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Synthetic LXXLL Peptides Antagonize 1,25-Dihydroxyvitamin D_3-Dependent Transcription

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Abstract The vitamin D receptor (VDR) is known to mediate the biological actions of 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) through its ability to regulate cellular programs of gene expression. We identified VDR- and retinoid X receptor (RXR)-interacting LXXLL peptides using a mammalian two-hybrid system and examined whether these molecules could block vitamin D and 9-*cis* retinoic acid (9-*cis* RA) response. Peptides were identified that were reactive to RXR alone as well as to both VDR and RXR. Peptide fusion proteins were then examined in MC3T3 E1 cells for their ability to block induction of the osteocalcin promoter by 1,25$(OH)_2D_3$ or stimulation of an RARE-TK reporter by 9-*cis* RA. Peptides that interacted with both VDR and RXR blocked 1,25$(OH)_2D_3$-dependent transcription by up to 75%. Peptides that interacted with RXR blocked 9-*cis* RA induced transcription. Two RXR-interacting peptides, however, were also found to block 1,25$(OH)_2D_3$ response effectively. These studies support the idea that comodulator recruitment is essential for VDR- and RXR-mediated gene expression and that RXR is required for 1,25$(OH)_2D_3$-induced osteocalcin gene transcription. This approach may represent a novel means of assessing the contribution of RXR in various endogenous biological responses to 1,25$(OH)_2D_3$. J. Cell. Biochem. 88: 252–258, 2003.

Key words: peptides; VDR; RXR; comodulators; transcription

The vitamin D receptor (VDR) is a member of the steroid and thyroid hormone receptor superfamily of transcription factors that mediates the biological actions of the calciotropic hormone 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) [Evans, 1989; Haussler et al., 1998]. Fundamental advances in our understanding of the mechanism of vitamin D action include the initial cloning and structural characterization of the VDR [Baker et al., 1988; Burmester et al., 1988] definition and characterization of DNA binding sites for the VDR on the OC and OP promoters [Kerner et al., 1989; Noda et al., 1990], and the discovery that VDR requires a partner protein subsequently identified as retinoid X receptor (RXR) [Sone et al., 1991b; Yu et al., 1991]. The central importance of VDR in calcium and phosphorus homeostasis has been highlighted in the human syndrome of hereditary 1,25$(OH)_2D_3$-resistant rickets [Malloy et al., 1999].

Active VDR is believed to be composed of a VDR/RXR heterodimer that binds to specific DNA sequences in response to 1,25$(OH)_2D_3$ [Mangelsdorf et al., 1995] Early biochemical as well as more recent molecular studies have revealed that the interaction between the VDR and RXR occurs via several α helical regions and is modulated by ligand much like that observed for other nuclear receptors [Whitfield et al., 1995; Jin et al., 1996]. The interaction between RXR and VDR is not dependent upon the presence of nucleic acids, suggesting that ligand-induced formation of the protein complex may precede its interaction with DNA [Sone et al., 1991a; Cheskis and Freedman, 1996; Love et al., 2002].

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As with other nuclear receptors, the RXR/VDR heterodimer functions to recruit additional factors that play an essential role in transcription [Rachez et al., 2000]. VDR is able to recruit a group of some 10–13 proteins termed D Receptor Interacting Proteins (DRIP complex) [Rachez et al., 1999; Ryu et al., 1999]. The single protein that mediates interaction with the VDR is $DRIP_{205}$ [Rachez and Freedman, 2000]. VDR is also known to interact with members of the p160 class of coactivators including Grip and Src-1, factors that are important to sex steroid receptor function. These factors appear to act directly to catalyze selective acetylation of histones and indirectly to recruit additional regulatory molecules such as histone acetyltransferases capable of eliciting related changes in chromatin structure [Rachez and Freedman, 2000]. The interaction of the VDR with comodulators such as $DRIP_{205}$ as well as Src-1 and Grip is mediated through LXXLL motifs that associate directly within the AF-2 region of nuclear receptors [McInerney et al., 1998; Perissi et al., 1999]. Recent crystallographic studies have defined the interaction between the LXXLL motif and the AF-2 cleft of the nuclear receptors created upon ligand-binding [Brzozowski et al., 1997; Shiau et al., 1998; Gampe et al., 2000].

Small LXXLL peptides have been identified that target the AF-2 domain of the nuclear receptor family [Chang et al., 1999; Hall et al., 2000]. These peptides mimic the LXXLL motifs found in at least three classes of comodulator proteins, display a certain degree of selectivity for members of the nuclear receptors family and are able to block ligand-induced receptor transactivation in reporter gene assays when linked to a large protein sequence such as the Gal4 DNA binding domain (DBD). We screened a subset of previously identified LXXLL peptides for reactivity with both VDR and RXR and show that several can block vitamin D response. Our results provide additional evidence that the AF-2 region of VDR is essential for $1,25(OH)_2D_3$ stimulated transcription and that RXR is a participant in vitamin D response.

MATERIALS AND METHODS

Reagents

Modified Eagles medium alpha (α-MEM) and Dulbecco's modified essential medium (DMEM) were purchased from Life Technologies (Grand Island, NY).

DNA Plasmids

pAVhVDR, pCMX-RXRα, pVDR-VP16, and pRXRα-VP16 were described earlier [Noda et al., 1990; Hall et al., 2000]. phOC 3900 and pGal4(5x)luc3 have been previously reported [Noda et al., 1990; Hall et al., 2000]. pTK-RXRE was a gift from Dr. David Mangelsdorf (University of Texas-Southwestern). pM-Src-1 (residues 621–765), pM-Grip (residues 629–760), pM-C33, pM-D47, pM- EBIP41, pM-EBIP44, pM-EBIP45, pM-EBIP53, pM-EBIP60, pM-EBIP66, pM-EBIP76, pM-EBIP87, pM-EBIP92, and pM-F6 prepared from the pM vector (Clontech, San Diego, CA) containing Gal4 (residues 1–147) were also previously described [Chang et al., 1999; Hall et al., 2000].

Cell Culture

COS7 cells were cultured in DMEM supplemented with 10% fetal bovine serum. MC3T3-E1 cells were cultured in α-MEM supplemented with 10% fetal bovine serum. Transient transfections were carried out in 24-well plates using Lipofectamine Plus as described by the manufacturer. A total of 500–600 ng of DNA per well was introduced into cells. Cells were harvested 24 h later, lysed, and extracts examined for both β-galactosidase and luciferase activities as previously described [Jin et al., 1996]. Luciferase activity was normalized for transfection efficiency using β-galactosidase activity. Individual transfection assays were performed at least three times.

Statistics

Triplicates were averaged and standard deviations determined. Statistical significance was determined using ANOVA.

RESULTS

Src-1 and Grip Peptides Inhibit $1,25(OH)_2D_3$ Induced Osteocalcin Promoter Transactivation

We first examined the ability of three tandem cognate LXXLL motifs derived from Src-1 and Grip fused to the larger Gal4 DBD to suppress $1,25(OH)_2D_3$ stimulated transactivation in the MC3T3 E1 cell line (Fig. 1a). As can be seen in Figure 1b, while $1,25(OH)_2D_3$ strongly induced the OC promoter-driven luciferase reporter, introduction of the expression vector for Grip NR boxes inhibited this transcriptional induction in a dose-dependent manner by as much as

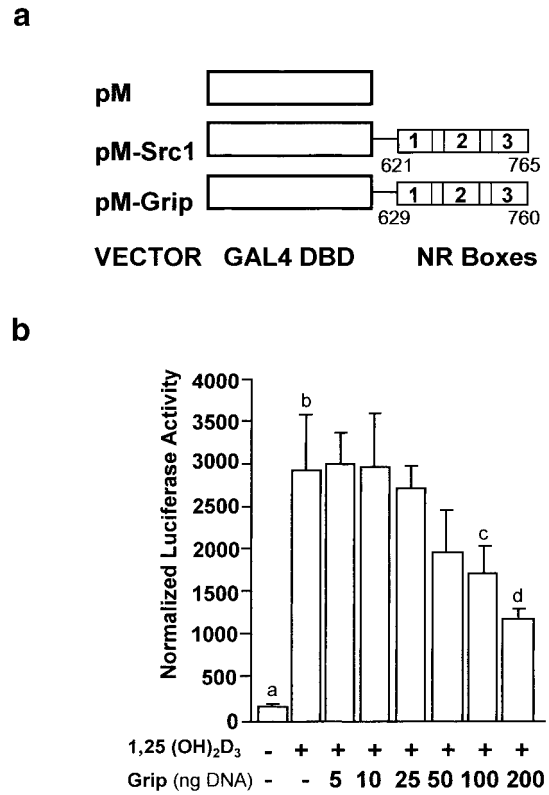

Fig. 1. Gal4 DBD-Src-1 and Gal4 DBD-Grip fusion proteins suppress $1,25(OH)_2D_3$ stimulated transcription from the osteocalcin promoter in MC3T3 E1 cells. **a**: Peptide expression constructs employed. Each construct encodes an amino terminal Gal4 DNA binding domain (residues 1–147) fused to either Src-1 (residues 621–765 containing three LXXLL (NR) boxes) or Grip (residues 629–760 containing three LXXLL (NR) boxes). **b**: A total of 500 ng of DNA/well comprised of 250 ng of phOC-3900, 50 ng RSV-βGal, and up to 200 ng of pM-Grip (adjusted with pM alone) was transfected into MC3T3 E1 cells plated 24 h earlier. Cells were treated with vehicle or $1,25(OH)_2D_3$ (10^{-8} M) for 24 h. Activities are the means of triplicate assays ± SD. b is significantly different from a, c, and d; $P < 0.05$.

60%. Since these peptides interact directly with the AF-2 domain of the nuclear receptor family [Hall et al., 2000], our results indicate that blockade of this region prevents transcriptional activation by the VDR (or RXR) and supports the prevalent idea that the receptor must recruit an additional factor(s) through that site to manifest function. They also establish a system, whereby LXXLL-containing peptides can be evaluated for antagonist properties.

LXXLL-Gal4 DBD Fusion Peptides Interact With VDR

Phage display screening was used previously with ERα and ERβ as nuclear receptor targets to identify short LXXLL containing peptides that were capable of selectively blocking the transactivating ability of ERα or ERβ when fused to the DBD of Gal4 [Hall et al., 2000]. Many of these peptides interacted with additional nuclear receptors as well [Hall et al., 2000]. We selected a subset of these peptide-Gal4 DBD fusion genes whose sequences have been previously reported [Chang et al., 1999; Hall et al., 2000] and assessed their capacity to produce proteins capable of interacting with either VDR or RXRα in a mammalian two-hybrid system. As is evident in Figure 2a (left panel), both the Src-1 and Grip NR boxes as well as a number of peptide-Gal4 DBD fusion proteins were able to interact with the VDR in the presence of $1,25(OH)_2D_3$ as indicated by levels of luciferase activity that are well above those observed with the Gal4 DBD control alone (pM). Positively interacting peptides, including C33, D47, and EBIP41 and EBIP44 (Fig. 2a, left panel) each varied in relative activity. Several peptides did

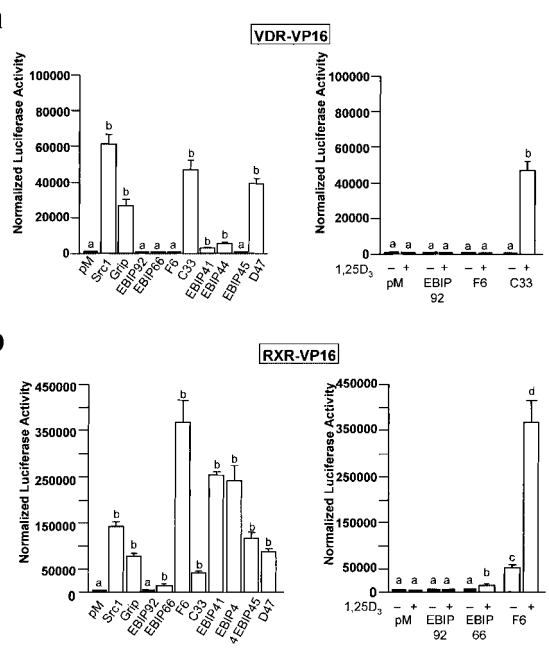

Fig. 2. Gal4 DBD LXXLL fusion proteins interact with VDR and/or RXRα in a mammalian two-hybrid system. A total of 600 ng of DNA/well comprised of 250 ng of pGal4(5x)luc3, 50 ng RSV-βGal, 50 ng of either pM vector or the indicated fusion proteins and 50 ng of either phVDR-VP16 (**a**) or pRXRα-VP16 (**b**) was transfected into MC3T3 E1 cells plated 24 h earlier. Cells were treated without or with $1,25(OH)_2D_3$ (10^{-8} M) (A) or 9-*cis* RA (10^{-6} M) (B) as indicated, harvested 24 h later and evaluated for both β-galactosidase and luciferase activities. Activities are the means of triplicate assays ± SD. A: b is different from a, $P < 0.05$. B: Left panel: b is significantly different from a, $P < 0.05$; right panel: b, c, and d are significantly different from a, $P < 0.05$ and c is significantly different from D, $P < 0.05$.

not interact with the VDR including EBIP45, EBIP66, EBIP92, and F6 (Fig. 2a (left panel). The activities of several of the chimeric peptides in the absence and presence of $1,25(OH)_2D_3$ are observed in Figure 2a (right panel) In the absence of ligand, the basal activity for these peptides as well as those seen in the left panel of Figure 2a was similar to that seen with the Gal 4 DBD alone (pM vector).

LXXLL-Gal4 DBD Fusion Peptides That Interact With RXRα

Examination of each of these peptides in the presence of RXRα-VP16 and 9-*cis* RA revealed that while several of the VDR reactive peptides also bound to RXR (C33, D47, EBIP41, and EBIP44 (Fig. 2b, left panel)), a number of peptides that were unreactive to VDR interacted significantly with RXRα including F6 and EBIP66 (Fig. 2b, left panel). As with VDR, EBIP92 showed no reactivity with RXR. Interestingly, while RXRα exhibited only basal activity with most of the peptides in the absence of 9-*cis* RA, its interaction with the F6 peptide was significantly above baseline (Fig. 2b, right panel). This suggests that the Gal 4 DBD-F6 protein can interact with unliganded and liganded RXRα. The results of these studies with VDR and RXRα indicate that the peptides examined exhibit three patterns of reactivity: interaction with (1) both RXRα and VDR, (2) RXRα alone, and (3) neither receptor.

Inhibition of $1,25(OH)_2D_3$ or 9-*cis* RA Response With LXXLL-Gal4 DBD Chimeric Proteins

Having identified several LXXLL peptides that appear to exhibit VDR and RXRα interaction selectivity, we then assessed whether these peptides could block either VDR- or RXRα-induced transactivation. We used the human OC promoter (as in Fig. 1) as a target for VDR, whereas we used the TK promoter containing three copies of the RXRE derived from the CRABPII gene [Mangelsdorf et al., 1991] as a target for RXR. RXR expression in MC3T3 E1 cells was insufficient to mediate 9-*cis* RA response such that endogenous levels were supplemented through the use of an RXRα expression vector. As seen in Figure 3, while $1,25(OH)_2D_3$-stimulated the OC promoter strongly in the absence of peptide (pM vector alone), the VDR-reactive LXXLL peptides Src-1, Grip, C33, D47, and EBIP41, and EBIP44 all suppressed the

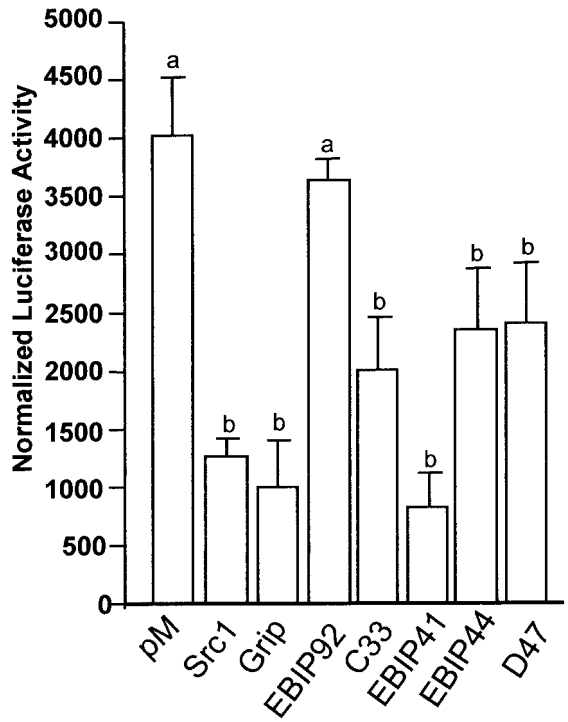

Fig. 3. Gal4 DBD fusion peptides C33, D42, D47 but not E92, suppress $1,25(OH)_2D_3$-stimulated transcription from the osteocalcin promoter in MC3T3 E1 cells. A total of 500 ng of DNA/well comprised of 250 ng of phOC-3900, 50 ng RSV-βGal, and 200 ng of either pM-Src-1, pM-Grip, pM-C33, pM-D47, pM-EBIP41, pM-EBIP44, or pM-EBIP92 (adjusted with pM alone) was transfected MC3T3 E1 cells plated 24 h earlier. Cells were treated with vehicle or $1,25(OH)_2D_3$ (10^{-8} M) for 24 h, harvested and evaluated for both β-galactosidase and luciferase activities. Activities are the means of triplicate assays ± SD. b is different from a, $P < 0.05$.

receptor's activity when used at a concentration of 200 ng of plasmid. The findings in Figure 4 reveal that peptide Src-1 as well as the RXRα-reactive peptides F6 and EBIP66 each suppressed 9-*cis* RA-induced activity compared to the unreactive control peptide EBIP92. These results indicate that single copies of receptor-specific LXXLL peptides are capable of interacting with nuclear receptor targets with sufficient affinity to antagonize normal transcriptional ability.

RXRα-Selective LXXLL Peptides Block VDR-Mediated Transactivation

RXR is believed to participate directly as a heterodimeric partner in VDR mediated transactivation [Sone et al., 1991a; Yu et al., 1991; Mangelsdorf et al., 1995; Whitfield et al., 1995; Jin et al., 1996]. Thus, a peptide capable of inhibiting 9-*cis* RA-activated, RXR-mediated

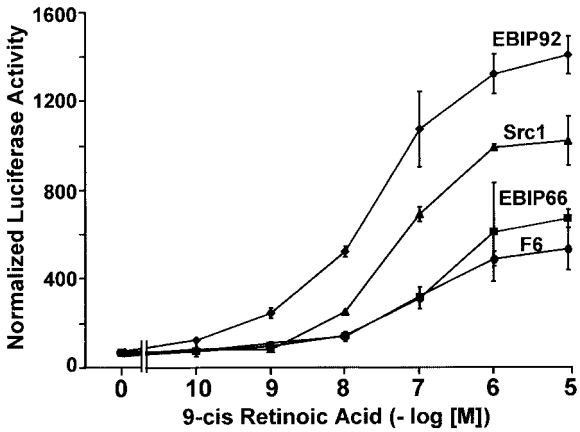

Fig. 4. Gal4 DBD fusion peptides Src-1, F6, and E66 inhibit 9-*cis* RA induced transcription in MC3T3 E1 cells. A total of 600 ng of DNA/well comprised of 250 ng of pTK-RXRE, 50 ng RSV-βGal, and up to 250 ng of either pM-Src-1, pM-E66, pM-F6, or pM-E92 (adjusted with pM to 600 ng) was transfected into MC3T3 E1 cells plated 24 h earlier. Cells were treated with vehicle or increasing concentrations of 9-*cis* RA for 24 h, harvested and evaluated for both β-galactosidase and luciferase activities. Luciferase activity in the presence of pM-E92 was not different from that with pM alone. Activities are the means of triplicate assays ± SD.

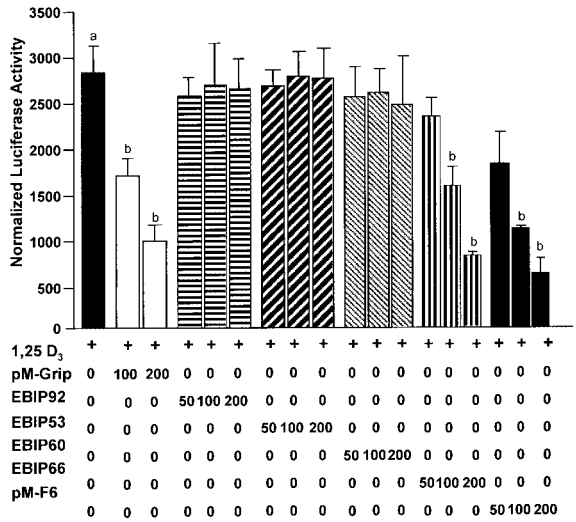

Fig. 5. Gal4 DBD fusion peptides F6 and E66 suppress $1,25(OH)_2D_3$-stimulated transcription from the osteocalcin promoter in MC3T3 E1 cells. A total of 500 ng of DNA/well comprised of 250 ng of phOC-3900, 50 ng RSV-βGal, and up to 200 ng of either pM-Grip, pM-E92, pM-E53, pM-E60, pM-E66, or pM-F6 (adjusted with pM to total 600 ng) was transfected into MC3T3 E1 cells plated 24 h earlier. Cells were treated with vehicle or $1,25(OH)_2D_3$ (10^{-8} M) for 24 h, harvested and evaluated for both β-galactosidase and luciferase activities. Activities are the means of triplicate assays ± SD. b is significantly different from a, $P < 0.05$.

transactivation should function also to inhibit $1,25(OH)_2D_3$-stimulated transcription. We therefore introduced the osteocalcin promoter/luciferase reporter gene into MC3T3 E1 cells in the absence or presence of increasing concentrations of EBIP53, EBIP60, EBIP66, or F6 and assessed the ability of these peptides to inhibit $1,25(OH)_2D_3$ response. As can be seen in Figure 5, EBIP53, EBIP60, and EBIP92 were unreactive. Both F6 and EBIP66, however, significantly suppressed VDR transactivation in a dose-dependent fashion. These experiments support the idea that RXR is an essential participant in $1,25(OH)_2D_3$-stimulated transcription of the human OC and 24OHase genes.

DISCUSSION

The VDR functions in response to $1,25(OH)_2D_3$ to alter the expression of specific genes in a tissue-selective fashion within cells [Evans, 1989; Haussler et al., 1998]. The nuclear receptor family, of which the VDR is a member, acts to recruit additional comodulators to regulated promoters [Lemon and Freedman, 1996]. This recruitment involves an activation domain on the receptor surface and an LXXLL motif located within the comodulator itself [McInerney et al., 1998; Hall et al., 2000]. We show here that several LXXLL motifs isolated from the Src1 and Grip comodulators as well as from a phage display screen can interact with the AF2 domain of the VDR and can function as antagonists when linked to a chimeric protein such as the Gal 4 DBD. We also demonstrate that LXXLL peptides directed specifically towards RXR and capable of suppressing 9-*cis* RA stimulated transcription also inhibit $1,25(OH)_2D_3$ response. Our observations support the current hypothesis that the AF2 domain of the VDR is essential for $1,25(OH)_2D_3$ stimulated transactivation and that RXR not only functions as a heterodimeric partner of VDR but may also facilitate transactivation.

RXR is widely believed to be a necessary partner for VDR activity in response to $1,25(OH)_2D_3$ [Sone et al., 1991a,b; Whitfield et al., 1995; Cheskis and Freedman, 1996; Jin et al., 1996; Lemon and Freedman, 1996; Thompson et al., 1998; Thompson et al., 2001]. Despite this evidence, the absolute requirement for RXR in diverse $1,25(OH)_2D_3$-stimulated responses has not been determined largely because the RXRs are derived from three different genes and

almost ubiquitously expressed in mammalian cells [Evans, 1989; Mangelsdorf et al., 1995]. Our current studies lend additional support for the above prevailing idea by showing that inhibition of endogenous RXR in cells (EBIP66 and F6) can limit vitamin D response. Several of the RXR-selective peptides (EBIP53 and EBIP60) did not inhibit vitamin D response. It is possible that these particular peptides do not display an affinity for RXR that is sufficient to prevent recruitment of endogenous coactivators and therefore cannot block vitamin D response.

RXR is also believed to participate directly in the transactivation process [Thompson et al., 2001]. Accordingly, mutations in the activation domain of RXR were found to blunt the ability of VDR to activate transcription, thus acting as dominant negative inhibitors of VDR action. These findings indicate that both receptors likely participate in comodulator interaction essential to transactivation. Our studies support this view in that LXXLL peptides can both interact with the RXR AF-2 and block VDR transcriptional activity. Interestingly, the residues within the RXR AF2 region that mediate RXR homodimer and RXR/VDR heterodimer activity are different. This may reflect a difference in the organization of the receptors on unique DNA sequence elements, promoter context or, alternatively, selectivity in the cofactors that are recruited to those surfaces. The ability of peptides to exhibit selectivity between VDR and RXR, as seen in these studies, may also be indicative of receptor preferences for endogenous comodulators.

Ligands activate nuclear receptors by inducing receptor conformations capable of subsequent interaction with LXXLL containing coactivators. In view of this, while the F6 peptide can be argued to interact with both unliganded and liganded RXR, it is surprising that peptides that interact in a 9-*cis* RA-dependent manner with RXR such as EBIP66 can block a vitamin D-induced response in the absence of the RXR ligand. This finding suggests the possibility that VDR itself may act to induce a conformation within RXR that is similar although perhaps not identical to that induced by 9-*cis* RA. This observation supports a similar conclusion drawn from the finding that specific mutations within the LXXLL binding site of RXR (different from those that inhibit 9-*cis* RA dependent RXR activity) block vitamin D response [Thompson et al., 2001].

Vitamin D exerts highly pleiotropic actions on a variety of cell types through the regulation of numerous genes [Haussler et al., 1998; DeLuca and Cantorna, 2001]. While the actions of VDR as an RXR heterodimer on genes such as OP, OC, and 24OHase appear well supported, it is possible that additional regulatory paradigms may exist where the VDR functions differently. For example, recent evidence suggests that the VDR may function in certain settings as a monomer. Accordingly, the VDR appears to bind to an unusual DNA sequence half site on the GM-CSF promoter and to interact with proteins other than RXR such as NFAT1 and c-Jun [Towers and Freedman, 1998]. In addition, VDRE sequences that mediate suppression in the human parathyroid hormone gene may also require a protein other than RXR [Mackey et al., 1996]. These studies support the idea that the VDR could function through several different mechanisms to regulate transcription. Thus, RXR-selective peptides that are capable of inhibiting responses that require RXR such as those identified herein may be useful in defining the role of RXR in a wide variety of individual vitamin D-induced activities. Current efforts focus on using these peptides to inhibit specific endogenous responses to $1,25(OH)_2D_3$ in cells and in vivo.

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