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Expression of the Vitamin D and the Retinoid X Receptors in *Saccharomyces cerevisiae:* Alternative In Vivo Models for Ligand-Induced Transactivation

Yvonne Berghöfer-Hochheimer,[1] Christian Zurek,[1] Gernot Langer,[2] and Thomas Munder[1]*

[1]Department of Cell and Molecular Biology, Hans-Knöll-Institut für Naturstoff-Forschung e.V., Jena, Germany
[2]Research Laboratories of Schering AG, Berlin, Germany

Abstract The transcription factors of the nuclear hormone receptor familiy regulate gene expression via a complex network of macromolecular interactions. The ligand dependent activity of the vitamin D receptor is of particular interest because it modulates gene expression by the heterodimeric interaction with retinoid X receptors. We report here that individual functions of the vitamin D receptor including DNA-binding, homo- and heterodimerization and transactivation can be reconstituted in the yeast *Saccharomyces cerevisiae.* Interestingly, the simultaneous expression of the native vitamin D receptor and the retinoid X receptor β resulted in a ligand independent transactivation of the *lacZ* reporter gene coupled to a mouse osteopontin vitamin D response element. However, homodimerization of the vitamin D receptor and heterodimerization were strongly enhanced upon ligand binding, when the receptors were expressed as fusion proteins with the Gal4 transcription factor in a yeast two-hybrid system. Furthermore, transactivating activity of a Gal4-fused vitamin D receptor was induced by vitamin D in a one-hybrid system devoid of retinoid X receptors. In addition, both Gal4-based systems behaved similar with regard to their dose-dependent response to vitamin D and related compounds when compared to the transcriptional activity of the vitamin D receptor in transiently transfected MCF-7 cells. Our results point out that specific ligands strongly enhanced receptor dimerization and induced transactivation in yeast and in MCF-7 cells. The constitutive transactivation by vitamin D receptor-retinoid X receptor heterodimers in yeast, depending on DNA binding of the receptors, strongly argues for the existence of cofactors, which are absent in yeast, but play a fundamental role in gene regulation in higher eukaryotic organisms. J. Cell. Biochem. 66:184–196, 1997.

Key words: vitamin D receptor; retinoid X receptor; transactivation systems; vitamin D regulation; *Saccharomyces cerevisiae*

The biological effects of vitamin D (1,25 dihydroxyvitamin D3) are mediated by a nuclear protein, the vitamin D receptor (VDR), a ligand-inducible transcription factor, which belongs to the superfamily of nuclear receptors [Evans, 1988; Mangelsdorf et al., 1995]. These receptors associate as homodimers or as heterodimers with other nuclear factors and bind to specific ligand response elements within the promoter region of target genes to regulate their transcription in a ligand dependent fashion [Tsai and O'Malley, 1994]. Vitamin D response elements (VDREs) consist of direct hexameric repeats, which are separated by a defined number of spacing nucleotides [Umesono et al., 1991]. Nonetheless, natural VDREs may diverge from the consensus sequence AGGTCA, presumably reflecting one of the parameters that determine the diversity of vitamin D controlled transcriptional networks [Carlberg, 1995]. In principle, VDR homodimers bind to specific VDREs. This protein-DNA interaction is strongly enhanced by heterodimerization of the VDR with the retinoid X receptor (RXR), which occurs in different

Abbreviations used: AD, activating domain; CRT, calreticulin; DBD, DNA-binding domain; DMSO, dimethyl sulfoxide; LBD, ligand-binding domain; 9-*cis* RA, 9-*cis*-retinoic acid; ONPG, o-nitrophenyl-β-D-galactoside; RXR, retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin D response element; vitamin D, 1,25-dihydroxyvitamin D_3; X-Gal, 5-bromo-4-chloro-3-indoyl β-D-galactoside.

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*Correspondence to: Thomas Munder, Hans-Knöll-Institut für Naturstoff-Forschung e.V., Department of Cell and Molecular Biology, Beutenbergstr. 11, 07745 Jena, Germany.

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isoforms [Mangelsdorf et al., 1990; Leid et al., 1992; Yu et al., 1991; Kliewer et al., 1992]. Additionally, RXRs have been shown to increase the transcriptional response to VDR activity in the presence of ligand [MacDonald et al., 1993; Ferrara et al., 1994].

Investigation of receptor dimerization and transcriptional activity has to be considered with respect to the background of endogenously and cell-type specific expressed RXRs. Furthermore, the interpretation of this regulatory network is rendered more difficult by coregulators, which modulate the activity of the nuclear receptors [Onate et al., 1995; Kurokawa et al., 1995; Chen and Evans, 1995]. To circumvent the complexity of mammalian systems, the activities of several mammalian nuclear receptors were reconstituted in yeast [Schena and Yamamoto, 1988; Sande and Privalsky, 1994; Allegretto et al., 1993; Heery et al., 1994; Hall et al., 1993; Wang et al., 1995]. Yeast possess the necessary cellular background for expression of heterologous proteins and are devoid of endogenous nuclear receptors, providing a well-defined eukaryotic in vivo system. Indeed, ligand activated VDR homodimers have been shown to increase transcriptional activity in the yeast *Saccharomyces cerevisiae* [McDonnell et al., 1989]. These studies were performed before the discovery of the RXRs.

Recent transactivation studies have shown that VDR-RXR mediated activation of transcription can be reconstituted in *Saccharomyces cerevisiae*, but the influence of the ligands on transactivation is strongly affected by the respective response elements and RXR isoforms [Jin and Pike, 1996; Kephart et al., 1996].

Using alternative yeast expression systems we now show, that the activity of the human VDR is strictly dependent on the presence of ligand(s). Our primary goal was to uncouple dimerization of the receptors from DNA-binding by taking advantage of the DNA-binding properties of the yeast Gal4 transactivator. We constructed chimeric receptors with a Gal4-moiety and investigated heterodimerization between the VDR and the RXR in the yeast two-hybrid system [Fields and Song, 1989]. This system utilizes the reconstituted Gal4 transcription factor, which drives transactivation of the reporter gene *lacZ*. In a one-hybrid system, lacking RXR, we investigated the intrinsic transactivation function of the VDR. In this system, the induction of transcription of *lacZ* was mediated only by the chimeric VDR. Our results indicated that binding of the natural ligand vitamin D at least promoted homo- and heterodimerization as well as transactivation of the VDR.

Finally, we analyzed three vitamin D–related drugs for their ability to induce activity of the VDR in the Gal4-based systems and in the mammalian MCF-7 cell line, expressing endogenous VDR and RXR(s). Our results indicated a similar responsiveness to vitamin D and its analoges in yeast and in MCF-7 cells. We, therefore, propose our ligand-inducible systems as in vivo tools for analyzing novel vitamin D–related drugs, which might expand the therapeutic applications for vitamin D in the fields of osteoporosis, psoriasis, and inhibition of cell proliferation.

MATERIALS AND METHODS

Vitamin D and Compounds

Vitamin D (1,25 Dihydroxyvitamin D3) was obtained from Solvay-Duphar (DA WEESP, The Netherlands). The compounds A, B, and C were synthesized and purified at Schering AG. The ligand 9-*cis*-retinoic acid was obtained from Biomol (Hamburg, Germany).

Plasmid Constructions

All two-hybrid constructs, containing the Gal4-DNA binding domain (DBD) or the Gal4 activation domain (AD), used the pGBT9 and pGAD424 yeast expression vectors [Bartel et al., 1993]. The one-hybrid constructs, containing the Gal4-DBD, were based on the yeast expression vector pAS2-1, a derivative of $pAS1_{CYH2}$ [Harper et al., 1993]. To produce pGBT9-VDR (DBD-VDR) and pGAD424-VDR (AD-VDR), human VDR cDNA [Baker et al., 1988] was amplified by polymerase chain reaction using the following primer pairs: 5′-TGATGGAATTCATGGCGGCCAGCACTTC-3′ and 5′-GGTTAGGATCCACAGGAGAGAGA-ATGGGCTG-3′. Restriction sites are underlined. The 1.44 kbp product was digested with *Eco*RI and *Bam*HI and inserted into pGBT9 and pGAD424. To obtain fusions between the Gal4-DBD and the VDR-DNA binding domain (VDR-DBD, amino acids 4–124) or the VDR ligand binding domain (VDR-LBD, amino acids 125–423), VDR cDNA was amplified with the following primer pairs: 5′-TGATGGAATTCATG-

GCGGCCAGCACTTC-3′ and 5′-CTGCTGGGATCCCAGCTTGGGCCG-3′ or 5′-GGCCCGAATTCTCTGAGGAGCAGC-3′ and 5′-GGTTAGGATCCACAGGAGAGAGAATGGGCTG-3′. The PCR products were digested with *Eco*RI and *Bam*HI and inserted into pGBT9 to produce pGBT9-VDR-DBD and pGBT9-VDR-LBD. To generate pGAD424-RXR (AD-RXR), the ligand binding domain of RXR β (amino acids 264–508) was amplified from a human skeletal muscle cDNA library (Clontech Laboratories, Palo Alto, CA) with the primers: 5′-CACTGAATTCAAGAGGGAGGCGGTACAG-3′ and 5′-CGATGGATCCGTCACCAATGAGCTTG-3′. The PCR product was digested with *Eco*RI and *Bam*HI and inserted into pGAD424. To produce pGAD424-Calreticulin (AD-CRT), CRT cDNA [Fliegel et al., 1989] was amplified with the following primers: 5′-CGGAATTCATGCTGCTCCCTGTGCCGCTG-3′ and 5′-AGCTCAGTCGACTTAGCGGATCCAGGTGCCTTTGT-3′. The 0.88 kbp PCR product was digested with *Eco*RI and *Sal*I and inserted into pGAD424. The Gal4-receptor chimeras for the one-hybrid studies were constructed as follows: VDR cDNA was amplified with the primer pairs 5′-CCGGAGAATTCCCCCGGATCTGTGG-3′ and 5′-GGTTAGGATCCACAGGAGAGAGATGGGCTG-3′. The product was digested with *Eco*RI and *Bam*HI and inserted into pAS2-1 to obtain pAS-VDR20-423 (AS-VDR20-423). The VDR insert of pGBT9 was excised and inserted into *Eco*RI-*Bam*HI-digested pAS2-1 to generate pAS-VDR (AS-VDR). These vectors generate in frame-fusions between the Gal4-DBD and full-length VDR or truncated VDR, lacking 20 amino acids at the amino terminus. The high copy number plasmids Yepubstu (a gift from R. Kölling) and pTM159, a derivative of Yep6 [Struhl et al., 1979] were used to drive transcription of the human VDR or mouse RXR β2 by the copper-responsive yeast metallothionein (*CUP1*) promoter. Yepubstu encodes ubiquitin, which is fused to the 5′-end of the receptors. After translation, the ubiquitin moiety is removed by host processing enzymes, releasing unfused receptor molecules [Butt et al., 1988]. An *Eco*RI-*Bam*HI-fragment of the full-length human VDR cDNA was inserted into the multiple cloning site of pTM159. Initiation in pTM159-VDR was from the natural AUG of the receptor. To create an RXR-ubiquitin fusion, mouse RXR β2 cDNA [Leid et al., 1992] was amplified with the following primer pairs: 5′-TCTGGGACCGGATTCCCGAAG-3′ and 5′-CTGCCCAGGTACCAGTCCTCCTCAGTG-3′. Amplified RXR cDNA (1.4 kbp) was digested with *Kpn*I and inserted into *Stu*I-*Kpn*I digested Yepubstu to obtain Yep-RXR. All cDNAs were amplified as follows: Template DNA was denatured at 94°C for 5 min prior to amplification (30 cycles). Cycle conditions were: denaturation at 92°C for 1 min, annealing for 1 min according to the melting point of the primers (between 50° and 60°C), polymerisation at 72°C for 2 min. The standard $MgCl_2$ concentration was used for amplification with *Pwo*-polymerase (Boehringer, Mannheim, Germany). The yeast reporter plasmid pTM108-DR3 was constructed by inserting annealed oligonucleotides with *Xho*I-overhangs upstream of the cytochrome c_1 TATA box of pTM108, a derivative of pSS [Schena and Yamamoto, 1988]. In pTM108, *lacZ* is used as reporter gene. The synthetic DR3-VDRE (sense strand 5′-TCGAGAA**GGTTCA**CGA**GGTTCA** CGTC-3′) was derived from a region of the mouse SSP-1 promoter [Noda et al., 1990]. Direct repeats are shown in bold letters. For construction of the mammalian reporter plasmid pDR3-luc, we obtained a synthetic vitamin D response element (ANF3) from C. Carlberg, which was derived from the rat atrial natriuretic factor 3 (sense strand 5′-CTAGAAG**AGGTCA**TGA**AGGACA** AGATC. Direct repeats are shown in bold letters. *Xba*I-overhangs for the insertion of the ANF3-VDRE into pDR3-luc are underlined. The chloramphenicol acetyl transferase, located downstream of the response element in a pBLCAT2 based construct was replaced by the luciferase reporter gene, isolated from pGL2-Basic (Serva, Heidelberg, Germany) by *Bgl*II-*Sma*I digestion, and cloned into the likewise digested pBLCAT2. Sequences of all PCR products were confirmed by dideoxy-sequencing with Sequenase 2.0 (Amersham, Braunschweig, Germany).

Strains

For cloning strategies *Escherichia coli* strain DH1 (a gift from J.P. Jost) was used. The expression plasmids pTM159-VDR and Yep-RXR together with pTM108-DR3 were transformed into yeast strain YJH487 (*MAT*α, *his3-Δ1, ade2, ura3-53, trp1-289, leu2-3*), a gift from H. Hegemann. The yeast two-hybrid and one-hybrid system were propagated in strains SFY526 (*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4-542, gal80-538,*

URA3::GAL1-lacZ) and Y190 (MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, LYS2::GAL1$_{UAS}$-HIS3$_{TATA}$-HIS3, URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ (Clontech Laboratories).

Yeast β-Galactosidase Assays

Yeast transformations were performed as previously described [Klebe et al., 1983] and transformants were selected on SD medium (2% glucose, 0.67% yeast nitrogen base without amino acids), supplemented with the appropriate nutrients. Individual clones were transfered onto selective SD media, supplemented with either 0.001 mM vitamin D or with 0.5% DMSO as a control and incubated at 30°C for 2 days. Enzymatic activity of β-galactosidase, the product of *lacZ*, was used to indicate reconstitution of the Gal4-transactivator via interaction of the fusion proteins in the two-hybrid system and to indicate transcriptional activities of fused and unfused receptors in the one-hybrid system and in the transactivation assay. For the qualitative detection of β-galactosidase activity, the colony-lift filter assay was used. The individual colonies were transfered to Whatman paper filters (Sigma, Deisenhofer, Germany), which were frozen with liquid nitrogen for a few seconds and thawed afterwards to permeabilize the cells. The filters were placed onto paper filters that were presoaked with X-Gal solution (60 mM Na_2 HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, 0.33 mg/ml X-Gal) and incubated at 30°C. For the quantitative analysis of β-galactosidase yeast cultures in the early log-phase were diluted to an $A_{600\,nm}$ of 0.05 and incubated, until an $A_{600\,nm}$ of 0.2 was achieved. Aliquots of 0.1 ml cell suspension were plated into 96-well culture dishes and supplemented with 0.005 ml aliquots of different concentrations of vitamin D or the related compounds, dissolved in 10% DMSO. For monitoring transactivation, 0–0.1 mM $CuSO_4$ (final concentrations) and/or different concentrations of vitamin D and 9-*cis* RA were added to the cells. The cultures were incubated with gentle shaking at 30°C in the dark. After 16 h, $A_{600\,nm}$ was determined. The cells were lysed with 0.025 ml lysis-detection buffer at 37°C and $A_{415\,nm}$ was read after 20–150 min. Lysis-detection buffer was the same as X-Gal solution with the following exceptions: X-Gal was replaced by ONPG (7.7 mg/ml) and Triton X-100 (0.6%), SDS (0.2%) and Tris-HCl (0.08 M; pH 7.5) were added. Normalized β-galactosidase values were determined from the mean value of 8 individual samples as follows: $A_{415\,nm}/A_{600\,nm} \times 1{,}000$/min developed. Each experiment has been performed at least three times.

Cell Culture, Transfections, and Luciferase Assay

The human breast cancer cell line MCF-7 (American Type Culture Collection HTB 22, Rockville, MD) was grown in Dulbecco's medium (DMEM) without phenol-red (Life Technologies, Bethesda, MA) and supplemented with 2 mM glutamine, penicillin (10 U/ml), streptomycin (0.01 mg/ml) and 10% fetal calf serum (FCS). For transfections, cells were harvested by trypsinization and resuspended in DMEM with 5% charcoal treated FCS. Cells (2×10^4) were seeded per well of a 96-well dish and grown for 6 h. For all transfections, a 1:5 ratio of the reporter gene plasmid and lipofectin was used. Two separate stock solutions were prepared containing the appropriate amount of plasmid DNA and lipofectin in 0.5 ml Opti-MEM (Life Technologies). The stock solutions were combined and incubated for 20 min at room temperature. Opti-MEM was added to reach a final concentration of 100 ng plasmid DNA and 500 ng lipofectin per 0.1 ml. Per individual well 0.1 ml was added to the cells, which were washed with phosphate-buffered saline (0.01 M phosphate-buffer, 0.0027 M KCl, 0.137 M NaCl). After 12 h, transfection medium was replaced by DMEM, supplemented with 5% charcoal treated FCS and the appropriate concentrations of vitamin D or compounds in 0.1% DMSO. Control cells only received 0.1% DMSO. Incubation of the cells was continued for 24 h. For detection of luciferase activity, cells were washed with phosphate-buffered saline and incubated with 0.03 ml of cell culture lysis reagent (Promega, Heidelberg, Germany) for 20 min at room temperature. Luminescence was measured after injection of 0.1 ml Luciferase assay reagent using the ML 3000 Luminometer (Dynatech, Chantilly, VA). Luminescence values (relative light units, RLU) were taken from triplicate samples.

RESULTS

Transactivation by Human VDR and Mouse RXR β2 Through the Synthetic VDRE DR3 Is Constitutive in Yeast

The interaction between human VDR and mouse RXR β2 was reconstituted in yeast on

response elements derived from the mouse osteopontin promoter (DR3). Surprisingly, the simultaneous expression of the VDR and the RXR resulted in a strong constitutive β-galactosidase activity through binding of both receptors to the DR3-VDRE. Fully induced receptor expression in YJH487 (0.1 mM Cu) resulted in a constitutive almost 4-fold increase of β-galactosidase activity (Fig. 1). As outlined in Figure 1, transcriptional activity of *lacZ* was enhanced only to a small extent, when the cotransformants of YJH487 were incubated with different concentrations of vitamin D. Addition of the RXR ligand, 9-*cis* RA 10^{-6} M, alone or combined with 10^{-6}M and 10^{-7}M vitamin D, did not further enhance reporter gene activity (data not shown).

To exclude, that a saturated expression of the receptors might mask a potential influence of the ligand(s), strain YJH487 was incubated with copper concentrations between 0.01 and 0.2 mM. Activity of β-galactosidase as response to ligand-receptor interaction was hardly affected by the concentration of copper. Constitutive transactivation in the absence of the ligands almost was independent of copper concentrations (data not shown). Uninduced cells, in which the expression of the receptors was not induced by the addition of copper, exhibited a background β-galactosidase activity, which was due to the leaky copper-responsive promoter of the expression plasmids (Fig. 1). However, cells that expressed either the VDR or the RXR alone, did not exhibit reporter gene activity in the absence or in the presence of copper. Furthermore, no β-galactosidase activity was detected in cells, which were transformed with a reporter gene construct, lacking the VDRE (data not shown). Thus, we conclude, that ligand-independent induction of transcription of the *lacZ* reporter gene was mediated by binding of VDR-RXR heterodimers to the mouse osteopontin VDRE.

Interaction Between Human VDR and the Ligand-Binding Domain of Human RXR β Is Strongly Dependent on the Ligand in the Yeast Two-Hybrid System

We examined the dimerization between the VDR and the RXR in the yeast two-hybrid system, which makes use of the yeast Gal4 transactivator. This system enabled us to study VDR-RXR heterodimerization independently from their DNA-binding properties.

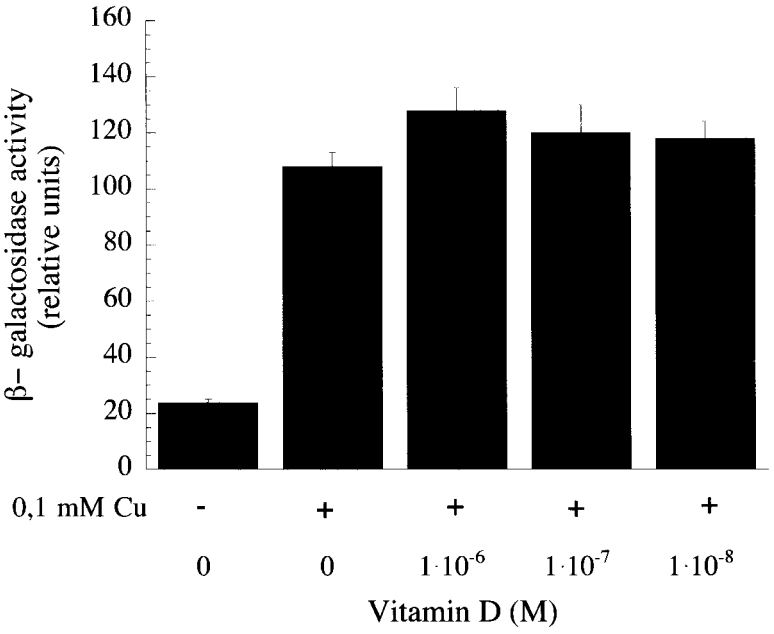

Fig. 1. Induction of β-galactosidase activity by VDR-RXR β2 transactivation in yeast strain YJH487. The DR3-VDRE containing reporter plasmid pTM108-DR3 was cotransformed with the yeast expression plasmids pTM159-VDR and Yep-RXR into strain YJH487. Cells were grown in the absence (0.5% DMSO as a control) or in the presence of 0.1 mM $CuSO_4$. The copper-induced cultures were incubated with different concentrations of vitamin D. Expression of the reporter gene *lacZ* was detected by β-galactosidase activity in 96-well dishes, as described in Materials and Methods, and corrected for cell density and for incubation time with X-Gal $A_{415\ nm}/A_{600\ nm} \times 1{,}000$/min.

Transformants, coexpressing Gal4-DBD-VDR and Gal4-AD-RXR fusion proteins (Fig. 2), within a few minutes developed a blue colour, indicating β-galactosidase activity in the colony-lift filter assay. Reporter gene activity, mediated by the VDR-RXR interaction, was only detected in the presence of 10^{-6}M (0.001 mM) vitamin D (Table I). Due to the intrinsic activating domain(s) of the VDR, a residual β-galactosidase activity was measured in colonies expressing the Gal4-DBD-VDR fusion in the presence of vitamin D (Table I). Neither the Gal4-AD-RXR fusion protein nor the Gal4-domains alone led to the induction of the reporter gene *lacZ*. The results of all control experiments are outlined in Table I. To further examine heterodimerization with the RXR, we constructed Gal4-VDR gene fusions, encoding either the VDR DNA-binding domain, Gal4-DBD-VDR124, or the ligand-binding domain, Gal4-DBD-VDR298 (Fig. 2). Expression of the ligand-binding domain of the VDR together with the Gal4-RXR chimera was sufficient for ligand-dependent heterodimerization. The strength of interaction, as indicated by β-galactosidase activity, was similar to that obtained by the interaction between the full-length VDR and RXR (Table I). Reporter gene activity was not induced by the Gal4-DBD-VDR298 hybrid construct in the presence of vitamin D, although the VDR-LBD harbors the carboxyterminal ligand-dependent activating domain of the receptor (Table I). The DNA-binding domain of the VDR (Gal4-DBD-VDR124) did not interact with the Gal4-RXR hybrid protein. Our results show that the Gal4-DBD-VDR fusion protein is activated by its natural ligand vitamin D. The introduction of the Gal4-AD-RXR fusion strongly enhances transactivation, mediated by the Gal4 transactivator, indicating a ligand-dependent VDR-RXR heterodimerization.

Human VDR Homodimerizes and Interacts With Calreticulin in a Ligand Dependent Manner

From various transactivation experiments in eukaryotes and from in vitro experiments it is known that VDR homodimerizes and, depending on the response elements, exhibits transactivation on target genes [McDonnell et al., 1989; Kephart et al., 1996; Cheskis and Freedman, 1994]. Transcriptional activity nonetheless is strongly enhanced by heterodimeric protein complexes. We investigated homodimerization in the two-hybrid system and furthermore the interaction between VDR and calreticulin (CRT) for comparison with VDR-RXR dimerization. Strain SFY526, coexpressing Gal4-DBD-VDR and Gal4-AD-VDR or Gal4-AD-CRT (Fig. 2), exhibited β-galactosidase activity in the colony-lift filter assay only in the presence of vitamin D (Table II). Thus, homodimerization of the VDR and its binding to CRT were shown to be dependent on vitamin D. Comparing the exposure times to the synthetic β-galactosidase substrate X-Gal (Tables I and II), we conclude that the heterodimer exhibited a much stronger protein-protein interaction than the homodimer species or the VDR-CRT interaction. VDR homodimerization and VDR-CRT interaction could not be reconstituted with the Gal4-receptor hybrid, encoding the VDR-DBD (Gal4-DBD-VDR124), although the VDR-DBD overlaps with the homodimerization function and is the target domain for CRT-binding (Table II). The controls listed in Tables I and II demonstrate that the proteins or fragments thereof, which were fused to the Gal4-AD, did not bind to the relevant *GAL1*-promoter sequences in strain SFY526 by chance and did not induce reporter gene activity.

Particular Yeast One-Hybrid System Exhibits Strong RXR-Independent Transactivation by the VDR

Due to its intrinsic transactivating function, the VDR stimulated *lacZ* gene expression in the absence of the Gal4-AD (Table I). DNA-protein contact was mediated by the Gal4-moiety of the fusion proteins, whereas VDR interacted with the basal transcription machinery to induce transcription. To develop a sensitive detection system for the activating function of the receptor, we placed the Gal4-VDR fused gene under control of a strong yeast promoter (Fig. 2).

Strain Y190, transformed with this Gal4-VDR construct, exhibited reporter gene activity only in the presence of vitamin D, indicating a conformational change, which exposed the activating domain of the VDR (Table III). In the absence of vitamin D, the fusion protein was transcriptional silent.

We furthermore examined if an aminoterminal deletion of the VDR interfered with the transactivating potency of the receptor. The residual VDR-cDNA was fused in frame to the Gal4-DBD to obtain AS-VDR20-423 (Fig. 2). Transformants, expressing the truncated version of the VDR, exhibited comparably strong

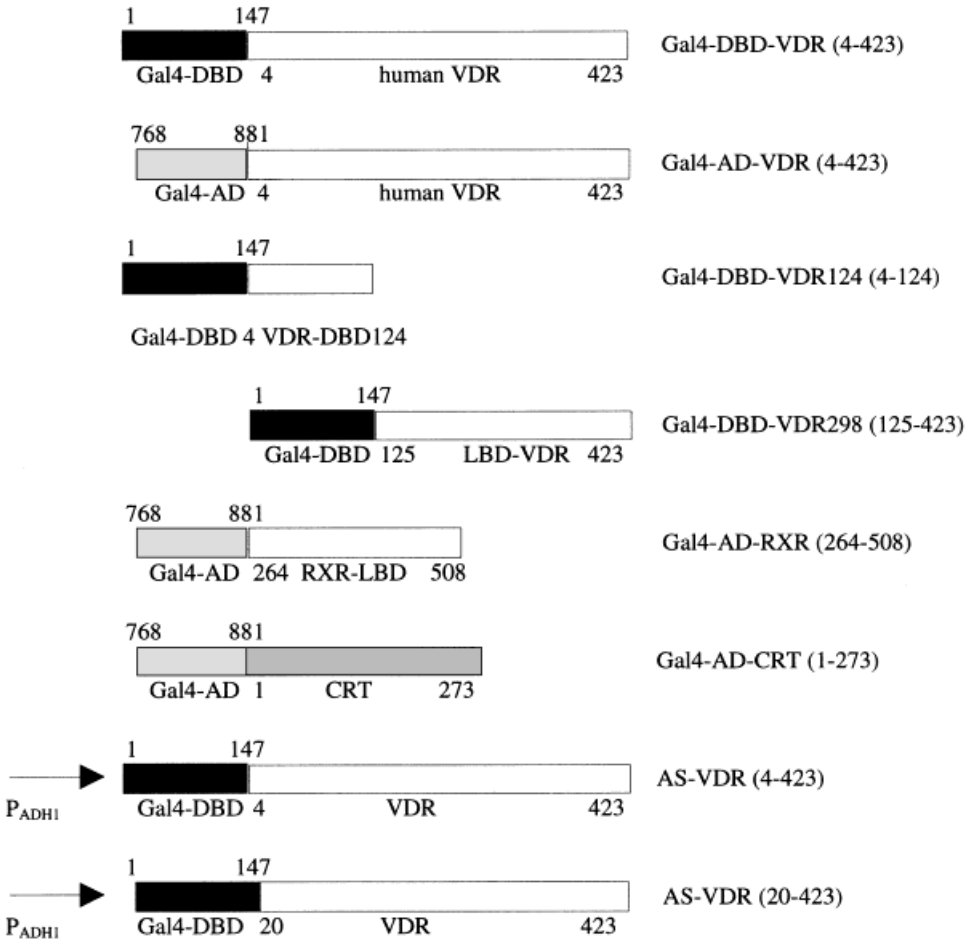

Fig. 2. Gal4-receptor hybrid constructs used in the yeast two-hybrid and one-hybrid systems. The Gal4-DNA binding domain (DBD) is encoded in the expression plasmids pGBT9 and pAS2-1 (AS-VDR). The Gal4-activating domain (AD) is contained in the expression plasmid pGAD424. Numbers indicate amino acids positions of the Gal4 domains and of the domains of the receptors (DBD, DNA-binding domain; LBD, ligand-binding domain) and of calreticulin (CRT). The promoter of the pAS2-1 constructs, P_{ADH1}, is the full-length fragment in contrast to the truncated promoter fragment used in the pGBT9 and pGAD424 expression plasmids.

β-galactosidase activity as the full-length construct (Table III). Induction of reporter gene activity was strictly dependent on the presence of vitamin D. Our results demonstrate an RXR-independent activity of the VDR in the one-hybrid system, which was intrinsic to the carboxyterminal ligand-dependent transactivating domain of the receptor.

Comparison of Mammalian MCF-7 Cells and the Yeast Systems Reveals Similarities With Regard to the Influence of VDR-Specific Ligands on Transactivation

We have shown that reporter gene activity in the Gal4-based systems was controlled by vitamin D mediated VDR activity. The two-hybrid system responded to a vitamin D concentration of 10^{-8} M with an increase of β-galactosidase activity, which was higher than the background activity in vitamin D deprived cells (Fig. 3A). Background enzyme activity was comparable to β-galactosidase activity in cells, treated with 10^{-9} M vitamin D. Transformants, which were incubated with 10^{-6} M vitamin D, exhibited a 3-fold induction of reporter gene activity in comparison to the background level. Activity of β-galactosidase was not further enhanced upon the addition of 10^{-5} M vitamin D, indicating saturation of the ligand-dependent VDR-RXR interaction in this system (data not shown).

We furthermore examined the influence of vitamin D on the one-hybrid system and on mammalian MCF-7 cells. Both systems responded to a vitamin D concentration of 10^{-6} M

TABLE I. Colony-Lift Filter Assay of Transformants Expressing Gal4-Fused VDR and RXR in the Yeast Two-Hybrid System*

SFY526 transformants	β-Galactosidase activity	
	+ Vitamin D	− Vitamin D
Gal4-DBD and Gal4-AD	−	−
Gal4-DBD-VDR	−/+	−
Gal4-AD-RXR	−	−
Gal4-DBD and Gal4-AD-RXR	−	−
Gal4-DBD-VDR and Gal4-AD-RXR	+	−
Gal4-DBD-VDR and Gal4-AD	−/+	−
Gal4-DBD-VDR124	−	−
Gal4-DBD-VDR298	−	−
Gal4-DBD-VDR124 and Gal4-AD-RXR	−	−
Gal4-DBD-VDR298 and Gal4-AD-RXR	+	−

*Strain SFY526 was transformed with expression plasmids, encoding Gal4-VDR and Gal4-RXR fused cDNAs, and transformants were incubated on solid media in the absence or in the presence of 0.001 mM vitamin D. Activity of β-galactosidase was detected by development of a blue colour of the transfered colonies in the filter assay, as described in Material and Methods. −/+, blue colonies were detected after 16 h incubation with X-Gal on the filters. +, colonies turned blue after 0.3 h of incubation with X-Gal on the filters, −, colonies did not develop a blue colour within 30 h.

TABLE II. Colony-Lift Filter Assay of Transformants Demonstrating VDR Homodimerization and VDR-Calreticulin Interaction in the Two-Hybrid System*

SFY526 transformants	β-Galactosidase activity	
	+ Vitamin D	− Vitamin D
Gal4-AD-VDR	−	−
Gal4-DBD and Gal4-AD-VDR	−	−
Gal4-DBD-VDR and Gal4-AD-VDR	+	−
Gal4-AD-CRT	−	−
Gal4-DBD and Gal4-AD-CRT	−	−
Gal4-DBD-VDR and Gal4-AD-CRT	+	−
Gal4-DBD-VDR124 and Gal4-AD-VDR	−	−
Gal4-DBD-VDR298 and Gal4-AD-VDR	−	−
Gal4-DBD-VDR124 and Gal4-AD-CRT	−	−

*Strain SFY526 was transformed with expression plasmids, containing Gal4-VDR and Gal4-CRT fused cDNAs, and tested for vitamin D responsiveness as described in Table I. +, cotransformants on the filters developed a blue colour after 3 h of incubation with X-Gal, −, colonies did not develop a blue colour within 30 h.

TABLE III. Colony-Lift Filter Assay of Transformants Expressing Gal4-VDR Fusion Proteins in the One-Hybrid System*

Y190	β-Galactosidase activity	
	+ Vitamin D	− Vitamin D
AS-VDR	+	−
AS-VDR20-423	+	−

*Strain Y190, containing a *GAL1*-promoter-*lacZ* unit, was transformed with Gal4-VDR gene fusions, either encoded by the expression plasmid AS2-1 or AS-VDR20-423 and the transformants were tested for vitamin D responsiveness as described in Table I. In AS-VDR20-423, the VDR lacks the first 20 amino acids of the amino terminus. +, colonies on the filters turned blue after 2 h of exposure to X-Gal, −, colonies did not develop a blue colour within 30 h.

with a 4-fold increase in β-galactosidase activity (Figs. 3B and 4). Compound A (Fig. 5) affected VDR-mediated transactivation in all three systems. The β-galactosidase activity in the yeast systems was stimulated almost to the same extent, as with vitamin D (Fig. 3), but did not increase further with higher concentrations of compound A (data not shown). In MCF-7 cells, compound A exhibited a higher sensitivity as vitamin D, because reporter gene activity was more increased with A at low concentrations of the ligand (Fig. 4). Compound B (Fig. 5) neither influenced the mammalian transactivation system nor enhanced β-galactosidase activity, mediated by VDR-RXR dimerization in the two-hybrid system (Figs. 3A and 4). Compound C (Fig. 5) exerted its influence on the VDR in MCF-7 cells only at elevated concentrations and thus was a weak inducer of VDR-RXR mediated transactivation (Fig. 4). This compound did not enhance VDR-RXR heterodimerization in the two-hybrid system in a concentration of 10^{-6} M (Fig. 3A).

DISCUSSION

In our report we presented different in vivo systems, investigating activity of the human VDR. The experiments were based on VDR-induced transactivation in yeast compared to mammalian MCF-7 cells and on yeast two-hybrid and one-hybrid systems. The VDR was expected to be more likely in its native conformation in the eukaryotic organism yeast than

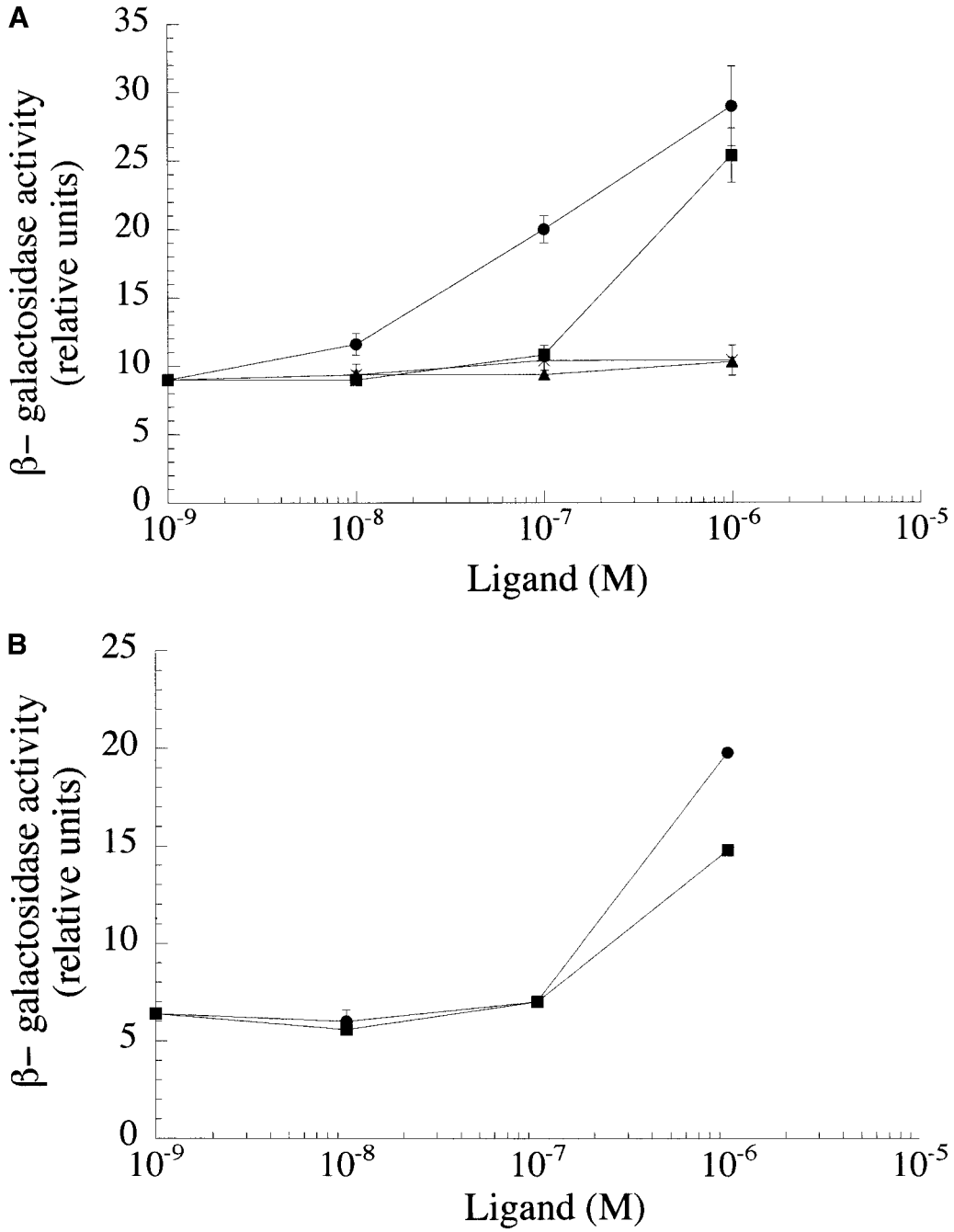

Fig. 3. Induction of β-galactosidase activity by ligand-induced activation of the VDR in the yeast two-hybrid and one-hybrid system. Strain SFY526, containing the *GAL1* promoter-*lacZ* transcription unit, was cotransformed with expression plasmids Gal4-DBD-VDR and Gal4-AD-RXR-LBD (**A**). Strain Y190 was transformed with expression plasmid AS-VDR as a one-hybrid system (**B**). Cells were grown and incubated with vitamin D (●) or the related compounds A (■), B (▲), or C (x) in different concentrations. Transcriptional activity of *lacZ* in the two-hybrid system (A) was induced by ligand-dependent VDR-RXR interaction, which reconstituted Gal4. The *lacZ* activity in the one-hybrid system was mediated by the Gal4-VDR fusion protein of AS-VDR (B). Reporter gene activity was quantified at $A_{415\ nm}$ in 96-well dishes, as described in Materials and Methods, and corrected for cell density and incubation time with X-Gal ($A_{415\ nm}/A_{600\ nm} \times 1{,}000$/min).

in in vitro systems. Therefore, we used the in vivo approach to analyze different features of the receptor such as DNA-binding, dimerization, and ligand-dependent transactivation.

VDR-mediated transactivation in yeast was shown to be independent of vitamin D. Moreover, strain YJH487 did not respond to different copper concentrations with a corresponding

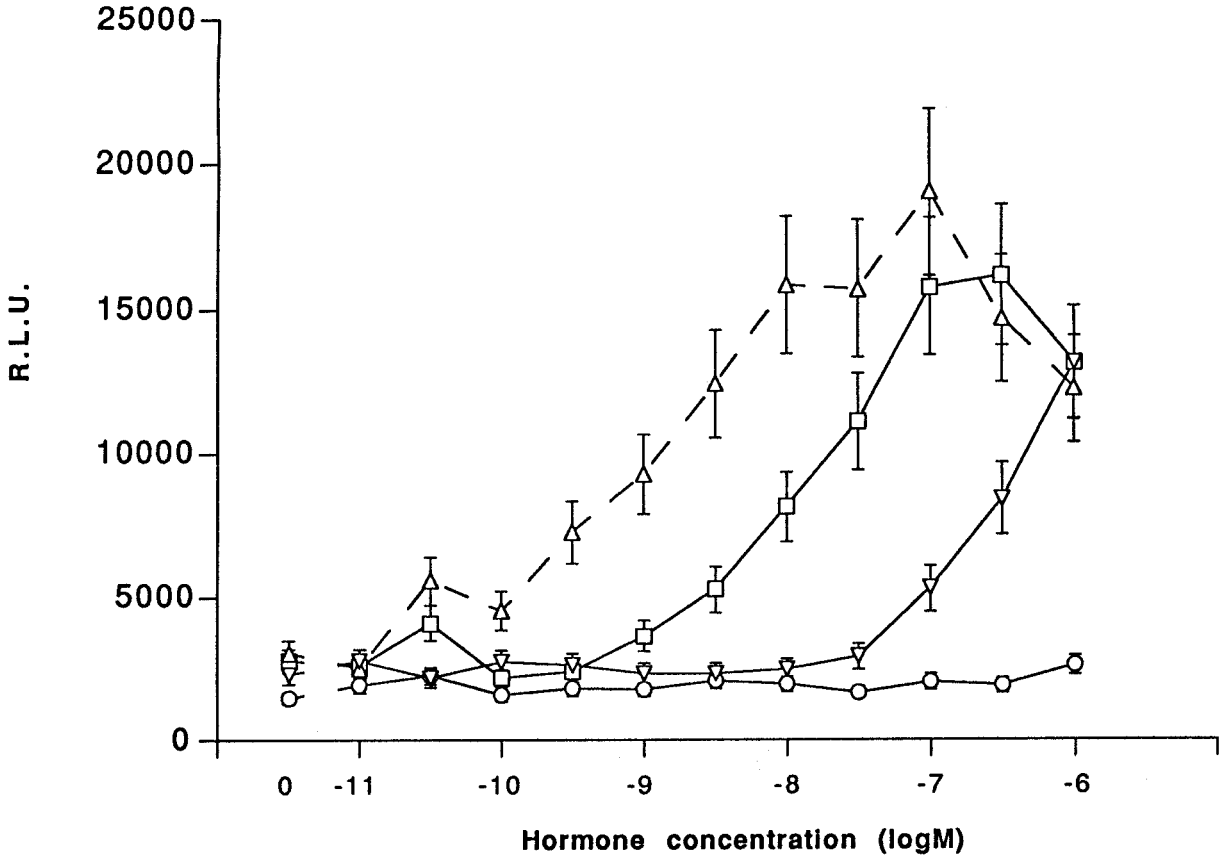

Fig. 4. Ligand-induced transactivation of the reporter gene *luc* in mammalian MCF-7 cells. MCF-7 cells were transfected with the reporter plasmid pDR3-luc and transformant cultures incubated with vitamin D (□) or the compounds A (△), B (○), or C (▽) in different concentrations. Induction of the reporter gene *luc* was quantified in 96-well dishes by determination of relative light units, as described in Materials and Methods. Vitamin D–treated cultures were measured in duplicates, whereas cultures incubated with the other compounds were measured in triplicates.

change in reporter gene activity. Thus, we excluded that the influence of the ligands was covered by a saturated expression of both receptors. Additionally, expression of the receptors in uninduced cells of YJH487 already was sufficient to induce β-galactosidase activity.

These results were consistent with previous studies, which showed a similar constitutive transactivation by VDR-RXR γ heterodimers in yeast [Jin and Pike, 1996]. Moreover, it was demonstrated that heterodimeric transactivation in yeast was strongly influenced by the respective RXR isoforms and by the nature of response elements [Kephart et al., 1996]. A maximum transactivation, mediated by the α and the γ isoforms of the RXR through the 24-hydroxylase VDRE, was detected in the presence of vitamin D and 9-*cis* RA. This natural RXR ligand did not affect transactivation mediated by VDR-RXR β heterodimers through the response element DR3 in our experiments. Thus, particular combinations of the receptors in the heterodimeric complexes and the nature of the response elements together seemed to determine responsiveness to the respective ligands vitamin D and 9-*cis* RA in yeast.

Ligand-independent heterodimerization between VDR and RXR as well as between RXR and retinoic acid receptors (RARs) previously has been shown in vitro and in vivo [Yu et al., 1991; Cheskis and Freedman, 1994; Nagpal et al., 1993]. Nonetheless, ligand was shown to strongly promote dimerization [MacDonald et al., 1993]. This enhanced interaction was mainly detected in our yeast two-hybrid system, which was highly ligand-inducible. The question remained, why transactivation by DNA-bound heterodimers in yeast was constitutive, but inducible by hormone in MCF-7 cells. Although this mammalian system used a high copy number reporter plasmid like the yeast transactivation system, transactivation was hardly de-

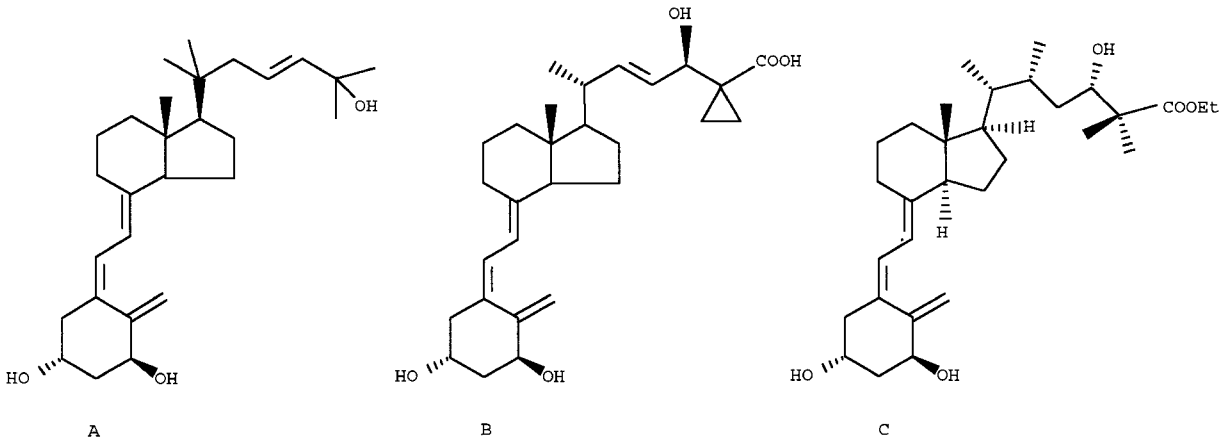

Fig. 5. Structural formulas of the vitamin D related compounds A, B, and C. **A:** 5-(2-[1(R)-(5-hydroxy-1,1,5-trimethyl-hex-3-enyl)-7a(R)-methyl-octahydro- 3a(R)inden-4-ylidene]-ethylidene-4-methylene-cyclohexan-1(R),3(S)-diol. **B:** 1-(4(R)-[4-[2-(3(S),4(R)-dihydroxy-2-methylene-cyclohexylidene)-ethylidene]- 7a(R)-methyl-octahydro-3a(R)-inden-1(R)-yl]-1(R)-hydroxy-pent-2-enyl)- cyclopropane-carboxylic acid. **C:** 6(R)-[4-[2-(3(S),5(R)-dihydroxy-2-methylene-cyclohexylidene)-ethylidene]- 7a(R)-methyl-octahydro-3a(R)-inden-1(R)-yl]3(S)-hydroxy-2,2,5(R)-trimethyl- heptanoic acid ethylester.

tected in vitamin D deprived cells. This strongly argued for the existence of cofactors in MCF-7 cells, which dissociated from the receptor(s) upon ligand binding. Influence of corepressors on DNA-bound RXR-RAR heterodimers or on thyroid receptor have been previously described [Kurokawa et al., 1995; Chen and Evans, 1995]. Thus, we assumed that in particular DNA-bound VDR-RXR dimers exhibited detectable transactivation in the absence of ligand and modulating cofactors in yeast.

If both receptors dimerized at the DR3-VDRE, which moiety of the proteins did mediate transactivation of the reporter gene in yeast? As already mentioned, we did not detect transactivation by VDR or RXR alone. Thus, we excluded that homodimers or monomers of the respective receptors were responsible for transactivation through DR3. Also putative modifications in yeast, such as constitutive phosphorylation of the receptors, did not seem to be probable because this led to constitutive activities of the VDR at least in the one-hybrid system. We assumed that heterodimerization was necessary for binding to the DR3-VDRE and that the RXR moiety alone exhibited transactivation by interacting with the basal transcription machinery. Nagpal and coworkers [1993] previously had demonstrated that the aminoterminal transactivating functions of the RXR isoforms were autonomous and drove transcriptional activation in the absence of ligands.

The comparison between the transactivation system and the two-hybrid system revealed that the Gal4-based systems were more sensitive with respect to ligand binding because the background activity of ligand independent dimerization was reduced. Moreover, the RXR moiety in the two-hybrid system was devoid of transactivating functions, which instead were mediated by the Gal-AD of the Gal4-RXR fusion protein.

The use of Gal4-receptor chimeras enabled us to investigate distinct functions of the receptors, independently of DNA-binding. Dimerization or transactivation, which was mediated by the VDR in the one-hybrid system, were dependent on binding of the ligand. We also investigated the necessity of the ligand for VDR-homodimerization and VDR-calreticulin interaction in the two-hybrid system. VDR-homodimerization alone was detected in the presence of vitamin D and was substantially weaker compared to ligand-dependent heterodimerization with RXR.

Calreticulin is a calcium-binding protein, which has been shown to interact with the DNA-binding domains of several receptors [Dedhar et al., 1994] and to inhibit vitamin D–mediated signal transduction [Wheeler et al., 1995]. In the two-hybrid system the aminoterminus of calreticulin interacted with full-length VDR only in the presence of vitamin D. In contrast to previous in vitro studies [Wheeler et al., 1995] we did not detect the interaction between calreticulin and the DNA-binding domain of the VDR, which is devoid of the ligand-binding function. Therefore we assumed rather that ligand-bound VDR was the target species for binding of calreticulin.

We also demonstrated, that the ligand-binding domain of the VDR was sufficient for a strong ligand-dependent interaction with RXR. Previous in vitro studies support this result [Nishikawa et al., 1995]. It is tempting to speculate that these results mirror the different strength of interactions in mammalian cells. This would confirm the necessity for a heterodimeric complex in VDR mediated transactivation. This assumption was limited by the fact that it was not clear whether heterodimerization was facilitated by using the truncated RXR in contrast to full-length constructs in the homodimeric complex. To examine this, we investigated homodimerization between full-length VDR and the DNA-binding domain of the VDR, which mediates homodimerization. Neither in the presence nor in the absence of vitamin D was activity of β-galactosidase detected. Thus, the influence of particular constructs of the receptors on reporter gene activity could be largely excluded.

Using the one-hybrid system, we were able to demonstrate the intrinsic transactivation function of the VDR. For the expression of the Gal4-VDR fusion protein, we used a vector that differed from the construct used in our two-hybrid system with respect to promoter strength [Legrain et al., 1994]. The increased expression of the Gal4-VDR chimera allowed a sensitive measurement of transactivation mediated by the VDR in the absence of RXR. Transactivation of the reporter gene *lacZ* essentially was dependent on the concentration of the ligand, indicating a ligand-induced conformational change of the receptor moiety.

We have shown that reporter gene activity in the Gal4-based systems was controlled by vitamin D–mediated VDR activity. To further investigate validity of these interactions with respect to their importance in higher eukaryotes, we investigated the effects of selected vitamin D–related compounds on dimerization in yeast and on transactivation in the yeast one-hybrid system and in mammalian MCF-7 cells. The results pointed out that the Gal4-receptor chimeras were suitable to mimick VDR mediated transactivation in the mammalian cell line. However, the effective concentrations of vitamin D and the compounds, applied to yeast, were much higher than the concentrations used in the mammalian systems. Probably this was due to unefficient penetration of the yeast cell wall. Studies investigating the activity of the estrogen receptor in yeast support this assumption [Wang et al., 1995]. Furthermore, it has been shown that yeast possess transport proteins, which decrease the potency of steroids by an active export [Kralli et al., 1995]. Additionally, it appeared that VDR-RXR-mediated transactivation in MCF-7 cells was more complex with respect to the RXR, which is expressed in different isoforms in mammalian cell lines [Ferrara et al., 1994].

In conclusion, our results point out that the VDR activity is a ligand-inducible event in yeast. Thus, the two-hybrid system and the one-hybrid system can be used in combination with mammalian systems for the analysis of vitamin D–related drugs. The transactivation system offers great opportunities to identify compounds or new proteins, which directly interfere with the DNA-binding of VDR-RXR heterodimers.

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