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Response Element and Coactivator-Mediated Conformational Change of the Vitamin D₃ Receptor Permits Sensitive Interaction with Agonists

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ABSTRACT

The vitamin D receptor (VDR) is the nuclear receptor for 1,25-dihydroxyvitamin D₃ [$1\alpha,25(OH)_2D_3$] that acts as a ligand-dependent transcription factor via combined contact with coactivator proteins (steroid receptor coactivator-1, transcriptional intermediary factor 2, and receptor associated coactivator 3) and specific DNA binding sites [vitamin D response elements (VDREs)]. Ligand-mediated conformational changes of the VDR contribute to the key mechanisms in this nuclear hormone signaling process. $1\alpha,25(OH)_2D_3$, MC1288 [20-epi-$1\alpha,25(OH)_2D_3$], ZK161422 [20-methyl-$1\alpha,25(OH)_2D_3$], and Ro27–2310 (also called Gemini, having two side chains at carbon 20) were used as model VDR agonists. The analysis of agonist-induced VDR conformations and coactivator interactions were found to be insufficient for extrapolating in vivo activities. In DNA-independent assays, such as classical limited protease digestions and glutathione S-transferase pull downs, Gemini seemed to be up to 10,000-fold and the other

VDR agonists 10- to 100-fold weaker than in functional in vivo assays. A more accurate description of the gene regulatory potential of VDR agonists was obtained with all tested VDR agonists by analyzing VDR conformations in the context of VDRE-bound VDR-retinoid X receptor heterodimers, in such assays as gel supershift, gel shift clipping, and limited protease digestion in the presence of DNA and cofactor. Coactivators were found to shift the ligand sensitivity (by a factor of 4 for Gemini) and the ratio of VDR conformations in the presence of DNA toward the high-affinity ligand binding conformation ($c1_{LPD}$). In conclusion, the induction of response element- and coactivator-modulated VDR conformations appears to be a key step for the gene regulatory function of a VDR agonist. The quantification of these effects would be of central importance for the evaluation of the cell-specific efficacy of systemically applied $1\alpha,25(OH)_2D_3$ analogs.

All genomic effects of 1α,25-dihydroxyvitamin D₃]1α,25(OH)₂D₃], which is the biologically active form of vitamin D₃, seem to be mediated through its nuclear receptor, the vitamin D receptor (VDR) (Carlberg and Polly, 1998). VDR preferentially binds as a heterodimer with the retinoid X receptor (RXR) to specific sequences in promoter regions of $1\alpha,25(OH)_2D_3$ target genes, commonly referred to as $1\alpha,25(OH)_2D_3$ response elements (VDREs) (Carlberg and Polly, 1998). Simple VDREs consist of two hexameric nuclear receptor binding sites that are arranged as a direct repeat with three spacing nucleotides (DR3-type VDREs) or an in-

verted palindrome with nine intervening nucleotides (IP9-type VDREs) (Carlberg, 1995). VDR contains two zinc finger structures that form the DNA binding domain (DBD) of 66 amino acids (aa) that is characteristic of all members of the nuclear receptor superfamily (Freedman, 1992), in addition to a carboxyl-terminal ligand binding domain (LBD) of 302 aa, which is formed by 12 α helices (Wurtz et al., 1996). The LBD has diverse functions in addition to ligand binding. These include interaction with other nuclear receptors for the formation of dimeric complexes and contact with nuclear mediator proteins, such as coactivators and corepressors, for modulation of transcriptional activities.

The general functional role of coactivators seems to be an enhancement of target gene transcription. In the past 4 years, several coactivators have been cloned and characterized; of these, the three members of the steroid receptor

ABBREVIATIONS: $1\alpha,25(OH)_2D_3$, 1,25-dihydroxyvitamin D₃; VDR, 1,25-dihydroxyvitamin D₃ receptor; VDRE, 1,25-dihydroxyvitamin D₃ response element; DR3, direct repeat spaced by 3 nucleotides; IP9, inverted palindrome spaced by nine nucleotides; DBD, DNA binding domain; aa, amino acids; LBD, ligand binding domain; SRC-1, steroid receptor coactivator-1; TIF2, transcriptional intermediary factor 2; RAC3, receptor associated coactivator 3; RXR, retinoid X receptor; PCR, polymerase chain reaction; GST, glutathione S-transferase; ANF, atrial natriuretic factor; DMEM, Dulbecco's modified Eagle's medium; LPD, limited protease digestion.

coactivator (SRC)-family, SRC-1/ERAP160/NCoA1, transcriptional intermediary factor 2 (TIF2)/Grip-1/NCoA2 and receptor-associated coactivator 3 (RAC3)/AIB1/ACTR/pCIP (reviewed in Chen and Li, 1998) seem to be the most prominent. The binding of $1\alpha,25(OH)_2D_3$ or its analogs induces a conformational change in the ligand binding domain (LBD) of the VDR, which then facilitates the interaction with coactivator proteins. Contact points for the members of the SRC-family have been mapped in the activation function 2 domain of helix 12 (Jurutka et al., 1997; Gill et al., 1998) and in helix 3 (Jimenez-Lara and Aranda, 1999; Kraichely et al., 1999) of the VDR. Modeling of the VDR-LBD structure (Norman et al., 1999) has confirmed cofactor contact points in these two helices, which were proposed by scanning surface mutagenesis of the LBD of the thyroid hormone receptor (Feng et al., 1998). This VDR-coactivator interaction then further facilitates recruitment of other factors to form a larger complex that modulates chromatin structure and initiates transcription (Spencer et al., 1997). This also involves the recently described DRIP/ARC cofactor complexes (Näär et al., 1999; Rachez et al., 1999), which seem to contact the VDR and other nuclear receptors preceding their interaction with SRC-family coactivators (Freedman, 1999).

The main physiological role of $1\alpha,25(OH)_2D_3$ is the regulation of calcium homeostasis and bone mineralization (De-Luca et al., 1990); however, the hormone is also involved in controlling cellular growth, differentiation, and apoptosis (Walters, 1992). Various analogs of $1\alpha,25(OH)_2D_3$, which mainly contain modifications of the side chain, have been developed with the goal to improve the biological profile of the natural hormone for a potential therapeutic application (Bouillon et al., 1995). However, several of these analogs represent interesting model agonists that are useful for studying the action of the VDR. These investigations focus on the interaction of $1\alpha,25(OH)_2D_3$ and its analogs with the receptor, via the induction of a conformational change in the LBD. Traditional competition assays using radiolabeled ligand provide an idea of the receptor-ligand interaction affinity but do not allow for the visualization of receptor conformational changes (Mørk Hansen et al., 1996). Therefore, the limited protease digestion assay, in which interaction of a nuclear receptor with ligand protects the LBD against protease digestion (Leng et al., 1993), has proven to be a powerful method for characterizing functional VDR conformations (Nayeri and Carlberg, 1997). The latest development that allows evaluation of ligand-induced conformations of DNA-bound VDR-RXR heterodimers is the gel shift clipping assay, which combines gel shift assays with limited protease digestion assays (Quack et al., 1998; Quack and Carlberg, 1999).

In this report, the effects of an agonist-triggered conformational change of the VDR have been studied by various in vitro and in vivo methods. The $1\alpha,25(OH)_2D_3$ analogs MC1288 [20-epi-$1\alpha,25(OH)_2D_3$] and ZK161422 [20-methyl-$1\alpha,25(OH)_2D_3$] were chosen as model VDR agonists, because their main structural characteristic, compared with the natural hormone, is an opposite orientation of their side chains. In addition, a novel $1\alpha,25(OH)_2D_3$ analog, called Gemini or Ro27-2310, which seems to be a "superimposed" hybrid structure of $1\alpha,25(OH)_2D_3$ and MC1288 by carrying two side chains at carbon 20, was studied. The results suggest that the induction of response element- and coactivator-modu-

lated VDR conformations is a key step for the action of a VDR agonist.

Materials and Methods

Compounds

$1\alpha,25(OH)_2D_3$ and MC1288 (Binderup et al., 1991) were dissolved in 2-propanol; ZK161422 (Neef et al., 1995) and Gemini [two side chains, one in the normal orientation and the other in 20-epi orientation, also called Ro27-2310 (Uskokovic et al., 1997)] were dissolved in ethanol. All compounds were further diluted in ethanol for use in assays. The structure of the side chains of all four compounds are shown in Fig. 1. Gemini was synthesized at Roche, $1\alpha,25(OH)_2D_3$ and MC1288 were obtained from LEO (Ballerup, Denmark), and ZK161422 was provided by Schering (Berlin, Germany).

DNA Constructs

In Vitro Translation/Mammalian Expression Constructs. The full-length cDNAs for human VDR (Carlberg et al., 1993), human retinoid X receptor (RXR) α (Levin et al., 1992) and human TIF2 (Voegel et al., 1996) were subcloned into the simian virus 40 promoter-driven pSG5 expression vector (Stratagene, Heidelberg, Germany). The interaction domains of SRC-1 (spanning aa 596–790) (Onate et al., 1995), TIF2 (spanning aa 646–926) (Voegel et al., 1996), and RAC3 (spanning aa 673-1106) (Li et al., 1997) were generated by polymerase chain reaction (PCR). The respective 5′-PCR primers each contained a T_7 promoter and a start codon, which allowed for in vitro transcription of the respective PCR products.

Glutathione S-Transferase (GST) Fusion Protein Constructs. The full-length cDNA of human VDR (aa 1–427) and the nuclear receptor interaction domain of human TIF2 (spanning aa 646–926) (Voegel et al., 1996) were subcloned into *Bam*HI and *Hin*dIII sites of the GST fusion vector pGEX (Amersham-Pharmacia, Freiburg, Germany).

VDRE-Driven Reporter Gene Constructs. Four copies of the DR3-type VDRE from the rat atrial natriuretic factor (ANF) gene promoter (Kahlen and Carlberg, 1996) and four copies of the IP9-type VDRE from the mouse c-*fos* promoter (Schräder et al., 1997)
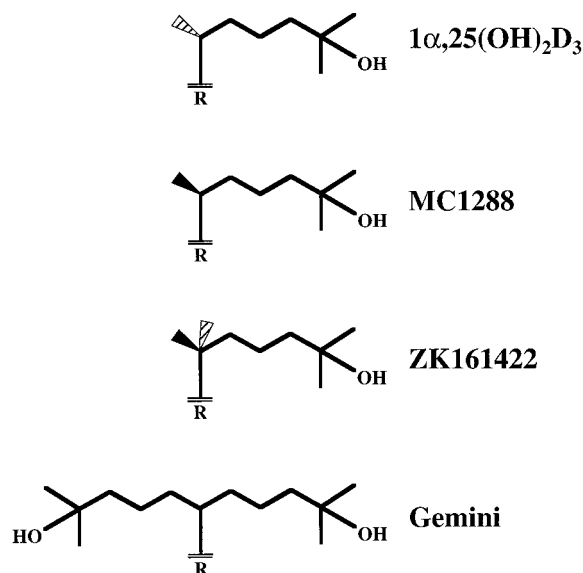

Fig. 1. The side chain structure of $1\alpha,25(OH)_2D_3$ and its analogs MC1288, ZK161422, and Gemini. Compared with $1\alpha,25(OH)_2D_3$, the side chain of MC1288 has a 20-epi conformation. An addition of a methyl group at position 20 created a similar conformation in ZK161422. The Gemini analog contains a complete second side chain at position 20. Gemini was synthesized at Roche, $1\alpha,25(OH)_2D_3$ and MC1288 were obtained from LEO and ZK161422 was provided by Schering.

were fused with the thymidine kinase (*tk*) minimal promoter driving the luciferase reporter gene. The core sequences of the VDREs are given in Fig. 4.

GAL4-Fusion Constructs. The DBD of the yeast transcription factor GAL4 (aa 1–147) was fused with the cDNA of the LBD of human VDR (aa 109–427). Three copies of the GAL4 binding site were fused with the *tk* promoter driving the luciferase reporter gene by for mammalian-one-hybrid assays (Hörlein et al., 1995).

In Vitro Protein Translation

In vitro translated VDR, RXR, $TIF2_{646-926}$, $SRC-1_{596-790}$, and $RAC3_{673-1106}$ proteins were generated by transcribing their respective linearized pSG5-based cDNA expression vectors or PCR products with T_7 RNA polymerase and translating these RNAs in vitro using rabbit reticulocyte lysate as recommended by the supplier (Promega, Mannheim, Germany).

Limited Protease Digestion Assay

In vitro translated, ^{35}S-labeled VDR protein (2.5 μl) and ligand in 10 to 20 μl of binding buffer [10 mM HEPES, pH 7.9, 1 mM dithiothreitol, 0.2 μg/μl poly(dI-C), and 5% glycerol] were preincubated for 15 min at room temperature. In the modified form of the limited protease digestion assay, complex formation with 2.5 μl of in vitro translated RXR, 1 ng of unlabeled rat ANF DR3-type VDRE, and 5 μl of bacterially produced $GST-TIF2_{646-926}$ was performed. The buffer was adjusted to 150 mM monovalent cations by addition of KCl. In both cases, trypsin (final concentration, 8.3 ng/μl; Promega) was then added and the mixtures were further incubated for 10 to 15 min at room temperature. The digestion reactions were stopped by adding 10 to 20 μl of protein gel loading buffer (0.25 M Tris, pH 6.8, 20% glycerol, 5% mercaptoethanol, 2% SDS, and 0.025% bromphenol blue). The samples were denatured at 95°C for 3 min and electrophoresed through a 15% SDS-polyacrylamide gel. The gels were dried and exposed to a Fuji MP2040S imager screen. The individual protease-sensitive VDR fragments were detected on a Fuji FLA2000 reader (Tokyo, Japan) using Image Gauge software (Raytest, Sprockhövel, Germany).

GST Pull-Down Assays

Bacterial overexpression of GST-VDR and $GST-TIF2_{646-926}$ was facilitated in the *Escherichia coli* BL21(DE3)pLysS strain (Stratagene). GST-VDR fusion protein expression was performed with isopropyl-β-D-thio-galactopyranoside (0.25 mM) for 3 h at 30°C and $GST-TIF2_{646-926}$ expression with isopropyl-β-D-thio-galactopyranoside (0.25 mM) for 3 h at 37°C. The fusion proteins were checked for equal loading by Coomassie Brilliant Blue staining. GST pull-down assays were performed by coincubation of a 50% GST-VDR- or $GST-TIF2_{646-926}$-Sepharose bead slurry with in vitro translated ^{35}S-labeled $TIF2_{646-926}$, ^{35}S-labeled $SRC-1_{596-790}$, ^{35}S-labeled $RAC3_{673-1106}$, or ^{35}S-labeled VDR and the respective VDR agonists (graded concentrations) in immunoprecipitation buffer (20 mM HEPES, pH 7.9, 200 mM KCl, 1 mM EDTA, 4 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 10% glycerol) for 20 min at 30°C. GST-fusion protein-Sepharose slurries were routinely preblocked in immunoprecipitation buffer containing BSA (1 μg/μl) before use in pull-down assays. In vitro translated proteins that were not bound to GST-fusion proteins were washed away with immunoprecipitation buffer. GST-fusion protein bound proteins were detected by electrophoresis through a 10% SDS-polyacrylamide gel and were quantified with the use of a Fuji FLA2000 reader.

Transfection and Luciferase Assays

Cos-7 simian virus 40-transformed African Green monkey kidney cells were seeded into six-well plates (10^5 cells/ml) and grown overnight in phenol-red–free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% charcoal-treated fetal bovine serum (FBS). Liposomes were formed by incubating 1 μg of the re-

porter plasmid, 1 μg of each pSG5-based receptor expression vector for VDR, RXR, and TIF2 (as indicated in the figure legends), and 1 μg of the reference plasmid pCH110 (Amersham-Pharmacia) with 15 μg of *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (Roche Diagnostics, Mannheim, Germany) for 15 min at room temperature in a total volume of 100 μl. After dilution with 900 μl of phenol-red-free DMEM, the liposomes were added to the cells. Phenol-red-free DMEM supplemented with 30% charcoal-treated fetal bovine serum (500 μl) was added 4 h after transfection. At this time, graded concentrations of VDR agonists were also added. HeLa human cervix carcinoma cells were cultured, seeded, and transfected under the same conditions as Cos-7 cells, but for the mammalian-one-hybrid assay, the expression vector for the $GAL4_{DBD}VDR_{LBD}$-fusion protein and a GAL4 binding site-driven luciferase reporter gene construct were used in transfections. The cells were lysed 16 h after onset of stimulation using the reporter gene lysis buffer (Roche Diagnostics) for both types of assay and the constant light signal luciferase reporter gene assay was performed as recommended by the supplier (Roche Diagnostics). The luciferase activities were normalized with respect to β-galactosidase activity and induction factors were calculated as the ratio of luciferase activity of ligand-stimulated cells to that of solvent controls.

Gel Shift Assays, Supershift Assays And Gel Shift Clipping Assays

In vitro translated VDR-RXR heterodimers were incubated in graded concentrations of VDR agonists for 15 min at room temperature in a total volume of 20 μl of binding buffer. The buffer had been adjusted to 150 mM by addition of KCl. For supershift assays, 3 μl of $GST-TIF2_{646-926}$ fusion protein were included in the incubation. Approximately 1 ng of ^{32}P-labeled rat ANF DR3-type VDRE or mouse c-*fos* IP9-type VDRE probe (50,000 cpm) was added to the protein-ligand mixture and incubation was continued for 20 min. For gel shift clipping assays, trypsin was added to a final concentration of 8.3 ng/μl and the incubation was continued for 15 min further at room temperature. Nondigested as well as partially digested protein-DNA complexes were resolved through an 8% nondenaturing polyacrylamide gel in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) and were quantified with the use of a PhosphorImager.

Results

Classical limited protease digestion assays were performed with graded concentrations of $1\alpha,25(OH)_2D_3$ and the model analogs MC1288, ZK161422, and Gemini (for structures, see Fig. 1) which provided two digestion products, $c1_{LPD}$ (LPD, limited protease digestion) and $c3_{LPD}$ (Fig. 2). In the case of MC1288, the additional digestion product $c2_{LPD}$ was observed. The VDR fragments $c1_{LPD}$, $c2_{LPD}$, and $c3_{LPD}$ have been characterized previously as containing major parts of the LBD and its carboxyl-terminal truncations, which represent the functional VDR conformations 1, 2, and 3, respectively (Peleg et al., 1995; Nayeri et al., 1996; Liu et al., 1997; Nayeri and Carlberg, 1997). $1\alpha,25(OH)_2D_3$ and the analogs MC1288 and ZK161422 predominantly stabilize 60 to 80% of all VDR molecules in $c1_{LPD}$ in a dose-dependent fashion. Conformation 1_{LPD} is known to be the most ligand-sensitive of the three functional VDR conformations (Nayeri et al., 1996). Half-maximal activation (EC_{50}) values of 8 nM for $1\alpha,25(OH)_2D_3$, 3 nM for MC1288, and 0.65 nM for ZK161422 were determined from the dose-response curves. In contrast, Gemini was found to stabilize predominantly 80% of all VDR molecules in $c3_{LPD}$ with an EC_{50} value of 90 nM.

GST pull-down assays were performed with bacterially

produced GST-VDR fusion proteins, in vitro translated TIF2 nuclear receptor interaction domains ($TIF2_{646-926}$), and graded concentrations of the four VDR agonists. $1\alpha,25(OH)_2D_3$, MC1288, and ZK161422 mediated a precipitation of up to 25% of $TIF2_{646-926}$ input in a dose-dependent fashion, with EC_{50} values of 9 nM for $1\alpha,25(OH)_2D_3$, 7 nM for MC1288, and 60 nM for ZK161422 (Fig. 3A). In contrast, Gemini was only able to precipitate 15% of $TIF2_{646-926}$ input with an EC_{50} value of 1000 nM. A direct comparison of the ability of the four VDR agonists to mediate an interaction of GST-VDR fusion proteins, at saturating concentrations (10 μM), with the nuclear receptor interaction domains of the three members of the SRC coactivator-family, $SRC-1_{596-790}$, $TIF2_{646-926}$, and $RAC3_{673-1106}$, demonstrated very similar results (Fig. 3B). $1\alpha,25(OH)_2D_3$, MC1288, and ZK161422 showed a similar high potency, whereas Gemini was found to be clearly less potent. The "inverse" pull-down experiment was performed as a control, in which VDR was in vitro translated and $TIF2_{646-926}$ was provided as a bacterially

produced GST fusion protein (Fig. 3C). In this experiment, Gemini also clearly demonstrated lower potency than the natural hormone, both at the level of the VDR's ligand sensitivity [EC_{50} values of 150 nM for Gemini versus 9 nM for $1\alpha,25(OH)_2D_3$] and amount of precipitated VDR at saturating concentrations [11% for Gemini versus 19% for $1\alpha,25(OH)_2D_3$].

Luciferase reporter gene assays were performed in Cos-7 cells that were transfected with luciferase reporter gene constructs driven by the DR3-type VDRE from the rat ANF gene promoter (Kahlen and Carlberg, 1996) or by the IP9-type VDRE from the mouse c-*fos* gene promoter (Schräder et al., 1997), together with expression vectors for VDR and RXR alone or additionally with an expression vector for full-length TIF2. Cells were stimulated for 16 h with graded concentrations of $1\alpha,25(OH)_2D_3$, MC1288, ZK161422, and Gemini. β-Galactosidase-normalized luciferase reporter gene activities were expressed as fold-induction and provided typical dose-response curves (Fig. 4). When coactivators were not
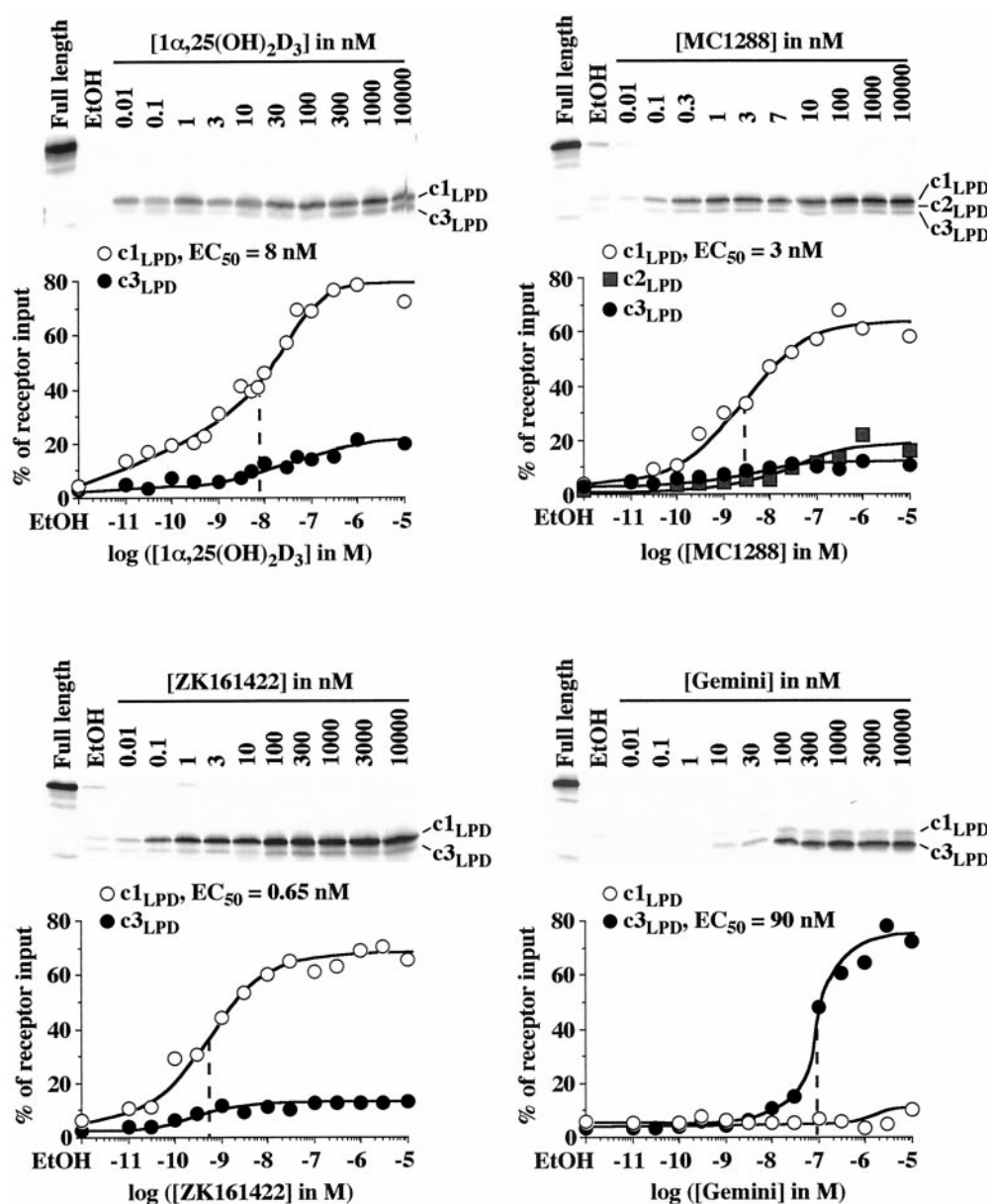

Fig. 2. Stabilization of VDR conformations in solution. Limited protease digestion assays were performed by preincubating in vitro synthesized ^{35}S-labeled VDR with graded concentrations of $1\alpha,25(OH)_2D_3$, MC1288, ZK161422, and Gemini. Trypsin was then added and the mixtures were further incubated at room temperature. Samples were electrophoresed through a 15% SDS-polyacrylamide gel. Representative experiments are shown. The amount of ligand-stabilized VDR in conformations 1 ($c1_{LPD}$, $\bigcirc$), 2 ($c2_{LPD}$, squares, only in case of MC1288) and 3 ($c3_{LPD}$, $\bullet$) was calculated in relation to VDR input. Each data point represents the mean of at least three experiments, the standard deviation was generally below 10%. The EC_{50} values for the stabilization of the respective predominant conformation were determined from the dose-response curves.

overexpressed in Cos-7 cells, the EC_{50} values that were determined from dose response curves demonstrated that ZK161422 (2 nM on the DR3-type VDRE and 1.8 nM on the IP9-type VDRE) was as sensitive as $1\alpha,25(OH)_2D_3$ (3 and 1.8 nM), whereas MC1288 (0.1 and 0.04 nM) and Gemini (0.8 and 0.4 nM) seemed to be approximately 30-fold and 4-fold

more sensitive than the natural hormone, respectively. The IP9-type VDRE showed slightly higher ligand sensitivity; this, however, did not represent significant promoter selectivity compared with $1\alpha,25(OH)_2D_3$ analogs such as EB1089 (Nayeri et al., 1995). The overexpression of TIF2 resulted in a shift of the respective EC_{50} values; the effect with the VDR
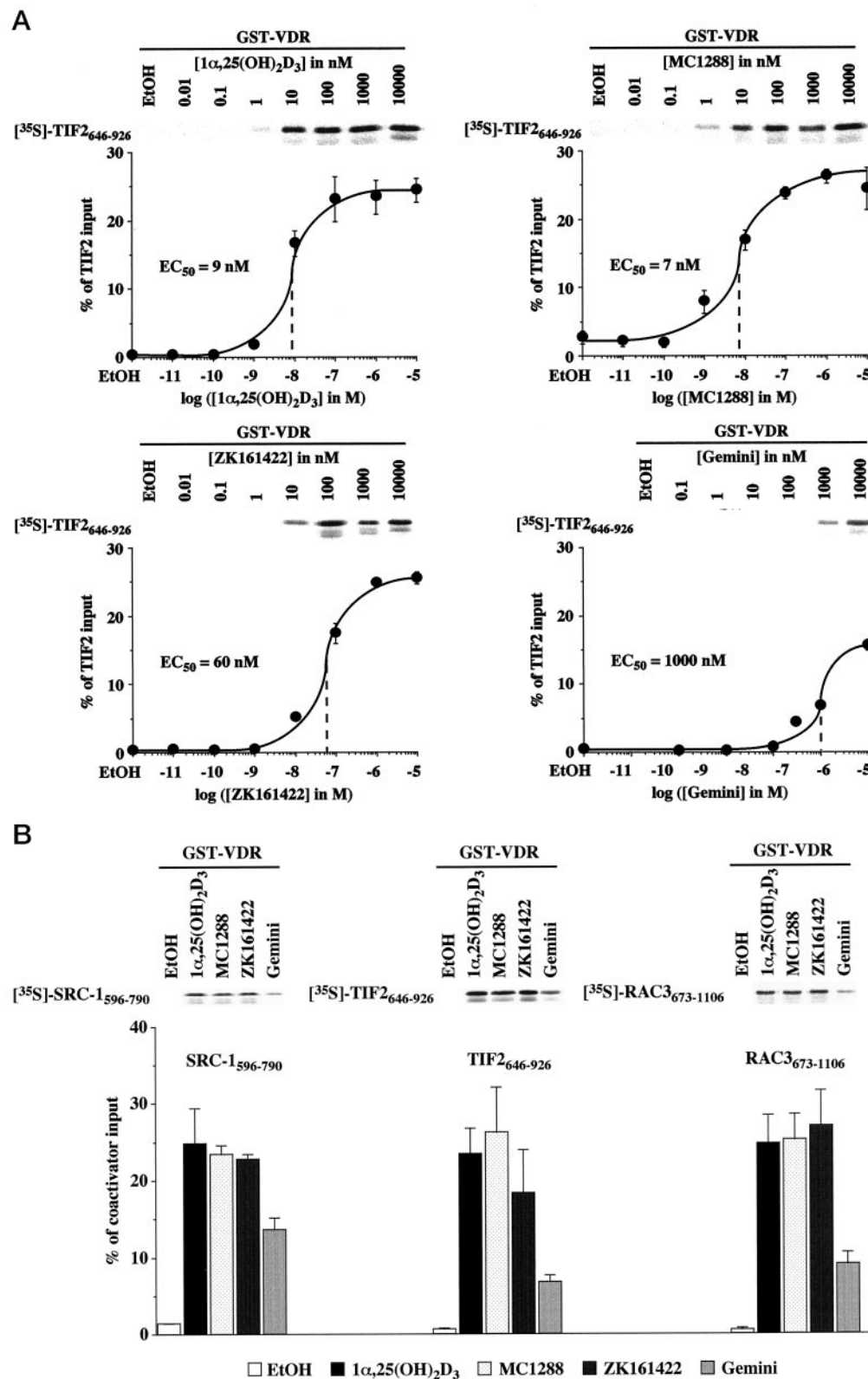

Fig. 3. Ligand-triggered VDR-coactivator interaction. GST pull-down assays were performed with in vitro translated [35S]-labeled TIF2$_{646-926}$ (A, B), [35S]-labeled SRC-1$_{596-790}$ (B) and [35S]-labeled RAC3$_{673-1106}$ (B), [35S]-labeled VDR (C), and bacterially expressed GST-VDR (A, B) or GST-TIF2$_{646-926}$ (C). Nuclear receptors were preincubated at room temperature with graded (A, C) or saturating (10 μM, B) concentrations of $1\alpha,25(OH)_2D_3$, MC1288, ZK161422, and Gemini. Representative experiments are shown. The percentage of precipitated coactivator (A, B) or VDR (C) with respect to input was quantified with the use of a PhosphorImager. Data points (A, C) or columns (B) represent mean values of triplicates and the bars indicate standard deviations. The EC_{50} values for the VDR-coactivator interaction were determined from the dose-response curves.

agonists $1\alpha,25(OH)_2D_3$ (1.6 nM on the DR3-type VDRE and 0.6 nM on the IP9-type VDRE) and MC1288 (0.1 and 0.022 nM) was only minor, whereas with ZK161422 (0.6 and 0.22 nM) and with Gemini in particular (0.022 and 0.06 nM), up to 36-fold higher ligand sensitivities were observed. The VDR agonists did not display reasonable promoter selectivity under these conditions. In addition to increasing ligand sensitivity, the overexpression of TIF2 provided higher inducibility of reporter gene activity, which varied between the VDR agonists and was found to be dependent on the type of VDRE. The combination of overexpressed TIF2 and Gemini on a DR3-type VDRE resulted in 2-fold "superactivation", which was not observed on the IP9-type VDRE. Interestingly, the overexpression of SRC-1 and RAC3 had effects very similar to those seen for TIF2 (data not shown).

Mammalian one-hybrid assays in HeLa cells were used as an alternative in vivo test system for the evaluation of the relative potency of the four VDR agonists. Cells were transiently transfected with an expression vector for a fusion protein containing the DBD of the yeast transcription factor GAL4 and the VDR-LBD, in addition to a GAL4 binding site driven luciferase reporter construct. After stimulating the cells for 16 h with graded concentrations of $1\alpha,25(OH)_2D_3$, MC1288, ZK161422, and Gemini, luciferase reporter gene activities were determined (Fig. 5). The dose-response curves indicated that all three $1\alpha,25(OH)_2D_3$ analogs (EC_{50} values of 0.036 nM for MC1288, 0.1 for ZK161422, and 0.11 nM for Gemini) were more sensitive in inducing gene activity than the natural hormone $1\alpha,25(OH)_2D_3$ (EC_{50} = 0.9 nM) in this in vivo assay.

Ligand-dependent gel shift assays were performed with VDR-RXR heterodimers bound to the rat ANF DR3-type VDRE or the mouse c-*fos* IP9-type VDRE in the presence of GST-TIF2$_{646-926}$ and saturating concentrations (10 μM) of the four VDR agonists (Fig. 6A). The addition of each agonist induced an interaction of VDR-RXR heterodimers with the coactivator protein GST-TIF2$_{646-926}$, which was indicated by a supershift. The quantification of the relative intensities of these supershifts suggested that the interaction of VDR-RXR heterodimers with TIF2 seems to be independent from the type of VDRE used. As a control, a supershift with GST-TIF2$_{646-926}$ was not observed in the absence of ligand (first lane in each of the representative gels shown in Fig. 6B). Supershifts were performed with graded concentrations of $1\alpha,25(OH)_2D_3$, MC1288, ZK161422, and Gemini on the rat ANF DR3-type VDRE (Fig. 6B), because saturating concentrations of the different VDR agonists provided very similar quantities of supershifted VDR-RXR heterodimers. The resulting dose response curves provided EC_{50} values of 0.1 nM for $1\alpha,25(OH)_2D_3$, MC1288 and Gemini and of 0.2 nM for ZK161422. This suggests that in this DNA-dependent assay, all four tested VDR agonists have a very similar potency in inducing a conformation in the VDR that results in an interaction with coactivator proteins.

As second DNA-dependent assay, gel shift clipping assays were performed with VDR-RXR heterodimers bound to the rat ANF DR3-type VDRE in the presence of graded concentrations of $1\alpha,25(OH)_2D_3$, MC1288, ZK161422 and Gemini (Fig. 7). Separation of the reaction products through a nondenaturing polyacrylamide gel resulted in two protein-DNA complexes (c1$_{GSC}$ and c2$_{GSC}$) that migrated faster, i.e., that appeared to be of lower molecular mass than undigested VDR-RXR heterodimers. These complexes were interpreted as being representatives of different conformations of DNA-bound VDR-RXR heterodimers (Quack and Carlberg, 1999). Also in this DNA-dependent assay, all four VDR agonists were found to provide a very similar EC_{50} values (with $1\alpha,25(OH)_2D_3$ 0.1 nM for c1$_{GSC}$ and 0.2 nM for c2$_{GSC}$, with MC1288 0.08 and 0.07 nM, with ZK161422 0.013 and 0.006 nM and with Gemini 0.06 and 0.2 nM), which confirmed the average value of 0.1 nM the supershift assay (Fig. 5A). Moreover at saturating concentrations, all four ligands stabilized approximately 60% of the VDR-RXR heterodimers in c1$_{GSC}$ and only 20% in conformation c2$_{GSC}$. Furthermore, gel shift clipping assays were also performed with $1\alpha,25(OH)_2D_3$-stabilized VDR-RXR heterodimers in the presence of GST-TIF2$_{646-926}$, but showed no effect on the EC_{50} value and the c1$_{GSC}$/c2$_{GSC}$ ratio (data not shown).

Finally, a modified form of the limited protease digestion assay, where VDR molecules were complexed with RXR, bacterially produced GST-TIF2$_{646-926}$, and the rat ANF DR3-
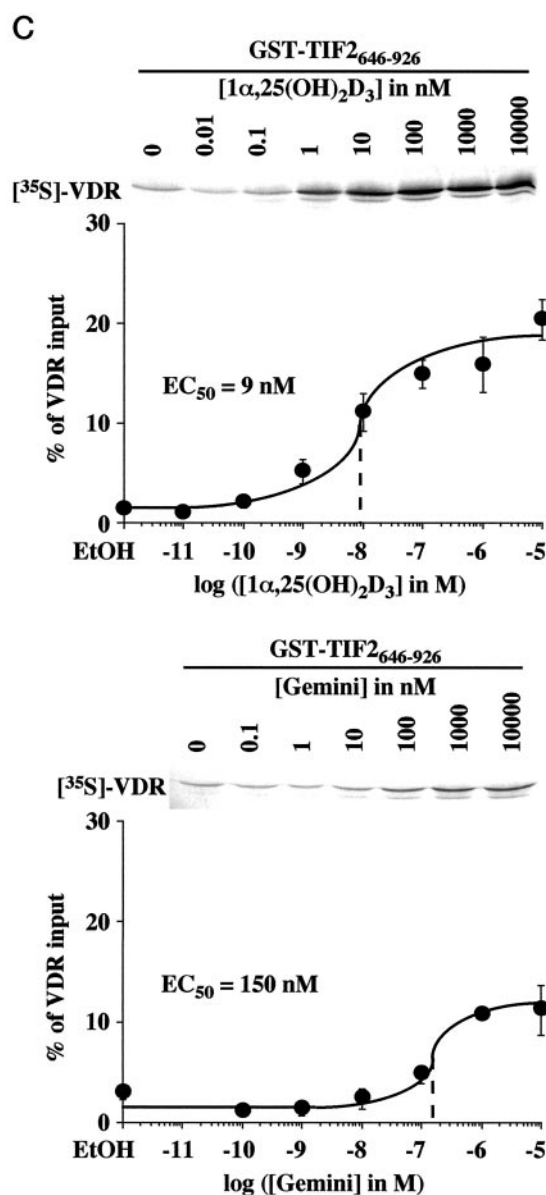

Fig 3. Continued.

type VDRE, was performed to assess the effect of graded concentrations of $1\alpha,25(OH)_2D_3$, MC1288, ZK161422 and Gemini on the functional conformations of ^{35}S-labeled VDR (Fig. 8A). When comparing the results from dose responses performed with the classical limited protease digestion assay that used VDR in solution (Fig. 2) with the dose responses from this modified assay (Fig. 8A), the RXR-driven complex

formation with a VDRE was found to clearly increase the ligand sensitivity of VDR conformation 1 ($c1_{LPD}$) for all four VDR agonists. For $1\alpha,25(OH)_2D_3$ the EC_{50} value decreased from 8 nM in the absence of DNA to 0.3 nM in the presence of DNA; for MC1288, the shift was from 3 to 0.3 nM; for ZK161422, from 0.65 nM to 0.09 nM, and for Gemini, from undetectable to 2 nM. An EC_{50} value for $c3_{LPD}$ was only
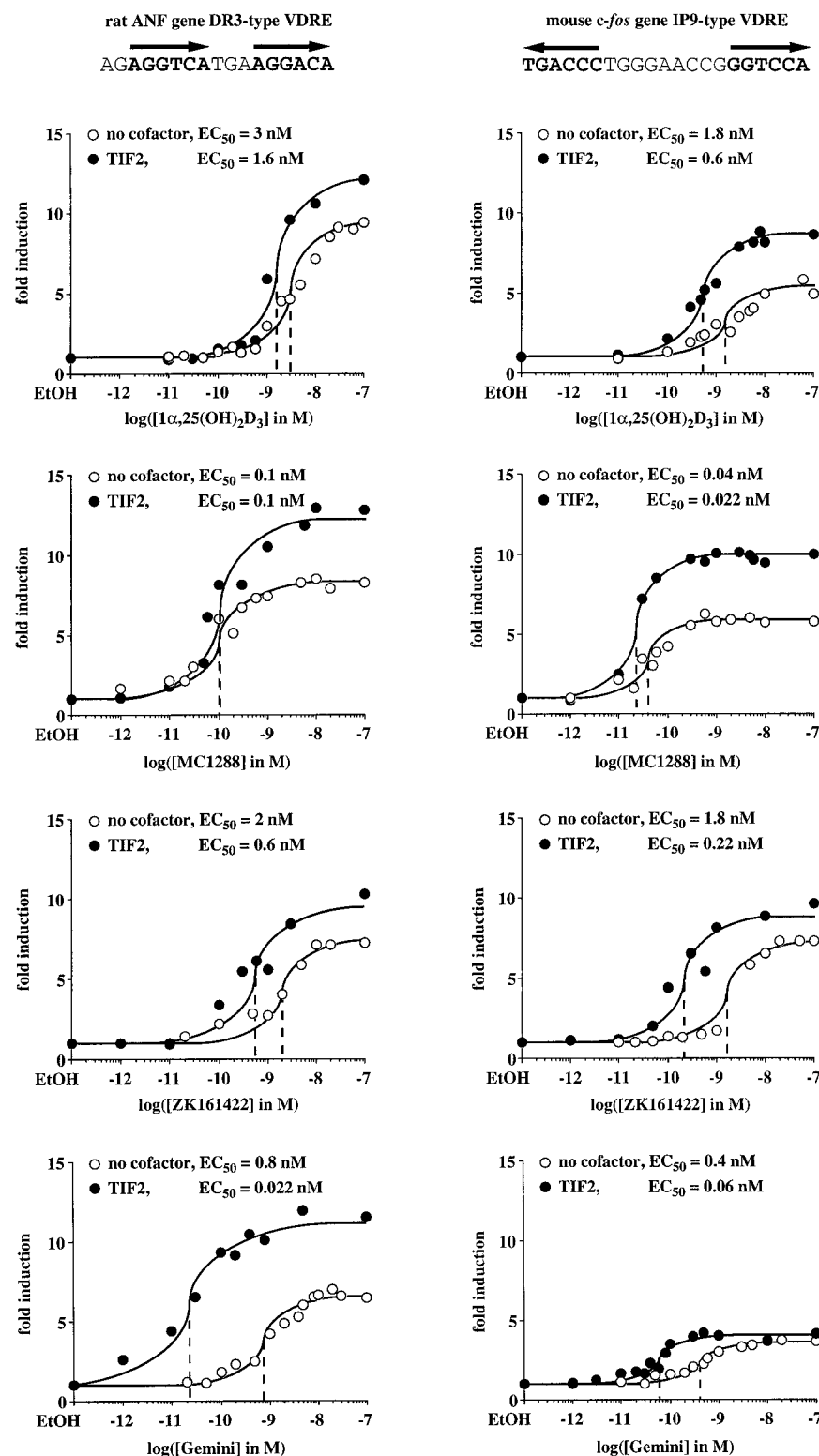
Fig. 4. TIF2-modulated functional activity. Luciferase reporter gene assays were performed with extracts from Cos-7 cells that were transiently transfected with reporter gene constructs driven by four copies of the DR3-type VDRE from the rat ANF gene promoter or the IP9-type VDRE from the mouse c-*fos* gene promoter, together with the expression vectors for VDR and RXR alone (○) or additionally with an expression vector for full-length TIF2 (●). The core sequences of the VDREs are given above. Cells were treated for 16 h with graded concentrations of $1\alpha,25(OH)_2D_3$, MC1288, ZK161422, and Gemini. Stimulation of β-galactosidase-normalized luciferase activity was calculated in comparison with solvent-induced controls. Each data point represents the mean of at least three experiments, the standard deviation was generally below 10%. The EC_{50} values for ligand-triggered reporter gene activities were determined from the dose-response curves.

detectable with Gemini where a shift from 90 to 1.5 nM was observed. Interestingly, when using $1\alpha,25(OH)_2D_3$, MC1288, and ZK161422, the complex formation of VDR with RXR and DNA did not result in a significant effect on the ratio of $c1_{LPD}$ to $c3_{LPD}$, whereas in the case of Gemini, the ratio of 10:75% shifted to a ratio of 45:55%. Modified limited protease digestion assays in the presence of GST-TIF2$_{646-926}$, did not demonstrate a reasonable effect on ligand sensitivity of $c1_{LPD}$ for $1\alpha,25(OH)_2D_3$, MC1288, and ZK161422 and the $c1_{LPD}/c3_{LPD}$ ratio (Fig. 8B). In contrast in the presence of GST-TIF2$_{646-926}$, $c1_{LPD}$ was protected by Gemini with higher sensitivity (decrease of the EC_{50} value from 2 to 0.5 nM) and $c3_{LPD}$ was protected with a lower sensitivity (increase of the EC_{50} value from 1.5 to 9 nM). In parallel, at saturating concentrations of Gemini, the ratio of conformations 1_{LPD} and 3_{LPD} increased to a ratio of 70:30%.

Discussion

A critical step in nuclear hormone signaling is the specific ligand-triggered induction of a conformational change within the nuclear receptor LBD (Moras and Gronemeyer, 1998; Torchia et al., 1998). This conformational change induces the dissociation of nuclear receptor-corepressor complexes and facilitates the interaction with coactivator proteins, which consequently results in stimulation of transcriptional activity via various additional protein-protein interactions. Therefore, the ligand-complexed nuclear receptor is still the main focus of molecular endocrinology and pharmacology. In this respect, the classical form of the limited protease digestion assay (Leng et al., 1993) has improved the understanding of receptor-ligand interaction by visualizing and differentiating functional (i.e., ligand-stabilized) receptor conformations. However, this report demonstrated that the characterization of such functional conformations of a nuclear receptor, such as the VDR, is insufficient for extrapolating their gene regulatory potential. It was shown that the model analog Gemini, when used in DNA-independent in vitro assays, such as

classical limited protease digestion or GST pull-down assays, behaves as a weak VDR agonist (100 times weaker than the natural hormone). In contrast, functional in vivo assays, such as reporter gene or mammalian one-hybrid assays, indicated a higher potency for Gemini than for $1\alpha,25(OH)_2D_3$. With monomeric VDR in solution, the Gemini analog was only able to stabilize VDR conformation 3 ($c3_{LPD}$), which was previously characterized as a low-affinity conformation (Nayeri et al., 1996), whereas $1\alpha,25(OH)_2D_3$ and the structurally related analogs MC1288 and ZK161422 preferentially stabilize the high affinity VDR conformation 1 ($c1_{LPD}$). Heterodimerization of VDR with RXR in solution did not demonstrate a significant effect on the ligand affinity and the ratio of VDR conformations (data not shown), however, complex formation of VDR-RXR heterodimers on VDREs was very effective in increasing the ligand sensitivity of the VDR. Modified limited protease digestion assays, performed in presence of RXR and DNA, demonstrated that the sensitivity of $c1_{LPD}$ for $1\alpha,25(OH)_2D_3$ increased by a factor of 27. Moreover, both supershift and gel shift clipping assays indicated that the affinity of VDR-RXR heterodimer conformations 1 and 2 ($c1_{GSC}$ and $c2_{GSC}$) for $1\alpha,25(OH)_2D_3$ was in the order of 0.1 to 0.2 nM. Interestingly, the complex formation of the VDR with DNA not only increased the affinity of the $c3_{LPD}$ for the Gemini analog by a factor of 60, but also enabled an interaction of $c1_{LPD}$ with the analog. Gel shift clipping assays confirmed this drastic increase in affinity of VDR-RXR heterodimer conformations for the Gemini analog, which was found to be in the same order as that for the natural hormone. In summary, using in vitro assays that take advantage of the fact that VDR-RXR complex formation on VDREs clearly enhances agonist affinity and can facilitate conformational changes of the VDR could solve the apparent discrepancy between in vitro and in vivo assays.

This study confirmed previous reports that VDR interacts in a ligand-dependent fashion with the three members of the SRC-coactivator family, SRC-1, TIF2, and RAC3. Moreover,
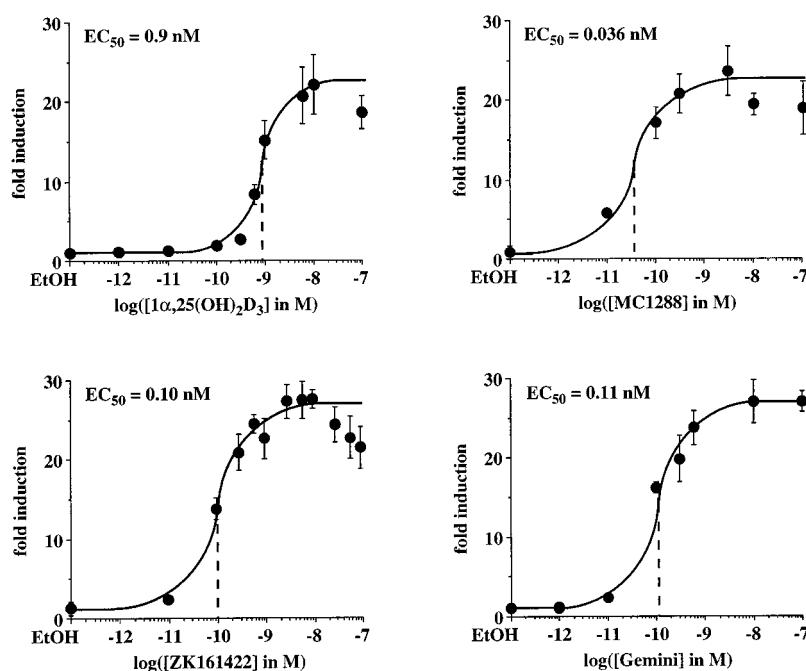

Fig. 5. Functional activity in a mammalian one-hybrid system. Luciferase reporter gene assays were performed from extracts with HeLa cells that were transiently transfected with a reporter gene construct driven by three copies of the GAL4 binding site and an expression vector for a GAL4$_{DBD}$VDR$_{LBD}$ fusion protein. Cells were treated for 16 h with graded concentrations of $1\alpha,25(OH)_2D_3$, MC1288, ZK161422 and Gemini. Stimulation of β-galactosidase-normalized luciferase activity was calculated in comparison with solvent-induced controls. Each data point represents the mean of triplicates and the bars indicate standard deviations. The EC_{50} values for ligand-triggered reporter gene activities were determined from the dose-response curves.
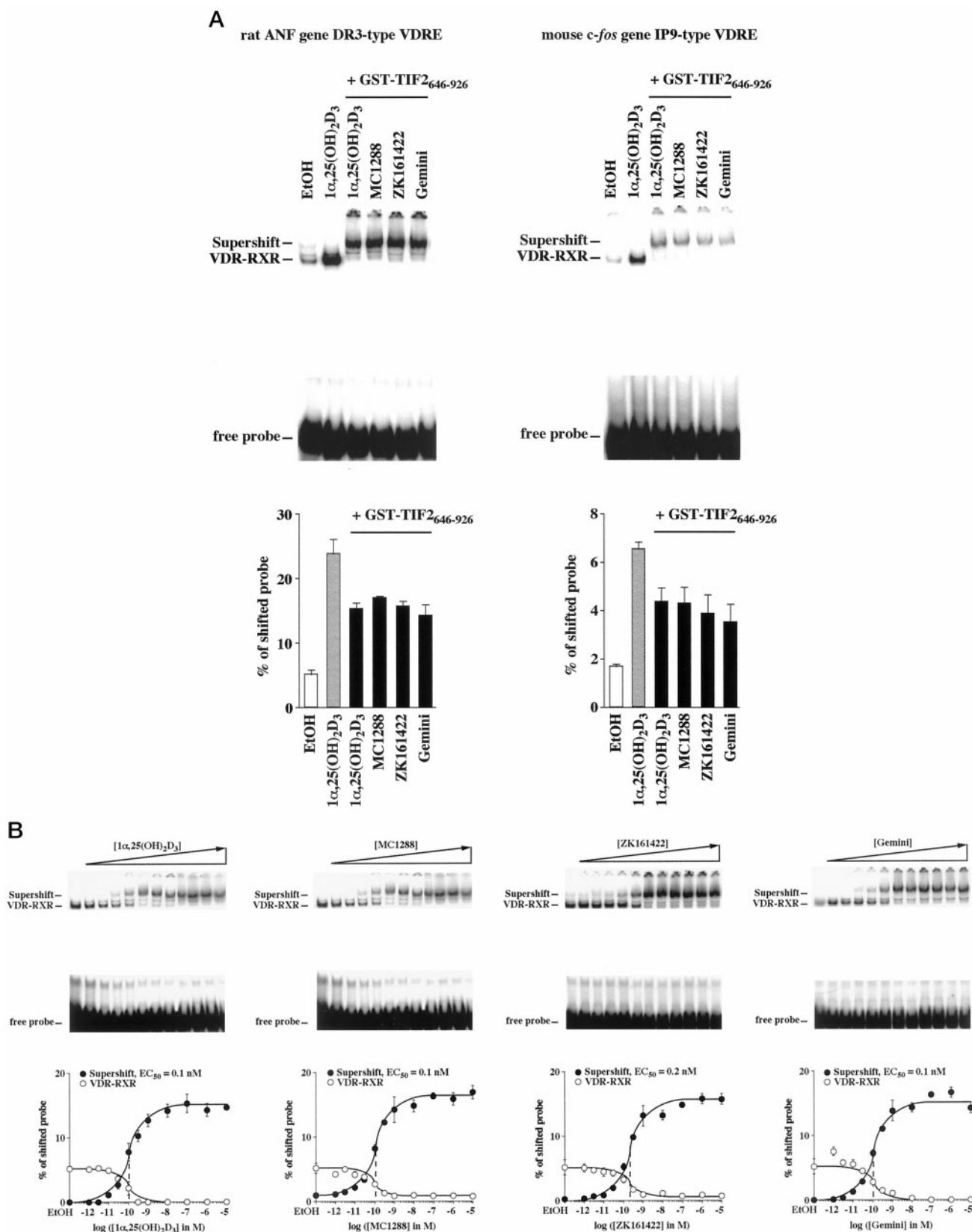
Fig. 6. Ligand-induced TIF2 supershift of DNA-bound VDR-RXR heterodimers. Gel shift experiments were performed with in vitro translated VDR-RXR heterodimers that were preincubated with bacterially expressed GST-TIF2$_{646-926}$, at saturating (10 μM, A) or graded concentrations (B) of $1\alpha,25(OH)_2D_3$, MC1288, ZK161422, and Gemini and the ^{32}P-labeled DR3-type VDRE from the rat ANF gene promoter (A, B) or the IP9-type VDRE from the c-*fos* promoter (A). VDR-RXR heterodimers were separated from free probe through an 8% nondenaturing polyacrylamide gel. Representative experiments are shown. The amount of VDR/RXR/VDRE (white columns/data points) or VDR/RXR/GST-TIF2$_{646-926}$/VDRE (supershift; black columns/ data points) complexes in relation to free probe was quantified with the use of a PhosphorImager. Columns (A) or data points (B) represent the mean of triplicates and bars indicate standard deviations.

the direct comparison of the affinity of the coactivator nuclear receptor interaction domains for a selection of VDR agonists did not provide significant evidence for a coactivator-analog "selectivity". In contrast to the recently suggested coactivator selectivity of the $1\alpha,25(OH)_2D_3$ analog OCT (Takeyama et al., 1999), the three members of the SRC-coactivator family appear to act similarly with the selection of VDR agonists used in this study and may therefore replace each other in the in vivo situation (Xu et al., 1998). However, compared with $1\alpha,25(OH)_2D_3$ and its 20-epi and 20-methyl analogs, the Gemini analog appeared to be quite ineffective in mediating an in vitro interaction of VDR with any of the
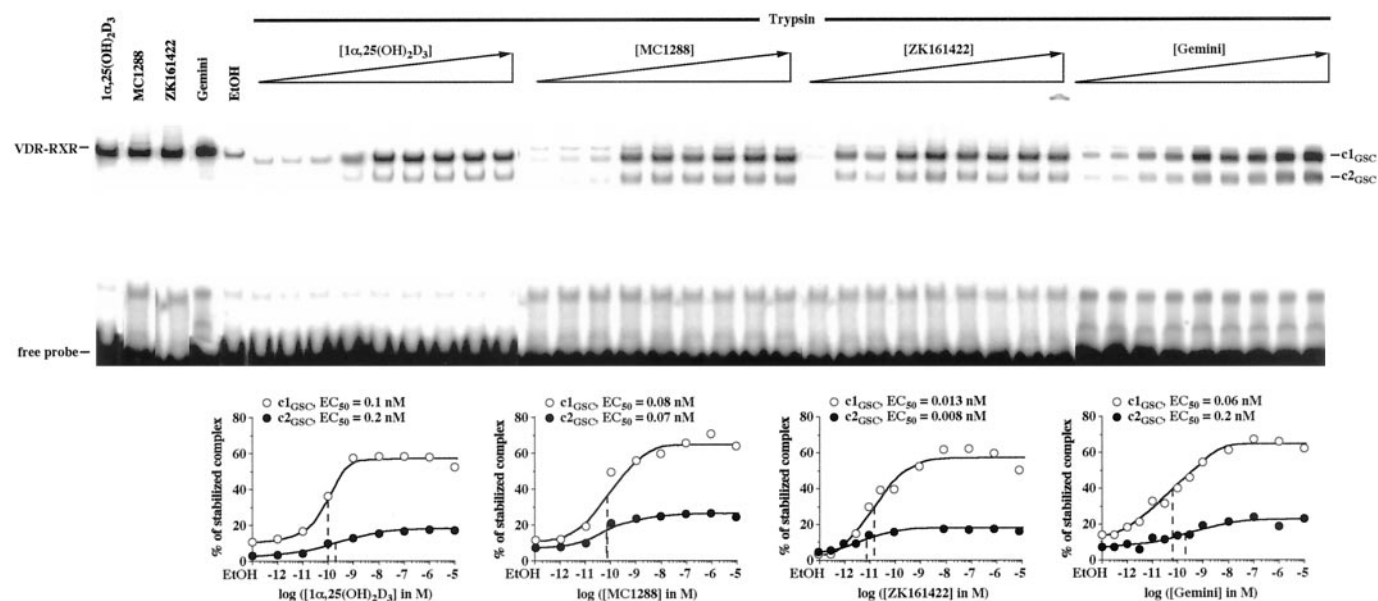

Fig. 7. Stabilization of VDR-RXR conformations on a DR3-type VDRE. Gel shift clipping assays were performed with in vitro translated VDR-RXR heterodimers, which were preincubated with graded concentrations of $1\alpha,25(OH)_2D_3$, MC1288, ZK161422 and Gemini and the ^{32}P-labeled DR3-type VDRE from the rat ANF gene promoter. Protein-DNA complexes were separated from free probe through an 8% nondenaturing gel. Representative experiments are shown. The amounts of digested VDR-RXR heterodimer-VDRE complexes in relation to the respective $1\alpha,25(OH)_2D_3$-induced, undigested VDR-RXR heterodimer (left two lanes) were quantified with the use of a PhosphorImager. Each data point represents the mean of triplicates and the standard deviation was generally below 10%. The EC_{50} values for stabilization of the two VDR-RXR heterodimer conformations, $c1_{GSC}$ and $c2_{GSC}$, were determined from the dose-response curves.
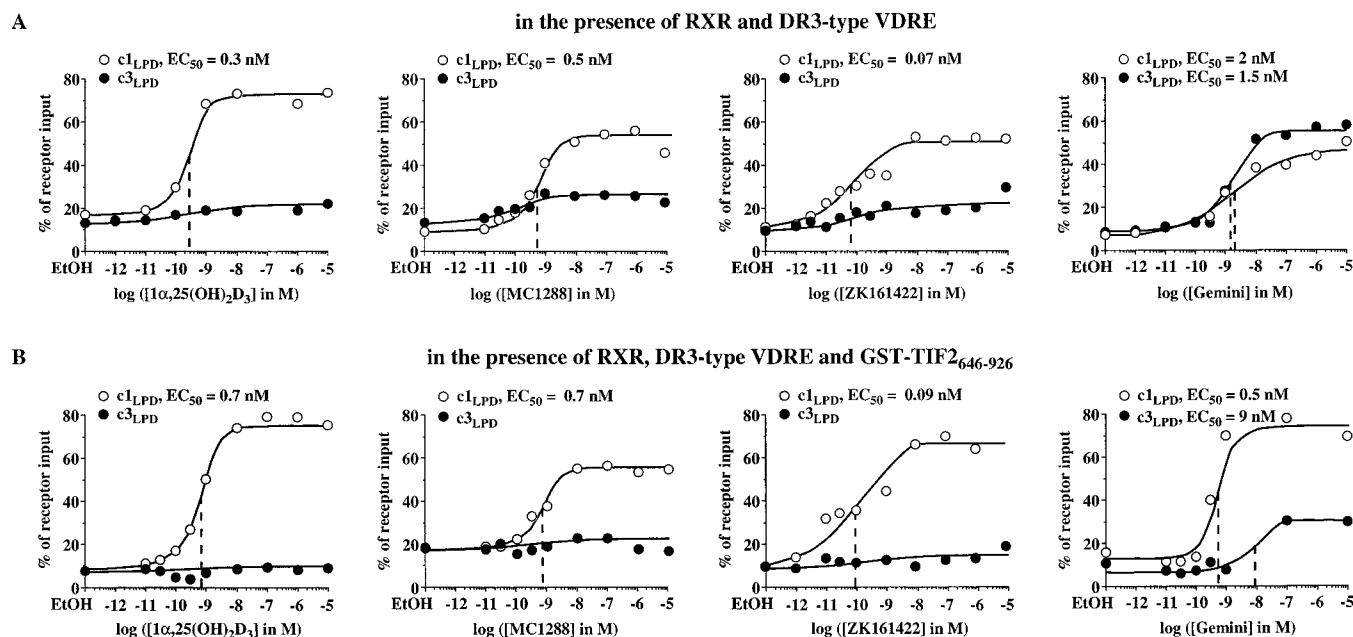

Fig. 8. Coactivator-modulated stabilization of VDR conformations on DNA. Limited protease digestion assays were performed by preincubating heterodimers of in vitro synthesized ^{35}S-labeled VDR and unlabeled RXR with graded concentration of $1\alpha,25(OH)_2D_3$, MC1288, ZK161422 and Gemini. Protein-DNA complexes were then formed on the unlabeled DR3-type VDRE from the rat ANF gene promoter in the absence (A) or presence (B) of bacterially expressed GST-TIF2$_{646-926}$. After digestion with trypsin, samples were electrophoresed through a 15% SDS-polyacrylamide gel. The amount of ligand-stabilized VDR in conformations 1 ($c1_{LPD}$, ○) and 3 ($c3_{LPD}$, ●) in relation to VDR input was quantified with the use of a PhosphorImager. Each data point represents the mean of triplicates, the standard deviation was generally below 10%. The EC_{50} values for stabilization of the VDR conformations, $c1_{LPD}$ and $c3_{LPD}$ (only in case of Gemini), were determined from the dose-response curves.

three coactivators in solution. However, in DNA-dependent assays TIF2 (as a representative coactivator family member) was able to enhance the effects of the Gemini analog. In the modified limited protease digestion assay (i.e., in the presence of DNA and RXR), the addition of TIF2 shifted the majority of the VDR molecules into $c1_{LPD}$ and as a consequence increased the ligand sensitivity of this conformation by a factor of 4. In reporter gene assays the overexpression of TIF2 even resulted in a 36-fold increased sensitivity for gene activation from DR3-type VDREs. Taken together, these results suggest that with some VDR agonists such as the Gemini analog, the complex formation of VDR-RXR heterodimers on a VDRE, which directs the VDR into $c1_{LPD}$, is the prerequisite for an efficient interaction with coactivators. In the case of VDR agonists that readily stabilize most VDR molecules in solution in $c1_{LPD}$, such as the natural hormone $1\alpha,25(OH)_2D_3$ and the analogs MC1288 and ZK161422, VDR-RXR-VDRE complex formation does not appear to be a prerequisite for the interaction with a coactivator. However, the complex formation with DNA can also increase the sensitivity of the receptor-ligand interaction in these cases by a factor of 7 to 80.

Traditional ligand binding assays, in addition to the classical form of the limited protease digestion assay, are performed with receptors in solution (i.e., in the absence of DNA and coactivators). The observation, that DNA and coactivators are able to modulate the structure of the VDR to favor an effective interaction with agonists is therefore important for accurate characterization of VDR agonists in vitro. Based on assays in solution, a $1\alpha,25(OH)_2D_3$ analog may be considered as weak which might actually be misleading, as it has been shown for Gemini as an example. A comparison of the potential of the four VDR agonists in the variant assays suggested that MC1288 seems to be the most potent compound in vivo, being 3- to 10-times more sensitive than ZK161422 and Gemini. However, at high coactivator expression levels, such as in transfected Cos-7 cells, Gemini proved to be at least as potent as MC1288. The cell systems that are used for the functional in vivo assays differ in their endogenous coactivator expression levels (May et al., 1996), which could be an explanation for observing differential potency of a VDR agonist in variant systems. However, the natural hormone, $1\alpha,25(OH)_2D_3$, demonstrated 10- to 30-times lower potencies in these in vivo assays than its analogs, which indicates that it is metabolically less stable than the synthetic compounds. In the cofactor- and DNA-dependent in vitro assays $1\alpha,25(OH)_2D_3$, MC1288 and Gemini demonstrated very comparable sensitivities, whereas ZK161422 proved to be approximately 10-fold more sensitive. This suggests that, in vivo, ZK161422 is metabolized faster than MC1288 and Gemini. Taken together, various factors modulate the efficacy of a systemically applied $1\alpha,25(OH)_2D_3$ analog, the most critical of which seem to be the cofactor expression level, the cell-specific rate of analog metabolism, and the individual selectivity for DNA-independent versus DNA-dependent $1\alpha,25(OH)_2D_3$ signaling pathways.

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