Large-scale expression and purification of the human vitamin D receptor and its ligand-binding domain for structural studies

Kari JUNTUNEN*, Natacha ROCHEL†, Dino MORAS† and Pirkko VIHKO*‡¹

*Biocenter Oulu and WHO Collaborating Centre for Research on Reproductive Health, University of Oulu, FIN-90220 Oulu, Finland, †Laboratoire de Biologie Structurale, Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, BP 163, 67404 Illkirch Cedex, Cité Universitaire de Strasbourg, France, and

‡Department of Biosciences, Division of Biochemistry, FIN-00014 University of Helsinki, Finland

We have expressed recombinant human vitamin D receptor and its ligand-binding domain in *Spodoptera frugiperda* (Sf9) insect cells with a 30-litre bioreactor. Both proteins were purified to apparent homogeneity with yields of 0.5–3.5 mg/l. Gel-filtration analyses indicated that the purified human vitamin D receptor and its ligand-binding domain were present as monomers in solution. The purified vitamin D receptor and its ligand-binding domain were demonstrated to bind 1 α ,25-dihydroxyvitamin D₃ with high affinity, the K_d values ranging from 0.9 to 1.2 nM. Neutron scattering studies of the ligand-binding domain demonstrated that the samples are homogeneous and contain monomeric species of polypeptides. The purified vitamin D receptor

binds to the vitamin D response elements of osteopontin and osteocalcin genes as a homodimer or as a heterodimer with the retinoid X receptor- α ΔAB and we were able to purify these complexes in quantities sufficient for crystallization studies. The results indicate that we can produce biologically active human vitamin D receptor and its ligand-binding domain in insect cells and purify them for functional and structural studies.

Key words: nuclear hormone receptors, neutron scattering, recombinant gene expression, retinoid × receptor, vitamin D response element.

INTRODUCTION

The biologically active form of vitamin D₃, 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃], has important physiological functions in calcium homeostasis, bone remodelling and the regulation of cell growth and differentiation in many target tissues. Most of the biological actions of 1 α ,25(OH)₂D₃ are mediated through its ability to modulate gene transcription. The mechanism of 1 α ,25(OH)₂D₃-regulated transcription requires that the hormone bind to the vitamin D receptor (VDR). This binding leads to a conformational change within the receptor and increases its affinity for a specific vitamin D response element (VDRE) located in the regulatory regions of vitamin D target genes [1–3]. In the case of VDR, retinoic acid receptor (RAR) and thyroid receptor (TR), the transactivation complex also typically contains the retinoid X receptor (RXR), although complexes comprising other combinations of receptors, including VDR–VDR, VDR–RAR, and VDR–TR, have been reported [4–7].

VDR is a member of the steroid/thyroid receptor superfamily to which VDR is related by its extensive nucleotide and amino acid sequence similarity. Steroid hormone receptor molecules generally consist of six distinct regions denoted A–F. The C region (DNA-binding domain) is the most conserved domain of the different members of the superfamily [8,9]. The DNA-binding domain is a globular structure that consists of a cysteine-rich cluster that contains two zinc-finger-like structures. The DNA-binding domain binds specifically to hormone-responsive elements in DNA. The structural basis of DNA recognition is well established from nuclear magnetic resonance and X-ray crystallography analyses [10–14].

The E region [ligand-binding domain (LBD)] is relatively large and not well conserved. It mediates the binding of the ligand, the homodimerization and/or heterodimerization of the receptor and the transcriptional activation or repression of target genes. Crystal structures have been determined for the apo-RXR α [15], holo-RAR γ [16] and holo-TR α [17] LBDs. However, no structure of any receptor LBD in both liganded and unliganded states has yet been solved. Each of these domains contains a folded structure, which is found to generate a conserved ligand-binding pocket. The results also suggest that the binding of ligand brings about several conformational changes, which are absolute requirements for receptor activation. Binding of the ligand to the receptor might also cause the dissociation of repressor molecules, allowing association with general transcription factors, other DNA-binding proteins or co-activators [15–19].

The three-dimensional structure of VDR bound to DNA, either as a homodimer or as a heterodimer, is critical for understanding the structural mechanism of the hormonal action of vitamin D. The structure of the RXR/VDR heterodimer would be valuable because recent studies suggest that the RXR/VDR heterodimer is the predominant transactivating complex [6].

The therapeutic value of vitamin D and its analogues has been under intensive evaluation in many laboratories around the world. Knowledge of the crystal structure of VDR would speed up the development of new vitamin D analogues that might be usable as pharmaceutical compounds in human diseases such as cancer, leukaemia and psoriasis. No rational drug design in the vitamin D area is possible until detailed three-dimensional analyses of receptor–ligand interaction and receptor–DNA interaction have been performed.

Abbreviations used: DTT, dithiothreitol; LBD, ligand-binding domain; 9-*cis*-RA, 9-*cis*-retinoic acid; 1 α ,25(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; RAR, retinoic acid receptor; RXR, retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin D response element.

¹ To whom correspondence should be addressed in Oulu (e-mail pviikko@whoccr oulu.fi).

The availability of purified protein is an absolute requirement for structural studies of VDR. In this study we have expressed recombinant human VDR and its LBD in insect cells, with the use of the baculovirus expression vector system. We have purified the proteins to homogeneity as soluble functionally active forms; 30-litre batch culture and our purification scheme provide sufficient amounts of human VDR protein and its LBD for structure-function studies.

EXPERIMENTAL

Construction of baculovirus transfer vectors and generation of recombinant viruses

The cDNA of human VDR amino acid residues 3–427 and its LBD (residues 108–427) were amplified by PCR. The 5' oligomers used for amplification of the LBD contained a *NotI* restriction site, a coding sequence of methionine, six histidine residues and an LBD sequence, whereas the 3' oligomer included an *EcoRI* restriction site and an LBD sequence. The 5' oligomers used for amplification of VDR amino acids 3–427 contained a *NotI* restriction site additional to the VDR sequence and the 3' oligomer included an *EcoRI* restriction site, a coding sequence of six histidine residues and a VDR sequence. The amplified VDR and its LBD were cloned into baculovirus transfer vector pVL 1392. The transfer vectors pVL 1392 VDR and pVL 1392 LBD were co-transfected with linearized wild-type BaculoGold-DNA (Pharmingen, San Diego, CA, U.S.A.) into *Spodoptera frugiperda* (Sf9) cells (CRL1711; A.T.C.C., Manassas, VA, U.S.A.) with a modification of the calcium phosphate precipitation technique.

Expression of VDR and its LBD in insect cells

Recombinant proteins were produced in exponentially growing Sf9 insect cells [$(1.5\text{--}2.5) \times 10^6$ cells/ml] at 27 °C infected by recombinant virus at a multiplicity of infection of 1 in 1000 ml spinner flasks (Belco, Vineland, NJ, U.S.A.) containing 10% (v/v) fetal calf serum in complete TMN-FH insect medium (Sigma, St. Louis, MO, U.S.A.) with antibiotics. For mass production of the recombinant proteins, Sf9 cells were first scaled up to a 2-litre bioreactor (Biostat MD) and subsequently to a 30-litre bioreactor (Biostat UD 30; Braun Biotech International, Melsungen, Germany) as described previously [20]. Infection in the Biostat UD 30 bioreactor was produced by adding recombinant virus at a multiplicity of infection of 1 with 2 litres of fresh medium once a cell concentration of $(1.5\text{--}2.0) \times 10^6$ per ml had been reached. The expression of recombinant proteins was monitored in Western blots by using anti-VDR monoclonal antibodies together with a ProtoBlot AP system (Promega, Madison, WI, U.S.A.).

Purification of VDR and its LBD

The cells were lysed by ultrasonication in buffer containing 30 mM Tris/HCl (pH 7.5)/200 mM NaCl/5 mM 2-mercaptoethanol/leupeptin (0.2 µg/ml)/pepstatin (0.5 µg/ml)/PMSF (1 mM)/aprotinin (0.2 µg/ml). The cell lysate was ultracentrifuged at 100 000 g for 40 min at 4 °C. The supernatant was precipitated by adding an equal volume of 10 mM Tris/HCl (pH 7.5)/3 M $(\text{NH}_4)_2\text{SO}_4$ for 2 h at 4 °C. The precipitated proteins were redissolved in 30 mM Tris/HCl (pH 8.0)/0.5 M NaCl/5 mM 2-mercaptoethanol and loaded on an immobilized metal-affinity column (Talon; Clontech, Palo Alto, CA, U.S.A.). After washing, the bound proteins were eluted from the column with 10 mM Tris/HCl (pH 8.0)/100 mM imidazole/50 mM NaCl/5 mM 2-mercaptoethanol. The eluate was diluted with an equal

volume of 10 mM Tris/HCl, pH 8.0, containing 50 mM NaCl and 14 mM 2-mercaptoethanol before being loaded on a Mono Q anion-exchange column (0.5 cm × 5 cm; Pharmacia Biotech AB, Uppsala, Sweden) and run at 0.5 ml/min. The sample was eluted from the column with a linear gradient of NaCl (50–400 mM). The fractions with the highest VDR or LBD contents were pooled and concentrated in a Macrosep 10 K centrifugal concentrator (Filtron) for gel-filtration chromatography. A Superdex 75 column (2.6 cm × 90 cm; Pharmacia) run at 1 ml/min was eluted with 10 mM Tris/HCl, pH 8.0, containing 0.5 M NaCl and 5 mM dithiothreitol (DTT). The fractions with the highest VDR or LBD contents were pooled. The purity of recombinant proteins was evaluated by SDS/PAGE and visualization by staining with Coomassie Brilliant Blue R-250. The protein concentration of purified receptors was measured by Bio-Rad Protein Assay (Bio-Rad, Richmond, CA, U.S.A.) with BSA as a standard.

Analytical gel filtration

A sample of VDR or its LBD purified by metal-affinity chromatography was dialysed in 10 mM Tris/HCl buffer, pH 7.6, containing 150 mM NaCl and 5 mM DTT. The sample was concentrated (1–2 mg/ml) and loaded on a Superdex 200 HR gel-filtration column connected to an FPLC system (Pharmacia Biotech). The column was pre-equilibrated with the same buffer as the sample and eluted at a flow rate of 200 µl/min. Fractions of 300 µl were collected and analysed by SDS/PAGE and Western blotting with anti-VDR antibodies. The column had previously been calibrated with proteins of known molecular mass.

Ligand binding assay

Extracts from baculovirus-infected Sf9 cells expressing VDR or LBD (approx. 40 µg of protein) were incubated in 50 mM Tris/HCl, pH 7.4, containing 150 mM NaCl and 5 mM DTT for 20 h at 4 °C with increasing concentrations of tritiated $1\alpha,25(\text{OH})_2\text{D}_3$ (174 Ci/mmol) (Amersham Life Science, Little Chalfont, Bucks., U.K.). Non-specific binding of ligand was measured by adding a 100-fold excess of unlabelled ligand (Calbiochem-Novabiochem Corporation, La Jolla, CA, U.S.A.). At the end of the incubation, the samples were filtered under vacuum on glass fibre filters (GF/C, Whatman, Maidstone, Kent, U.K.) and washed three times with 10 ml of 50 mM Tris/HCl, pH 7.4, containing 150 mM NaCl and 0.01% (v/v) Triton X-100. The radioactivity retained on the filters was determined by liquid-scintillation counting. The dissociation constants were determined by Scatchard analysis.

Formation and (w/v) purification of receptor-DNA complexes

Synthetic oligonucleotides based on the VDREs of the genes for osteopontin (5'-AGGTGAATGAGGACA-3') or osteocalcin (5'-AGGTCAAGGAGGTCA-3') were purified by reverse-phase and ion-exchange chromatography. The reverse-phase R1H column (Poros) was washed with 6 column vol. of ammonium acetate. The oligonucleotide was directly detritylated on the column with 2% (v/v) trifluoroacetic acid. The detritylated oligonucleotide was eluted with an acetonitrile gradient and loaded on an anion-exchange column (HqH; Poros). Fractions of interest were pooled, dialysed against water, freeze-dried and resuspended in 2 ml of annealing buffer [5 mM cacodylate (pH 6.5)/50 mM NaCl]. The purified single-stranded oligonucleotides were combined in equimolar amounts and annealed.

To form DNA–receptor complexes, approx. 2–5 mg of purified VDR or VDR and RXR α Δ AB were mixed with an equimolar amount of double-stranded VDRE in an excess of 1 α ,25(OH) $_2$ D $_3$ or 1 α ,25(OH) $_2$ D $_3$ and 9-*cis*-retinoic acid (9-*cis*-RA). Monomeric RXR α lacking the N-terminal A/B domain (RXR α Δ AB) was obtained by overexpression in *Escherichia coli* and purified as described previously [21]. The homodimeric or heterodimeric DNA samples were dialysed overnight in buffer [10 mM Tris/HCl (pH 8.0)/100 mM KCl/10 mM DTT/10 mM MgCl $_2$] at 4 °C. The receptor–DNA complexes were purified by Superdex 200 16/60 gel-filtration chromatography (Pharmacia Biotech). The column was pre-equilibrated with the same buffer as in the dialysed sample and eluted at a flow rate of 1 ml/min. Complexes were separated by SDS/PAGE and native PAGE, which was performed on a PhastSystem (Pharmacia Biotech) with PhastGel gradient medium (8–25 %) and Coomassie-stained.

Small-angle neutron scattering measurements of VDR LBD

The VDR LBD samples, with or without vitamin D ligand, were subjected to gel filtration on an analytical Superdex 200 HR column equilibrated with a buffer containing 10 mM Tris/HCl, pH 8.0, 500 mM NaCl, 1 mM DTT, 14 mM 2-mercaptoethanol and 0.1 mM di-isopropyl fluorophosphate. The samples were dialysed extensively in the same buffer in 2 H $_2$ O at 4 °C. The concentrations determined from absorbance measurements were 2 mg/ml for the apo and holo VDR LBDs.

Neutron scattering data were obtained in one session on Instrument D11 at the Institut Laue-Langevin (Grenoble, France) [22]. The size of the detector was 64 cm $\times$ 64 cm. Sample–detector distance was 2.5 m and the wavelength of the beam was 10 Å. The corresponding Q range was 0.01–0.03 Å $^{-1}$. Samples were measured at 4 °C in Hellma rectangular quartz cells 2 mm thick. The data acquisition time was 30 min. Data were processed with Grenoble software (RNILS, XPOLLY, RGUI and RPLLOT) [23]. The radius of gyration was derived from a Guinier analysis [24]:

$$\ln I(Q) = \ln I(0) - R_g^2 Q^2 / 3$$

with

$$Q = 4\pi \sin \theta / \lambda$$

where Q is the scattering vector for a scattering angle of 2θ and λ is the wavelength.

For monodisperse solutions, the Guinier plot is a straight line with a slope of R_g , the radius of gyration, in the small-angle region.

RESULTS

Expression and purification of human VDR and its LBD

We expressed full-length human VDR fused to the C-terminal hexahistidine tag as well as its LBD (residues 108–427) fused to the N-terminal hexahistidine tag in Sf9 insect cells using a 30-litre bioreactor. A shorter construct of the LBD (residues 136–427) was also expressed but this polypeptide was not capable of binding the ligand (Figure 1). Full-length VDR or LBD was purified from cell lysates to apparent homogeneity by using (NH $_4$) $_2$ SO $_4$ precipitation followed by metal-chelating affinity chromatography, anion-exchange chromatography and gel filtration. Samples taken at each purification step were subjected to SDS/PAGE under reducing conditions and revealed by

Figure 1 Binding analysis of tritiated 1,25(OH) $_2$ D $_3$ to VDR, LBD 108–427 and LBD 136–427

A crude extract of baculovirus infected cell lysate (20–40 μ g) was incubated for 20 h at 4 °C with increasing concentrations of tritiated 1,25(OH) $_2$ D $_3$. The unbound ligand was separated on glass fibre filters. The values have had non-specific binding subtracted. Symbols: ●, VDR 1–427; □, 108–427; ▼, 136–427.

Coomassie staining (Figure 2). The yields of purified VDR and LBD were 1.5–3.5 and 0.2–0.5 mg/l respectively. Thus the yield of the full-length receptor was consistently higher.

Characterization of recombinant human VDR and its LBD

Recombinant human VDR and its LBD were expressed in insect cells and purified to near homogeneity by metal-chelating affinity chromatography. Aliquots were analysed by analytical gel filtration with and without incubation with 1 α ,25(OH) $_2$ D $_3$ before gel filtration. The gel-filtration fractions were analysed by immunoblots, which showed that VDR eluted as one peak

Figure 2 Coomassie-stained SDS/PAGE of VDR at various stages of purification

Lane 1, the soluble fraction of cell lysate, when the cells were harvested 3 days after infection; lane 2, proteins precipitated with 1.5 M (NH $_4$) $_2$ SO $_4$; lane 3, pool of VDR eluted from the Co $^{2+}$ affinity column; lane 4, pool of VDR eluted from the anion-exchange column; lane 5, pool of VDR after gel filtration; lane 6, purified LBD. The positions of molecular mass markers are indicated at the left.

Figure 3 Guinier plots of neutron scattering data of apo ($\diamond$) and holo ($\triangle$) LBDs in $^2\text{H}_2\text{O}$

Figure 4 Scatchard analysis of the binding of $1\alpha,25(\text{OH})_2\text{D}_3$ to LBD 108–427

Crude extracts from baculovirus-infected Sf9 cells expressing LBD were used for binding assays as described in the Materials and methods section. The equilibrium dissociation constant was determined as 1.19 nM.

corresponding to the 50 kDa monomeric form, whether or not it was preincubated with $1\alpha,25(\text{OH})_2\text{D}_3$ (results not shown). The LBD was also eluted as one peak corresponding to the 38 kDa monomer in the presence or absence of $1\alpha,25(\text{OH})_2\text{D}_3$ (results not shown).

To study the quality and structural aspects of the LBD, purified LBD samples were characterized further by using small-angle neutron scattering. The Guinier plots of the scattering intensities of the apo and holo LBD species (Figure 3) showed that the samples were monodisperse, with no deviation from linearity at the smallest scattering angles. The calculated R_G values from these plots in $^2\text{H}_2\text{O}$ were $21.1 \pm 0.3 \text{ \AA}$ and $20.2 \pm 0.6 \text{ \AA}$ for the apo and holo forms respectively. These values are consistent with monomeric species. Furthermore, the difference in gyration radius in the two forms indicated that the ligand induced a conformational change of the LBD.

The ligand-binding properties of recombinant VDR, VDR with the C-terminal hexahistidine residues (VDR-His₆) and LBD with the N-terminal hexahistidine residues (His₆-LBD) were studied by Scatchard analysis (Figure 4). The binding studies

Figure 5 Native PAGE (8–25%) analyses of VDR homodimer and VDR/RXR $\alpha\Delta\text{AB}$ heterodimer DNA complexes

Lane 1, VDR homodimer complex with a 22-mer osteocalcin-based DNA and $1\alpha,25(\text{OH})_2\text{D}_3$; lane 2, VDR/RXR $\alpha\Delta\text{AB}$ heterodimer complex with a 22-mer osteocalcin-based DNA plus $1\alpha,25(\text{OH})_2\text{D}_3$ and 9-*cis*-RA; lane 3, VDR/RXR $\alpha\Delta\text{AB}$ heterodimer complex with a 22-mer osteocalcin-based DNA and $1\alpha,25(\text{OH})_2\text{D}_3$.

demonstrated that all three proteins had similar dissociation constants, ranging from 0.9 to 1.2 nM. Thus the hexahistidine tag located in the N-terminus or the C-terminus of VDR did not affect its ligand-binding properties. The K_d was about an order of magnitude more than that of native VDR in cell studies, but the measured K_d value agreed well with previous values for recombinant VDR obtained from *E. coli*, yeast and insect cells [25–27].

Sequence-specific DNA-binding activity of VDR was studied *in vitro* by incubating it with double-stranded DNA oligonucleotides (17-mer to 23-mer) containing the VDR-binding sequence of the gene for osteocalcin or osteopontin, in the presence of $1\alpha,25(\text{OH})_2\text{D}_3$. The VDR molecules formed stable homodimeric complexes with both binding sequences (VDREs). The homodimeric VDR–DNA complexes were purified by gel-filtration chromatography and analysed by native PAGE. Purified VDR–VDRE complexes migrated as one band on a native PAGE gel (Figure 5, lane 1). The binding of VDR to DNA increased the solubility and stability of the receptor.

Because VDR needs to be complexed with RXR for biological activity, we decided to investigate the association of these proteins by using modified human RXR $\alpha\Delta\text{AB}$ [21]. This was done by incubating equimolar amounts of purified VDR, human RXR $\alpha\Delta\text{AB}$ monomers and VDRE with either $1\alpha,25(\text{OH})_2\text{D}_3$ alone or with $1\alpha,25(\text{OH})_2\text{D}_3$ and 9-*cis*-RA, the RXR ligand. The protein–DNA complexes thus formed were purified by gel-filtration chromatography. The protein elution profile indicated that over 90% of the molecules took part in complex formation (Figure 6a). The purified complexes were present only as VDR–RXR $\alpha\Delta\text{AB}$ heterodimers containing the VDRE of the osteocalcin or osteopontin gene (Figures 6b and 6c). 9-*cis*-RA did not affect the formation of the VDR–RXR $\alpha\Delta\text{AB}$ –VDRE complex but the mobility of the complex was slightly higher on native PAGE gel in the presence of 9-*cis*-RA (Figure 5, lanes 2 and 3). Formation of the heterodimeric complex was also studied in the absence of DNA. The complexes formed under these conditions were eluted in gel filtration as a broad peak and gave rise to several bands in native PAGE (results not shown).

Figure 6 Purification and analysis of VDR/RXR α Δ AB complexes

(a) Protein elution profile of the heterodimer VDR/RXR α Δ AB complexed with a 23-mer osteocalcin-based DNA and 1 α ,25(OH) $_2$ D $_3$. The receptor–DNA complexes were purified by Superdex 200 16/60 gel-filtration chromatography (Pharmacia Biotech). The column was pre-equilibrated with buffer [10 mM Tris/HCl (pH 8.0)/100 mM KCl/10 mM DTT/10 mM MgCl $_2$] and run at a flow rate of 1 ml/min. (b) Coomassie-stained SDS/PAGE (15% gel) of the gel-filtration fractions. The lanes correspond to fractions (2 ml) of the elution volume between 62 and 76 ml. The positions of molecular mass markers are indicated (in kDa) at the left. (c) Native PAGE (8–25%) analyses of purified VDR/RXR α Δ AB heterodimer complexed with DNA. Lane 1, complex with 18-mer osteocalcin-based DNA; lane 2, complex with 21-mer osteocalcin-based DNA; lane 3, complex with 23-mer osteocalcin-based DNA.

DISCUSSION

We have developed a large-scale expression system for the production of recombinant human VDR and its LBD in Sf9 cells. A hexahistidine affinity tag improved the yield and purity

of VDR during purification. We have been able to purify soluble recombinant VDR at a yield of 1–3.5 mg/l and LBD at 0.2–0.5 mg/l; this difference in yields was due to differences in expression levels rather than differential losses during purification. Recombinant human VDR has previously been

expressed in insect cells [25,26], yeast [27] and *E. coli* [26]. One problem with expression in *E. coli* was the accumulation of insoluble VDR in inclusion bodies. In baculovirus and yeast expression systems the amount of purified protein has been low [25–27]. The yield of purified LBD obtained in the present study is approximately the same as the yield of His₆-hRXR α LBD [28] and thyroid hormone receptor LBD [29] produced in *E. coli*.

The approx. 38 kDa LBD of human VDR encompasses two-thirds of the receptor molecule. Earlier investigators have identified the N-terminal boundary of the LBD as being somewhere between residues 114 and 166 [30] but the results did not reveal the exact residues required for ligand binding. We made two constructs for the production of LBD fragments, one corresponding to residues 136–427 and the other to residues 108–427. Fragment 136–427 did not bind the ligand at all, indicating that the N-terminal boundary of LBD is before residue 136. This result is consistent with the sequence alignment of nuclear receptor LBDs published by Wurtz et al. [18], on the basis of information provided by the superposition of the RXR α and RAR γ LBD crystal structures. The extension of the similarity in three-dimensional structure to the nuclear receptor superfamily through sequence alignments strongly supports the existence of a canonical LBD structure composed of helices H1 and H3–H10 linked to a flexible C-terminus. The N-terminal deletion of residues 1–135 breaks the predicted α -helix H1 of the LBD and this seems to affect the three-dimensional structure in such a way that ligand binding is abolished. The three-dimensional structures of apo-RXR α and holo-RAR γ LBD show that H1 is an integral part of the LBD module, interacting with helices H3, H5 and H8 [15,16]. These structures and the results of protease sensitivity experiments on VDR [31] also indicate that binding of the ligand to the receptor causes conformational changes in the receptor molecules, which lead to a more compact structure than in the unbound state. Our scattering measurements of the apo and holo forms of the LBD showed that the binding of ligand induced conformational changes within the LBD that led to a decrease in R_g . This result supports earlier findings that the holo receptor is more tightly packed than the apo receptor.

Only monomeric VDR and its LBD were detected by analytical gel filtration at a physiological salt concentration. This contrasts with a previous report that VDR might also exist as a dimer in solution. Nishikawa et al. [32] found weak dimerization activity of VDR by using a maltose-binding-protein-based protein-binding assay with radiolabelled proteins. Cheskis and Freedman [33] detected VDR homodimers and determined their K_d as being 600 nM by surface plasmon resonance. The differences between these later findings and those in the present study are most probably due to methodological differences.

It is known that VDR binds to target sequences in DNA as a homodimer or as a heterodimeric complex with RXR; however, it has been suggested that only the latter complex is physiologically relevant [6]. We were able to establish conditions for the formation of these complexes in the presence of oligonucleotides representing known VDREs and were able to isolate and purify both complexes in quantities sufficient for crystallization studies. The VDR homodimer forms in the presence of the ligand, 1 α ,25(OH)₂D₃; this dimerization increases the stability and solubility of the receptor in solution. This does not prove that homodimers also form *in vivo*, because the receptor and ionic concentrations used in these experiments differ significantly from those *in vivo*. VDR also formed heterodimeric complexes with RXR in the presence of 1 α ,25(OH)₂D₃; this complex is considerably more stable than the homodimer. The addition of 9-*cis*-RA to the preformed 1 α ,25(OH)₂D₃-occupied VDR–RXR–VDRE complex slightly increased its mobility on native PAGE

gels. This observation could mean that the preformed VDR–RXR–VDRE complex is able to bind 9-*cis*-RA and that this binding causes a conformational change in the complex. This would be in contrast with results of Thompson et al. [34], who postulated that VDR, if previously bound to 1 α ,25(OH)₂D₃, influences the conformation of RXR in the heterodimer in a manner that makes it resistant to binding by 9-*cis*-RA. Previous findings about the binding of VDR to VDRE have been inconsistent. For example, it was reported that VDR expressed in baculovirus and yeast was unable to interact directly with VDREs; these results were interpreted to mean that VDR cannot form homodimers [25,27]. In a study that used gel-shift assays, purified VDRs were shown to bind to the osteopontin VDRE in the absence of RXR; however, RXR was required for binding to osteocalcin VDRE [35], whereas opposite results were obtained in a study in which VDR translated *in vitro* was used [36]. The conflicting results are likely to be due to differences in sample quality and purity as well as in methodology.

The transcriptional activation of target genes by the VDR–RXR heterodimer is likely to be a complex process that involves an interplay between the DNA-binding domains and the LBDs of VDR, RXR, VDRE and the receptor ligands and a host of other components that remain to be identified. The availability of the crystal structure of the VDR–RXR complex on VDRE would be useful in the elucidation of the mechanism of transcriptional activation by steroid hormone receptors.

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