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The Impact of Functional Vitamin D_3 Receptor Conformations on DNA-Dependent Vitamin D_3 Signaling

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ABSTRACT

The vitamin D_3 receptor (VDR) is the nuclear receptor for 1α,25-dihydroxyvitamin D_3 (VD) that acts primarily as a heterodimer with the retinoid X receptor (RXR) on different types of VD response elements, i.e., DNA-bound VDR-RXR heterodimers are the molecular switches in nuclear VD signaling pathways. In this study, DNA-dependent limited protease digestion assays and gel shift clipping assays were used for the analysis of VDR conformations and showed the same high ligand sensitivity for VD response element-bound VDR-RXR heterodimers (EC_{50} of 0.1 nM for VD). In contrast, DNA-independent limited protease digestion assays clearly demonstrated a reduced ligand sensitivity for monomeric VDR in solution. Interestingly, the relative amount of reduction was found to be specific for each VDR agonist. Moreover, complex formation of the VDR on DNA resulted in a shift from the receptor's low-affinity ligand binding conformation ($c3_{LPD}$) to its high affinity conformation ($c1_{LPD}$). Finally, the characterization of the conformations of N- and C-terminally truncated VDR proteins defined the high-affinity ligand binding domain of the VDR as being positioned between amino acids 128 and 427. Taken together, the analysis of VDR conformations in solution in comparison to those of DNA-complexed VDR-RXR heterodimers allows a differentiation to be drawn between DNA-dependent and DNA-independent VD signaling pathways that can in turn be used for the identification of pathway selective VDR agonists.

The transcription factor vitamin D receptor (VDR) is the nuclear receptor for 1α,25-dihydroxyvitamin D_3 (VD), which is the physiologically active form of vitamin D_3 and is thus the mediator of all genomic actions of VD and its analogues (Carlberg and Polly, 1998). VD is the main regulator of calcium homeostasis and is therefore very critical in bone formation (DeLuca et al., 1990), which was ultimately confirmed by the rickets phenotype of VDR knockout mice (Yoshizawa et al., 1997). Moreover, VD is also involved in controlling cellular growth, differentiation, and apoptosis (Walters, 1992), which makes VD analogues with a low calcemic profile interesting for therapy of hyperproliferative diseases such as different types of cancer and psoriasis (Bouillon et al., 1995).

The VDR is a member of the nuclear receptor superfamily that also contains structurally related receptors for other nuclear hormones, such as steroids, retinoic acid (RA), and thyroid hormone, and many orphan nuclear receptors (Mangelsdorf et al., 1995). Nuclear receptors contain a highly conserved DNA binding domain (DBD) of 66 to 70 amino acids that form two zinc finger structures (Freedman, 1992) and a moderately conserved ligand binding domain (LBD) of ~250 amino acids that form 11 to 12 α-helices in their C-terminal part (Moras and Gronemeyer, 1998). Most nuclear receptors form homo- or heterodimers with other nuclear receptors to facilitate specific and high-affinity binding to DNA binding sites, referred to as response elements (Glass, 1994). The main dimerization partner of the VDR is the retinoid X receptor (RXR), which is the receptor for 9-*cis* RA (Carlberg, 1996). Simple VD response elements (VDREs) are formed by two hexameric binding sites, and VDR-RXR heterodimers bind preferentially to directly repeated binding site arrangements with three intervening nucleotides (DR3-type VDRE) or to inverted palindromes spaced by nine nucleotides (IP9-type VDRE). In addition, VDREs with direct repeats spaced by four or six nucleotides have been described (Carlberg, 1995).

The crystal structure of the six nuclear receptor LBDs that have been published until now show a rather conserved three-dimensional structure (Moras and Gronemeyer, 1998). The inner surface of these LBDs forms a cavity for highly specific ligand binding, whereas the outer surface serves as a interface for the interaction with other proteins. Helices 3, 5, and 12 of the LBD form an interface for the interaction with

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ABBREVIATIONS: VDR, vitamin D receptor; AF-2, activation function-2; ANF, atrial natriuretic factor; c, conformation; DBD, DNA binding domain; DR3, direct repeat spaced by 3 nucleotides; GSC, gel shift clipping; LBD, ligand binding domain; LPD, limited protease digestion; RA, retinoic acid; RAR, all-*trans* RA receptor; RXR, retinoid X receptor; VD, 1α,25-dihydroxyvitamin D_3; VDRE, VD response element.

coactivator proteins. The comparison of the apo-structure of RXRα (Bourguet et al., 1995) with the holo-structure of the closely related RA receptor (RAR)γ (Renaud et al., 1995) suggested that ligand binding induces a conformational change within the LBD that resulted in a changed orientation of helix 12. This helix contains the activation function 2 (AF-2) domain, which is exposed to coactivators, like in a closing mouse-trap. In this way, the ligand-induced conformational change of the LBD allows an interaction with coactivators and finally results in the onset of transactivation.

The investigation of conformational changes of the VDR, i.e., the characterization of functional VDR conformations, is of central importance for understanding VD signaling. For this purpose, two in vitro methods have been developed. In the limited protease digestion assay (Leng et al., 1993), ligand-bound monomeric VDR is incubated with an endoprotease such as trypsin, which allows for the detection of up to three different protease-resistant VDR fragments (Nayeri et al., 1995; Peleg et al., 1995, 1996b). The VDR fragments that were obtained are interpreted as representatives of different ligand-induced VDR conformations ($c1_{LPD}$, $c2_{LPD}$, and $c3_{LPD}$) (Nayeri et al., 1996a; Liu et al., 1997; Nayeri and Carlberg, 1997). However, with most VDR agonists, only conformations $c1_{LPD}$ and $c3_{LPD}$ are observed (Nayeri et al., 1996b). In the second method, referred to as a gel shift clipping (GSC) assay, the ligand-dependent gel shift assay, as a detection method for protein-DNA interactions, was combined with the limited protease digestion (LPD) assay (Quack et al., 1998c; Quack and Carlberg, 1999). In this assay, VDR-RXR heterodimers are formed on a VDRE and consequently incubated with an endoprotease, which provides, when trypsin is used, two truncated DNA-bound VDR-RXR complexes that are interpreted as conformations of ligand-stabilized VDR-RXR heterodimers ($c1_{GSC}$ and $c2_{GSC}$).

In this study, DNA-dependent LPD assays and GSC assays were used for the analysis of high-affinity ligand binding conformations of the VDR. DNA-independent LPD assays demonstrated a clearly reduced ligand sensitivity of the VDR in solution. Moreover, conformations of N- and C-terminally truncated VDR proteins defined the high-affinity LBD of the VDR, thus allowing differences between DNA-dependent and DNA-independent VD signaling pathways to be drawn.

Materials and Methods

Compounds. VD and EB1436 [(25S),26*S*-OH,1(S),3(R)-dihydroxy-20(R)-(5′-ethyl-5′-hydroxy-hepta-1′(E),3′(E)-dien-1′-yl)-9,10-secopregna-5(Z),7(E),10(19)-triene] were synthesized at Leo Pharmaceutical Products (Ballerup, Denmark). The ligands were dissolved in isopropanol at 4 mM, and dilutions were performed in dimethyl sulfoxide.

Generation of Truncated VDR Protein. The cDNA for human wild-type VDR ($VDR_{1–427}$) was subcloned into the expression vector pSG5 (Stratagene, Heidelberg, Germany) (Carlberg et al., 1993). For the generation of the N-terminal VDR truncations $VDR_{125–427}$, $VDR_{128–427}$, $VDR_{131–427}$, and $VDR_{132–427}$ and the C-terminal truncations $VDR_{1–413}$, $VDR_{1–401}$, $VDR_{1–390}$, and $VDR_{1–388}$, PCR was performed for 35 cycles with a profile of 0.5 min at 94°C, 1 min at 60°C, and 1.5 min at 72°C using respective combinations of the following primers: VDR_1, TAATACGACTCACTATAGGGCCATGGAGGCAATGGCGGCCA; VDR_{376}, TAATACGACTCACTATAGGGCCATGGAGGAGCAGCAGCGCATCATT; VDR_{385}, TAATACGACTCACTATAGGGCCATGCAGCGCATCATTGCCATACT; VDR_{394}, TAATACGACTCACTATAGGGCCATGATTGCCATACTGCTGGACGC; VDR_{397}, TAATACGACTCACTATAGGGCCATGGCCATACTGCTGGACGCCCAC; VDR_{1284}, TCAGGAGATCTCATTGCC; VDR_{1239}, CTTCATGCTGCACTCAGGCT; VDR_{1203}, GTACTGCTTGGAGTGCTCCT; VDR_{1170}, CAGGTCGGCTAGCTTCTGGA; and VDR_{1164}, GGCTAGCTTCTGGATCATCT. VDR_1, VDR_{376}, VDR_{385}, VDR_{394}, and VDR_{397} were used as forward primers and contain a T_7-promoter and a ATG start codon. Linearized cDNA of wild-type VDR ($VDR_{1–427}$) and PCR-generated DNA templates were transcribed with T_7 RNA-polymerase and translated as recommended by the supplier (Promega, Mannheim, Germany).

LPD Assays. In DNA-independent LPD assays, 2.5 μl of in vitro-translated, [^{35}S]methionine-labeled wild-type VDR or indicated VDR truncations and 2.5 μl of unprogrammed lysate were incubated with 2 μl of graded concentrations of VD or EB1436 (or solvent as control) in a total volume of 20 μl of binding buffer (10 mM HEPES, pH 7.9, 1 mM dithiothreitol, 0.2 μg/μl poly(dI-C), and 5% glycerol) for 15 min at room temperature. The buffer was adjusted to 150 mM monovalent cations by addition of KCl. For DNA-dependent LPD assays, 2.5 μl of in vitro-translated RXR was used instead of unprogrammed lysate and ~1 ng of nonlabeled double-stranded oligonucleotide containing the DR3-type VDRE of the rat atrial natriuretic factor (ANF) gene promoter (core sequence AG**AGGTCA**TGA**AGGACA**) (Kahlen and Carlberg, 1996) was added to the receptor-ligand mixture. Incubation was then continued for 20 min. In both cases, the endoprotease trypsin (Promega; final concentration 8.3 μg/ml) was then added, and incubation was continued for 10 min at room temperature. The digestion reaction were stopped by adding 1 volume protein gel loading buffer (0.25 M Tris, pH 6.8, 20% glycerol, 5% mercaptoethanol, 2% SDS, 0.025% (w/v) bromophenol blue) and denaturation at 95°C for 5 min. Protease-resistant VDR fragments were electrophoresed through 15% SDS-polyacrylamide gels, exposed to a Fuji MP2040S imager screen, and quantified on a Fuji FLA2000 reader (Tokyo, Japan) using Image Gauge software (Raytest, Sprockhövel, Germany).

GSC Assays. Equal amounts of in vitro-translated wild-type VDR (or indicated VDR truncations) and in vitro-translated RXR protein were mixed and incubated in the presence of indicated concentrations of VD or EB1436 (or solvent as control) for 15 min at room temperature in a total volume of 20 μl binding buffer. The buffer was adjusted to 150 mM monovalent cations by addition of KCl. The rat ANF DR3-type VDRE was labeled by a fill-in reaction using [^{32}P]dCTP and the Klenow fragment of DNA polymerase I (Promega). Approximately 1 ng of labeled probe (50,000 cpm) was added to the receptor-ligand mixture, and incubation was continued for 20 min. Trypsin was then added (final concentration 8.3 μg/ml), and incubation was further continued for 10 min at room temperature. Protein-DNA complexes were resolved through 8% nondenaturing polyacrylamide gels (at room temperature) in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) and exposed to a Fuji MP2040S imager screen. The ratio of protein-complexed probe to free probe was quantified on a Fuji FLA2000 reader using Image Gauge software. "Reverse" GSC assays were performed under principally identical conditions but required [^{35}S]methionine-labeled VDR protein, nonlabeled DR3-type VDRE, and an extra 4% stacking gel. In the reverse GSC assay, DNA-complexed VDR protein was quantified in relation to VDR input.

Results

LPD assays were performed with in vitro-translated, [^{35}S]-labeled wild-type VDR and the endoprotease trypsin (Fig. 1). In the presence of saturating concentrations of VD (10 μM), two typical VDR fragments of a molecular mass of ~28 and 23 kD were obtained (Peleg et al., 1995; Nayeri and Carlberg, 1997), which were interpreted as being representative of the VDR conformations 1 ($c1_{LPD}$) and 3 ($c3_{LPD}$). In this reference experiment, 40% of the VDR input was stabilized in $c1_{LPD}$, which, in comparison with the solvent control, represents a ligand inducibility of a factor of 11. In contrast, <5% of all

receptor molecules were stabilized in $c3_{LPD}$ and did not demonstrate reasonable ligand inducibility.

The dose-dependent stabilization of functional conformations of wild-type VDR in solution (Fig. 2*A*) or bound as a heterodimer with RXR to the rat ANF DR3-type VDRE (Fig. 2*B*) was compared by LPD in the presence of graded concentrations of VD or the VD analog EB1436. EB1436 is a metabolite of the potent VD analog EB1089 (Kissmeyer et al., 1997; Quack et al., 1998b), which was chosen here as a representative of those analogues that stabilize VDR in conformation 3 ($c3_{LPD}$). The natural hormone VD was found to stabilize conformation 1 ($c1_{LPD}$) of monomeric VDR with a half-maximal activation (EC_{50}) value of 1.5 nM, whereas EB1436 displayed an EC_{50} value for $c1_{LPD}$ of only 30 nM (Fig. 2*A*). A dose-dependent increase of VDR conformation 3 ($c3_{LPD}$) could not be detected with VD, whereas EB1436 stabilized $c3_{LPD}$ with an EC_{50} of 60 nM. As expected, EB1436 stabilized, at saturating concentrations, 30% of VDR input in either $c1_{LPD}$ or $c3_{LPD}$ (Fig. 2*A*). Interestingly, the sensitivity of $c1_{LPD}$ in DNA-bound VDR-RXR heterodimers for VD (EC_{50} of 0.15 nM) was found to be 10-fold higher than that of monomeric VDR (Fig. 2*B*). With EB1436 (EC_{50} of 1.2 nM), the complex formation on DNA resulted in a 25-fold increase of the ligand sensitivity of $c1_{LPD}$, whereas under the same conditions the ligand sensitivity of $c3_{LPD}$ for EB1436 (EC_{50} value of 300 nM) decreased by a factor of 5. In parallel, at saturating concentrations of EB1436 the percentage of VDR molecules that are stabilized in $c1_{LPD}$ are increased to 40% of VDR input, whereas those in $c3_{LPD}$ decreased to 10% (Fig. 2*B*).

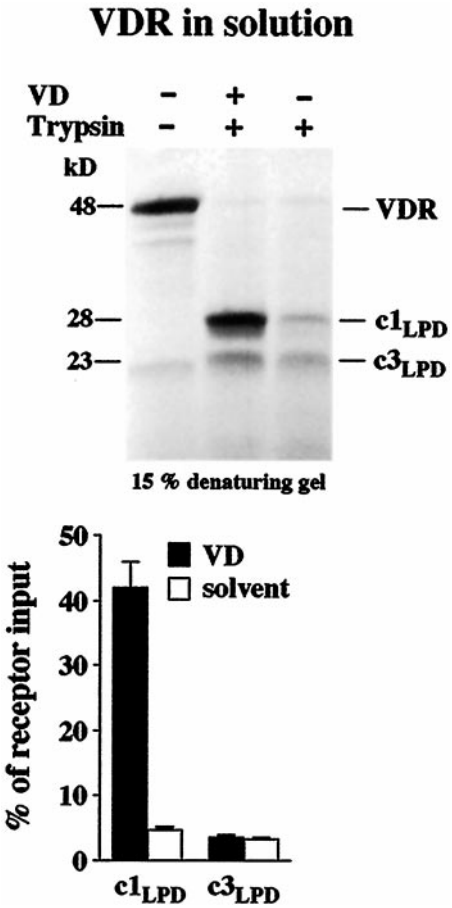

Fig. 1. Ligand-dependent stabilization of VDR conformations in solution. DNA-independent LPD assays were performed by preincubating in vitro-translated, [^{35}S]methionine-labeled wild-type VDR with 10 μM VD or solvent for 15 min at room temperature. Then trypsin was added (final concentration 8.3 μg/ml), and incubation was continued for 10 min. Samples were electrophoresed through a 15% SDS-polyacrylamide gel. A representative experiment is shown, where the molecular mass of full-length VDR and VDR fragments 1 and 3 (interpreted as VDR conformations $c1_{LPD}$ and $c3_{LPD}$) is indicated. The amount of $c1_{LPD}$ and $c3_{LPD}$ was quantified in relation to VDR input by phosphorimaging. Each column represents the average of triplicates and bars indicate S.D.

GSC assays with VDR-RXR heterodimers bound to the rat ANF DR3-type VDRE were performed to confirm the results from the LPD of DNA-bound VDR molecules with a second DNA-dependent assay (Fig. 3). In this assay, the typical two protein-DNA complexes ($c1_{GSC}$ and $c2_{GSC}$) were obtained that are interpreted as the representatives of two different VDR-RXR heterodimer conformations (Quack et al., 1998c; Quack and Carlberg, 1999). The dose-dependent stabilization of these conformations with VD provided EC_{50} values of 0.1 and 0.15 nM for $c1_{GSC}$ and $c2_{GSC}$, respectively, and with EB1436 an EC_{50} value of 1 nM for both VDR-RXR conformations. Moreover, at saturating concentrations (10 μM) both ligands stabilize ~60% (VD) and 50% (EB1436) of all DNA-bound VDR-RXR heterodimers in $c1_{GSC}$ and only 17% (VD) and 12% (EB1436) in $c2_{GSC}$.

For a direct comparison of the results from LPD and GSC assays, a "reverse" GSC assay was performed (Fig. 4). In contrast to the normal GSC assay, in the "reverse" assay the VDR protein and not the DNA was radioactively labeled. The protein-DNA complexes were separated on nondenaturing gels, as normal, but in parallel the total VDR input was determined on denaturing gels (data not shown, but comparable with the results from Fig. 1). Therefore, it was determined that in the presence of saturating VD concentrations 43% of the VDR input formed heterodimers with RXR on DNA, from which approximately half (21% of input) participated in VDR-RXR conformation 1 ($c1_{GSC}$), but less than a quarter (7% of input) occupied conformation 2 ($c2_{GSC}$). In the absence of VD, only 26% of the VDR input formed heterodimers on DNA, 9% were in $c1_{GSC}$, and only 4% in $c2_{GSC}$.

A more detailed investigation of VDR and VDR-RXR conformations used N-terminally truncated VDR proteins $VDR_{125-427}$, $VDR_{128-427}$, $VDR_{131-427}$, and $VDR_{132-427}$ and C-terminally truncated VDR proteins VDR_{1-413}, VDR_{1-401}, VDR_{1-390}, and VDR_{1-388}, which were produced by in vitro transcription/translation of PCR-generated DNA templates. These proteins were first analyzed in "classical" LPD assays, i.e., in the absence of DNA (Fig. 5). VDR conformation 1 ($c1_{LPD}$) of the N-terminal VDR truncation $VDR_{125-427}$ produced a similar pattern to $c1_{LPD}$ of wild-type VDR (Fig. 1), but a further truncation of 3 or 6 amino acids ($VDR_{128-427}$ and $VDR_{131-427}$) provided a clear reduction of stabilized $c1_{LPD}$ and a truncation of 7 amino acids ($VDR_{132-427}$) finally resulted in a complete loss of the ligand inducibility of $c1_{LPD}$ (Fig. 5*A*). The ligand inducibility of $c1_{LPD}$ of the C-terminal truncations VDR_{1-413}, VDR_{1-401}, and VDR_{1-390} was found to decrease with increasing number of truncated amino acids and was abolished when 39 amino acids were truncated from the C terminus of the VDR (VDR_{1-390}) (Fig. 5*B*). Interestingly, VDR conformation 3 ($c3_{LPD}$) was found to be more prominent with both N- and C-terminal truncations than with wild-type VDR (Fig. 1) and demonstrated, with excep-

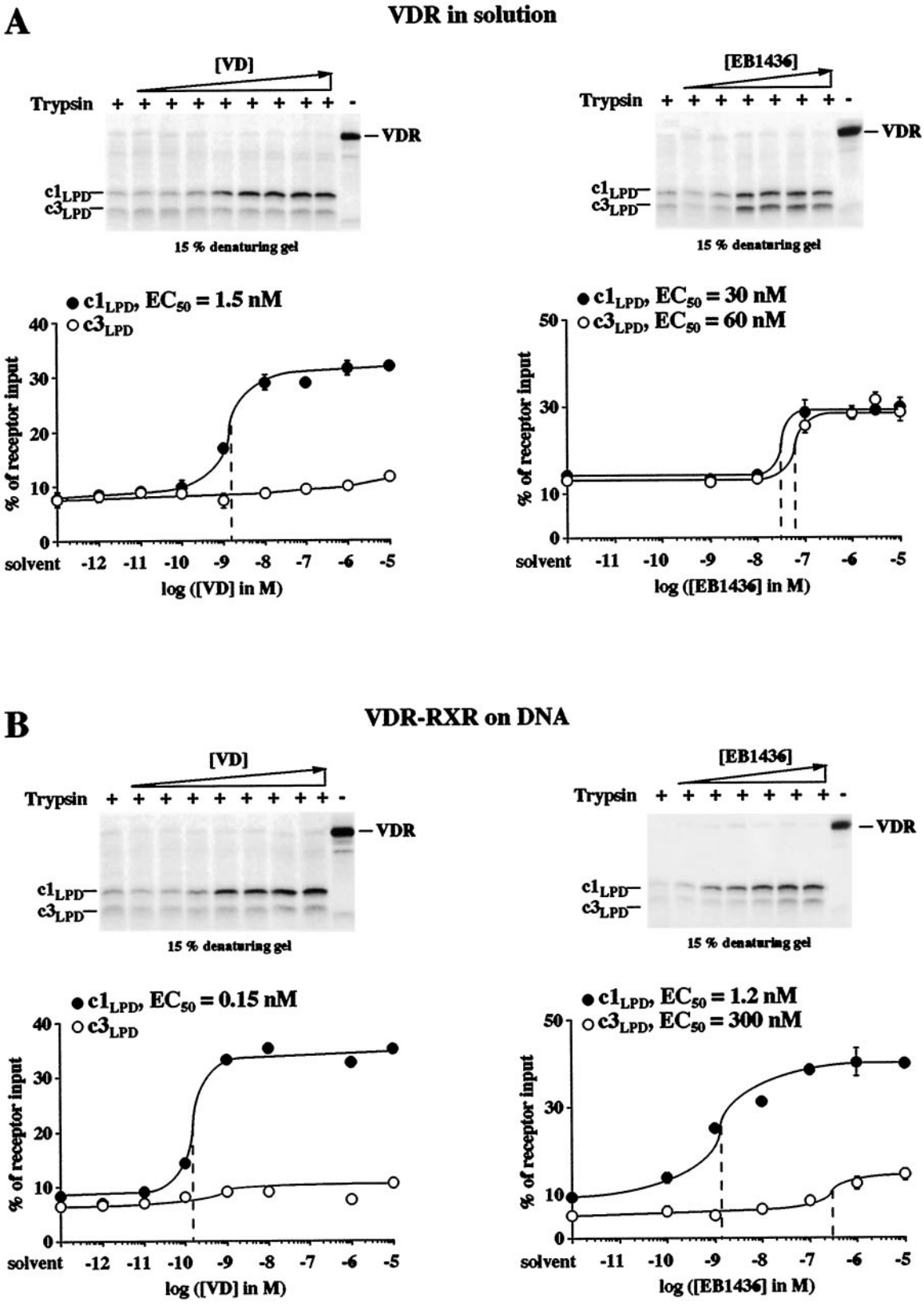

Fig. 2. Heterodimer complex formation on DNA enhances ligand sensitivity of VDR conformations. DNA-independent (*A*) and DNA-dependent (*B*) LPD assays were performed by preincubating in vitro-translated, [^{35}S]methionine-labeled wild-type VDR alone (*A*) or as a heterodimer with in vitro-translated RXR (*B*) with graded concentrations of VD and the analog EB1436 for 15 min at room temperature. VDR-RXR heterodimers were further incubated with the nonlabeled rat ANF DR3-type VDRE. In both cases, trypsin was added (final concentration 8.3 μg/ml), and incubation continued for 10 min. Samples were electrophoresed through 15% SDS-polyacrylamide gels. Representative experiments are shown. The amount of the VDR conformations 1 ($c1_{LPD}$, ●) and 3 ($c3_{LPD}$, ○) was quantified in relation to VDR input by phosphorimaging. Each data point represents the average of triplicates and bars indicate S.D. The EC_{50} values for the stabilization of VDR conformations were determined from dose-response curves.

tion of $VDR_{132-427}$ and VDR_{1-388}, some residual ligand inducibility.

The ligand sensitivity of the N-terminally truncated, monomeric VDR proteins $VDR_{125-427}$, $VDR_{128-427}$, and $VDR_{131-427}$ was analyzed in LPD assays (Fig. 6). An EC_{50} value of 1 nM was determined for the VD-induced stabilization of VDR conformation 1 ($c1_{LPD}$) with both $VDR_{125-427}$ and $VDR_{128-427}$, whereas with $VDR_{131-427}$ a value of only 7 nM was found. The lack of a DBD in these N-terminal truncations did not permit any DNA-dependent assays to be performed.

DNA-independent (Fig. 7*A*) and DNA-dependent (Fig. 7*B*) LPD assays were performed with the C-terminally truncated VDR proteins VDR_{1-413} and VDR_{1-401} in the absence and presence of the rat ANF DR3-type VDRE, respectively. With monomeric VDR_{1-413}, EC_{50} values of 45 and 50 nM were determined for the VD-induced stabilization of the VDR conformations 1 ($c1_{LPD}$) and 3 ($c3_{LPD}$), respectively, whereas with monomeric VDR_{1-401} EC_{50} values of 100 and 150 nM were found for $c1_{LPD}$ and $c3_{LPD}$ (Fig. 7*A*). DNA-complexed VDR_{1-413} provided EC_{50} values of 1 and 10 nM for $c1_{LPD}$ and $c3_{LPD}$, whereas under these conditions with VDR_{1-401} an EC_{50} value of only 100 nM was determined for both VDR conformations (Fig. 7*B*). GSC assays were performed with VDR_{1-413} and VDR_{1-401} proteins, which were complexed with RXR on the rat ANF DR3-type VDRE (Fig. 7*C*). With VDR_{1-413}, an EC_{50} value of 2 nM was determined for both VDR-RXR heterodimer conformations, whereas with VDR_{1-401} EC_{50} values of 100 and 50 nM were found for $c1_{GSC}$ and $c2_{GSC}$, respectively. Moreover, at saturating VD concentrations (10 μM), ~60% (VDR_{1-413}) or 50% (VDR_{1-401}) of all DNA-bound VDR-RXR heterodimers were stabilized in $c1_{GSC}$, whereas only 12% (VDR_{1-413}) and 17% (VDR_{1-401}) were found in $c2_{GSC}$.

Discussion

The activation of VDR-RXR heterodimers by ligand is the key reaction in nuclear VD signaling (Carlberg, 1996). Therefore, DNA-bound VDR-RXR heterodimers can be considered as the molecular switches in the VD endocrine system. The main goal of developing LPD and GSC assays was to produce a more detailed understanding of these molecular switches (Quack et al., 1998c), as both assay systems can also promise a fast and most accurate in vitro evaluation of VD analogues (Quack and Carlberg, 1999). The assays differ in their per-

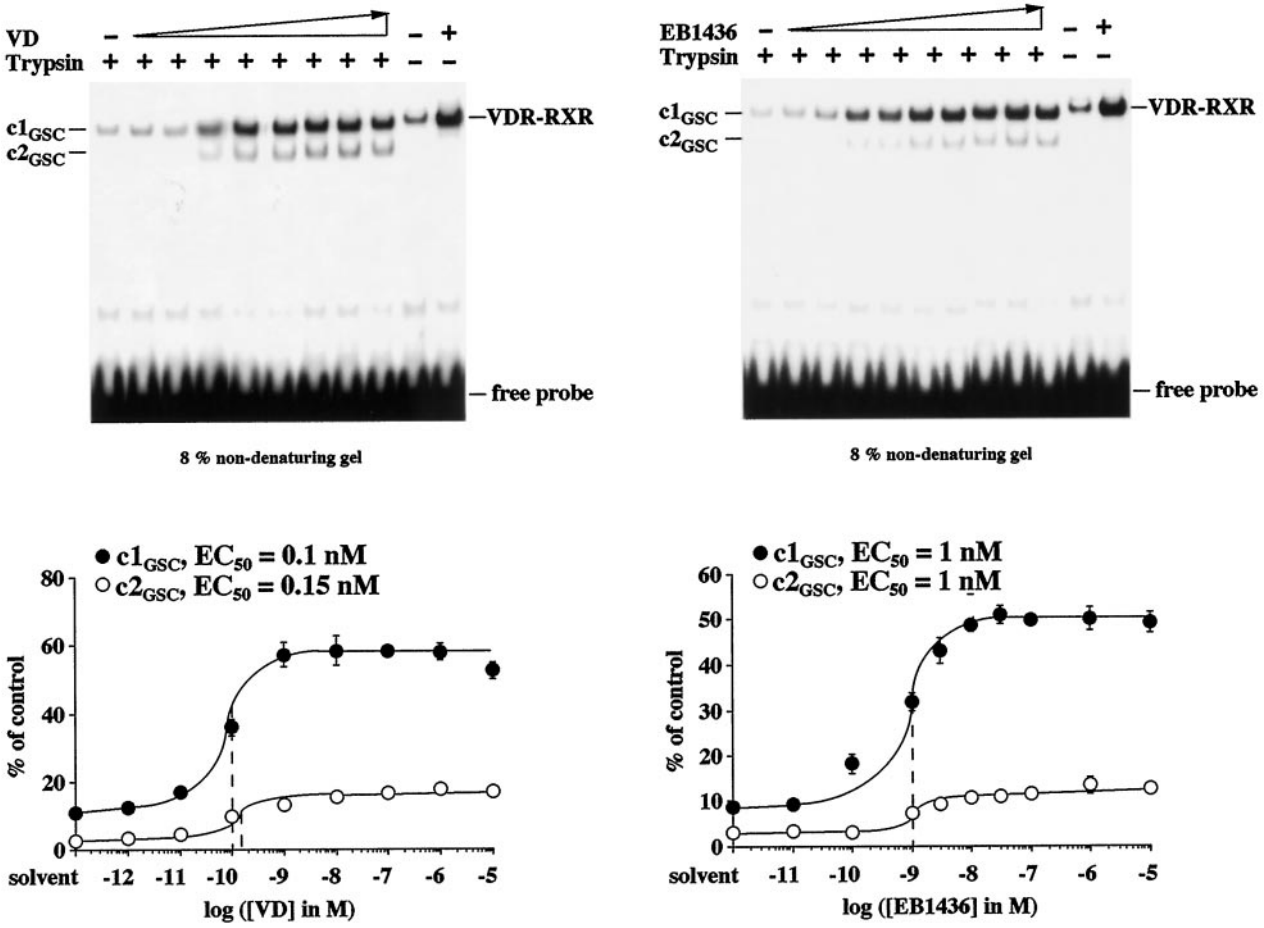

Fig. 3. Ligand-triggered stabilization of VDR-RXR heterodimer conformations on DNA. GSC assays were performed by preincubating heterodimers of in vitro-translated wild-type VDR and RXR proteins with graded concentrations of VD and EB1436 on the [^{32}P]-labeled rat ANF DR3-type VDRE for 15 min at room temperature. Then trypsin was added (final concentration 8.3 μg/ml), and incubation continued for 10 min. Protein-DNA complexes were separated from free probe on 8% nondenaturing polyacrylamide gels. Representative experiments are shown. The amount of digested VDR-RXR heterodimer-DNA complexes 1 and 2 (interpreted as VDR-RXR heterodimer conformations $c1_{GSC}$ and $c2_{GSC}$) were quantified in relation to respective ligand-induced, nondigested VDR-RXR heterodimers by phosphorimaging. Each data point represents the average of triplicates and bars indicate S.D. The EC_{50} values for the stabilization of VDR-RXR heterodimer conformations were determined from dose-response curves.

spectives on ligand-induced VDR conformations. The DNA-independent LPD assay studies monomeric VDR in solution, whereas the GSC assay analyses DNA-bound VDR-RXR heterodimers. However, in this study LPD was also performed with DNA-bound VDR-RXR heterodimers. The first surprising result was that DNA-dependent LPD assays and GSC assays provided the same highly sensitive EC_{50} value (0.1 nM in the case of the natural ligand VD), whereas in the DNA-independent LPD assay a clearly lower ligand sensitivity was observed. This also indicates that complex formation on DNA is a necessary prerequisite for high-affinity ligand binding of the VDR. With VD, the increase in affinity was only 10-fold, whereas with the model analog EB1436 a 25-fold increase in affinity was already apparent and some other VD analogues provide even higher factors (our unpublished observations). This suggests that for the most accurate analysis of the binding affinity of a VDR agonist to the receptor, the respective assays are preferably performed with DNA-complexed VDR.

With most VD analogues, the LPD assay allows the characterization of the two VDR conformations 1 ($c1_{LPD}$) and 3 ($c3_{LPD}$) (Peleg et al., 1995; Nayeri et al., 1996a, 1996b). Most potent agonists preferentially stabilize $c1_{LPD}$ of monomeric VDR, like VD in this study, but other agonists, such as EB1436, also stabilize $c3_{LPD}$. The latter observation is generally interpreted as a sign for a weak agonist, as the stabilization of conformation 3 requires rather high ligand concentrations (Quack et al., 1998a, 1998b). However, in this study it was observed that complex formation of the VDR on DNA results in a shift from conformation 3 to 1, i.e., the relative amount of VDR molecules that are stabilized in $c3_{LPD}$ are decreased in favor for those that occupy $c1_{LPD}$. This shift may also explain why the heterodimerization on DNA was found to have a more pronounced effect, for EB1436, on the increase of ligand sensitivity than with the natural ligand VD. Moreover, this suggests that a VDR agonist that appears to be weak in a DNA-independent assay may show unexpected potency in a DNA-dependent assay. However, even the two DNA-dependent assays characterize EB1436 as a VDR agonist that is ~10 times weaker than VD, which fits quite well its biological profile (Kissmeyer et al., 1997; Quack et al., 1998b).

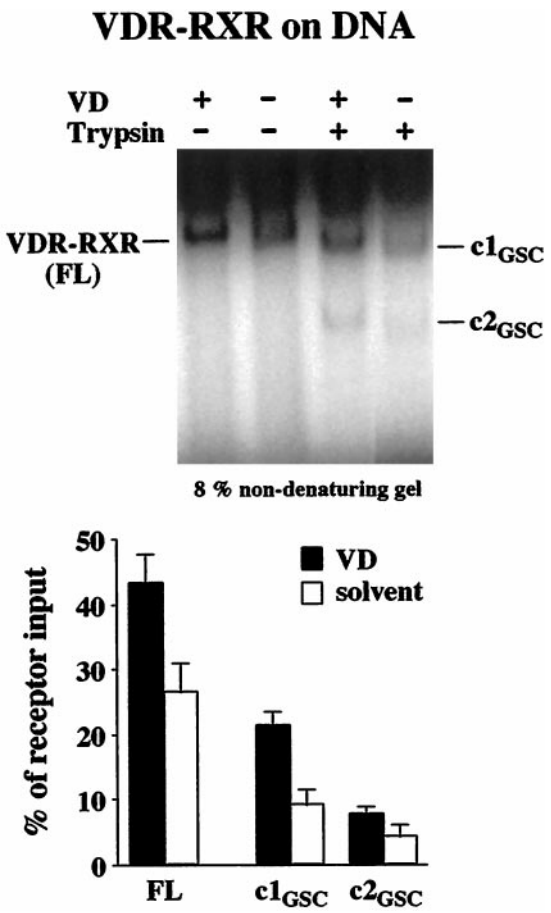

Fig. 4. VDR-RXR heterodimer conformations on DNA. "Reverse" GSC assays were performed by preincubating heterodimers of in vitro-translated [^{35}S]methionine-labeled wild-type VDR and nonlabeled RXR proteins with 10 μM VD on the nonlabeled DR3-type rat ANF VDRE for 15 min at room temperature. Then trypsin was added (final concentration 8.3 μg/ml), and incubation continued for 10 min. Protein-DNA complexes were separated on 8% nondenaturing polyacrylamide gels (using 4% stacking gels). A representative experiment is shown. The amount of nondigested DNA-complexed VDR-RXR heterodimers and of VDR-RXR heterodimer conformations 1 ($c1_{GSC}$) and 2 ($c2_{GSC}$) were quantified in relation to VDR input by phosphorimaging. Each column represents the average of triplicates and bars indicate S.D.

There is growing evidence that the VDR not only acts as a DNA-bound transcription factor, but also as a DNA-independent modulator of other nuclear signaling pathways. One example is the repression of IL-2 gene expression by VD, for which it was suggested that the VDR inhibits the complex formation of the T cell transcription factor nuclear factor of activated T cells on its specific binding site in the IL-2 promoter (Alroy et al., 1995). However, DNA-independent effects of the VDR are presently not as intensively studied as that of the RAR, for which agonists have been described that specifically block the action of the transcription factor activated protein-1 without activating "classical" retinoid signaling through RA response elements (Fanjul et al., 1994). Interestingly, also fast, nongenomic actions of VD are known (Norman, 1998), for which a membrane receptor has been postulated. However, an alternative hypothesis would be that the VDR modulates in a ligand-dependent fashion the activity of membrane-associated proteins, such as protein kinase C. VD analogues have been identified that are selective for a putative membrane signaling pathway (Norman et al., 1997), which may alternatively be explained through a selectivity of these analogues for a conformation of cytosolic VDR. Taken together, there are several indications that the characterization of VDR conformations in solution in comparison to those in DNA-complexed heterodimers may allow the identification of VDR agonists with a selective functional profile.

LPD assays showed that at saturating ligand concentrations up to 40% of all VDR molecules were found to be stabilized in VDR conformation $c1_{LPD}$, whereas "reverse" GSC assays demonstrated that ~20 and 10% of all VDR molecules participate in VDR-RXR conformations $c1_{GSC}$ and $c2_{GSC}$, respectively. This finally suggests that $c1_{GSC}$ and $c2_{GSC}$ (in sum ~30%) in DNA-bound heterodimers represent the same subset of VDR molecules than those that were found in VDR conformation $c1_{LPD}$ (~40%). A comparison of the effective ligand concentrations that were determined in reporter gene assays and other functional in vivo assays (Nayeri et al., 1995; Danielsson et al., 1996, 1997) with the in vitro evaluation of VD analogues in DNA-dependent LPD and GSC assays (this study, see also Quack and Carlberg,

1999) suggested that $c1_{LPD}$ and $c1_{GSC}$ and $c2_{GSC}$ represent active VDR-RXR heterodimers that act as the molecular switches of VD signaling. In contrast, this suggests that $c3_{LPD}$ appears not to be of relevance for the characterization of an agonistic behavior of a VDR ligand.

In the second part of this study, wild-type VDR was compared with different N- and C-terminal receptor truncations to further characterize these VDR conformations. The VDR truncations were chosen to span over critical regions of the LBD, which are the positions 125–132 and 388–427. In DNA-independent LPD assays, the N-terminal truncation $VDR_{125-427}$ demonstrates a similar behavior as the wild-type

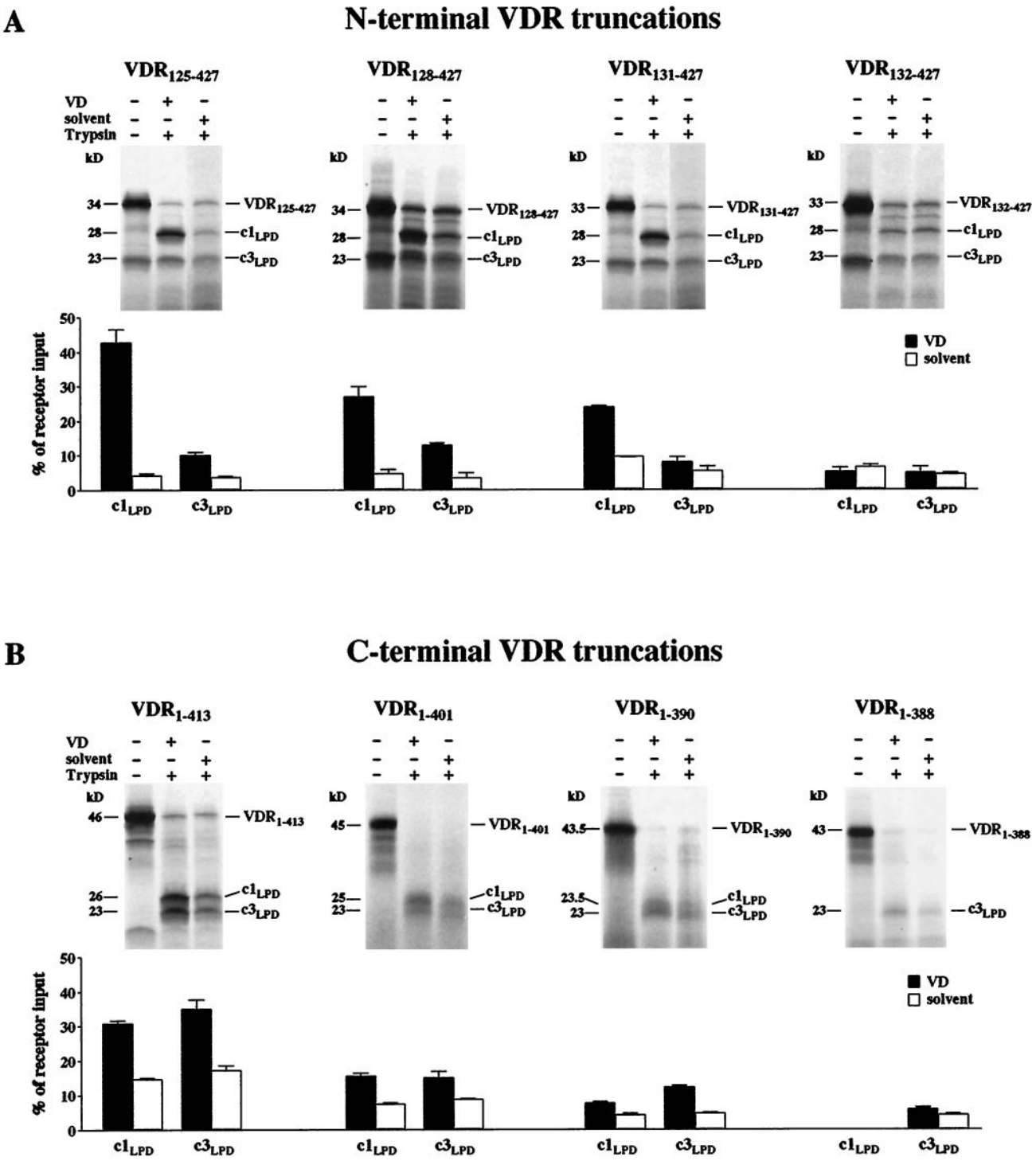

Fig. 5. Defining the border of the VDR-LBD. LPD assays were performed by preincubating in vitro-translated, [^{35}S]methionine-labeled N-terminal VDR truncations $VDR_{125-427}$, $VDR_{128-427}$, $VDR_{131-427}$, and $VDR_{132-427}$ (*A*) or C-terminal VDR truncations VDR_{1-413}, VDR_{1-401}, VDR_{1-390}, and VDR_{1-388} (*B*) with 10 μM VD or solvent for 15 min at room temperature. Then trypsin was added (final concentration 8.3 μg/ml), and incubation was continued for 10 min. Samples were electrophoresed through 15% SDS-polyacrylamide gels. Representative experiments are shown, where the molecular mass of truncated VDRs and VDR conformations 1 ($c1_{LPD}$) and 3 ($c3_{LPD}$) is indicated. The amount of $c1_{LPD}$ and $c3_{LPD}$ was quantified in relation to VDR input by phosphorimaging. Each column represents the average of triplicates and bars indicate S.D.

VDR in ligand sensitivity as well as maximal stabilization of $c1_{LPD}$ and $c3_{LPD}$. A further truncation by three amino acids ($VDR_{128-427}$) also showed no effect on ligand sensitivity, but resulted in a reduced amount of stabilized $c1_{LPD}$. Three and four additionally truncated amino acids ($VDR_{131-427}$ and $VDR_{132-427}$) nearly or completely abolished the ligand-dependent stabilization of $c1_{LPD}$. These results indicate that ligand effects on monomeric VDR do not require the DBD (positions 24–89), but the LBD from position 128. Amino acids 128–131 appear to be quite critical for the structural integrity of the LBD, which corresponds with the proposed start of helix 1 of the LBD at position 125 (Wurtz et al., 1997; Norman et al., 1999).

C-terminal truncations of the VDR also appear to be quite critical. A truncation of the 14 most C-terminal amino acids (VDR_{1-413}), i.e., of the AF-2 domain containing helix 12, resulted in a clear loss in ligand sensitivity of VDR conformation 1 (by a factor of 30 in the DNA-independent and by a factor of 10 in the DNA-dependent LPD assay). Truncation of 12 additional amino acids (VDR_{1-401}) resulted in further loss of ligand sensitivity (EC_{50} value of 100 nM) and in a drastic decrease in the amount of VD-stabilized $c1_{LPD}$. Both outcomes could not be improved by complex formation with DNA. The results for both truncations were confirmed by GSC assays. Finally, a truncation of in total 37 or 39 amino acids (VDR_{1-390} and VDR_{1-388}) resulted in a near complete or complete loss of the ligand sensitivity of $c1_{LPD}$. Interestingly, with an increasing number of amino acids that were truncated from the C terminus, the size of the VDR fragment 1 that represents $c1_{LPD}$ decreased in relation to the constant size of VDR fragment 3 (representing $c3_{LPD}$). This suggests that the major difference between $c1_{LPD}$ and $c3_{LPD}$ is the 40 most C-terminal amino acids, which would perfectly explain the size difference between VDR fragments 1 and 3. However, a high-affinity ligand interaction requires an intact C terminus of the LBD including the AF-2 domain. Point mutations within the AF-2 domain, in particular at the phenylalanine 422, have already indicated that this domain is essential for a most effective receptor-ligand interaction (Nayeri et al., 1996a; Liu et al., 1997; Nayeri and Carlberg, 1997).

Taken together, the analysis both of N- and C-terminal truncations of the VDR by biochemical assays allows the position of the high-affinity LBD of the VDR to be defined between amino acids 128 to 427. Interestingly, the VDR fragments that represent $c1_{LPD}$ (28 kD) and $c3_{LPD}$ (23 kD) do not contain all of these 300 amino acids. Microsequencing has indicated that the VDR fragments are generated by a digestion between amino acids 173 and 174 (Väisänen et al., 1997). This cutting site is located within a subdomain of the LBD that shows no homology to any other member of the nuclear receptor superfamily (Wurtz et al., 1996). Thus, it is likely that this subdomain is not involved in complexing the ligand in the ligand binding cleft and may therefore be exposed enough for accessing the protease.

Finally, the comparison of the EC_{50} values that were obtained with the VDR-RXR conformations $c1_{GSC}$ and $c2_{GSC}$ suggests that at least with the natural hormone VD both conformations have a very similar, if not identical ligand sensitivity. This makes it difficult to find a difference between both conformations. The truncation experiments clarified that the VDR half of both VDR-RXR complexes should be identical, otherwise different ligand sensitivity would be expected. Therefore, the remaining explanation is that the VDR-RXR conformations differ in their RXR moiety. The migration and apparent size difference between both VDR-RXR conformations is very likely due to the enzymatic truncation of the N-terminal domain of the RXR (~150 amino acids), whereas the faint migration difference between nondigested VDR-RXR heterodimers and VDR-RXR conformation 1 ($c1_{GSC}$) could be due to the truncation of the N-terminal region of the VDR (23 amino acids). However, there are VD analogues that selectively stabilize VDR-RXR conformations, such as the potent analog EB1089 that preferentially stabilizes $c1_{GSC}$ of VDR-RXR heterodimers bound to an IP9-type VDRE (Quack and Carlberg, 1999). The observation is taken as the explanation for the previously reported promoter selectivity of EB1089 (Nayeri et al., 1995). The improved understanding of VDR-RXR conformations would now suggest investigating further details of the promoter selectivity at the RXR moiety of the complex.

In conclusion, the results of this study provided several novel outcomes and important information on VDR and VDR-RXR heterodimer conformations that will allow for a more detailed understanding of the molecular switches of VD signaling.

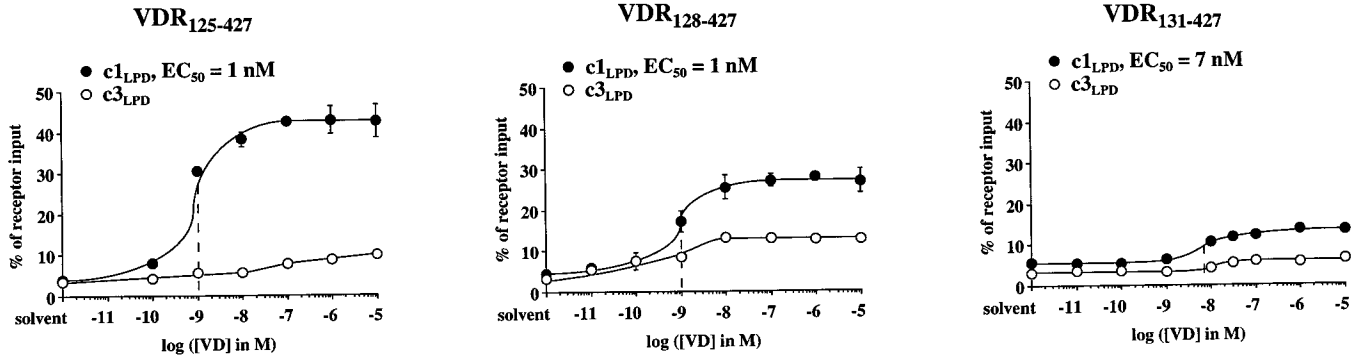

Fig. 6. Stabilization of VDR conformations in N-terminally truncated VDR proteins in solution. LPD assays were performed by preincubating in vitro-translated, [^{35}S]methionine-labeled VDR truncations $VDR_{125-427}$, $VD_{128-427}$, and $VDR_{131-427}$ with graded concentrations of VD for 15 min at room temperature. Then trypsin was added (final concentration 8.3 μg/ml), and incubation continued for 10 min. Samples were electrophoresed through 15% SDS-polyacrylamide gels. The amount of the VDR conformations 1 ($c1_{LPD}$, ●) and 3 ($c3_{LPD}$, ○) was quantified in relation to VDR input by phosphorimaging. Each data point represents the average of triplicates and bars indicate S.D. The EC_{50} values for stabilization of VDR conformations were determined from dose-response curves.

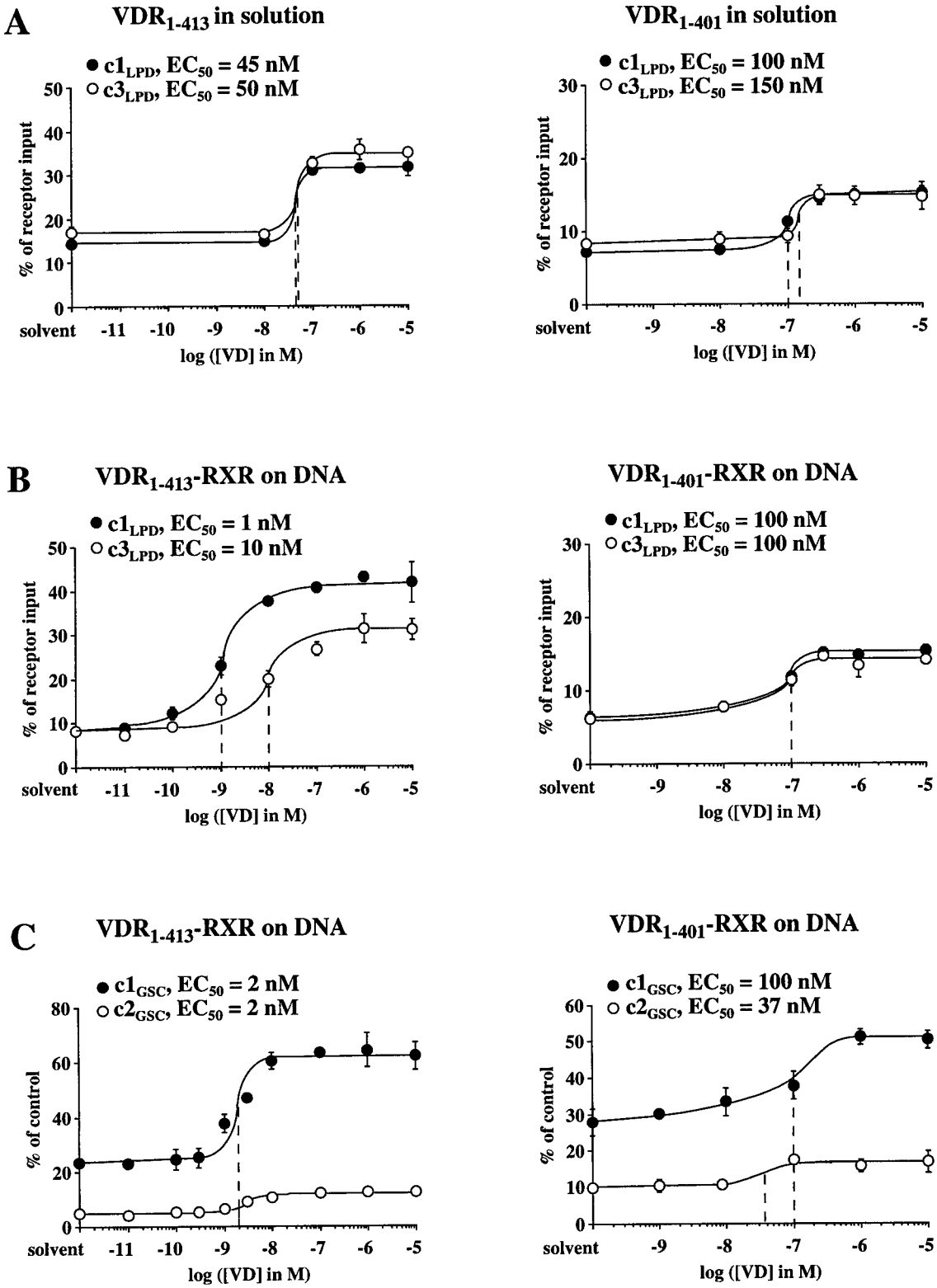

Fig. 7. Stabilization of VDR conformations in C-terminally truncated VDR proteins in solution and on DNA. LPD assays (*A* and *B*) were performed by preincubating in vitro-translated, [^{35}S]methionine-labeled VDR truncations VDR_{1-413} and VDR_{1-401} alone (*A*) or as heterodimers with in vitro-translated RXR (*B*) with graded concentrations of VD for 15 min at room temperature. VDR-RXR heterodimers were further incubated with the nonlabeled rat ANF DR3-type VDRE. GSC assays (*C*) were performed by preincubating heterodimers of in vitro-translated VDR truncations VDR_{1-413} and VDR_{1-401} and RXR proteins with graded concentrations of VD on the [^{32}P]-labeled rat ANF DR3-type VDRE for 15 min at room temperature. In all cases, trypsin was added (final concentration 8.3 μg/ml), and incubation continued for 10 min. Samples were electrophoresed through 15% SDS-polyacrylamide gels (*A* and *B*) or through 8% nondenaturing polyacrylamide gels (*C*). The amount of the VDR conformations 1 ($c1_{LPD}$, ●) and 3 ($c3_{LPD}$, ○) was quantified in relation to VDR input by phosphorimaging (*A* and *B*). Each data point represents the average of triplicates and bars indicate S.D. The amount of VDR-RXR heterodimer conformations 1 ($c1_{GSC}$) and 2 ($c2_{GSC}$) were quantified in relation to respective ligand-induced, nondigested VDR-RXR heterodimers by phosphorimaging (*C*). The respective EC_{50} values were determined from dose-response curves.

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