable

VDR lipophilic ligands, cell-specific VDR phosphorylations perhaps resulting from cross-talk actions of cell surface acting hormones, cell-specific *trans*factors, and gene promoter selective VDRE DNA sequence platforms.

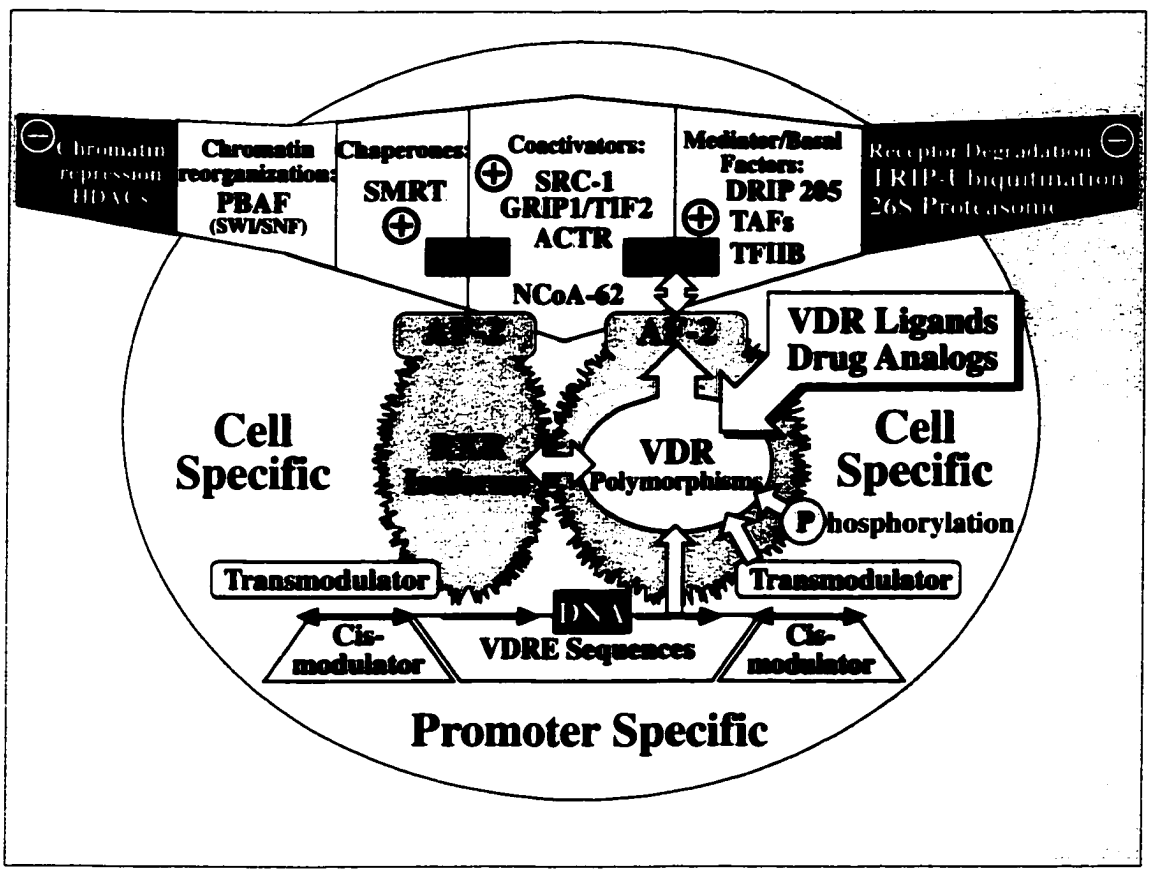


Fig. 48. Allosteric model showing the regulatory influences on VDR as a ligand-DNA-protein supercomplex.

As has been shown throughout this report, in order for the vitamin D receptor to activate the expression of genes, it must to interact with comodulator proteins that possess a variety of activities to aid in transducing the signal to RNA polymerase II. Most of these proteins do so through the association with the VDR's AF-2/helix-12 and,

as demonstrated in this dissertation, other regions of VDR such as helix-3 and the N-terminus. Clearly the above outlined allosteric influences on VDR also impinge on the ability of the receptor to attract the sequence of comodulators illustrated from left to right across the top of Fig. 48. There are several categories of cofactors that associate with VDR, and they are classified according to their actions: chromatin re-organizational proteins (PBAF, SWI/SNF), chaperones/"corepressors" (SMRT), coactivators (SRC-1, GRIP1, ACTR, NCoA-62), mediators (DRIPs, TRAPs), TAFs/basal transcription factors ($TFII_{135}$, TFIIB) and degradation triggering factors (Trip1). The cycle pictured in Fig. 47 presents a working hypothesis as to how this plethora of comodulators can sequentially interact with VDR to achieve control of gene transcription, and Fig. 48 reveals the allosteric influences discussed above that may also affect the VDR molecular life cycle.

This present study was conducted with the goal of increasing our understanding of the control of gene expression mediated by VDR, and the cofactors that help transduce the $1,25(OH)_2D_3$ signal. The project was accomplished utilizing specific conditions, some *in vitro* and some in intact cells, but all of them with the purpose of simplifying the elements that represent the overall factors that somehow execute the actions mediated by VDR. As stated above, for this research project, only one polymorphic variant of VDR and one ligand were employed, four target cell lines from four different tissues were studied, and only three vitamin D controlled promoters were used, while no studies of VDR phosphorylation were carried out. Nevertheless, this project was designed to serve as an elementary framework to which any other condition

may be added to investigate and better understand the mechanism by which VDR mediates the transcription of a select target gene in a specific tissue.

Future work

The present study was conducted with the use of a combination of cell culture and biochemical techniques to characterize the aforementioned VIPs and evaluate the influence they exert on the transcriptional activation of genes regulated by VDR. A logical progression to expand this present work will be to test the proposed hypothesis and model in the context of an organism, *in vivo*.

On problem with evaluating the *in vivo* significance of SRCs is the functional redundancy of their isoforms, and variable patterns of expression thereof in cells. Thus, SRC-1 knockout mice respond nearly normally to estrogen in terms of the reproductive function, and both SRC-1 and SRC-2 (GRIP1) must be knocked out to abolish estrogen responsiveness via ERα,β (Apostolakis *et al.* 2002). Apparently, SRC-2 is induced in SRC-1 null mice, and serves to replace SRC-1 in ER actions. Similarly, both SRC-1 and SRC-2 must be inactivated to generate thyroid hormone-resistant mice (Bert O'Malley, personal communication via Mark Haussler). Moreover, the present experiments with cotransfection/overexpression of SRC-1, $DRIP_{205}$, and other coactivators yield statistically significant, but not quantitatively large, effects on VDR-mediated transcription. It is likely that coactivator-null target cells would generate more impressive results when the relevant coactivator is added back. Cells with all isoforms of, for example, SRC, knocked out would be difficult to obtain, and perhaps acute

antisense (Apostolakis *et al.* 2002) or RNA interference technology (Elbashir *et al.* 2001) could be used to eliminate endogenous VDR comodulators. Recently a system for stable expression of short interfering RNAs in mammalian cells has been reported (Brummelkamp *et al.* 2002).

In addition to the SRC/p160 family of coactivators, components of the DRIP/Mediator complex have also been knocked out in experimental animals. For instance, $TRAP_{220}$-null fibroblasts are refractory to PPARγ-stimulated adipogenesis (Ge *et al.* 2002), and ARC_{105}-null *Xenopus laevis* embryos lack responsiveness to TGFβ (Kato *et al.* 2002). Thus, there may exist specific molecular roles in transcription for various Mediator subunits, and to date, $DRIP_{205}$ has not been knocked out to determine the effect on VDR signaling.

A second group of proposed experiments would be the use of the chromatin immunoprecipitation assay (ChiP) to evaluate the model for VDR action presented in Fig. 47, and help to resolve further the controversy between the sequential (Shang *et al.* 2000) and simultaneous (Sharma and Fondell 2000) proposed actions of coactivators on gene expression. With the use of this technique, it would be possible to assay in real time, in the natural environment of the cell, which cofactors associate with the DNA-bound receptor at a particular time, in a select cell line, and with a specific ligand at the relevant promoter. Unpublished ChIP assays of ER cofactors (Leonard Freedman, personal communication via Mark Haussler) suggest oscillatory and alternating behavior of p160 and DRIP binding to the receptor, supporting the cyclic model illustrated for VDR in Fig. 47. Coimmunoprecipitation experiments of VDR and comodulators would

be an alternative approach to this problem of defining the order in which VIPs interact with VDR in transducing the $1,25(OH)_2D_3$ signal at target VDREs in natural promoters.

A third group of future experiments in this case would be the development and utilization of an *in vitro* transcription system (IVT) containing chromatin to address the effect that a group, or a specific, coactivator exerts on VDR-mediated transcription. This *in vitro* system could be patterned after that of Lemon *et al.* (Lemon *et al.* 2001), and may not allow one to make a distinction between the aforementioned sequential and simultaneous models for VIP-VDR interaction, but via reconstitution studies, it would permit the biochemical evaluation of the significance of each putative comodulator in VDR action.

Finally, a fourth set of experiments would be the identification and characterization of the amino acid sequence of VDR involved in the ubiquitination of the receptor and the degradation process that accompanies it. For this analysis it would be necessary to develop a radioactive ubiquitin labeling assay for VDR, as well as site directed mutagenesis to create (as in the use of the R49K synthetic mutant) and destroy sites to which the E2/E3 adds ubiquitin moieties to the receptor, tagging it for degradation. VDR also apparently contains a ubiquitin-interaction motif (UIM) (Polo *et al.* 2002) to which E3 binds, and this could be identified by deletion and point mutagenic analysis of VDR.

Other accomplishments

During the course of this dissertation research program, several papers were published or submitted from our laboratory on VDR, in which the candidate had an opportunity to contribute to the research design and experiments, and therefore to be a co-author. These accomplishments were related to the dissertation research reported herein, but not central to the theme of characterizing the transcriptional life cycle of VDR, and were reported in primary articles, review articles, and chapters or abstracts of presentations at scientific meetings. Of these eight publications, one was an early chapter (Haussler *et al.* 1997b) reporting preliminary data and insights on VDR cofactors that established a foundation for the present dissertation research, and two were review articles on VDR (Haussler *et al.* 1998; Haussler *et al.* 2002). Two other co-authored publications were a chapter (Thompson *et al.* 2000) and a primary article (Thompson *et al.* 2001) reporting on the functional significance of the AF-2 in the VDR partner RXR in terms of $1,25(OH)_2D_3$ signaling. An abstract (Jurutka *et al.* 2001a) was published on the relevance of helix-1 and helix-3 in hVDR mediation of transcriptional activation by $1,25(OH)_2D_3$. Finally, two publications, a chapter (Whitfield *et al.* 2000), and a primary article (Whitfield *et al.* 2001), included data on the mechanistic significance of common hVDR polymorphisms in variable $1,25(OH)_2D_3$ signal transduction. Recently, two other accomplishments were submitted for publication, namely the cloning by RT-PCR of the gecko VDR for purposes of evolutionary comparison of VDR functional domains (Whitfield *et al.* 2002), and the demonstration by Western blotting that $1,25(OH)_2D_3$ induces human CYP3A4 proteins as a detoxification enzyme in human colon cancer

cells (Thompson *et al.* 2002). In summary, in addition to elucidating the VDR transcriptional life cycle as reported in this dissertation, the author has also cloned an evolutionarily ancient gecko VDR, probed the structure/function of helix-1 and helix-3 regions of hVDR, studied hVDR common gene polymorphisms that affect transcriptional activation potency, investigated the transcriptional stimulation role of the VDR "silent" partner, RXR, and provided evidence for a novel gene target in the human CYP3A4 detoxification enzyme that may be relevant to the known colon cancer preventative effects of vitamin D.

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