Journal of Cellular Biochemistry 85:435–457 (2002)

Isolation of Baculovirus-Expressed Human Vitamin D Receptor: DNA Responsive Element Interactions and Phosphorylation of the Purified Receptor

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Abstract Two controversial aspects in the mechanism of human vitamin D receptor (hVDR) action are the possible significance of VDR homodimers and the functional role of receptor phosphorylation. To address these issues, milligram quantities of baculovirus-expressed hVDR were purified to 97% homogeneity, and then tested for binding to the rat osteocalcin vitamin D responsive element (VDRE) via electrophoretic mobility shift and half-site competition assays in the presence or absence of a CV-1 nuclear extract containing retinoid X receptor (RXR). Methylation interference analysis revealed that both the hVDR homodimer and the VDR–RXR heterodimer display similar patterns of VDRE G-base protection. However, in competition studies, the relative dissociation of the homodimeric hVDR complex from the VDRE was extremely rapid ($t_{1/2} < 30$ s) compared to the dissociation of the heteromeric complex ($t_{1/2} > 5$ min), thus illustrating the relative instability and low affinity of homodimeric VDR binding to DNA. These results indicate that VDR–RXR heterodimers are the preferred VDRE binding species. Further, two dimensional gel electrophoresis of hVDR demonstrated several isoelectric forms of the receptor, suggesting that it is subject to multiple phosphorylation events. In vitro kinase assays confirmed that purified hVDR is an efficient substrate for protein kinases A and Cβ, as well as casein kinase II. In vivo studies of the expressed receptor in intact cells, namely baculovirus vector infected Sf9 insect cells and transfected mammalian COS-7 cells, demonstrated that hVDR was phosphorylated in a hormone-enhanced fashion. Functional consequences of hVDR phosphorylation were suggested by the observations that: (i) potato acid phosphatase (PAP)-treated hVDR no longer interacted with the VDRE as either a homodimer or a heteromeric complex with RXR, and (ii) treatment of transfected COS-7 cells with a phosphatase inhibitor (okadaic acid) along with 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) resulted in a synergistic enhancement of both hVDR phosphorylation and transactivation of a VDRE-linked reporter gene, compared to the effect of treatment with either agent alone. These studies point to a significant role for phosphorylation of VDR in regulating high-affinity VDR–RXR interactions with VDREs, and also in modulating $1,25(OH)_2D_3$-elicited transcriptional activation in target cells. J. Cell. Biochem. 85: 435–457, 2002.

Key words: nuclear hormone receptor; 1,25-dihydroxyvitamin D_3; transcriptional activation; heterodimerization

The biological effects of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) are mediated by a soluble receptor protein termed the vitamin D receptor, or VDR [Haussler et al., 1998; Jones et al., 1998; Jurutka et al., 2001; MacDonald et al., 2001]. Based on sequence similarities, the VDR is classified as a member of the superfamily of nuclear receptors for steroid hormones, thyroid hormone (TR), and retinoids [Whitfield et al., 1999]. These nuclear receptors alter gene expression by binding to specific cis-acting DNA elements in the regulatory regions of hormone responsive genes [Glass and Rosenfeld, 2000]. For the subfamily of classic steroid hormone

Grant sponsor: National Institutes of Health (M.R.H.).
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Received 9 November 2001; Accepted 9 January 2002
DOI 10.1002/jcb.10134

receptors, such as the glucocorticoid, progesterone, and estrogen receptors, these cis-acting hormone responsive elements (HREs) in DNA are generally palindromic, and the receptors bind as ligand-dependent cooperative homodimers in the promoter region of the target gene [O'Malley, 1990; Luisi et al., 1991; Schwabe et al., 1993].

In contrast, nuclear receptors in the thyroid hormone, vitamin D, and retinoid ligand-binding subfamily generally associate as retinoid X receptor (RXR) heterodimers with directly repeated elements in DNA [Mangelsdorf and Evans, 1995]. These tandem repeat HREs [Perlmann et al., 1996], present in the promoter region of target genes, are known to consist of six nucleotide half-elements separated by a variable nucleotide spacer, corresponding to two or five base pairs for the retinoic acid receptor (RAR)–RXR heterodimer, three base pairs for the VDR–RXR complex, and four base pairs for the TR–RXR heterodimer [Umesono et al., 1991]. However, TR also appears to function as a homodimer on palindromic and variably spaced directly repeated elements [Umesono et al., 1988; Sjoberg and Vennstrom, 1995; Nomura et al., 1996; Tomic-Canic et al., 1996].

Once bound to HREs, nuclear hormone receptors belonging to either subfamily mediate ligand-dependent activation of gene transcription by associating progressively with coactivators of the p160 class (e.g., SRC-1), cointegrators like CBP that remodel chromatin, and an RNA polymerase II recruiting complex known as mediator [Kornberg, 1999]. A model for the specific case of VDR-mediated, $1,25(OH)_2D_3$-stimulated transcription consists of the following sequence of events: VDR ligand binding, RXR-heterodimerization and vitamin D responsive element (VDRE) association [Thompson et al., 1998], SRC-1/CBP [Gill et al., 1998] recruitment for chromatin remodeling in the target promoter region catalyzed by the histone acetyl transferase (HAT) activity intrinsic to the coactivator/cointegrator, SKIP-related coactivator NCoA-62 binding [Baudino et al., 1998], attraction of RNA polymerase II via the VDR-mediator/D-receptor interacting protein (DRIP) network [Rachez et al., 1999], and, finally, stabilization of the preinitiation complex through delivery of TFIIB by VDR [Blanco et al., 1995; MacDonald et al., 1995; Jurutka et al., 2001; Lian et al., 2001]. By activating (or, in some cases, repressing) target gene expression, $1,25(OH)_2D_3$-VDR controls cell functions such as intestinal calcium absorption, bone remodeling, and cell differentiation at sites including skin and the hair follicle [Haussler et al., 1998]. The present communication addresses two unresolved issues in the above paradigm for VDR action: (i) the possible role of VDR homodimers in binding to VDREs and transducing the $1,25(OH)_2D_3$ ligand signal, and (ii) the potential functional involvement of VDR phosphorylation/dephosphorylation in modulating receptor activity.

With respect to the first of these unresolved issues, receptor targeted VDREs have been identified in several genes that are induced by $1,25(OH)_2D_3$, namely rat [Markose et al., 1990; Terpening et al., 1991; Breen et al., 1994] and human [Ozono et al., 1990] osteocalcin, mouse osteopontin [Noda et al., 1990], and rat 25-hydroxyvitamin D_3 24-hydroxylase [Ohyama et al., 1996]. As stated above, these VDREs are consistent with the pattern of imperfect direct repeats separated by a 3-nucleotide spacer (DR3), and considerable evidence indicates that vitamin D receptors bind to these direct repeat motifs as heteromeric complexes in association with RXR or possibly with other unidentified nuclear factors. For example, enriched overexpressed VDR apparently does not bind to VDREs in DNA binding assays unless a mammalian nuclear extract is present [MacDonald et al., 1991; Sone et al., 1991; Ross et al., 1992]. A large body of data suggests that the major receptor auxiliary factors (RAFs) facilitating VDR DNA binding are the RXRs [Kliewer et al., 1992; MacDonald et al., 1993; Munder et al., 1995; Jin and Pike, 1996; Staal et al., 1996; Lemon et al., 1997].

However, it has also been reported by several groups that VDR can bind as a homodimer to certain DR3 sequences. These include a VDRE that is a perfect direct repeat [Nishikawa et al., 1994], and also a DR3 VDRE that contains the AGTTCA half-site proposed to represent a consensus sequence for VDR–VDR homodimeric binding [Cheskis and Freedman, 1994; Freedman et al., 1994] or consisting of artificial DR3 constructs containing the AGGTCA motif [Takeshita et al., 2000]. In addition, a number of reports have suggested the existence of VDR homodimeric binding sequences that are comprised of a DR6 type element located within the promoters of the human osteocalcin [Carlberg et al., 1993], rat 25-hydroxyvitamin

D_3 24-hydroxylase [Kahlen and Carlberg, 1994], and murine fibronectin [Polly et al., 1996] genes. Furthermore, it has been proposed that the differential effects of certain pharmacologically active vitamin D analogs may be mediated through VDR homodimers [Koszewski et al., 1999]. Therefore, considerable literature reporting VDR–DNA binding studies, in vitro, implicates the receptor homodimer in $1,25(OH)_2D_3$ signaling, raising the possibility that VDR does not function exclusively as a heterodimer with RXR.

The second unresolved issue in transactivation by VDR is the potential role of phosphorylation in regulating this process. The activity of other nuclear receptors is known to be modulated by direct phosphorylation events. As one example, ligand-dependent phosphorylation of the human progesterone receptor (hPR) at several sites is required for full transcriptional activity in response to progesterone [Knotts et al., 2001]. Also, the human estrogen receptor α (ERα) is rendered active in a ligand-independent fashion via phosphorylation in either the N-terminal [Kato et al., 1995] or C-terminal [Arnold et al., 1995] domains. Similarly, mouse RARα can be activated by protein kinase A (PKA) via phosphorylation of a serine residue near the C-terminal activation function-2 (AF-2) [Rochette-Egly et al., 2000]. Apparent PKA catalyzed phosphorylation of the human glucocorticoid receptor (GR) increases its DNA binding activity [Rangarajan et al., 1992], although site-directed mutagenesis of all seven phosphorylation sites in the mouse GR yields only a 22% decrease in ligand-dependent transcriptional activity [Mason and Housley, 1993]. More recently, it has been shown that both basal and hormone-stimulated phosphorylation of GR are cell cycle-dependent [Bodwell et al., 1998].

It has been established that VDR is a phosphoprotein, phosphorylated primarily on serine residues [Haussler et al., 1988], at least in mouse 3T6 cells, and is the substrate for a minimum of four protein kinases [Haussler et al., 1997]. In two $1,25(OH)_2D_3$-independent phosphorylations of hVDR, the receptor is attenuated in its DNA-binding and transactivation via phosphorylation by protein kinase C (PKC) [Hsieh et al., 1993] and PKA [Jurutka et al., 1993a; Nakajima et al., 2000], respectively. In addition, there is evidence for two $1,25(OH)_2D_3$-stimulated phosphorylations of hVDR, one catalyzed by casein kinase-II (CK2) that amplifies hVDR transcriptional activity [Jurutka et al., 1996], and a second with unknown functional significance [Haussler et al., 1994]. All four hVDR phosphorylation events have been shown to occur in intact cells [Haussler et al., 1994], and three of the phosphorylation sites have been localized by deletion mapping and point mutation of hVDR. PKC phosphorylates hVDR at ser-51 in the α-helical region within the zinc finger domain that recognizes the VDRE in DNA [Hsieh et al., 1993], whereas PKA phosphorylates hVDR between residues 134 and 202 in the unconserved and putatively unstructured [Rochel et al., 2001] loop between helix-1 and helix-2 of the ligand binding domain [Jurutka et al., 1993a]. Recently, Hsieh et al. [2001] have localized the PKA site in hVDR to serines 182–185. CK2 phosphorylates hVDR at Ser-208 in the ligand binding domain [Jurutka et al., 1993b; Hilliard et al., 1994], and this reaction is stimulated by $1,25(OH)_2D_3$ as demonstrated by phosphopeptide sequencing [Hilliard et al., 1994]. The three identified protein kinases appear to be important for control of VDR function, because point mutation of the relevant serines [Hsieh et al., 1993, 2001; Jurutka et al., 1996], and overexpression or activation of the respective kinase in intact cells [Hsieh et al., 1991; Jurutka et al., 1993a, 1996], alters hVDR activity.

Despite emerging knowledge of specific hVDR phosphorylation events, conflicting conclusions exist in the area of the functional significance of VDR phosphorylation. Matkovits and Christakos [1995] have reported that, in the presence of the phosphatase inhibitor okadaic acid, hVDR is capable of ligand-independent transactivation from a VDRE reporter in transfected CV-1 cells, implying that hyperphosphorylated hVDR is constitutively active. Conversely, Desai et al. [1995] have observed that endogenous rat VDR in ROS 17/2.8 osteosarcoma cells is inhibited in both its VDRE binding and mediation of $1,25(OH)_2D_3$-stimulated transcription functions when these cells are exposed to okadaic acid, indicating that hyperphosphorylated rat VDR is unable to heterodimerize with RXR and bind to DNA. One explanation for the above disparity could be species differences between the phosphorylation sites in the human and rat VDR amino acid sequences, although cell-specific protein kinase expression in CV-1 versus ROS 17/2.8 cells may also account for this incongruity.

In the current investigation, hVDR overproduced in the insect baculovirus expression vector system (BEVS) [MacDonald et al., 1991] was purified to near homogeneity in order to examine the receptor as a substrate for various protein kinases, in vitro. Also, the influence of the hVDR phosphorylation state on VDRE binding and transactivation was assessed, as was the potential role of hVDR homodimers compared to hVDR–RXR heterodimers in VDRE binding. Basically, it is concluded that hVDR functions to bind the VDRE and apparently to stimulate gene transcription as a ligand-stimulated phosphoreceptor complexed in a heterodimer with RXR, but that a subset of independent phosphorylation events, catalyzed by either PKC, PKA, or both, effectively silences hVDR activity by precluding DNA binding.

MATERIALS AND METHODS

Purification of BEVS-Expressed hVDR

Spodoptera frugiperda (Sf9) cells (1500 ml at 1.2×10^6cells/ml) were infected with an hVDR-expressing recombinant baculovirus for 48 h using standard techniques. A cell extract was prepared by sonication of pelleted cells in four packed cell volumes of KETZD-0.3 (10 mM Tris-Cl, pH 7.6, 1 mM EDTA, 0.3 mM $ZnSO_4$, 5 mM DTT, and the indicated molar concentration of KCl, in this case 0.3 M) followed by centrifugation at 200,000*g* for 30 min. The supernatant was labeled for 1 h at 4°C with $1,25(OH)_2[^3H]D_3$ (final concentration of approximately 5 μM at 0.02 Ci/mmol). The extract was diluted with 2 volumes of KETZD-0 and centrifuged at 20,000*g* to pellet insoluble material. The supernatant was applied to a Blue Dextran–Sepharose column (2.5×19 cm) equilibrated in KETZD-0.1. The flow rate was approximately 1 ml/min and 3.5 ml fractions were collected. Following sample application, the column was washed with 100 ml of KETZD-0.1 and batch eluted with KETZD-0.8. Fractions containing $1,25(OH)_2[^3H]D_3$ were pooled and dialyzed against KETZD-0 to the appropriate conductivity. The dialyzed material (25 ml) was centrifuged at 20,000*g* and applied to a 2.5×8 cm DNA-cellulose column (Pharmacia, Piscataway, NJ) at 1 ml/min, and 3 ml fractions were collected. The column was washed with 75 ml KETZD-0 and then eluted with a 150 ml linear gradient of 0–0.3 M KCl in KETZD. The fractions containing $1,25(OH)_2[^3H]D_3$ were pooled and dialyzed into DE-52 buffer (20 mM Tris-Cl (pH 8.0 at 22° C), 1 mM EDTA, 0.3 mM $ZnSO_4$). This material was applied to a DEAE–Sephadex (DE-52, Whatman) column (2.5×12 cm) and VDR was eluted with a 150 ml gradient of Tris-Cl (pH 8.0) from 0.02–0.5 M. Fractions were pooled (32 ml volume), dialyzed into 20 mM potassium phosphate (pH 7.5), 1 mM DTT, and applied to a hydroxylapatite column (2.5×2.5 cm). Purified hVDR was eluted with a 50 ml gradient of potassium phosphate (0.02–0.25 M). The pooled material was dialyzed against 20 mM HEPES (pH 7.9), 20% glycerol, 1 mM DTT and concentrated by Amicon filtration (from 36–1 ml). Aliquots were snap-frozen in liquid nitrogen and stored at −70°C.

DNA Binding Studies

Nuclear extracts were obtained from CV-1 Green monkey kidney cells as described earlier [Shapiro et al., 1988]. Electrophoretic mobility shift assays were carried out utilizing those conditions and probes described previously [MacDonald et al., 1993]. Briefly, 200 ng of hVDR alone or in combination with 250 ng of nuclear protein obtained from CV-1 cells and/or unlabeled competitor DNAs were preincubated in buffer containing 0.15 M KCl at room temperature for 15 min. The [^{32}P]-labeled rat osteocalcin VDRE-WT probe (0.5 ng) was added and, after a 15 min incubation, the mixture was resolved on a low-ionic strength, nondenaturing polyacrylamide gel. The dried gel was used to expose X-ray film. Highly purified preparations of the 4A5 and 9A7 monoclonal antibodies raised against avian VDR [Pike et al., 1983] were used in this study, and the nonspecific IgG was an affinity purified rabbit anti-sheep IgG obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Methylation interference experiments were performed using a partially methylated, [^{32}P]-labeled rat osteocalcin VDRE probe and conditions described previously [MacDonald et al., 1991].

Phosphatase Treatment of Purified VDR

Potato acid phosphatase (PAP) was obtained from Roche (Indianapolis, IN) and was dissolved in PAP reaction buffer (20 mM Tris-Cl, pH 7.2, 1 mM EDTA, 2 mM β-mercaptoethanol, 10% glycerol). Purified hVDR (100 ng) was treated with increasing amounts of PAP (0.22–1.76 U) at 25°C for 15 min in a final volume of 5 μl.

Control incubations contained 100 mM sodium phosphate as an inhibitor of PAP. Following the 15 min incubation, sodium phosphate (final concentration 100 mM) and water were added to the PAP reactions and control reactions, respectively. The incubation was continued for an additional 15 min. This material was then assayed for VDRE interactions in gel mobility shift assays as described above.

In Vitro Phosphorylation

One microgram of purified BEVS–hVDR was incubated with 0.01–0.30 U purified bovine heart PKA (catalytic subunit), mouse brain PKCβ, or bovine testis casein kinase II (CK2) in the presence of 40 μCi [γ-^{32}P]ATP for 5 min at 30°C in the appropriate kinase buffer. For PKA, the kinase buffer included 10 mM Tris-Cl, pH 7.4, 25 mM $MgCl_2$, and 5 mM DTT. PKC buffer contained 20 mM Tris-Cl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 25 mg of phosphatidylserine per milliliter, and 100 ng of phorbol 12-myristate 13-acetate per milliliter. CK2 buffer contained 0.15 M KCl, 50 mM Tris-Cl, pH 7.4, 15 mM $MgCl_2$, and 5 mM DTT. All reactions were terminated by the addition of 2X final sample buffer (4% SDS, 10% β-mercaptoethanol, 125 mM Tris-Cl, pH 6.8, and 20% glycerol).

In Vivo Phosphorylation of VDR in Sf9 and COS-7 Cells

Sf9 cells were plated at 2.5×10^6 cells per 60 mm plate and infected with a VDR-expressing recombinant baculovirus (MOI = 1). Sixty-four hours post-infection, the monolayer was rinsed twice with 5 ml of phosphate-free EX-CELL 401 (JRH Biosciences). The cells were incubated for 30 min with 0.5 mCi of ^{32}P-orthophosphate in 1.5 ml of phosphate-free medium. Ethanol vehicle or $1,25(OH)_2D_3$ (10^{-7} M) was added and the incubation continued for an additional 2 h. The medium was removed, the monolayers were rinsed in Tris-buffered saline, and the cells lysed in 1 ml of KETZD-0.3 (0.3 M KCl, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 0.3 mM $ZnCl_2$, and 5 mM DTT) containing 0.5% Triton X-100, phosphatase inhibitors (20 mM sodium fluoride, 10 mM sodium molybdate, 100 μM sodium ortho-vanadate, and 50 nM okadaic acid) and protease inhibitors (2 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 50 μg/ml trypsin inhibitor). Lysates were immunoprecipitated with 4A5 monoclonal antibody linked to Sepharose and subjected to denaturing gel electrophoresis as described below. ^{32}P-labeled proteins were visualized by exposing X-ray film to the dried gels.

COS-7 monkey kidney epithelial cells were transfected with 1.0 μg wild type pSG5-hVDR expression plasmid by the calcium phosphate coprecipitation method as previously described [Jurutka et al., 1993b]. Forty-eight hours post-transfection, the cells were metabolically labeled with [^{32}P]orthophosphate as described earlier [Jones et al., 1991], except that the preincubation time with orthophosphate was 1 h followed by 2 h of treatment with 15 nM $1,25(OH)_2D_3$, ethanol vehicle and/or okadaic acid (50 nM). Cells were then lysed in KETZD-0.3 buffer containing 0.5% Triton X-100, phosphatase inhibitors and protease inhibitors, and immunoprecipitated as described below.

Immunoprecipitation and Gel Electrophoresis

Cell lysates were immunoprecipitated with monoclonal anti-VDR 4A5 antibody [Pike et al., 1983] linked to Sepharose beads. Typically, 500 μl aliquots of lysate were incubated with 10 μl of 4A5-Sepharose (≈1 mg mAb/ml) overnight at 4°C with gentle shaking. The Sepharose beads were then washed extensively with detergent-based wash buffers, essentially as described previously [Mangelsdorf et al., 1987]. The washed beads were resuspended in 30 μl 2X final sample buffer, boiled 4 min and electrophoresed on 10% SDS–polyacrylamide. The gels were fixed in 30% methanol/10% trichloroacetic acid/10% acetic acid, washed in water, impregnated with fluor (1 M sodium salicylate), dried and fluorographed at −70°C on Kodak X-OMAT AR film.

Transcription Assay

COS-7 cells (700,000 cells/60 mm plate) were transfected with 0.5 μg of wild-type hVDR expression plasmid and 10 μg of a reporter plasmid [$(CT4)_4$-TKGH] containing four copies of the rat osteocalcin VDRE [Terpening et al., 1991] inserted upstream of the viral thymidine kinase promoter-growth hormone reporter gene (Nichols Institute, San Juan Capistrano, CA) by the calcium phosphate-DNA coprecipitation method as described previously [Jurutka et al., 1993b]. The pTZ18U plasmid was used as carrier DNA and each transfection contained a constant amount of total DNA (20 μg). The transfected cells were washed, then refed in

Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 10^{-7} M $1,25(OH)_2D_3$ in ethanol vehicle and/or 50 nM okadaic acid (phosphatase inhibitor). After 24 h of incubation at 37°C, the level of growth hormone secreted into the culture medium, which serves as an index of hVDR transcriptional activity, was assessed by radioimmunoassay using a commercial kit (Nichols Institute).

RESULTS

Purification of Baculovirus-Expressed hVDR

We have previously described a baculovirus-mediated overexpression system (BEVS) for the human vitamin D receptor (hVDR) as well as a biochemical characterization of the unfractionated protein generated in this system [MacDonald et al., 1991]. To allow for further evaluation of the functional properties of the BEVS-expressed hVDR, the receptor protein was isolated by a 4-column purification protocol. The pooled material from each chromatographic step was analyzed on a denaturing polyacrylamide gel and the proteins were visualized by silver staining (Fig. 1). In addition, Western blot analysis revealed that BEVS-generated, full-length hVDR protein displayed electrophoretic and immunologic properties similar to those of mammalian VDRs (data not shown; [MacDonald et al., 1991]). From 1.5 L of infected Sf9 cells ($1-2 \times 10^6$ cells/ml), the combination of Blue Dextran–Sepharose, DNA-cellulose, anion exchange, and hydroxylapatite chromatography typically yielded 1.0–1.5 mg of hVDR that was over 97% pure as estimated by scanning densitometry of the stained gel (Fig. 1). Thus, the hVDR can be generated in the BEVS, and milligram quantities of the protein can be purified to near homogeneity by conventional column chromatography.

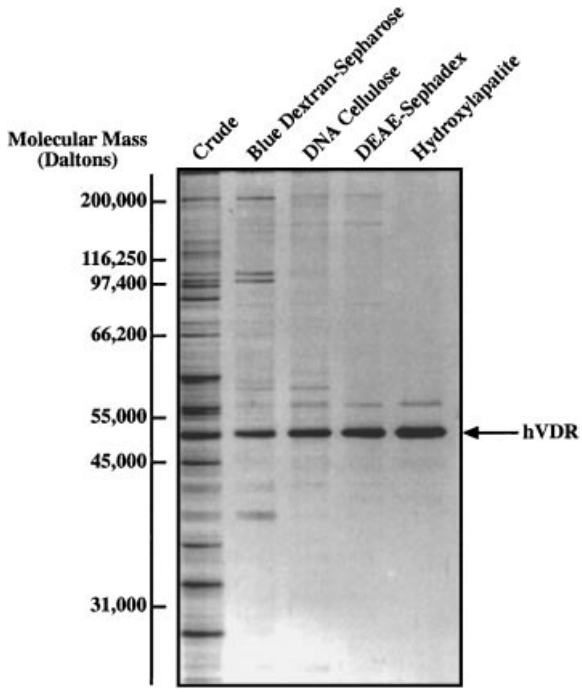


Fig. 1. Purification of baculovirus-expressed hVDR. VDR was isolated from 1.5 L of *S. frugiperda* (Sf9) cells infected with the hVDR recombinant baculovirus. The isolation procedure included a 4-column protocol employing Blue Dextran–Sepharose followed by DNA-cellulose, DEAE–Sephadex and hydroxylapatite-based chromatography (see Materials and Methods). Six micrograms of protein from the crude extract (applied to the first column) and 2.2 μg of protein from each column pool (peak fractions) were analyzed on a 10% polyacrylamide gel. The gel was fixed and proteins were visualized by silver staining. The migration positions of hVDR at approximately 52 kDa (right) and of protein molecular weight standards (left) are shown.

Interaction of Purified hVDR With a VDRE

The purified VDR was examined for its ability to bind the VDRE of the rat osteocalcin gene in an electrophoretic mobility shift analysis (Fig. 2A). Purified VDR formed a distinct complex with the VDRE (lane 1). Further, this complex was dependent on an intact VDRE sequence based on effective competition with unlabeled wild type VDRE probe (VDRE-WT; lanes 2–4) and less effective competition with a mutant VDRE probe (VDRE-MT; lanes 5–7). The VDRE-MT competitor contains a point mutation in each half element of the VDRE [MacDonald et al., 1991]. Comparison of lanes 3 and 6 showed complete competition with a 30-fold molar excess of the VDRE-WT probe and no effect with the mutant competitor; modest competition with the VDRE-MT probe was observed only at a 100-fold molar excess (lane 7). The protein:DNA complex was affected differentially by two monoclonal antibodies against the VDR. The 4A5 antibody disrupted the formation of the VDR:VDRE complex (lane 8) while the 9A7 antibody generated a supershifted species (lane 9). A nonspecific antibody did not affect the complex (lane 10). Therefore, the data in Figure 2A illustrate that abundant quantities (≥ 200 ng) of highly purified hVDR exhibited sequence-specific binding to the rat osteocalcin VDRE.

To determine if purified VDR interacted with the DR3 VDRE as a monomer or dimer, competition experiments were conducted with half-site oligonucleotide probes (Fig. 2B). These studies revealed that the VDR:VDRE complex was not competed by unlabeled probes containing only the 5′ (1/2 VDRE-A) or 3′ (1/2 VDRE-B) half-element of the rat osteocalcin VDRE. As illustrated in Figure 2B, the addition of a 100-fold molar excess of unlabeled competitors containing only the 5′ half-element (lane 6), or a separate DNA containing the 3′ half-element (lane 9), had no effect on the complex generated with purified VDR and the VDRE-WT probe. In contrast, a 100-fold molar excess of the unlabeled WT VDRE (lane 4) that contains both half-elements completely abolished VDR:VDRE interaction. Thus, based upon the relative ineffectiveness of VDRE half-sites to act as competitors in DNA binding assays (Fig. 2B), and the absence of a shifted complex of intermediate mobility compared to the migration position of the VDR homodimer (Fig. 2A), we conclude that VDR does not associate as a monomer with either of the VDRE half-sites alone, and binds to the DR3 only as an apparent homodimer.

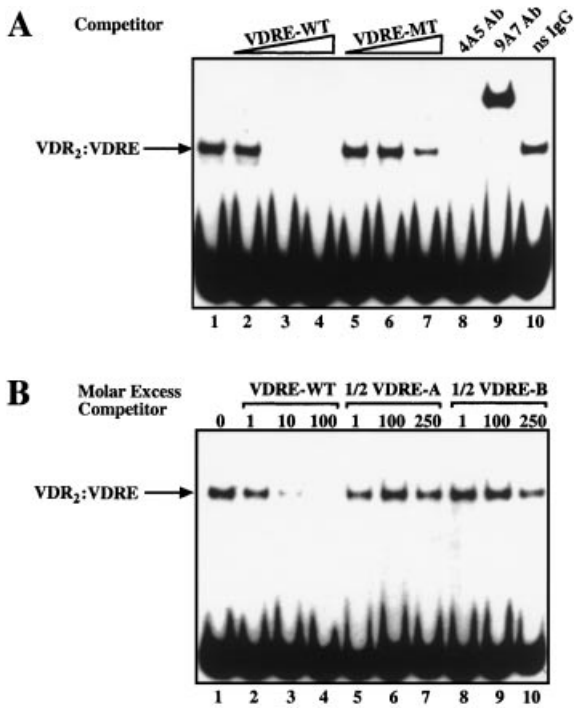


Fig. 2. Electrophoretic mobility shift analysis of purified BEVS-expressed hVDR. **A**: Purified hVDR was incubated with a [^{32}P]-labeled DNA probe containing the rat osteocalcin VDRE sequence (VDRE-WT) and then analyzed on a 4% nondenaturing polyacrylamide gel. **Lane 1** contains 0.2 μg of VDR and 0.5 ng of the labeled VDRE. **Lanes 2–4** contain a 10-, 30-, or 100-fold molar excess of unlabeled VDRE-WT competitor. **Lanes 5–7** contain a 10-, 30-, or 100-fold molar excess of an unlabeled VDRE probe having a point mutation in each of the half-sites in the rat osteocalcin VDRE (VDRE-MT). **Lanes 8** and **9** contain 4 μg of anti-VDR monoclonal antibody 4A5 or 9A7, respectively, whereas **lane 10** contains 4 μg of a nonspecific antibody. **B**: Purified VDR was examined in gel mobility shift analysis with [^{32}P]-labeled osteocalcin VDRE-WT probe (as in panel A) and the indicated molar excesses of unlabeled VDRE-WT (**lanes 2–4**) or unlabeled half-site competitors (**lanes 5–10**). 1/2 VDRE-A contains the 5′ half-element (GGGTGA) and 1/2 VDRE-B contains the 3′ half-element (AGGACA) of the rat osteocalcin VDRE. The sequences of these half-site competitors correspond exactly to the sequences of CT1 and CT2 as described earlier [Terpening et al., 1991].

Interaction of VDR as a Heterodimer With a VDRE

Next, we examined the effect of a nuclear extract from CV-1 cells on the interaction of BEVS-expressed hVDR with the VDRE. Incubation of the nuclear extract alone with the labeled rat osteocalcin VDRE did not generate a significant protein:DNA complex under the conditions used herein (Fig. 3A, lane 2). However, the combination of purified VDR and the nuclear extract yielded, in addition to the complex observed with VDR alone (lane 1), a distinct slower migrating complex (lane 3) that represents VDR heterodimerization with a second protein on DNA. Both heterodimeric and homodimeric complexes bound in a sequence-specific fashion based on competition with unlabeled VDRE-WT oligomers (lanes 4–6) and weak competition with VDRE-MT probes (lanes 7–9). Moreover, both complexes contained VDR as they were disrupted by the 4A5 antibody (lane 10). Thus, hVDR interacts with a RAF present in CV-1 cell nuclei to generate a protein:DNA complex that is distinct from the homodimeric complex formed by purified VDR, and likely represents a heterodimer containing VDR and an RXR isoform, as has been demonstrated with nuclear extracts from HeLa cells [MacDonald et al., 1993] and pig intestine [Munder et al., 1995]. Recent experiments in our laboratory have shown the presence of RXRα in a very similar complex generated using nuclear extracts from COS-7 cells [Thompson et al., 2001]. Given that COS-7 cells were derived from CV-1 cells [Gluzman, 1981], it is probable that the complex seen here also is a heterodimer of hVDR and primate RXRα.

When the relative stability of the VDR and VDR:RAF/RXR complexes was examined in a

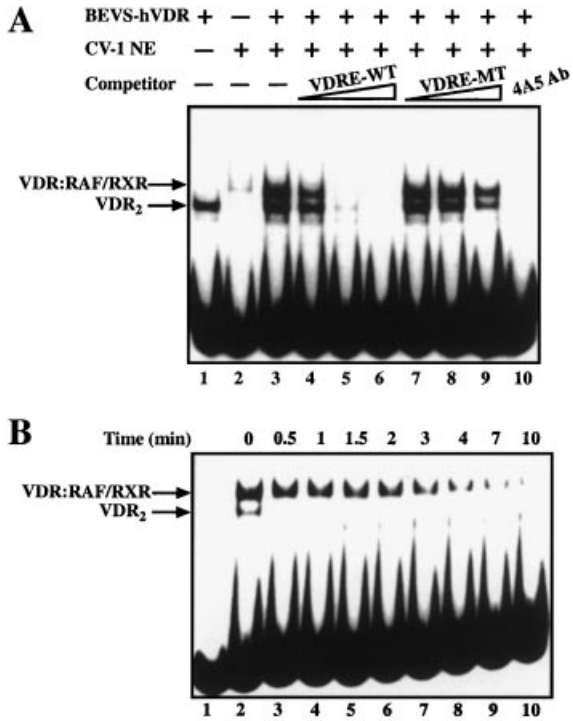


Fig. 3. Electrophoretic mobility shift analysis of purified BEVS-expressed hVDR in the presence of a mammalian cell nuclear extract. **A**: The effect of a nuclear extract from CV-1 cells on the interaction of VDR with the VDRE. Conditions, including amounts of BEVS–VDR and labeled probe, were similar to those described in the legend to Figure 2. **Lanes 2–10** contain 0.25 μg of nuclear protein obtained from CV-1 cells. Arrows indicate the positions of VDR homodimer (VDR_2), as well as a slower migrating species likely to be a VDR heterodimer with RXRα (see text). Competitions with VDRE-WT and VDRE-MT (lanes 4–9) were as described in the legend to Figure 2. The inhibitory monoclonal 4A5 antibody was included in lane 10. **B**: Comparison of the rate of dissociation of the VDR homodimer and the VDR:RAF/RXR heterodimer from the VDRE. Purified BEVS-expressed hVDR (0.2 μg protein) was incubated with a CV-1 nuclear extract (0.25 μg protein) and 0.5 ng of [^{32}P]-labeled VDRE-WT probe for 20 min at 22°C. A 100-fold molar excess of unlabeled VDRE-WT probe was added and aliquots were removed at the indicated times and subjected to electrophoretic mobility shift analysis.

mobility shift dissociation assay, a fundamental difference was noted. Purified hVDR was incubated with a CV-1 nuclear extract and labeled VDRE-WT probe. Following complex formation, a 100-fold excess of unlabeled VDRE-WT probe was added, and at various times following competitor addition, an aliquot of the incubation was analyzed on an electrophoretic mobility shift gel. As illustrated in Figure 3B, the VDR:RAF/RXR complex dissociated gradually from the labeled probe, exhibiting a half-life on the order of 5 min compared to the very rapid dissociation of the VDR homodimer (estimated to be < 30 s). Thus, in the absence of additional factors, purified VDR bound specifically to the VDRE as a homodimer with relatively low affinity. The heterodimeric VDR:RAF/RXR attains a more stable association with the VDRE, likely because of a much higher affinity imparted by a slower off-rate, compared to VDR homodimers bound to the VDRE. These results indicate not only that RAF/RXR imparts high affinity VDRE binding properties to VDR, but also that the VDR homodimer complex on DNA is not sufficiently stable to be consistent with the generation of a biologically relevant complex that initiates $1,25(OH)_2D_3$ signaling in transcriptional activation.

Defining the Nucleotide Contacts Between VDR and its VDRE Binding Site

In order to delineate further the interaction of purified hVDR with DNA, the base contacts between the receptor and a VDRE were determined utilizing DNA methylation interference assays. As shown in Figure 4, methylation protection of the specific guanine bases in the VDRE region of the rat osteocalcin promoter was assessed in the presence of the following purified proteins: baculovirus expressed hVDR alone (Fig. 4A, B1); BEVS–hVDR along with a CV-1 nuclear extract containing RAF (Fig. 4A, B2), *Escherichia coli*-expressed hVDR along with *E. coli*-expressed hRXRα (Fig. 4B, right side); and, finally, an *E. coli*-expressed truncated hVDR (Δ134) containing the core zinc-finger DNA-binding domain and its C-terminal extension (CTE) required for VDRE association (Fig. 4B, left side). As summarized schematically in Figure 4C considering only the DR3 VDRE bases, both homodimeric and RAF/RXR heterodimeric VDRs protect all of the G-bases in the 5′ VDRE half-element. However, there is a small, but significant difference between the RAF contained in CV-1 extracts and purified, *E. coli*-expressed hRXRα in that, when complexed to *E. coli*-expressed hVDR, the latter heteropartner does not protect the fourth G residue in the 5′ half-element (Fig. 4A–C). Strikingly, the purified *E. coli*-expressed truncated Δ134 hVDR, which is incapable of strong heterodimerization with RXR because it lacks the ligand binding domain, elicits no protection of any of the four G-bases on the sense strand in the 5′ half-element of the VDRE under the conditions of this methylation interference assay. Because

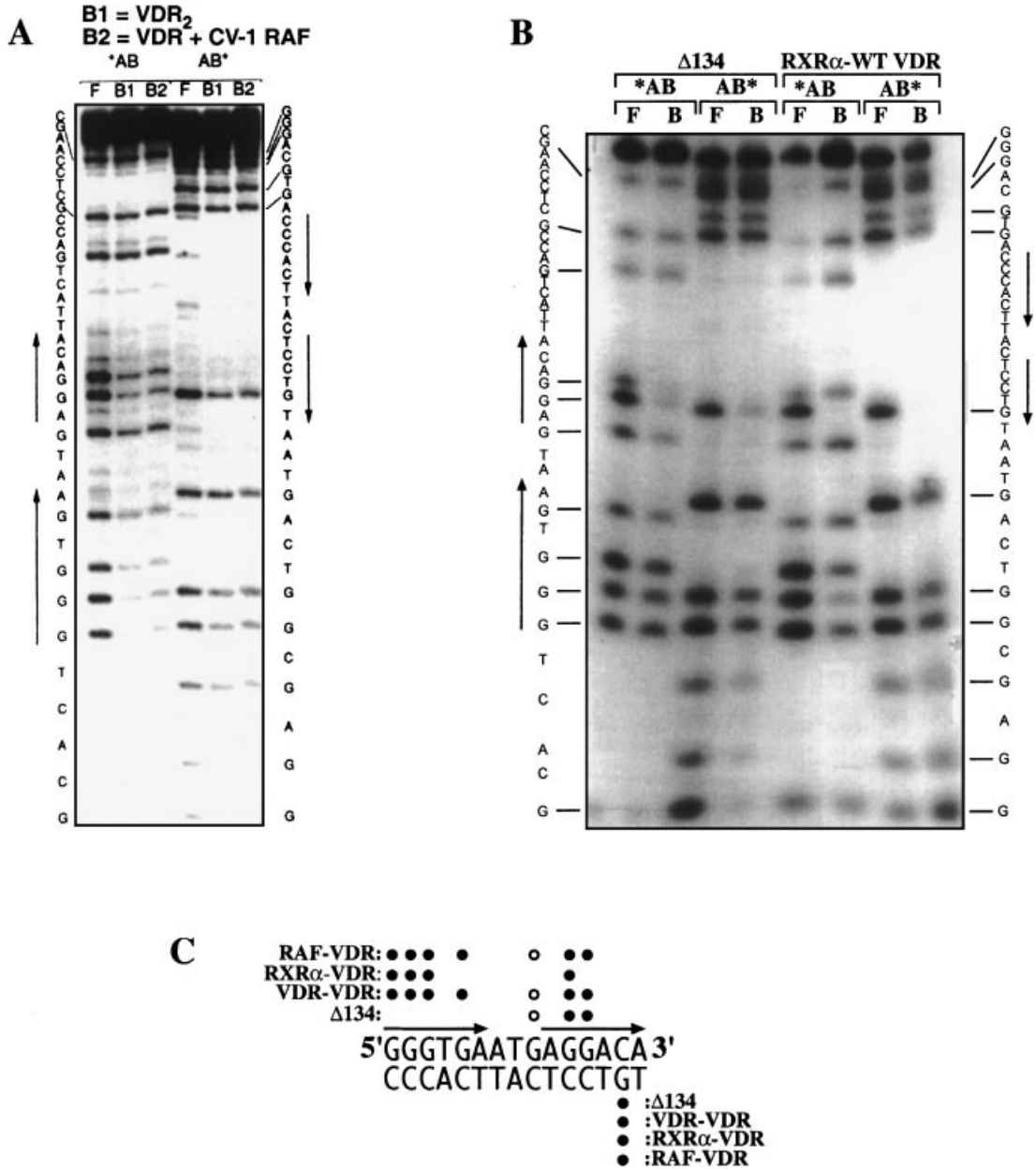


Fig. 4. Methylation interference analysis defines the nucleotide contact sites of the VDR and VDR:RAF/RXR complexes. **A**: A partially methylated, [^{32}P]-labeled probe corresponding to nucleotides −467 to −428 of the rat osteocalcin promoter was used in electrophoretic mobility shift analysis of purified VDR, and of VDR in combination with a CV-1 nuclear extract. DNA was isolated from the VDR homodimeric complex (**lane B1**), from the VDR:RAF/RXR complex (**lane B2**), and from unbound probe (**lane F**). The DNAs were cleaved with piperidine and analyzed on a 15% polyacrylamide sequencing gel containing 8.3 M urea. Probes labeled on either the sense strand (*AB) or the antisense strand (AB*) were examined. The sequences of the sense and antisense strands are illustrated on the left and right, respectively. The arrows mark the imperfect direct repeats of the VDRE. **B**: Methylation protection of the guanine bases in the VDRE that contact the Δ134 and full-length hVDRs. The Δ134 truncation (hVDR amino acids 4–133; [Hsieh et al., 1995]) contains the core DNA binding domain (DBD) and a CTE of the zinc finger region required for DNA association [Hsieh et al., 1999]. The 5′-labeled oligonucleotide containing the VDRE (as in A) was treated with dimethyl sulfate (DMS). The partially DMS-modified upper sense strand (*AB) and lower antisense strand (AB*) were incubated with 400 ng of purified Δ134 hVDR expressed in *E. coli* [Hsieh et al., 1995] (**lanes 1–4**) or 400 ng each of purified full-length hVDR and hRXRα that were expressed in *E. coli* [Hsieh et al., 1995] (**lanes 5–8**), respectively, and subjected to a gel mobility shift assay. The free (F) as well as bound complexes (B) were recovered by electroelution, cleaved with 1.0 M piperidine, and analyzed on a 15% sequencing gel. **C**: Summary of the bases in the rat osteocalcin VDRE exhibiting methylation interference by each of the receptor species tested in panels A and B. Significant interference is designated by closed circles and partial interference by open circles.

Δ134 hVDR generates methylation interference of the G-bases in the 3 nt spacer and the 3′ half-element of the VDRE (Fig. 4B,C), we conclude that core VDR binding occurs preferentially on the 3′ half-element. This finding is consistent with and extends the results of Jin and Pike [1996], who identified 3′ half-element occupation by VDR and 5′ half-element occupation by RXR when VDR–RXR binds to several DR3 VDREs. The present results (Fig. 4B,C) show that the rat osteocalcin VDRE represents another VDRE in which VDR preferentially asso-ciates with the 3′ half-element. Of course, at very high (supraphysiologic) concentrations, VDR also binds to the 5′ half-element as evidenced by the protection of all G-bases by the VDR homodimer (Fig. 4A,C). Koszewski et al. [1996] observed a pattern of homodimeric VDR protection of all the G-bases in the sense strand of the mouse osteopontin DR3 VDRE, which is analogous to the homodimeric VDR protection of G-bases in the rat osteocalcin VDRE (Fig. 4C).

With respect to heterodimeric receptor VDRE binding, we observe that the pattern of G-base protection within both half-sites of the rat osteocalcin VDRE is identical when one compares BEVS expressed hVDR in the absence and presence of endogenous RAF/RXR (Fig. 4A, C). Very similar methylation interference results were obtained by Markose et al. [1990] employing rat osteoblast nuclear extracts (containing endogenous VDR and RAF/RXR) and by MacDonald et al. [1991] using BEVS–hVDR and kidney cell nuclear extracts containing RXR as sources of rat osteocalcin VDRE binding proteins.

Interestingly, in the present results (Fig. 4A–C) the G-base protection pattern generated on the 3′ half-element and G in the spacer immediately upstream is identical for baculovirus-expressed homodimeric VDR, *E. coli*-expressed Δ134 hVDR, and BEVS–hVDR heterodimerized with endogenous CV-1 RAF/RXR. However, similar to the 5′ half-element results discussed above, the *E. coli*-expressed hVDR/hRXRα heterodimer fails to protect two G-bases that are occluded by the other hVDR preparations, specifically the G residue in the spacer and the third G residue in the 3′ half-element (Fig. 4A–C). Therefore, the data in Fig. 4A–C suggest that *E. coli*-expressed hVDR is fundamentally different from baculovirus expressed hVDR in that the former lacks the capacity to protect all of the G-bases in the 5′ and 3′ half-elements as well as in the VDRE spacer, at least when full-length *E. coli*-expressed hVDR is complexed with hRXRα also overexpressed in *E. coli*. One explanation for this observation is that hRXRα may not be the natural isoform complexed with VDR on the osteocalcin VDRE in osteoblasts, or that CV-1 cell nuclei contain other RXR isoforms or endogenous RAFs that are required for optimal VDRE binding. A plausible alternative explanation is that, unlike the case of BEVS expressed hVDR, which is phosphorylated (see Fig. 5, below), expression of hVDR in *E. coli* precludes endogenous phosphorylation(s) that may be required for optimal RXR heterodimerization and consequent VDRE binding.

BEVS-Expressed hVDR is a Phosphoprotein in Sf9 Cells and a Substrate for Protein Kinases In Vitro

In order to address the question of BEVS–hVDR phosphorylation in relation to DNA binding, we next determined the phosphorylation state of the overexpressed receptor. When purified BEVS–hVDR protein was analyzed by 2-dimensional polyacrylamide gel electrophoresis, the receptor appeared as multiple isoforms differing in their isoelectric points (Fig. 5A), even though all of the isoforms had the appropriate molecular mass of 52,000 Da based on their co-migration with the one-dimensional SDS–PAGE control hVDR (Fig. 5A, lane C). Thus, the BEVS-expressed hVDR protein exists as at least three isoforms of varying isoelectric points (see arrows at top of figure), consistent with a post-translational modification such as phosphorylation. Indeed, previous studies have demonstrated that the VDR is a phosphoprotein, and the extent of receptor phosphorylation is dependent on the presence of $1,25(OH)_2D_3$ [Jurutka et al., 1993b; Hilliard et al., 1994]. To determine whether phosphorylation events occurred in the insect expression system, infected cells were pulse-labeled with [^{32}P]-orthophosphate and cell extracts were immunoprecipitated with monoclonal antibody directed against the VDR. SDS–PAGE analysis of the immunoprecipitates revealed a major ^{32}P-labeled protein band of approximately 52,000 Da (Fig. 5B, lanes 1 and 2). This ^{32}P-labeled species was specific, as it was competed effectively with an excess of soluble monoclonal antibody in the immunoprecipitation reactions (lanes 3 and 6). When cells were treated with

$1,25(OH)_2D_3$, a 2-fold increase in the amount of incorporated label was noted (lanes 4 and 5). A duplicate gel from the same experiment revealed that the relative amount of silver-stained VDR in each group was similar and thus, the increased label was not the result of an enhancement in the levels of VDR protein or stability in the presence of hormone (data not shown). In Figure 5B, a minor, slower migrating phosphorylated protein appears just above the major hVDR band. This species is immunoprecipitated by the specific 4A5 VDR monoclonal antibody, competed with excess free antibody, and enhanced in its phosphorylation by $1,25(OH)_2D_3$ (Fig. 5B), all suggesting that it is a form of hVDR. We suspect that this band represents an N-terminally extended form of hVDR that may result from premature translation initiation at an upstream in-frame methionine in the mRNA generated from the incorporated hVDR plasmid. Interestingly, an identically migrating band appears also on the silver stained gel of the purified hVDR (far right lane, Fig. 1) as the principal copurifying "contaminant" (2–3% of the total purified protein fraction), providing independent evidence that the BEVS–hVDR expression system yields minor amounts of an N-terminally extended form of the native VDR which likely possesses near-identical functional properties to the major expressed hVDR species.

In order to probe hVDR phosphorylation further, we next determined if the isolated

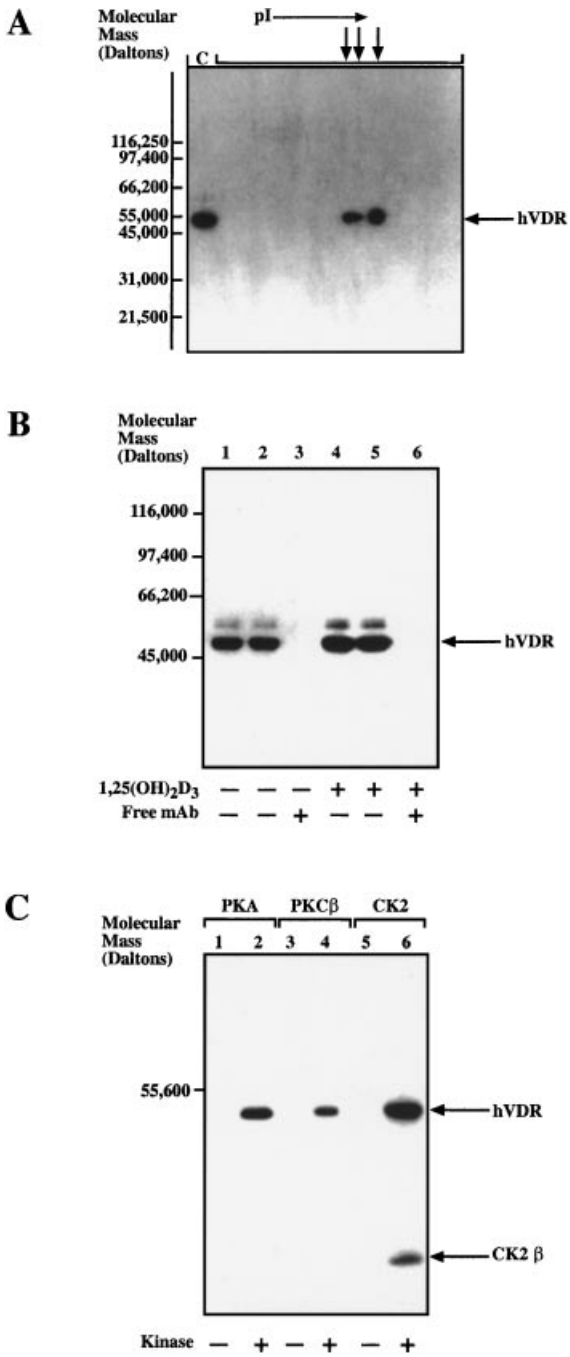


Fig. 5. Purified, BEVS-expressed hVDR exists in multiple isoelectric forms, and is phosphorylated in intact Sf9 cells, as well as in vitro. **A**: BEVS–hVDR was purified through the hydroxylapatite step (see Fig. 1) and was subjected to two-dimensional polyacrylamide gel electrophoresis. The proteins were transferred to Immobilon and the membrane was processed by Western blotting procedures with the 9A7 monoclonal antibody raised against VDR. Note the existence of multiple isoforms (arrows) of immunoreactive hVDR with varying isoelectric points (increasing pI from left to right) that co-migrate with the one-dimensional SDS/PAGE hVDR control (**lane C**). **B**: Sf9 cells were infected with the hVDR-recombinant virus for 64 h. The infected cells were labeled with 0.5 mCi [^{32}P]-orthophosphate in the absence or presence of 10^{-7} M $1,25(OH)_2D_3$ for 2 h. Cell lysates were immunoprecipitated with 4A5 antibody coupled to Sepharose in the absence and presence of excess free 4A5 antibody, as indicated at the bottom of the gel. The immunoprecipitates were analyzed by SDS–PAGE and the dried gel was exposed to X-ray film (as in C). **C**: In vitro phosphorylation of purified BEVS-expressed VDR. One microgram of purified BEVS–hVDR was incubated with either 0.10 U of purified bovine heart PKA (**lane 2**), 0.30 U of mouse brain PKCβ (**lane 4**) or 0.01 U of bovine testis CK2 (**lane 6**). Mock-incubated samples contained all assay components except the indicated enzyme (**lanes 1**, **3** and **5**). The reactions were allowed to proceed for 5 min at 30°C in the presence of 40 μCi [γ-^{32}P]ATP and then terminated by addition of 2X final sample buffer. The samples were then analyzed by 10% SDS/PAGE and the dried gel was exposed to film for 0.5 h at −70°C. The CK2 phosphorylated samples (lanes 5 and 6) were exposed to film for 2 h at −70°C. The upper and lower arrows indicate the migration positions of hVDR and the autophosphorylated β subunit of CK2, respectively.

receptor obtained from the baculovirus overexpression system could serve as a substrate for selected purified protein kinases, in vitro. As depicted in Figure 5C, purified hVDR is efficiently phosphorylated by PKA (lane 2), PKCβ (lane 4) and CK2 (lane 6). Note the autophosphorylation of the β subunit (arrow) of CK2, which serves as an internal control for the activity of this enzyme. Therefore, these in vitro protein kinase assays utilizing purified hVDR confirm that the receptor is a bona fide substrate for PKA [Jurutka et al., 1993a; Nakajima et al., 2000], PKCβ [Hsieh et al., 1991, 1993], and CK2 [Jurutka et al., 1993b; Hilliard et al., 1994], consistent with a possible functional role for VDR phosphorylation and crosstalk regulation by other signal transduction systems.

VDR Phosphorylation and VDRE Binding

A potential fundamental effect of phosphorylation on specific DNA binding was examined by treating purified BEVS-expressed hVDR with PAP. As illustrated in Figure 6A, the low affinity homodimer complex formed with pure VDR and the VDRE was extremely sensitive to pretreatment of the purified VDR with acid phosphatase. Treatment of VDR with as little as 0.22 U for 15 min completely eliminated VDR:VDRE complex formation. This effect was blocked when 0.1 M sodium phosphate was included as a phosphatase inhibitor (lanes 6–10). Treatment of VDR with low amounts (0.44 U) of phosphatase also impacted the ability of VDR to interact with RAF/RXR and the VDRE to generate the high affinity heteromeric protein:DNA complex (Fig. 6B, lanes 1–5). Again, sodium phosphate inhibited this effect, suggesting that it was phosphatase-mediated and not due to contaminating protease activity. These results demonstrate that the phosphorylation state of hVDR is crucial for complex formation on the VDRE, and possibly also for heterodimerization with RAF/RXR.

Level of Phosphorylation of VDR in COS-7 Cells Affects Transcriptional Activation by $1,25(OH)_2D_3$

To determine if hVDR phosphorylation affects the functional ability of the receptor to mediate ligand-activated gene transcription, VDR-deficient COS-7 cells were transfected with an hVDR expression plasmid and incubated in the absence and presence of the nonspecific phosphatase inhibitor, okadaic acid.

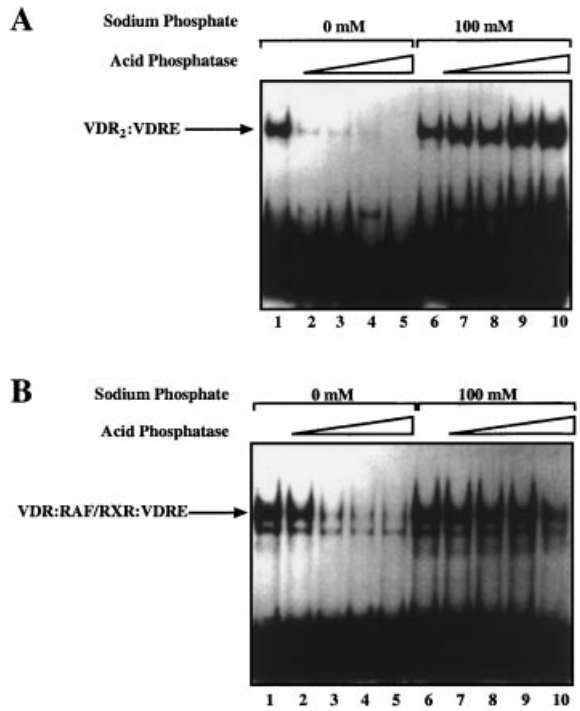


Fig. 6. Effect of phosphatase treatment of hVDR on VDRE association. **A**: BEVS-expressed VDR (100 ng of protein) was incubated for 15 min at 25°C with increasing amounts of PAP beginning at 0.22 U (**lanes 2** and **7**) and escalating to 0.44 U (**lanes 3** and **8**), 0.88 U (**lanes 4** and **9**), and 1.76 U (**lanes 5** and **10**) in the absence and presence of 100 mM sodium phosphate, respectively. Sodium phosphate was used as a phosphatase inhibitor control to ensure that the 25°C incubation was not accelerating the destruction of VDR by proteolysis. The PAP-treated VDR was used in gel mobility shift analysis with [^{32}P]-labeled VDRE-WT probe employing the same assay conditions as in Figure 2. **B**: Conditions were similar to panel A except that 0.25 μg of nuclear extract from CV-1 cells were added to the gel mobility shift assay to examine the effect of phosphatase treatment on the ability of the VDR to form a heterodimeric, high affinity complex on the VDRE.

The results in Figure 7A illustrate that the phosphorylation level of expressed hVDR is enhanced either by $1,25(OH)_2D_3$ ligand binding (lane 2; 5-fold), or by okadaic acid (lane 3; 4-fold). In combination, okadaic acid treatment potentiates $1,25(OH)_2D_3$-stimulated phosphorylation (Fig. 7A, lane 4; 9-fold). Similarly, as depicted in Figure 7B (bar 4), okadaic acid amplifies $1,25(OH)_2D_3$-stimulated transcription of a VDRE-reporter construct in cotransfected COS-7 cells. Strikingly, although okadaic acid treatment enhances hVDR phosphorylated protein concentrations almost as effectively as $1,25(OH)_2D_3$ exposure of the cells (Fig. 7A, compare lanes 2 and 3), the phosphatase

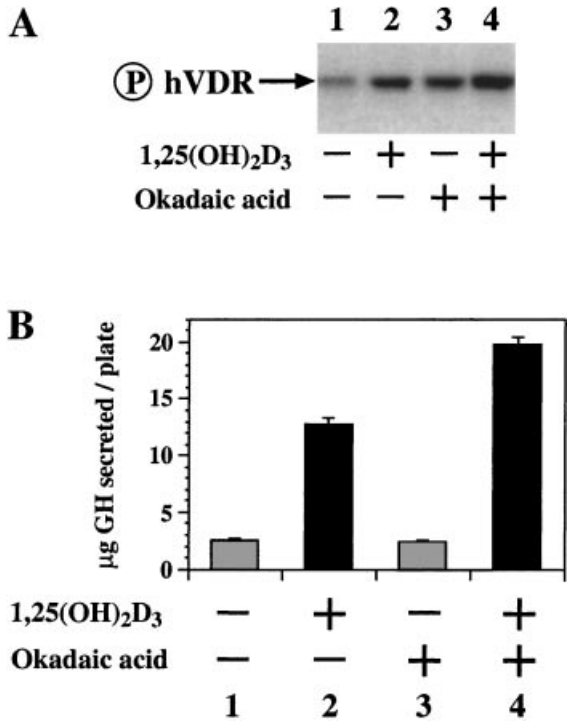


Fig. 7. Effect of $1,25(OH)_2D_3$ hormone and okadaic acid phosphatase inhibitor treatments on phosphorylation of, and transactivation by, hVDR in transfected COS-7 cells. **A**: Phosphorylation of the hVDR protein in intact COS-7 cells. Transfected cells (5 µg of hVDR expression vector per plate) were labeled with [^{32}P]orthophosphate for 2 h and then treated with the indicated combinations of either 50 nM okadaic acid (phosphatase inhibitor), 10^{-7} M $1,25(OH)_2D_3$ or ethanol (vehicle) control for an additional 2 h. The cells were then lysed and hVDR was immunoprecipitated with anti-VDR monoclonal antibody 4A5 linked to Sepharose beads. The ^{32}P-labeled hVDR protein was resolved by electrophoresis on 10% denaturing polyacrylamide gels and visualized by autoradiography for 5 h at −70 °C. **B**: $1,25(OH)_2D_3$-mediated transcriptional activation of a VDRE-linked reporter gene. COS-7 cells were cotransfected with 5 µg of wild type hVDR expression plasmid and 10 µg of a reporter vector containing four copies of the rat osteocalcin VDRE linked upstream of the human growth hormone gene. The cells were then treated (in triplicate) with the indicated combinations of either 50 nM okadaic acid, 10^{-7} M $1,25(OH)_2D_3$ or ethanol (vehicle) control for 24 h post-transfection. The levels of growth hormone secreted into the media, which serves as an index of transcriptional activity, were measured by radioimmunoassay. The results in A and B are representative of at least three independent experiments, with n = 3 and values ± SD for the particular experiment pictured in B.

inhibitor alone is incapable of inducing VDR-mediated transcription (Fig. 7B, bar 3). Therefore, only in the presence of the $1,25(OH)_2D_3$ ligand is there a correlation between hVDR phosphorylation state and transactivity. These data suggest that $1,25(OH)_2D_3$-dependent kinases, or more likely a $1,25(OH)_2D_3$-dependent alteration of the hVDR protein conformation to render it a suitable substrate for protein kinases like CK2 [Jurutka et al., 1996], are required for maximal transcriptional response to the hormone.

DISCUSSION

The present report describes the successful engineering of the full-length hVDR into a baculovirus vector, overexpression in Sf9 cells, and purification of milligram quantities of the recombinant receptor to near homogeneity employing conventional column chromatography (Fig. 1). Other full-length nuclear receptors, for example the GR, have been purified to homogeneity via column chromatography [Warren et al., 1996]. Another group [Juntunen et al., 1999] recently purified baculovirus expressed hVDR to homogeneity in preparation for crystallization, and hVDR also has been isolated using amylose resin affinity purification of an *E. coli*-overexpressed hVDR-maltose binding protein fusion construct [Mottershead et al., 1996]. To probe specific hVDR–DNA interactions in the current study, purified BEVS overexpressed receptor was utilized in electrophoretic mobility shift assays to evaluate the binding of hVDR, both alone and in conjunction with nuclear extracts, to the rat osteocalcin VDRE, an imperfect DR3 element with the sequence GGGTGAatgAGGACA. The purified BEVS–hVDR binds as both an apparent homodimer (Fig. 2) or as a heterodimer with RAF/RXR (Fig. 3A), with the latter species possessing a greater affinity for a DR3 responsive element based on competition/dissociation analysis (Fig. 3B). This observation is in concert with findings for RAR [Roy et al., 1995] and TR [Claret et al., 1996], where, likewise, RXR heterodimers are the more stable DNA binding species. Therefore, consistent with other nuclear receptors that bind to directly repeated DNA elements, VDR complexed to RXR as a heterodimer is the preferred VDRE binding species, in vitro, at least in the case of the rat osteocalcin DR3.

Other VDREs have been touted as candidate VDR-homodimer targets, such as the mouse osteopontin DR3 VDRE, which is a perfect direct repeat with a T present as the third base in each half-element, a feature that has been proposed to distinguish this element for VDR homodimeric binding [Freedman et al., 1994].

Previous reports utilizing an artificial osteopontin-like VDRE [Cheskis and Freedman, 1994; Freedman et al., 1994], and the actual mouse osteopontin VDRE [Nishikawa et al., 1994], GGTTCAcgaGGTTCA, have shown that it is possible, under certain experimental conditions, for VDR to form homodimers on DNA. However, a relevant point raised by Thompson et al. [1998], and emphasized herein, is that any conclusions concerning receptor-DNA complex formation must consider the experimental conditions employed, both in terms of receptor amounts and also salt concentrations, since low salt stringency can be permissive for nonphysiologic interactions. In these previous studies, salt concentrations were often not specified and vast excesses of VDR were utilized during gel mobility shift assays [Freedman et al., 1994; Nishikawa et al., 1994], and gel filtration experiments [Cheskis and Freedman, 1994]. Thompson et al. [1998] have shown, using gel mobility shift analysis mimicking the ionic strength of the cell, that no homodimeric form of VDR binds to the mouse osteopontin VDRE when limiting amounts of receptor are employed, and only at supraphysiological levels of receptor can a homodimeric species be observed to form on this element. Another observation that argues against a role of VDR homodimers has been provided by MacDonald et al. [1995] with the demonstration that VDR does not interact with itself when employing yeast two-hybrid technology, whereas VDR and RXR associate strongly in this system. Craig and coworkers have also demonstrated that VDR, in the presence of its cognate ligand, binds to the osteopontin VDRE as a heterodimer with RXR and not a homodimer by using the technique of microelectrospray ionization mass spectrometry [Craig et al., 1999]. Moreover, a strong argument against a functional role for VDR homodimers in binding to an osteopontin-type DR3 element has been provided by Freedman and coworkers who used a cell free in vitro transcription assay system to show that $1,25(OH)_2D_3$ signaling from such an element is mediated exclusively through a VDR–RXR heterodimeric complex [Lemon et al., 1997].

Finally, there exist two compelling lines of evidence from in vivo experiments that argue in favor of VDR–RXR heterodimer action over VDR homodimeric response to $1,25(OH)_2D_3$. First, naturally occurring point mutations in hVDR that confer vitamin D resistance in patients map in some cases exclusively to the RXR heterodimerization domain of VDR [Whitfield et al., 1996]. Second, temporally controlled knockout of all RXR isoforms in mouse epidermis elicits alopecia [Li et al., 2000], identical to the skin phenotype observed in VDR null mice [Li et al., 1997; Yoshizawa et al., 1997], and in the subset of hypocalcemic vitamin D resistant patients who are refractory to massive doses of $1,25(OH)_2D_3$ [Malloy et al., 1999]. These in vivo observations indicate that the VDR–RXR heterodimer is the obligate mediator of vitamin D signaling for processes such as intestinal calcium absorption and hair cycling. Such biological insights are in concert with the present findings (Figs. 2 and 4) that the VDR–RXR heterodimer, rather than an apparent VDR homodimer, is the high-affinity receptor species bound to the VDRE.

Having established that VDR binds to DNA as an obligate RXR heterodimer to effect its biological actions, the question remained as to the role of hVDR phosphorylation in signaling by the $1,25(OH)_2D_3$-occupied heterodimer. Methylation interference experiments (Fig. 4) suggested that unphosphorylated, *E. coli*-expressed hVDR and hRXRα [Hsieh et al., 1995] bound to the VDRE when present in excess but elicited a pattern of G-base protection that was incomplete compared to that observed with purified hVDR expressed via the baculovirus system in insect cells. Consequently, the phosphorylation of BEVS–hVDR was examined (Fig. 5), and the receptor was found to be phosphorylated at multiple sites in a $1,25(OH)_2D_3$-enhanceable fashion in intact Sf9 cells. The purified BEVS–hVDR also served as an efficient substrate for additional phosphorylation catalyzed by several protein kinases, in vitro, namely PKA, PKC, and CK2. Notably, studies in *Drosophila* have revealed the presence of apparent homologs for PKA [Kalderon and Rubin, 1988], PKC [Schaeffer et al., 1989], and CK2 [Saxena et al., 1987], suggesting that hVDR produced via the BEVS method may have been phosphorylated endogenously by insect homologs of the relevant mammalian protein kinase enzymes. Consistent with this notion, unliganded BEVS-generated hPR is phosphorylated at both hormone-inducible and basal sites, all of which are phosphorylated in the BEVS to the same extent as in the hormone treated receptor in mammalian cells [Beck et al., 1996]. These phosphorylations of hPR appear to be

catalyzed predominantly by protein kinases that recognize Ser/Thr residues immediately followed by a proline, but also by CK2 [see Knotts et al., 2001 and references therein]. Given these results with PR, it seems plausible to conclude that the pattern of hVDR phosphorylation in BEVS might be a reasonable approximation of what would occur in vivo in a mammalian cell in the presence of $1,25(OH)_2D_3$.

Previously described phosphorylation of hVDR by either PKA or PKC has been demonstrated to negatively impact receptor function. PKA catalyzed phosphorylation of hVDR has been shown to attenuate $1,25(OH)_2D_3$-mediated transactivation [Jurutka et al., 1993a; Nakajima et al., 2000], and more recently, Hsieh et al. [2001] have demonstrated that PKA catalyzed phosphorylation of the receptor occurs in the exon V loop of the LBD, specifically serines 182–185, blunting heterodimerization with RXR and consequently attenuating transactivation. Independently, PKC catalyzed phosphorylation of hVDR, at a site between the two zinc fingers in the DNA-recognition α-helix, blocks nuclear translocation of hVDR and the binding of hVDR–RXR to the VDRE [Hsieh et al., 1993]. Conversely, phosphorylation of hVDR by CK2 at Ser-208 in the LBD has a positive effect on receptor function. Jurutka et al. [1996] demonstrated that CK2 action potentiates hormone-dependent transactivation, perhaps by facilitating the association of VDR with a coregulator from the mediator complex, such as DRIP205 [Barletta et al., 2000]. Alternatively, analogous to the phosphorylation of the MADS-box transcription factor MEF2C by CK2, which stimulates its DNA-binding activity 5-fold [Molkentin et al., 1996], CK2 may potentiate VDR heterodimerization/DNA binding.

Which of the negative or positive effects on hVDR phosphorylation might predominate to modulate hVDR function was tested by treatment of BEVS receptor with PAP to, in essence, create a dephospho-hVDR. PAP treatment of the dioxin receptor obliterates its DNA binding capacity [Pongratz et al., 1991], and similar phosphatase incubation essentially abolished hVDR activity by preventing hVDR association as an RXR heterodimer with the VDRE (Fig. 6B). Utilizing an independent approach, okadaic acid treatment of COS-7 cells to block phosphatase action was shown to yield a hyperphosphorylated hVDR with an enhanced ability to mediate $1,25(OH)_2D_3$-dependent transcription (Fig. 7). The latter result is consistent with the above-cited Ser-208 phosphorylation, which would presumably be increased in the presence of okadaic acid, thereby boosting hVDR activity. However, current knowledge of hVDR phosphorylation does not explain the observed loss of receptor activity upon acid phosphatase treatment (Fig. 6). This unexpected result implies the existence of an obligatory rather than modulatory phosphorylation event, perhaps catalyzed by an unknown fourth hVDR kinase, that is essential for hVDR DNA binding activity. The following observations may provide clues as to the identity of this unidentified kinase. First, biochemical evidence derived from alkaline phosphatase treatment of rat VDR from ROS 17/2.8 cells has demonstrated a significant hormone-enhanced phosphorylation of the rat receptor distinct from that catalyzed by CK2 [Jurutka et al., 1993c]. Second, it has been found that mitogen activated protein kinase (MAPK) is essential for $1,25(OH)_2D_3$-induced differentiation of HL-60 cells along the monocyte/macrophage lineage [Marcinkowska et al., 1997]. In addition, $1,25(OH)_2D_3$ acutely activates MAPK in normal human keratinocytes [Gniadecki, 1996] and in HL-60 cells [Marcinkowska et al., 1997], providing a rapid mechanism for the hormone to promote its own receptor-mediated action.

The potential involvement of MAPK in hVDR phosphorylation has ample precedent with other nuclear receptors. MAPK is a proline-directed kinase, indicating that it phosphorylates Ser/Thr residues that are immediately followed by a proline residue. Such Ser-Pro or Thr-Pro motifs are common sites for phosphorylation in other nuclear receptors. As one example, TR has been observed to activate MAPK and elicit MAPK-mediated serine phosphorylation of TRβ1 and subsequent dissociation of the corepressor SMRT [Davis et al., 2000], leading to a potentiation of transactivation by TR. In another example, mouse steroidogenic factor 1 (SF-1) is phosphorylated by the MAPK signaling pathway at Ser-203 in its activation function-1 domain, an event that boosts recruitment of coactivators for transactivation [Hammer et al., 1999]. Among the classic steroid hormone receptors, human PR contains 14 Ser/Thr-Pro motifs, mainly in its N-terminal domain. Most of these motifs have been shown to be phosphorylated [Knotts et al., 2001]. The kinase responsible for this phosphorylation is proposed to be

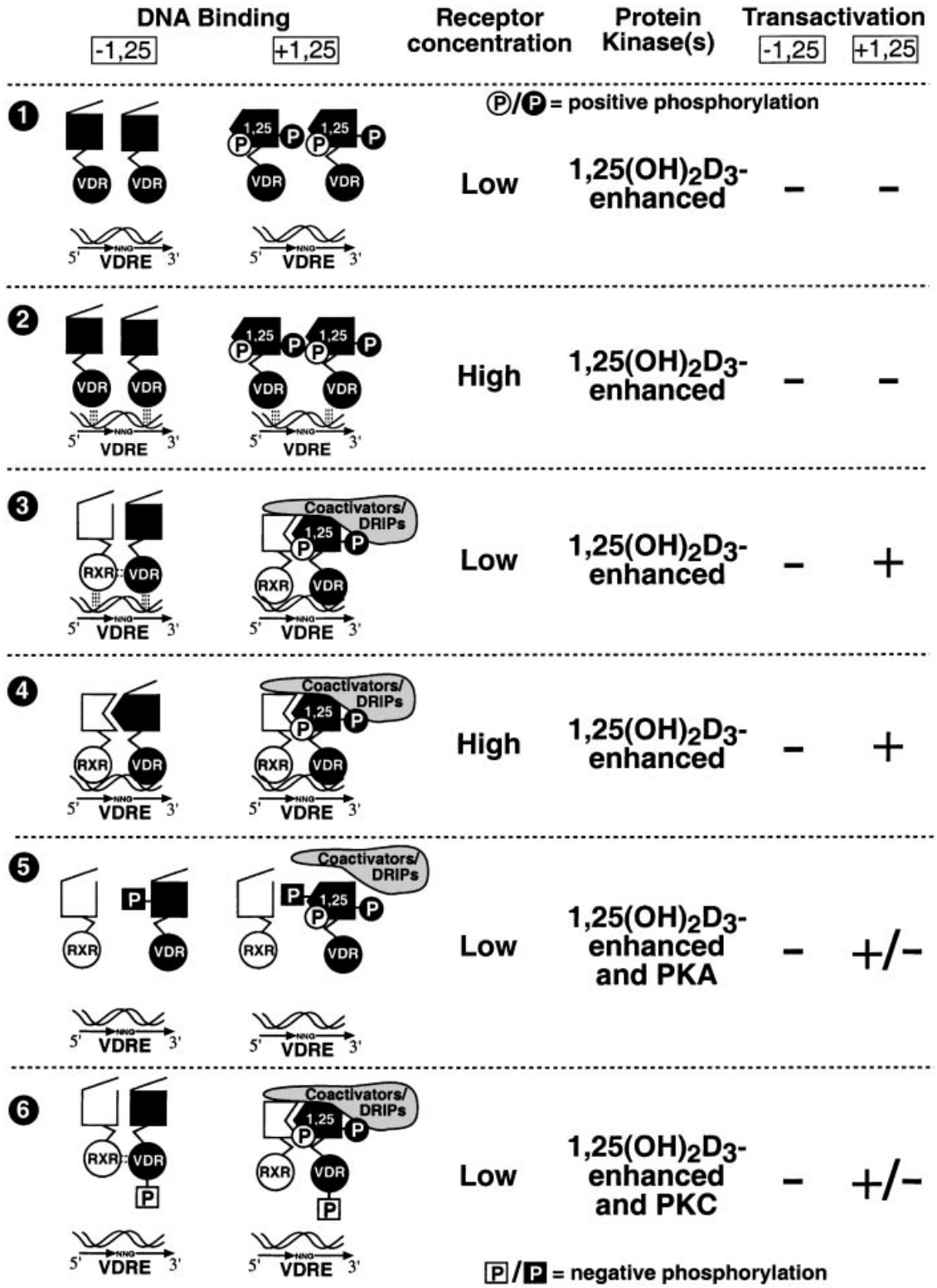
cyclin dependent kinase-2 (cdk2), a proline-directed kinase that requires stimulation by the MAPK [Chiariello et al., 2000] pathway. With human ERα, Ser-118 in the AF-1 is phosphorylated in a ligand-dependent manner, resulting in increased ER-mediated transactivation [Chen et al., 2000]. The mechanism of this effect involves the AF-2-dependent recruitment of TFIIH and catalysis by associated CDK7. Interestingly, this same Ser-118 site in human ERα is phosphorylated by MAPK [Kato et al., 1995], intimating that ERα can be activated not only by ligand-stimulated phosphorylation in the nucleus, but also via the Ras–MAPK cascade initiated at the cell surface by growth factors. Finally, MAPK plays a key role in androgen receptor (AR)-dependent transcription [Zhou et al., 1995] and androgen-dependent apoptosis in prostate cancer cells [Abreu-Martin et al., 1999].

Assuming that at least four protein kinases modulate hVDR activity, possibly including MAPK, it is reasonable that cell-specific expression and activation of these kinases, at least one of which (CK2) may be cell-cycle dependent [Bosc et al., 1999], could account for the reported differences in the functional impact of VDR phosphorylation [Darwish et al., 1993; Hsieh et al., 1993; Jurutka et al., 1993a, 1996; Desai et al., 1995; Matkovits and Christakos, 1995; Nakajima et al., 2000]. Despite the fact that in previous studies VDRs from different species were employed, as were a number of distinct promoter-reporter constructs, it is still possible to rationalize the current results within the context of the conflicting observations of Desai et al. [1995] and Matkovits and Christakos [1995]. Thus, in the Desai study, in which rat ROS 17/2.8 osteoblast-like cells were treated with okadaic acid, the phosphatase inhibitor may have preserved the phosphorylation of PKA and/or PKC sites, which would maintain the rat VDR inactive in bone cells expressing predominantly these kinases. Conversely, in the Matkovits and Christakos study of hVDR in transfected CV-1 kidney cells, it is possible that kinases such as CK2 and putative obligatory MAPK are predominant, explaining why these investigators observed a ligand-independent activation of hVDR-mediated transcription by okadaic acid treatment. In the present experiments with hVDR expressed in transfected COS-7 kidney cells (Fig. 7), we found no basal effect of okadaic acid, but we did observe a strong potentiation of the $1,25(OH)_2D_3$ effect by the phosphatase inhibitor. This implies that in COS-7 cells, the putative obligatory MAPK phosphorylation event requires prior membrane activation by the $1,25(OH)_2D_3$ hormone, rendering this system completely analogous to the TR-MAPK situation [Davis et al., 2000] outlined above. Regardless of the above complexities, it is likely that VDR phosphorylation is integral to $1,25(OH)_2D_3$ signaling, as also indicated by studies demonstrating that VDR phosphorylation occurs prior to $1,25(OH)_2D_3$-induced calbindin-D_{28k} induction and calcium absorption in chick intestine [Brown and DeLuca, 1990], and correlates positively with the dose response to the hormone in activating osteocalcin transcription in rat osteoblast-like cells [Jurutka et al., 1993c].

Based upon the present results, and data from the literature, a summary model can be constructed depicting the relative VDRE binding strengths of VDR:VDR homodimers and VDR:RXR heterodimers, along with a hypothetical conception of how these associations and the multiple-site phosphorylation of hVDR are translated into transactivation in response to the $1,25(OH)_2D_3$ ligand. As shown in Figure 8, Row 1, physiologic amounts (20 ng in a gel shift assay) of hVDR, in the absence of RXR coreceptor, do not occupy the VDRE in in vitro gel shift assays, even in the presence of $1,25(OH)_2D_3$ [Thompson et al., 1998]. However, as depicted in

Fig. 8. Model summarizing hVDR–DNA binding, phosphorylation and transactivation potential under various experimental conditions. VDR–VDRE binding is depicted when hVDR is present in low (physiologic) concentrations and high (overexpressed) concentrations, either in the absence (Rows 1 and 2) or presence (Rows 3–6) of RAF/RXR. Also shown are two $1,25(OH)_2D_3$-enhanced hVDR phosphorylations in the ligand binding domain (LBD), one at serine-208 (shown on right of LBD as a white ''P'' in a black circle) that assists in coactivator recruitment, and a second phosphorylation at an unknown site (shown on left of LBD as a black ''P'' in a white circle) that facilitates dimerization and DNA binding. Although not illustrated in the absence of $1,25(OH)_2D_3$ ligand (Rows 1–6, left), BEVS hVDR is assumed to be basally phosphorylated at the $1,25(OH)_2D_3$-enhanced sites. Rows 5 and 6 illustrate respective negative modulation (+/–) of receptor activity by ligand independent PKA-catalyzed phosphorylation at serines 182–185 in the VDR LBD domain (shown on left of LBD as a white ''P'' in a black box), and by ligand independent PKC-catalyzed phosphorylation of hVDR at serine-51 in the DNA binding domain (shown on DBD as a black ''P'' in a white box). PKA- and PKC-catalyzed phosphorylations of hVDR attenuate transactivity by inhibiting receptor heterodimerization and DNA binding, respectively.

Figure 8, Row 2, when the amount of receptor is increased well above the physiologic range (100 ng in a gel shift assay), there is a low-affinity (designated by dotted lines) interaction of hVDR with the VDRE as an apparent homodimer (Figs. 2, 4A, and as reported previously [Thompson et al., 1998]). Note that homodimer association is not enhanced by the introduction of



ligand (Fig. 8, Row 2, right; see also Fig. 3A and Thompson et al. [1998]). Furthermore, this homodimeric species, even if it should occur physiologically, would likely be inactive transcriptionally since RXR is obligatory for VDR-mediated transactivation as demonstrated in a yeast system devoid of RXR [Jin and Pike, 1996]. In contrast, as illustrated in Figure 8, Row 3, physiologic (low) levels of VDR and RXR associate weakly to form a VDR:RXR complex that binds with low affinity to the VDRE in the absence of ligand (Row 3, left). This association is significantly enhanced by $1,25(OH)_2D_3$, whose binding to VDR promotes high affinity VDRE binding of the heterodimer in the major groove of DNA and also reconfigures the activation function-2 (AF-2) helix 12 domain of VDR into the active conformation (depicted as a "closing of the lid" in VDR) for coactivator contact and transactivation (Row 3, right; see also Thompson et al. [1998]). In this setting (Row 3, right), the AF-2 of RXR is also thought to be repositioned into an active (closed) conformation through allosteric interactions with $1,25(OH)_2D_3$-VDR [Thompson et al., 2001]. Recall that the $1,25(OH)_2D_3$:VDR-RAF/RXR complex is a stable, high affinity species compared to the VDR homodimer (Fig. 3B). Finally, as hypothesized in Fig. 8, Row 4, supraphysiologic concentrations of VDR:RXR have the ability to overcome the requirement for $1,25(OH)_2D_3$ in order to heterodimerize and bind DNA with high affinity (Row 4, left; Figs. 3A and 4ABC) because, even in the absence of $1,25(OH)_2D_3$ there is sufficient receptor in the correct allosteric form to associate with the VDRE. However, even when bound in a heterodimer to the VDRE, the VDR AF-2 is still not positioned in the active (closed) conformation, and therefore does not bind to coactivators or stimulate transcription until the addition of ligand (Row 4, right). Therefore, the schematic summary in the first four rows of Figure 8 provides a conceptualization of how VDR homodimers can be visualized during in vitro gel shift experiments where excessive amounts of receptor are employed, and also how apparent VDR–RXR heterodimers are observed bound to the VDRE in the absence of hormonal ligand under these nonphysiologic conditions, in vitro. However, only in the presence of $1,25(OH)_2D_3$ ligand [Breen et al., 1994], and its associated enhancement of LBD phosphorylation, are transcriptionally productive VDR–RXR complexes generated on the VDRE.

Regarding the role of phosphorylation, we propose that hVDR is phosphorylated in the LBD (Fig. 8, Rows 1–4, depicted by a black "P" in a white circle) by an unidentified, $1,25(OH)_2D_3$-stimulated protein kinase that facilitates the DNA binding capacity of the receptor, perhaps by promoting heterodimerization with RXR. Also in response to ligand, CK2 phosphorylates hVDR at Ser-208 in the LBD (Fig. 8, Rows 1–4, depicted by a white "P" in a black circle) to enhance hormone-dependent transactivation [Jurutka et al., 1996]. At this stage, the heterodimer, primed by these two ligand-stimulated phosphorylations of hVDR, is now optimally suited to attract coactivators and DRIPS, [Rachez et al., 1999], thereby mediating transcriptional initiation (Fig. 8, Rows 3 or 4, right-hand complex). The two positive phosphorylation events outlined above may be counteracted under appropriate regulatory conditions by PKA- and/or PKC-catalyzed phosphorylation of hVDR (Fig. 8, Rows 5 and 6, respectively). PKA action to phosphorylate hVDR in the LBD at serines 182–185 (Fig. 8, Row 5, depicted by a white "P" in a black box), in response to stimulators of intracellular cAMP, appears to downregulate heterodimer formation on the VDRE by hVDR, evidently by inhibiting the interaction with RXR [Hsieh et al., 2001]. In analogous fashion, PKC action, in response to extracellular stimuli, leads to phosphorylation at Ser-51 in the DNA binding domain (Fig. 8, Row 6, depicted by a black "P" in a white box), resulting in an attenuation of DNA binding [Hsieh et al., 1993]. As discussed above, the significance of these phosphorylation events would differ according to specific cell type and regulatory circumstances, providing a potential for fine-tuning the response to $1,25(OH)_2D_3$ in reaction to extracellular signals. Further research will be required to refine and verify this model of VDR action to stimulate transcription, but what is clear from the present study are the crucial roles played by heterodimerization and phosphorylation in determining VDR function.

One possibility not excluded by the present experiments is that VDR action is influenced by phosphorylation of cooperating proteins instead of VDR itself. For example, nuclear receptor coactivators like SRC-1 [Rowan et al., 2000], and cointegrators like CBP [Zanger et al., 2001]

also can be phosphorylated to facilitate further stimulation of gene transcription. In fact, in response to ligand binding to surface receptors coupled to G_s, CREB is phosphorylated [Andrisani, 1999], and in turn recruits the CBP cointegrator to activate gene transcription [Zanger et al., 2001]. Therefore, phosphorylation/dephosphorylation of transcription factors is central to their molecular signaling, often constituting the final step in a signal transduction cascade initiated at the cell surface. Phosphorylation of STATs [Darnell, 1997] and SMADs [Kretzschmar and Massague, 1998] triggered by cognate cell surface receptors bound to GH and TGFβ, respectively, is required for these transcription factors to dimerize, bind DNA, and control target cell gene expression. Thus, considering the above examples, it is not surprising that the nuclear VDR also constitutes a transcription factor for which nuclear localization, dimerization/DNA binding, and transactivation are modulated by reversible phosphorylation.

ACKNOWLEDGMENTS

We thank Dr. Milan Uskokovic of Hoffmann-LaRoche, Inc. for kindly providing 1,25-dihydroxyvitamin D_3 for our studies. The authors express appreciation to Sanford H. Selznick for his technical expertise and patience in the preparation of figures for this manuscript and to Carlos Encinas Dominguez for helpful discussions. We also wish to thank Michelle Thatcher for excellent technical assistance.

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