Heterodimeric DNA Binding by the Vitamin D Receptor and Retinoid X Receptors Is Enhanced by 1,25-Dihydroxyvitamin D3 and Inhibited by 9-cis-Retinoic Acid

EVIDENCE FOR ALLOSTERIC RECEPTOR INTERACTIONS*

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Gel mobility shift analysis was utilized to investigate the molecular function of 1α,25-dihydroxyvitamin D3 (1,25-(OH)2D3) and 9-cis-retinoic acid (9-cis-RA) ligands in the binding of the vitamin D receptor (VDR) and retinoid X receptor (RXR) to mouse osteopontin and rat osteocalcin vitamin D-response elements (VDREs). At physiological ionic strength and reduced concentrations of expressed proteins, efficient binding to either VDRE occurs as a VDR-RXR heterodimer, not as a VDR homodimer. 1,25-(OH)2D3 dramatically enhances heterodimer-VDRE interaction, whereas somewhat higher concentrations of 9-cis-RA inhibit this association, perhaps related to the role of this retinoid in facilitating RXR homodimer formation. Interestingly, if VDR is occupied by 1,25-(OH)2D3 prior to complexing with RXR, the resulting heterodimer is relatively resistant to dissociation and diversion to other pathways by 9-cis-RA. Therefore, a proposed molecular action of 1,25-(OH)2D3 is to generate an allosteric switch in VDR to a form that not only binds to the VDRE with high affinity and specificity as a heterodimer with RXR, but also interacts with the RXR partner to conformationally restrict the action of its cognate ligand.

The active metabolite of vitamin D, 1α,25-dihydroxyvitamin D3 (1,25-(OH)2D3),1 exerts its hormonal properties predominantly on intestine, bone, and kidney, where it plays a crucial role in calcium and phosphate homeostasis, bone mineralization, and the prevention of rickets (1). 1,25-(OH)2D3 also has effects on calcium and phosphate homeostasis, bone mineralization, and inhibited by 9-cis-Retinoic Acid

9-cis-retinoic acid, and thyroid hormone receptors (5). Transcriptional control of specific 1,25-(OH)2D3-responsive genes is achieved by binding of the receptor, via its DNA binding domain, to a specific vitamin D-response element (VDRE) located in the 5′ promoter region of the regulated gene. VDREs have been identified in positively controlled genes such as human and rat osteocalcin (6–8), mouse osteopontin (9), avian integrin β3 (10), and rat vitamin D 24-hydroxylase (11, 12), and comparison of these sequences indicates that they generally consist of a hexanucleotide direct repeat with a spacer of three nucleotides (DR+3).

In many studies with natural DR+3 VDREs, VDR has been reported to bind as a heterodimer with the retinoid X receptor (RXR) (reviewed in Ref. 13). Thyroid hormone receptor (TR) and retinoic acid receptor (RAR) also form RXR-containing heterodimers on positively controlled DR+4 and DR+5 response elements, respectively (14). Evidence has been provided, however, implying that VDR may also bind as a homodimer to certain DR+3 elements, primarily those containing the osteopontin-like half-site, AGTTCA (15, 16).

In addition to this issue of VDR homodimeric binding to VDREs, controversy exists as to the precise molecular role (if any) of 1,25-(OH)2D3 in VDR-RXR heterodimer (or homodimer) formation and DNA binding. 1,25-(OH)2D3 has been proposed to elicit high affinity association of the VDR with its heterodimeric partner (17, 18), as well as induce a conformational change in VDR-RXR inferred from an increase in the mobility of the VDR-RXR-VDRE complex (8). Other studies indicated that the presence of both RXR and 1,25-(OH)2D3 is required for optimal binding of VDR to a DR+3 from the osteocalcin VDRE (19) and to a 24-OHase VDRE (20). In contrast, it has been proposed that initial VDR-RXR DNA interaction occurs in a ligand-independent fashion (21). Still another variation was postulated by Freedman and co-workers (22), in which the role of 1,25-(OH)2D3 was hypothesized to be the dissociation of the DNA-bound homodimer by decreasing the rate of conversion of DNA-bound monomer to homodimer, and ultimately promoting the formation of a DNA-bound VDR-RXR heterodimer.

There are also conflicting experimental data on the role played by 9-cis-retinoic acid (9-cis-RA), the cognate ligand for RXR, in the binding and transcriptional regulation of 1,25-(OH)2D3-responsive genes by the VDR-RXR heterodimer. 9-cis-RA has been observed to repress the 1,25-(OH)2D3-dependent accumulation of osteocalcin mRNA in osteoblast-like ROS 17/2.8 cells (19). Further analysis of VDRE binding in mobility shift assays (19) demonstrated that heterodimeric interactions between VDR and RXR were enhanced by 1,25-(OH)2D3, and were inhibited by high concentrations of 9-cis-RA, consistent with the observation that RXR-specific ligands can induce RXR

1 The abbreviations used are: 1,25-(OH)2D3, 1α,25-dihydroxyvitamin D3; VDR, vitamin D receptor; VDRE, vitamin D response element; TR, thyroid hormone receptor; RAR, retinoic acid receptor; RXX, retinoid X receptor; m, mouse; h, human; 9-cis-RA, 9-cis-retinoic acid; BEVS, baculovirus expression vector system; CRBP II, cellular retinol-binding protein II; RXRE, retinoid X receptor response element; ROC, rat osteocalcin; MOP, mouse osteopontin.

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homodimer formation in solution (23). In contrast, treatment of a variety of transfected cell types with both 1,25-(OH)2D3 and 9-cis-RA was reported to enhance the expression of an osteopontin VDRE-linked reporter gene over levels observed with 1,25-(OH)2D3 alone (24, 25). An apparent synergistic co-stimulation by 1,25-(OH)2D3 and 9-cis-RA in transfection systems employing AGTTCGTA synthetic DR-3 constructs has also been reported (26, 27), and in at least one instance, 9-cis-RA was observed to have no effect on 1,25-(OH)2D3-stimulated transcription (28). Because of the wide range of cell types (insect to mammalian) utilized in these transfection studies plus the untold effects of variable receptor/coreceptor expression, as well as different levels of reporter plasmid, it is difficult to interpret the general significance of these findings.

Therefore, to define at the biochemical level how 1,25-(OH)2D3, VDR, RXR and DNA interact ultimately to stimulate the transcription of hormone-responsive genes, the following three areas of conflicting experimental data were addressed: (i) the relative importance of VDR homodimer versus VDR-RXR heterodimer binding to DR+3 VDREs, including elements consisting of osteopontin half-sites; (ii) the effect of 1,25-(OH)2D3 on VDR binding to RXR or VDR-RXR binding to DNA; and (iii) the role, if any, of 9-cis-RA on VDR-RXR DNA binding. To resolve these issues, we have utilized an electrophoretic mobility shift assay system that includes physiological salt and limited receptor concentrations to investigate the effects of cognate ligands on possible VDR homodimer and VDR-RXR heterodimer binding to their target response elements.

**EXPERIMENTAL PROCEDURES**

**Enriched Receptors**—The human RXRα (hRXRα), murine RXRβ (mRXRβ), and hVDR cDNAs were incorporated into a baculovirus expression vector system (BEVS) as described previously (8, 19). All recombinant baculoviruses were isolated and plaque-purified using standard procedures (29), then used to infect SF9 cultures (Spodoptera frugiperda ovary cells). After 48 h, whole cell extracts were prepared by sonication in KETZD-0.3 (0.3 M KCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.8, 0.3 mM zine acetate, 5 mM dithiothreitol), followed by centrifugation at 200,000 g for 30 min. Supernatants containing hVDR, hRXRα, or mRXRβ were enriched to at least 70% purity by blue dextran-Sepharose chromatography as described in detail for VDR (8), then snap-frozen and stored at −70 °C. Human RXRα was expressed in Escherichia coli and enriched to approximately 70% purity as described previously (30). Human RXRβ (hRXRβ) was obtained from Affinity BioReagents (Golden, CO).

**Antibodies**—A monoclonal antibody to the vitamin D receptor, 9A7 (31), an hRXRα polyclonal antibody (32), a monoclonal antibody against mRXRβ, designated MOK 13.17 (33), and an RXRβ polyclonal antibody obtained from Affinity BioReagents were utilized.

**Reagents**—Klenow enzyme was purchased from Promega Corp. (Madison, WI). Poly(dI-dC) was obtained from Boehringer Mannheim. Synthetic oligonucleotides corresponding to the respective hormone response elements were synthesized by Integrated DNA Technologies Inc. (Corvalle, IA). [-32P]dCTP (3000 Ci/mmol) was purchased from NEN Life Science Products.

**Oligonucleotide Labeling**—Double-stranded versions of each of the test response elements (direct repeat hexanucleotides are underlined) with HindIII overhangs (lowercase) were created by annealing the following single-stranded synthetic oligonucleotides together with their complements (not shown). For the mouse osteopontin VDRE (9), the oligonucleotide was 5'-agctACAGTTTCAGGGTTTCCGTTT-3'. For the rat osteocalcin VDRE (7), the oligonucleotide was 5'-agctGCTTCCGATACGAGGACACACAGTTA-3'. For the rat cellular retinol-binding protein (CRBP) II RXRE (34), with a potential third half element indicated by a double underline, the oligonucleotide was 5'-agctGCTTCCGATACGAGGACACACAGTTA-3'.

The double-stranded oligonucleotides were labeled with [-32P]dCTP to a specific activity of >10⁶ cpm/μg as described by Nakajima et al. (35).

**Ligand-dependent Gel Mobility Shift Assays**—Previous VDR-RXR gel shift protocols were altered to reduce concentrations of receptors to approximate physiologic levels. Accordingly, hVDR (10–100 ng = 5–50 nM) 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 RXRα or RXRβ equivalent to that of hVDR. Prior to the addition of receptor(s), 1,25-(OH)2D3 was added to the reaction mixture in ethanol vehicle to give a final ligand concentration of 10⁻¹⁰ to 10⁻⁰ M. In experiments that involved the additional inclusion of 9-cis-RA (10⁻¹⁰ M), ethanol vehicle controls were included and the binding portion of the reaction (45 min at 22 °C) was shielded from light. One μl of radioactively labeled DNA probe (containing 0.5 ng DNA, or approximately 0.8 nM, and 50,000–100,000 cpm) was added to give a final volume of 40 μl, then incubated for an additional 30 min. Thereafter, the entire reaction was subjected to nondenaturing electrophoresis on a 4% polyacrylamide gel. The dried gels were used to expose Kodak X-Omat films at −70 °C. The autoradiographs of a number of assays were quantitated by densitometric analysis using an Apple OneScanner with Ofoto™ scanning software developed by Light Source, Inc.

A protocol identical to that described immediately above was utilized for the homodimeric association of RXRβ with the CRBP II RXRE, except that 9-cis-RA was substituted for 1,25-(OH)2D3 and no VDR was included. Finally, a separate set of experiments was carried out in which the order of addition of each of the receptors and their respective ligands to the reaction mixture was varied, with the total time of each reaction, including preincubation of receptor(s) with ligand, heterodimer formation, and DNA binding, not exceeding 110 min.

**Preparation of Nuclear Extract from ROS 17/2.8 Cells**—Cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1) supplemented with 5% fetal bovine serum and 5% newborn calf serum. Log phase cultures were harvested by trypsination and collected by centrifugation at room temperature for 10 min at 1000 x g, followed by washing in five volumes of phosphate-buffered saline at 4 °C. The nuclear extract was then prepared as described previously (36), with all steps performed at 4 °C.

**RESULTS**

**VDR Ligand Enhances Binding of RXR-VDR Heterodimers to the Mouse Osteopontin and Rat Osteocalcin VDREs**—Fig. 1A depicts a gel mobility shift experiment using the mouse osteopontin (MOP) VDRE and baculovirus-expressed VDR in the presence and absence of hRXRα or mRXRβ (20 ng of each receptor). At limiting concentrations, neither the VDR alone (lanes 1 and 2) nor RXR alone (data not shown) (35) produce an observable DNA binding complex either in the absence or presence of 1,25-(OH)2D3. The only major DNA binding complex observed in this experiment is that produced by incubation of VDR plus either RXRα or RXRβ, and, by densitometric analysis, this complex is enhanced 23-fold in the presence of hormone (compare lane 3 to lane 4, and lane 7 to lane 8). The average degree of binding enhancement elicited by 1,25-(OH)2D3 observed from more than 30 such experiments carried out at limiting receptor concentrations was 20-fold, although effects of ligand apparently as low as 2.5-fold have been recorded (see Fig. 4). The hormone effect is selective for 1,25-(OH)2D3, since the relative inactive natural vitamin D metabolite 24(R)-25-(OH)2D3, its epimer, 24(S)-25-(OH)2D3, as well as the 25-(OH)D3, 1α-(OH)D3, and vitamin D3 hormone precursors, do not enhance DNA-binding complexes at 10⁻⁸ M (data not shown). The hormone-stimulated, heterodimeric bands seen in lanes 4 and 8, are eliminated (lanes 5 and 9) by inclusion of the VDR-specific monoclonal antibody 9A7, which recognizes an epitope within the DNA binding domain. Moreover, the addition of an RXR-specific polyclonal antibody (lane 6) caused a majority of the complex to supershift, confirming the presence of RXRs in the complex. Interestingly, the RXRβ-specific monoclonal antibody MOK 13.17 (lane 10) showed little interaction with the VDR-RXRα DNA binding complex. As discussed below, the MOK 13.17 antibody may not be able to access its epitope when RXRβ is heterodimerized with VDR.

Fig. 1B depicts complexes formed on the rat osteocalcin (ROC) VDRE by hVDR in combination with baculovirus-expressed mRXRβ. These heterodimeric DNA binding species are consistently less intense than complexes formed on the MOP VDRE, but an enhancing effect of 1,25-(OH)2D3 is still appar-
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FIG. 1. Gel mobility shift analysis of baculovirus-expressed hVDR, hRXRα, and mRXRβ binding to VDREs. A, baculovirus-expressed hVDR, hRXRα, and mRXRβ (20 ng of each receptor) were analyzed for their ability to form DNA binding complexes on the MOP VDRE (0.5 ng), in the absence and presence of 10−8 M 1,25-(OH)2D3 as described under “Experimental Procedures,” using a salt concentration of 150 mM KCl. Lanes 1 and 2 show DNA complexes formed by VDR alone in the absence (−) or presence (+) of hormone. Lanes 3 and 4 display DNA complexes formed by VDR in combination with RXRα with or without hormone, whereas lanes 5 and 6 show the effects of a VDR-specific monoclonal antibody, 9A7 (0.6 µg; added to the reaction after VDR and RXR are allowed to heterodimerize in the presence of 1,25-(OH)2D3), and an RXRα-specific polyclonal antibody (1.0 µg; preincubated with RXRα alone), respectively. Lanes 7–10 repeat the conditions of lanes 3–6, but using mRXRβ as the VDR partner and 1 µg of an RXRβ-specific monoclonal antibody, MOK 13.17 (1 µg; added to the reaction after VDR and RXR are allowed to heterodimerize in the presence of 1,25-(OH)2D3). B, the ability of BEVS hVDR and mRXRβ (20 ng of each receptor) to form DNA binding complexes on the ROC VDRE (0.5 ng). Lanes 1 and 2 depict complexes formed with or without 10−7 M 1,25-(OH)2D3. Lane 3 shows the effect of preincubation of an RXRβ-specific polyclonal antibody with RXRβ prior to addition to the incubation mixture. C, the effects of 1,25-(OH)2D3 on the binding of endogenous receptors to the MOP VDRE. Lanes 1 and 2 illustrate reactions containing a ROS 17/2.8 cell nuclear extract (3.0 µg of protein) ± 10−8 M 1,25-(OH)2D3. Lane 3 shows the effect of adding VDR-specific monoclonal antibody, 9A7 (1.0 µg), to an incubation mixture containing 1,25-(OH)2D3.

ent (12-fold by densitometry of lane 2 versus lane 1). It should be noted that two heterodimeric complexes are observed (Fig. 1B) with some preparations of mRXRβ, presumably because of a second methionine translational start site present in the mRXRβ cDNA that leads to the production of two alternative forms of RXRβ protein. The occurrence of RXRβ in these complexes is confirmed by the appearance of a strong supershifted band in the presence of RXRβ-specific polyclonal antibody (lane 3), although non-shifted complexes appear to be stabilized by the inclusion of the proteinaceous antibody preparation.

FIG. 2. Ligand concentration dependence of VDR-RXR binding to the MOP and ROC VDREs. The ability of 1,25-(OH)2D3 to promote VDR-RXR heterodimer binding to the MOP (A) and ROC (B) VDREs was determined in the absence (lane 1) and presence of 1,25-(OH)2D3 over a concentration range of 10−9 to 10−10 M (lanes 2–7). The gel mobility shift assay was carried out using 20 ng each of baculovirus expressed hVDR and hRXRβ, employing a salt concentration of 150 mM KCl as described under “Experimental Procedures.”

the effect of 1,25-(OH)2D3 on the binding to the MOP VDRE of endogenous VDR and its presumed RXR partner in a rat osteoblast-like (ROS 17/2.8) cell nuclear extract. Using 3.0 µg of protein extract per reaction, the presence of 1,25-(OH)2D3 consistently and repeatedly facilitates a 2–3-fold increase in the binding of the endogenous VDR and RXR to the MOP VDRE. Specific inhibition of this complex by VDR antibody (lane 3) confirms the occurrence of VDR in this complex. Hence, in a natural situation (Fig. 1C), efficient association of endogenous VDR with DNA displays a requirement for the presence of 1,25-(OH)2D3, although the magnitude of ligand-enhanced complex formation does not attain that seen with enriched receptors (Fig. 1A).

Concentration Dependence of the 1,25-(OH)2D3 Effect on VDR-RXR Heterodimer Binding to the MOP and ROC VDREs—Fig. 2 depicts one of three replicate experiments testing the influence of different concentrations of 1,25-(OH)2D3 on VDR-RXR binding to the MOP (Fig. 2A) or ROC (Fig. 2B) VDRE. Binding to the MOP VDRE (Fig. 2A) exhibits a maximum at 10−8 M 1,25-(OH)2D3, diminishing to a minimal but significant enhancement at 10−10 M 1,25-(OH)2D3. The ROC
VDRE (Fig. 2B) displays a slightly different pattern with an optimum at $10^{-6}$ M hormone, followed by a more gradual decrease in binding as the ligand concentration is lowered. Den-sitometric scanning of these two gels, however, revealed that the ED$_{50}$ of 1,25-(OH)$_2$D$_3$ for both VDREs was approximately $10^{-8}$ M, representing a 13-fold increase in receptor heterodimer binding to the ROC VDRE and a 15-fold increase on the MOP element. Accordingly, the concentrations of 1,25-(OH)$_2$D$_3$ chosen for most subsequent experiments were either $10^{-9}$ or $10^{-8}$ M.

Effect of 1,25-(OH)$_2$D$_3$ to Reduce Dissociation of the VDR-RXR Heterodimer from a VDRE—Labeled MOP VDRE was incubated with VDR and RXR$\beta$ in the absence and presence of 1,25-(OH)$_2$D$_3$, followed by inclusion of a 100-fold excess of competing unlabeled MOP VDRE at time zero. As shown in Fig. 3A, the presence of ligand not only greatly potentiates initial (time 0) DNA binding, as seen previously, but also results in an apparent slower rate of dissociation of the heterodimeric receptor complex from the labeled response element. Densitometric analysis of this experiment (Fig. 3B) indicates that the liganded VDR-RXR complex exhibits a half-life of $\approx$11 min (by extrapolation), whereas the unliganded VDR-RXR heterodimer dissociates with an estimated half-life of only 3.0 min.

Higher Receptor Levels Generate Apparently Artifactual VDR Homodimers and Attenuate the VDR Ligand Dependence of VDR-RXR Binding to the ROC and MOP VDREs—The experiment depicted in Fig. 4 was performed using both 20 ng and 100 ng of VDR, to determine if homodimeric binding, which was not observable using limiting receptor concentrations (Fig. 1A), could be elicited at receptor concentrations significantly in excess of physiologic levels. On the weaker ROC response element (Fig. 4, left panel), neither 20 ng (lanes 1 and 2) nor 100 ng (lanes 3 and 4) of VDR exhibited any observable monomeric or homodimeric DNA binding species, even in the presence of 1,25-(OH)$_2$D$_3$. We have previously observed VDR homodimer binding to the ROC VDRE at very high VDR amounts of 260 ng (35), but the stability of such VDRE complexes is quite minimal, with an apparent half-life of less than 0.5 min when assayed in a mobility shift dissociation assay similar to that shown in Fig. 3. By densitometric analysis, the putative homodimeric binding seen in lane 3 (right panel) displayed virtually no enhancement (1.3-fold) in the presence of hormone (lane 4 versus lane 3). As a positive control in these experi-

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FIG. 3. Dissociation of VDR-RXR and 1,25-(OH)$_2$D$_3$-VDR-RXR complexes from a mouse osteopontin VDRE. A, formation of VDR-RXR heterodimeric DNA complexes was carried out in the absence and presence of 1,25-(OH)$_2$D$_3$ with standard binding conditions (see “Experimental Procedures”) employing the MOP VDRE (0.5 ng) and RXR$\beta$ as the heterodimeric partner of VDR (20 ng of each receptor). After complex formation, but before undergoing non-denaturing gel electrophoresis, a 100-fold excess (50 ng) of unlabeled competing MOP probe was added and individual reactions were allowed to proceed for the indicated time intervals, followed by electrophoresis on polycrylamide gels. B, densitometric scanning results of top bands in A are shown, expressed as percent of maximum VDR-RXR binding. EtOH, ethanol vehicle.

FIG. 4. Limiting concentrations of VDR bind to VDREs as a heterodimer rather than as a homodimer. Standard gel mobility shift assays were carried out on two different VDREs (0.5 ng) using the indicated amounts of receptors. Each receptor combination was tested in the presence (+) or absence (−) of 1,25-(OH)$_2$D$_3$. Note that, as observed in Fig. 1B and 3A, and discussed under “Results,” a second, faster migrating DNA complex appears with mRXR$\beta$, which likely represents a species composed of VDR and truncated mRXR$\beta$.

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P. N. MacDonald and M. R. Haussler, unpublished observation.
Although it was noted in Fig. 1A that the MOK 13.17 RXRβ antibody had relatively little effect on VDR-RXRβ binding to a VDRE, an apparent stabilization as well as robust supershift of the DNA-bound, unliganded, and ligand-enhanced RXR homodimeric species by this antibody was observed (Fig. 5A, lanes 3 and 4). Thus, RXRβ appears to be more accessible to the MOK 13.17 antibody when configured in its homodimeric form than when it exists in a heterodimer with VDR. As expected, the ligand-dependent RXR homodimer complex with the RXRE was neither inhibited nor supershifted in the presence of a VDR antibody (Fig. 5A, lanes 5 and 6). Fig. 5B depicts the RXRE binding displayed by E. coli-expressed hRXRα in the presence of varying concentrations of 9-cis-RA. At concentrations of 10⁻⁶, 10⁻⁷, and 10⁻⁸ M, the presence of 9-cis-RA enhances homodimeric binding to the RXRE 5.1-, 4.0-, and 1.4-fold, respectively, as determined by densitometric scanning.

Having demonstrated that, under our conditions, 9-cis-RA can facilitate RXR homodimerization, we next addressed the question of its influence on RXR-VDR heterodimers. As expected, 10⁻⁹ M 1,25-(OH)₂D₃ effectively promotes VDR-RXR binding to the MOP response element (Fig. 6A, lane 2 versus lane 1). Although 9-cis-RA alone does not have a significant effect on VDR-RXR binding to DNA (data not shown), this retinoid, at concentrations of 10⁻⁵ and 10⁻⁶ M, suppresses 1,25-(OH)₂D₃-stimulated DNA binding by the VDR-RXR heterodimer by 97% and 53%, respectively. Concentrations of 10⁻⁷ and 10⁻⁸ M each attenuate heterodimeric binding to the VDRE by 40%, whereas concentrations of 10⁻⁹ M or below have no observable suppressive effect (Fig. 6A). In Fig. 6B, a ROS 17/2.8 nuclear extract (3.0 μg of protein) was used to show that 9-cis-RA also diminishes the effect of 1,25-(OH)₂D₃ on endogenous VDR-RXR complexing to a VDRE. Lanes 1 and 2 demonstrate the 2–3 fold enhancement of VDRE binding by the endogenous receptors elicited by the addition of hormone (10⁻⁶ M), whereas lane 3 illustrates an 80% reduction in the 1,25-(OH)₂D₃-dependent enhancement by 10⁻⁶ M 9-cis-RA. A similar reduction to that observed with hRXRα and endogenous rat RXR is seen when baculovirus-expressed mRXRβ is used as the heterodimeric partner (data not shown). The inhibitory effect of 9-cis-RA on 1,25-(OH)₂D₃-induced binding can be overcome, however, by increasing the amount of RXR (Fig. 6C). The results in Fig. 6 (plus five other replicate experiments similar to that shown in Fig. 6A, along with the data in Fig. 5), are consistent with an ability of high concentrations of 9-cis-RA (10⁻⁵ M, 10⁻⁶ M and, to a lesser extent, 10⁻⁷ M and 10⁻⁸ M) to inhibit 1,25-(OH)₂D₃-dependent enhancement of VDRE binding by both overexpressed and endogenous VDR, most likely by occupying a limiting concentration of RXR and conformationally diverting it from heterodimerization with VDR toward homodimerization.

**Effect of Different Orders of Receptor-Ligand Assembly**—In a typical experiment depicted in Fig. 7, we employed baculovirus expressed mRXRβ as the heterodimeric partner for VDR. In lanes 1–3, the individual receptors and the indicated ligands were added simultaneously to the reaction mixture. Lanes 4–6 contain reactions in which VDR and RXR were first allowed to associate for 30 min and were then added to a reaction mixture containing either 1,25-(OH)₂D₃ (lane 5) or a combination of 1,25-(OH)₂D₃ and 9-cis-RA (lane 6). Finally, each receptor was individually preincubated with the indicated ligands and, after a 30-min period, the respective receptor partner was added to the reaction (lanes 7–10). Densitometric analysis of four such independently performed experiments indicated 9-cis-RA inhibits 1,25-(OH)₂D₃-mediated VDRE binding by an average of 78% when each component is added simultaneously to the reaction (Fig. 7, lane 3 versus lane 2). Similar repression of
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**Fig. 7.** Preincubation of VDR with its ligand blunts inhibition by 9-cis-RA. Ligand-dependent gel mobility shift assays were carried out using different orders of receptor and ligand addition to the incubation mixture. VDR and RXR, (20 ng each) were used in reactions together with 0.5 ng of labeled MOP under standard binding conditions (see "Experimental Procedures"). Lanes 1–3 display reactions in which the receptors and the indicated ligands are added simultaneously to the incubation mixture. In lanes 4–6, a preincubated VDR-RXR mixture was added to a reaction that contained the indicated ligands. In lanes 7 and 8, VDR was allowed to preincubate for 30 min in the absence (lane 7) or presence of $10^{-9} \text{M} 1,25-(\text{OH})_2\text{D}_3$ (lane 8) and then RXR was added to the reaction mixture. In lane 9, a indicates that VDR was preincubated for 30 min in a reaction mixture containing both $1,25-(\text{OH})_2\text{D}_3$ and 9-cis-RA and then RXR was added to the reaction. In lane 10, b indicates that RXR was allowed to incubate in a mixture containing $1,25-(\text{OH})_2\text{D}_3$ and 9-cis-RA and, after 30 min, VDR was added to the reaction.

**DISCUSSION**

An experimental system has been devised to investigate the interaction of VDR, RXR, $1,25-(\text{OH})_2\text{D}_3$, and 9-cis-RA with DNA, and to assess the contribution of each component to overall complex formation. Previous studies investigating the role of ligand in the interaction of VDR with DNA have produced somewhat conflicting results and conclusions (see Introduction). Indeed, even in our hands, VDR complex formation on a VDRE can become ligand- and RXR-independent either when salt concentrations are non-stringent (<100 mM KCl; data not shown) or when receptor levels are increased by ≥50 nM (Fig. 4).

The parameters used in the present gel mobility shift assay system were carefully chosen to approach those found within the cell, including 150 mM KCl and receptor concentrations in the range of 5–10 nM. Under these conditions, association between VDR and the MOP or ROC VDREs occurs only in the presence of RXR, and is greatly enhanced by $1,25-(\text{OH})_2\text{D}_3$ (Fig. 1, A and B). Further, the fact that both types of VDRE display an average of >10-fold ligand-dependent enhancement of receptor-DNA binding under near-physiologic conditions suggests that this effect is not gene- or element-specific. Endogenous VDR and RXR from a ROS 17/2.8 cell nuclear extract (Figs. 1C and 6B) also exhibit a ligand-dependent enhancement of binding to the VDRE, although the magnitude of the effect is lower (2–3-fold). This lower degree of ligand stimulation possibly reflects the presence of proteins that nonspecifically bind to $1,25-(\text{OH})_2\text{D}_3$ or interfering DNA-binding proteins in the crude nuclear preparations. It is also conceivable that ligand
acts on overexpressed receptors to facilitate their folding into a native conformation, thereby enhancing hormone-dependent DNA binding. Nevertheless, the fact that we observe a consistent effect of ligand using endogenous VDR in ROS 17/2.8 cell nuclear extracts argues that the enhancement by ligand of receptor binding to VDREs is biologically relevant. This conclusion is independently supported by in vivo footprinting experiments (38), which reveal that 1,25-(OH)2D3 is required for VDR occupation of the VDRE in the native rat osteocalcin promoter.

The present results indicate that VDR homodimerization is not a physiologically relevant event on either the ROC or MOP response elements. Previous studies utilizing an artificial osteopontin-like VDRE (15, 22), and the actual mouse osteopontin VDRE (16), suggested that these elements represented a target for VDR homodimeric binding, although the salt concentrations used during gel mobility shift assays were not specified in every case, and very high (micromolar) concentrations of VDR were utilized in gel filtration experiments (22). We have used both the natural MOP and ROC VDREs (Fig. 4), which typify the two main subtypes of VDREs reported to date. ROC is an imperfect direct repeat with a G present as the third base in each half element, whereas MOP is a perfect direct repeat with a T present in this position. It is this latter feature of the MOP element that explains previous reports that VDR binds ROC, the actual mouse osteopontin ROC VDRE (15, 22), and the teopontin-like VDRE (15, 22), and the actual mouse osteopontin ROC VDRE (16), both of which have been utilized to examine the ligand responsiveness of RXR, the heterodimeric DNA complexes such as those with TR-RXR and RAR-RXR, do not require ligand for specific DNA binding. We propose a model (see Fig. 8) in which VDR-RXR heterodimers exist in equilibrium between two distinct states, with the addition of 1,25-(OH)2D3 shifting the equilibrium of the heterodimer to a form that binds to the VDRE with not only increased specificity, but also greater affinity (Fig. 3). A supraphysiological concentration of receptor would, by mass action, artifactually increase the amount of receptor species already in the allosteric state that binds DNA, thus explaining the lack of hormone effect under conditions of vast VDR and RXR excesses.

The ligand-dependent gel mobility assay system has also been utilized to examine the ligand responsiveness of RXR, the heterodimeric partner of VDR, in binding as a homodimer to its cognate response element. Employing an RXRE of the DR+1 type (34), we find that RXRa and RXRa, RXRb, homodimers form a

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*Fig. 8. Model for different allosteric pathways for VDR-1,25-(OH)2D3 binding to the VDRE.* It is proposed that both VDR and RXR, upon binding their respective cognate ligands, undergo distinct conformational changes, creating two alternative allosteric pathways for VDR-RXR association and response to ligand. A, an unliganded VDR binds its RXR partner to generate an apoheterodimer, which, upon subsequent binding of 1,25-(OH)2D3, undergoes a conformational change, presumably in its ligand binding domain, that results in enhanced association of the VDR-RXR-1,25-(OH)2D3 complex with its specific DNA target sequence. In this form of the heterodimer, RXR is proposed to exist in a conformational state in which it can be readily dissociated from the VDR-RXR heterodimer by the addition of 9-cis-RA, leading to the formation of RXR homodimers that mediate distinct retinoid-responsive pathways. This 9-cis-RA-receptive conformation is proposed to exist also in monomeric RXR or in the apoxRXR-VDR heterocomplex. B, when VDR binds 1,25-(OH)2D3 before heterodimer formation, it is postulated to acquire a conformation distinct from that in pathway A. This 1,25-(OH)2D3-generated conformational change in monomeric VDR is then proposed to influence the state of the RXR ligand binding domain after heterodimerization. Essentially, occupied VDR is postulated to conformationally switch the RXR partner, rendering it hyposensitive to the effects of its own ligand and thus making this form of the heterodimer resistant to 9-cis-RA-elicited dissociation.
DNA-bound complex preferentially in the presence of 9-cis-RA (Fig. 5, A and B). In contrast to 1,25-(OH)₂D₃, the concentrations of 9-cis-RA (10⁻⁵ to 10⁻⁶ M) required to produce a striking ligand-dependent effect are higher than what are normally considered physiological concentrations (low nanomolar) of this retinoid. However, it should be noted that the Kᵦ (1.2 x 10⁻⁸ M) for 9-cis-RA binding to RXR (40) is 2 orders of magnitude higher than that for 1,25-(OH)₂D₃ binding to VDR. It is also possible that the extreme photolability of 9-cis-RA may lower the effective concentration present in these in vitro reactions, and in vivo, target tissues may require local concentrations that are much higher than circulating levels for a hormonal response. Nevertheless, our results agree qualitatively with a previous report that 9-cis-RA enhances the binding of RXRα homodimers to a DR+1 element (41).

A persistent area of controversy has been the effect of 9-cis-RA on VDR-RXR heterodimer binding to DNA and, hence, regulation of gene transcription. Previous studies from a number of laboratories have produced conflicting results ranging from evidence that 9-cis-RA can destabilize VDR-RXR heterodimers and presumably induce RXR homodimer complex formation (19), to a conclusion that this ligand interacts synergistically with 1,25-(OH)₂D₃ to transcriptionally activate the VDR-RXR heterodimer (24, 25). Our data from the gel mobility shift assay system tend to confirm that the effect of 9-cis-RA is to dissociate the VDR-RXR heterodimer and divert RXR to form homodimers. In combination with 10⁻⁹ M 1,25-(OH)₂D₃, 9-cis-RA at concentrations of 10⁻⁷ M to 10⁻⁶ M, equal to or slightly lower than those that elicit homodimerization of RXR (Fig. 5), cause significant dissociation of the VDR-RXRA heterodimer (Fig. 6A). The inhibitory effect of 9-cis-RA is seen even in the absence of an RXRE (data not shown), consistent with previous reports that RXR homodimerization takes place in solution (23). Importantly, using endogenous receptors present in a ROS 17/2.8 cell nuclear extract, 10⁻⁶ M 9-cis-RA inhibits 1,25-(OH)₂D₃ stimulated binding to the VDRE by 50% (Fig. 6B). The fact that this inhibitory effect of 9-cis-RA can be overcome by the subsequent addition of excess RXR (Fig. 6C) argues against the possibility that 9-cis-RA may be altering the affinity of the heterodimer for the VDRE and suggests that this inhibition is due to ligand-induced diversion of limiting RXR from the VDR-RXR complex toward homodimers. These findings can be generalized to propose the existence of hormonal crosstalk between metabolites of vitamins A and D in controlling VDR-mediated transcriptional regulation.

The present results further suggest that, in addition to the availability of target sequences, receptors, and hormonal ligands, another parameter regulating the transcriptional response to hormone may be the sequential order in which these components assemble. As can be seen from Fig. 7, the ability of 9-cis-RA to shift the equilibrium from DNA-bound VDR-RXR heterodimer to RXR homodimer formation depends either on 9-cis-RA first occupying RXR monomers to facilitate their homodimerization, or on VDR and RXR forming an apophetoderimer. Evidence that VDR and RXR can form apophetoderimers in solution is supported by yeast two-hybrid interaction studies (39), and protein-protein association assays using either VDR or RXR expressed as glutathione S-transferase fusion proteins (data not shown). Taken together, these findings suggest the existence of at least two allosteric pathways for VDR-RXR association and response to ligand, as presented in Fig. 8. This model predicts two allosterically distinct forms of the 1,25-(OH)₂D₃-occupied heterodimer: (i) a preformed VDR-RXR heterodimer that subsequently binds 1,25-(OH)₂D₃ and hence is induced to bind its specific DNA target sequence, but can be dissociated by the addition of 9-cis-RA (Fig. 8, pathway A) and (ii) a pre-ligated form of VDR that creates a heterodimer, which is largely resistant to 9-cis-RA-induced dissociation (Fig. 8, pathway B). This model may explain why 9-cis-RA is observed only to partially suppress 1,25-(OH)₂D₃ induced transcription in transfection studies (19), since the intact target cell likely contains a mixed population of the proposed two allosteric forms of the heterodimer.

Recently, a type of converse regulation has been reported with RXR homo- and heterodimers (42). It was found that unliganded RAR and TR suppressed RXR-mediated transcriptional response to an RXR specific-ligand, indicating that RAR and TR function as attenuating partners of RXR. Further, TR, when bound to its cognate ligand, completely suppresses RXR ligand binding, similar to what is observed with pre-ligated VDR (Fig. 8, pathway B). However, in contrast to both VDR-RXR and TR-RXR heterodimers, 9-cis-RA binding to RXR is restored when its partner RAR binds its cognate ligand, resulting in a synergistic response to both ligands. It was therefore proposed (42) that the addition of the RXR ligand transmits a positive allosteric signal to the RXR heterodimeric partner, whereas similar addition of ligand to TR prior to heterodimerization transmits a negative allosteric signal to RXR, which prevents it from binding 9-cis-RA. As summarized in Fig. 8, the current experimental evidence for VDR suggests that only the pre-ligated form of VDR can effectively mediate a negative allosteric change in RXR, such that it can neither bind 9-cis-RA nor readily dissociate to form RXR homodimers.

In conclusion, we propose that a role of 1,25-(OH)₂D₃ is to drive VDR-RXR heterodimers to an allosteric form which displays high affinity and specificity for its target response element. We also have presented evidence that the sequential order of assembly of ligand, receptor, and DNA apparently can result in distinct allosteric forms of the heterodimer which respond differently to the presence of 9-cis-RA, and perhaps reflect allosteric changes in VD that are conveyed to its heterodimeric partner. This proposed conformational switch may be fundamental to transcriptional regulation by the VDR subfamily of nuclear receptors.

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