Retinoid X Receptor (RXR) Ligands Activate the Human 25-Hydroxyvitamin D₃-24-hydroxylase Promoter via RXR Heterodimer Binding to Two Vitamin D-responsive Elements and Elicit Additive Effects with 1,25-Dihydroxyvitamin D₃

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We have previously shown that RNA levels of kidney 25-hydroxyvitamin D₃-24-hydroxylase (24(OH)ase), a key metabolic enzyme for 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), are up-regulated by retinoids in mice within hours. Deletion analysis of ∼5500 base pairs of the human 24(OH)ase promoter showed that the sequence between −316 and −142 contained the information necessary and sufficient for retinoid-induced activation of the promoter. This region contains two previously defined vitamin D-responsive elements (VDREs) at −294 to −274 and −174 to −151. Mutation of either VDRE diminished responsiveness of the −316 to −22 promoter sequence to retinoids or 1,25(OH)₂D₃, while mutation of both VDREs essentially abolished the activity of the ligands via the heteromer. The promoter vectors driven by the VDREs were responsive to a retinoid X receptor (RXR)-selective ligand (LG100268), a retinoic acid receptor (RAR)-selective ligand (TTNPB), or 1,25(OH)₂D₃, while combinations of LG100268 with either TTNPB or 1,25(OH)₂D₃ resulted in additive increases in activity. Band shift analyses showed that vitamin D receptor, RAR, or RXR alone did not bind to the VDREs; however, the combination of either vitamin D receptor or RAR with RXR led to retardation of each of the labeled probes. Treatment of non-transfected CV-1 cells with retinoids or 1,25(OH)₂D₃ resulted in induction of 24(OH)ase RNA, and ligand combinations led to increased RNA levels. These data imply that either or both of the heterodimer partners can be occupied with ligand to induce this enzyme, with dual receptor occupation leading to increased activation.

The retinoic acid receptors (RARs)¹ and retinoid X receptors (RXRs) are nuclear transcription factors that are activated by their retinoid ligands, all-trans-retinoic acid (tRA) and 9-cis-retinoic acid (9cRA) (1). While RARs bind to both tRA and 9cRA with high affinity, RXRs bind to 9cRA but not to tRA (2–5).

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¹ The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoid X receptor; tRA, all-trans-retinoic acid; 9cRA, 9-cis-retinoic acid; VDR, vitamin D receptor; VDRE, vitamin D-responsive element; 24(OH)ase, 25-hydroxyvitamin D₃-24-hydroxylase; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; kb, kilobase pair(s); PCR, polymerase chain reaction; DR, direct repeat.

Both receptor subfamilies are thought to mediate the biological actions of retinoids in processes such as cellular growth and differentiation and development by altering the production of certain proteins in various cells at the level of gene transcription (1, 6). The ligand-occupied receptors generally act by binding to retinoid receptor-responsive elements in the promoter regions of target genes. Ligand-bound RAR cooperates with RXR to form a heterodimer that is an efficient and high affinity binder of retinoic acid response elements, thereby activating transcription of certain tRA-responsive genes such as RARβ (7–9) and cellular retinoic acid binding protein II (10). RXR is postulated to function by at least two modes of action. RXR has been shown to act as a silent (nonliganded) partner with a number of other intracellular receptors, including RAR, vitamin D receptor (VDR), and thyroid hormone receptor, in response to their respective ligands (11–16). RXR has also been shown to form homodimers in a complex with DNA upon binding to 9cRA (17). However, evidence for RXR homodimers as functional units in the transcription of biologically relevant target genes has yet to be demonstrated.

For example, while RXR binds to, and stimulates transcription from, an element within the cellular retinol binding protein II gene promoter in cotransactivation assays in mammalian cells (18) and in yeast cells (which do not contain RAR; Ref. 2), this gene has not been shown to be regulated by retinoids in nontransfected cells or in the animal. However, evidence is emerging that RXR may not function solely as a silent partner in hormone signaling pathways. Recently, 9cRA has been shown to activate RXR in heterodimeric interactions with two orphan receptors, LXR (19) and NGFI-B (20), to stimulate transcription from synthetic response elements in cotransfection assays. Additionally, RXR ligands have been shown to increase the effects of RAR ligands to induce certain RNA species (21). In these experiments, however, while an RXR ligand modulated the effects of an RAR ligand to activate certain genes, the RXR ligand alone did not induce RNA levels.

We show here that an RXR-selective ligand alone is able to activate transcription of the human 25-hydroxyvitamin D₃-24-hydroxylase (24(OH)ase) promoter by binding to the RXR partner of RXR-VDR or RXR-RXR heterodimers and acting through previously defined VDRE sequences (22, 23) within the promoter. Either the RXR ligand alone or the VDR ligand alone leads to stimulation of the promoter, while the presence of both ligands leads to additive or more than additive induction of luciferase activity. These data indicate that ligand occupation of either or both heterodimeric receptor partners leads to a productive transcriptional event at this promoter, with maximal induction observed upon occupation of both receptors. Additionally, ligand-occupied RAR also activates this promoter through these sequences by binding with RXR, either with or
with its ligand. Therefore, these two previously defined VDRs within the human 24(OH)ase promoter can also serve as retinoid acid response elements, since they are able to confer responsiveness of the promoter to retinoids.

**EXPERIMENTAL PROCEDURES**

**Ligands—**LG100268 (24), TTNPB (25, 26), and 9cRA (25) were synthesized and purified at Ligand Pharmaceuticals, Inc. (San Diego, CA). 1,25(OH)2D3 was from Solvay Duphar (The Netherlands).

**Receptor and Reporter Vectors—**Receptor expression vectors (pRSXKRXr, pRSXRΔN14, pRSXRΔN17, and pRSXRΔN23) were described previously (2, 27). 24(OH)ase promoter-driven reporter constructs were derived from an ~6-kb human genomic clone (23) that included sequence 3’ to the start site of transcription. The 6-kb promoter fragment (~5500 to +455) was cloned into a promoterless luciferase expression vector, pLUCpl (2), at Sall and PstI sites upstream of the translation start site of the luciferase coding region of the plasmid. Deletion and mutant promoter constructs were generated from the 6-kb sequence with common 3’ ends by digestion with NsiI at position −22 in the promoter sequence (~5500 to −22)-LUC. (~1177 to −22)-LUC was generated by digesting (~5500 to +455)-LUC with Van911 (blunt) and NsiI, and the resultant fragment was subcloned into pLUCpl digested with Xhol (blunted) and PstI (~316 to −22)-LUC, (~261 to −22)-LUC, and (~22)-LUC, (~890 to −22)-LUC. (~22)-LUC was generated by PCR utilizing 5’ primers, some with an overhanging Sall site immediately upstream of the 5’-most base of the deletion construct (others without an enzyme site) and a common 3’ primer encompassing the NsiI site. PCR products were digested with Sall and NsiI and subcloned into pLUCpl cut with Xhol and PstI. (~316 to −22)-LUC was generated by digestion of (~316 to −22)-LUC with HindIII/BsrEII, digestion of (~261 to −22)-LUC with BsrEII/BamHI, and ligation of the two fragments into pLUCpl digested with HindIII/BamHI. (~316 to −22)-LUC was digested by PCR utilizing 5’ primers and sites immediately upstream of position −316 and the 3’ primer containing the NsiI site at −22. The results of this reaction were cloned into pLUCpl with HindIII/BsrEII and (~261 to −22)-LUC with BsrEII/BamHI and combining the two fragments in pLUCpl digested with HindIII/BamHI. (~316 to −22)-LUC was generated by PCR utilizing the MORPH™ site-specific plasmid DNA mutagenesis kit (5 Prime → 3 Prime, Inc., Boulder, CO). (~261 to −22)-LUC with (~168CCC mutated to GTT) was created utilizing the MORPH™ promoter mutagenesis kit (27). Therefore, two regions within the promoter conferred responsiveness to the RXR-selective ligand LG100268, one being upstream of position −316 and the other between −261 and −143

**CV-1 Cell Studies—**CV-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with l-glutamine and 10% fetal bovine serum. Cells at ~40% confluency were incubated in media containing 10% charcoal-stripped fetal bovine serum for 48 h prior to treatment with ligands. Cells were ~80% confluent upon the addition of ligands in 0.1% ethanol vehicle. 1,25(OH)2D3, LG100268, TTNPB, 9cRA, or combinations thereof were added in fresh media containing cholate and insulin and incubated for 43 to 72 h. Cells were then harvested into 0.1% Triton X-100 prior to harvest. Concentrations of ligands are indicated in the figure legends. Total cellular RNA was extracted, and Northern analysis was performed as per standard methodology. Probes included a 900-base pair EcoRI/XbaI DNA fragment of the rat 24(OH)ase cDNA (30) and a 1.4-kb human glyceraldehyde-3-phosphate dehydrogenase fragment (Clonetech). Hybridization was performed at 65°C in Quik-Hyb solution (Amersham Corp.) for 2 h followed by washing (0.1 × SSC, 0.1% SDS at 65°C for 1 h). Quantitation was by PhosphoImager analysis (Molecular Dynamics).

**RESULTS**

Two regions of the human (OV/4/hase promoter are responsive to retinoids and 1,25(OH)2D3. The cloning of the rat (31, 32) and human (22, 23) 24(OH)ase promoters has been reported previously. Two VDRs have been defined within both the rat and the human promoters that confer responsiveness of the promoters to 1,25(OH)2D3 (22, 32). We have previously reported that retinoids induce kidney 24(OH)ase activity in mice (27), and cursory experiments indicated that the human 24(OH)ase promoter was stimulated by retinoids in CV-1 cell cotransfection assays (27). The *in vivo* effects were observed with a synthetic RXR-selective ligand, LG100268 (24), a synthetic RXR-selective compound, TTNPB, and the endogenous retinoid ligands, 9cRA and tRA. To determine the mechanism of retinoid activation of the 24(OH)ase promoter, an in depth promoter study was undertaken. Various deletions of the previously cloned ~6-kb human 24(OH)ase promoter (23) were constructed into a promoterless luciferase reporter vector (2) and tested for the ability to respond to various ligands in kidney cell lines cotransfected with retinoid receptors and/or VDR. The promoter fragment containing ~5.5 kb upstream of the start site of transcription (~5500 to −22)-LUC yielded 3-fold induction of luciferase activity by the RXR-selective ligand, LG100268, in either of two kidney cell lines, CV-1 (data not shown) or COS-1 (Fig. 1A). COS-1 cells were used for the remainder of the study, since they gave identical results to CV-1 cells and were more stable in the transfection assays than CV-1 cells. The magnitude of the response (~3-fold) to LG100268 (~5500 to −22)-LUC yielded a less efficacious response (~1.8-fold activation; p < 0.05) with LG100268, and the addition of DNA probe. Reactions performed with ligand present included 9cRA (1 μM, 0.1% ethanol) or ethanol vehicle and RXR-containing extracts; incubation was at 4°C for 1 h prior to the DNA addition. Protein-DNA binding reactions were carried out in buffer containing 50–100 mM KCl, 20 mM Hepes, pH 7.4, 20% glycerol, 12.5 mM MgCl2, Oligonucleotide probe (~20,000 dpmm) was added to protein mixtures along with 1 μg of poly(dI-dC) competitor DNA/reactivation and incubated on ice for 20 min, followed by 2 min at room temperature. Electrophoresis of protein-DNA complexes was performed on 6% acrylamide, 0.5 × TBE gels (Novex, San Diego) in 0.5 × TBE running buffer at 4°C (~15 mA). Gels were dried and exposed to autoradiographic film for 1–2 h at ~80°C.
Interestingly, these two regions are also responsive to 1,25(OH)2D3 (Fig. 1B) and contain previously defined VDREs (see Fig. 2; Refs. 21 and 22). Sequence upstream of −2117 may contain an additional VDRE (Fig. 1B). The two LG100268-responsive regions also confer responsiveness of the promoter to TTNPB (Fig. 1C; p, 0.05) and to 9cRA treatment (Fig. 1D). COS-1 cells contain endogenous VDR, RAR, and RXR proteins (data not shown), and ligand-induced luciferase activity driven by the 24(OH)ase promoter sequences was also observed without transfected receptor expression vectors. The -fold induction varied from 20 to 80% of that in the presence of transfected receptors, depending on the ligand (Fig. 3). The endogenous retinoid receptor pan-agonist, 9cRA, elicited greater induction of luciferase activity through each of the promoter constructs in the presence (−6-fold; Fig. 1D) and in the absence (−3-fold; Fig. 3) of transfected receptors, than either TTNPB or LG100268 alone, implying that occupancy of both RAR and RXR leads to greater activation of the promoter than either individual liganded retinoid receptor.

RXR Binds as a Heterodimer with VDR or RAR, but Not as a Homodimer, to Retinoid-responsive Sequences of the Human 24(OH)ase Promoter—Upon identification of the retinoid-responsive regions of the human 24(OH)ase promoter (see Fig. 2), the ability of those sequences to bind directly to RXR and RAR was tested. It was previously demonstrated that RXR-VDR heterodimers could bind to these regions (22, 23). Oligonucleotides were synthesized spanning the regions from −294 to −274 and from −174 to −151. Wild type nontransformed yeast extracts did not display DNA binding activity via either of these sequences (Fig. 4, A–C). Extracts from yeast transformed with a human RXRa expression vector (2) were able to bind to each of these sequences in the presence of extracts from yeast expressing human VDR or human RARa (Fig. 4, A–C) or human RARγ (data not shown). However, RXR did not bind alone to these sequences (Fig. 4, A–C) or to the entire responsive region between −314 and −121 (Fig. 4D) in the absence or presence of 9cRA (Fig. 4D and data not shown). Conversely, RXR was able to bind to an oligonucleotide containing a consensus direct repeat separated by 1 base pair (DR1) (Fig. 4D), as previously shown (17, 18). Therefore, we conclude that RXR is able to bind as a homodimer to the retinoid-responsive elements of the 24(OH)ase promoter but that it does form heterodimers with either VDR or RAR. Also, VDR alone or RAR alone or the combination of the two receptors did not bind to any of the DNA probes (Fig. 4, A–C, and data not shown). Binding of RXR:RAR heterodimers to the human 24(OH)ase VDRE sequences was surprising in that the DR3 motifs that they contain are thought to bind preferentially to RXR-VDR

![Transactivational analysis of 5' deletion mutant human 24(OH)ase promoter sequences.](image-url)
with the glucocorticoid response element deleted) receptor vector. Fig. 6 shows that either VDRE alone as a single copy (−294 to −274)-LUC or (−174 to −151)-LUC) was able to drive increased luciferase activity in response to 1,25(OH)2D3 (panels A and C) or LG100268 (panels A–C). 1,25(OH)2D3 treatment resulted in 12- and 11.3-fold induction of luciferase activity from the 3′ and 5′ VDREs, respectively (Fig. 6A). LG100268 alone yielded 4- and 3.5-fold induction from the 3′ and 5′ VDREs, respectively, while the combination of 1,25(OH)2D3 and LG100268 resulted in 17.4- and 16-fold responses from the 3′ and 5′ VDREs, respectively, which represent additive increases in luciferase activity (Fig. 6A). TTNPB also acted through these elements on its own and additively increased the activation elicited by LG100268 alone (Fig. 6B). In these experiments (Fig. 6, A and B), receptor plasmids were used at 0.1 μg/ml, and reporter constructs were at 5 μg/ml, typical concentrations used in our cotransfection experiments. To ensure that the additive effects observed were not due to monomeric receptor activation of individual reporter templates instead of heterodimer action on common templates, the amount of reporter used was decreased to 0.1 μg/ml. Fig. 6C shows that, using the VDRE1-ΔMTV-LUC reporter construct at this concentration, the overall luciferase values fall substantially, as expected (Fig. 6, compare panels A and B with panel C). However, the additive effect of 1,25(OH)2D3 with LG100268 or 9cRA is still observed, implying that the activity is on common templates and most likely through heterodimers. Therefore, these data show that each of the human 24(OH)ase VDREs is able to confer retinoid and 1,25(OH)2D3 responsiveness to a heterologous promoter and that saturating concentrations of each ligand in combination elicit greater reporter activity than either compound alone, implying that both receptors can be occupied with ligand to yield greater activation of the promoter.

24(OH)ase mRNA Is Induced by LG100268, TTNPB, 9cRA, or 1,25(OH)2D3 in Nontransfected CV-1 Cells: Combinations of Ligands Elicit Additive Effects—To determine if the additive effects of retinoids and 1,25(OH)2D3 in stimulation of the 24(OH)ase promoter observed in cotransfection assays were borne out in an endogenous setting, nontransfected CV-1 cells (which contain VDR, RARs, and RXRs; our data not shown) were treated with various concentrations of LG100268, TTNPB, 9cRA, or 1,25(OH)2D3 alone and in combination, and 24(OH)ase mRNA levels were assessed. Following ligand treat-
ment for 6 h, cells were harvested, and total RNA was isolated and analyzed via Northern blotting with a rat 24(OH)ase cDNA probe (30). Fig. 7 illustrates that 1,25(OH)2D3 at 10 nM was as efficacious as 125(OH)2D3 alone at nanomolar concentrations. Furthermore, the data also show that combination treatment with retinoids and 1,25(OH)2D3 or two selective retinoids leads to increased levels of 24(OH)ase RNA, as was also demonstrated in the cotransfection assays. This information taken together with the DNA-binding data lead to the conclusion that ligand occupation of either or both receptor partners results in activation of 24(OH)ase, with increased levels of stimulation observed upon liganding of both receptors.

**DISCUSSION**

The experiments described here indicate that RXR-selective ligands are able to activate the human 24(OH)ase promoter in cotransactivation assays in COS-1 cells through binding to RXR, which has the ability to form heterodimers with either VDR or RAR. These heterodimers form on previously defined VDREs (22, 23), which as demonstrated here, also act as retinoic acid response elements. RXR is not observed to homodimerize on the retinoid-responsive sequences of the promoter as determined by electrophoretic mobility shift assays. Activation via the VDREs is achieved by specific ligands for either receptor, and the presence of both ligands leads to increased stimulation. Pan-agonists such as 9cRA lead to greater activation than either retinoid receptor-specific ligand alone. While we describe ligand-bound RXR interacting with VDR or RAR to activate this promoter, we cannot rule out the possibility of the occurrence of another partner for RXR in vivo, such as an orphan receptor (19, 20). However, the experiments in nontransfected CV-1 cells (Fig. 7) showing that a combination of
point mutants of VDRE 1 (22) and 10 treated with vehicle (22) and 1,25(OH)2D3 and 9cRA were approximately equipotent activation of both ligands gave an increased effect. Additionally, 9cRA yielded a greater induction of RNA than either saturating doses gave additive or superadditive increases in the amount of 24(OH)ase RNA that was produced. Addition ally, 9cRA yielded a greater induction of RNA than either site 3 (−316 to −22) promoter sequence by retinoids is retained with mutations in the 3′ VDRE, it is diminished, and mutations in both VDREs essentially destroy the ability of retinoids to initiate transcription from the promoter. This work on the dissection of the human 24(OH)ase promoter in cotransfection/cotransactivation assays corroborates the effects previously observed in vitamin D-deficient and normally fed mice (27), i.e. an RXR-selective ligand is able to activate 24(OH)ase in the absence or presence of 1,25(OH)2D3.

The doses of 1,25(OH)2D3 that were administered to the mice maximized the induction of 24(OH)ase RNA, and the addition of LG100268 with 1,25(OH)2D3 had no effect on RNA levels at 32 h postdose (27). To test the effects of combinations of retinoids and 1,25(OH)2D3 at shorter times post dosing, we used CV-1 kidney cells as a model. CV-1 cells were found to produce 24(OH)ase RNA, and the levels of the RNA were modulated by the amount of 24(OH)ase RNA that was produced. Addition ally, 9cRA yielded a greater induction of RNA than either receptor-selective retinoid alone at the same concentrations. The combination of the two selective retinoids (LG100268 and TTNPB) also elicited an additive effect. Therefore, from these experiments it was demonstrated that while either a retinoid or 1,25(OH)2D3 alone induced 24(OH)ase RNA, the combination of both ligands gave an increased effect. Additionally, 1,25(OH)2D3 and 9cRA were approximately equipotent activators of 24(OH)ase in nontransfected CV-1 cells, since each ligand at 10−8 M gave a similar induction of RNA levels (9.1- and 9.4-fold, respectively).

We have concluded from the data described herein that saturating amounts of 1,25(OH)2D3 and an RXR ligand yields increased levels of 24(OH)ase RNA is difficult to reconcile with the involvement of another partner.

The formation of RXR-VDR and RXR-RAR heterodimers occurs with approximately equal affinity on each of the VDREs within the human 24(OH)ase promoter as determined by electrophoretic mobility shift assays. This was somewhat surprising, since the VDREs contain DR3 motifs that have been shown to be preferential binders of RXR-VDR heterodimers rather than other receptor combinations (33). However, Umesono et al. (33) used consensus DR sequences for their experiments. Upon comparison of consensus DR3-containing oligonucleotides and the nonconsensus 24(OH)ase VDREs, it was apparent that while the perfect DR3 sequence did have a higher affinity for RXR-VDR heterodimers than for RXR-RAR heterodimers, the human 24(OH)ase VDREs bound both heterodimer pairs with approximately equal affinity (Fig. 4). These VDRE sequences also confer responsiveness of the promoter to retinoids and 1,25(OH)2D3 in cotransactivation assays. Therefore, the VDREs within the human 24(OH)ase promoter also function as retinoic acid response elements.
Retinoids Induce 24(OH)ase through RXR Heterodimers

1,25(OH)2D3 and retinoids exert additive effects through RXR heterodimers at the VDREs within the 24(OH)ase promoter. To rule out the possibility that retinoids induce VDR or that vitamin D up-regulates retinoid receptors in these cells, CV-1 cells were treated for 6 h with 10 and 100 nM 9cRA or 1,25(OH)2D3 and 100 nM and 1 μM LG100268 (concentrations that gave effects in both analyses), and receptor levels were quantitated by ligand binding assays. Neither retinoid increased VDR levels, as assayed by specific binding of the extracts to tritiated 1,25(OH)2D3 (data not shown). Additionally, 1,25(OH)2D3 treatment of the cells did not increase RARs or RXRs as assayed by specific binding of the extracts to tritiated 9cRA (data not shown). Therefore, the additive effects of retinoids and 1,25(OH)2D3 via this promoter are not due to ligand-induced up-regulation of the receptor proteins.

Two other vitamin D target genes have also been shown to be regulated by retinoids: osteopontin (34, 35) and osteocalcin (36–38). Osteopontin RNA was induced in rats after a 4-h treatment with tRA regardless of vitamin A or D status and cooperated with 1,25(OH)2D3 to induce increased levels of osteopontin (34). Others have used a heterologous promoter construct containing two copies of the osteopontin VDRE to show differential effects of retinoids in cotransfection assays (35). Osteocalcin production has been shown to be stimulated by retinoids in primary human osteoblasts, and synergistic induction was observed with tRA and 1,25(OH)2D3 (37), although others have reported down-regulation of osteocalcin by 9cRA in cultured ROS17/2.8 osteosarcoma cells (36). Additionally, the human osteocalcin promoter has been shown to be stimulated by retinoids in cotransactivation assays in osteosarcoma cells through a sequence containing the VDRE (38). RXR ligands may have the ability to regulate a number of vitamin D (and thyroid hormone) target genes through perturbation of the structure of
the heterodimer, which may lead to activation or repression. The potential for dual hormone regulation may depend on a number of factors including hormonal status of the organism, cellular receptor complement, promoter context, and the presence of specific receptor-interacting cofactors.

Interestingly, it has been shown that combinations of retinoids and vitamin D compounds have additive or synergistic effects in promoting apoptosis or growth inhibition in breast cancer cells (39), prostate cancer cells (40), and leukemia cells (41) and in growth inhibition and differentiation of leukemia cells (42–44). Therefore, lower concentrations of two ligands together may achieve efficacies that would require increased amounts of either compound alone. Clinically, combination therapy of retinoid and vitamin D analogues may potentially provide a drug treatment regimen that would exhibit a greater therapeutic index than either agent could achieve alone in diseases such as cancer and leukemia.

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