1,25-Dihydroxyvitamin D_3 Modulates Phosphorylation of Serine 205 in the Human Vitamin D Receptor: Site-Directed Mutagenesis of This Residue Promotes Alternative Phosphorylation

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ABSTRACT: The vitamin D receptor (VDR) from a variety of animal species is a hormone-modulated substrate for phosphorylation *in vivo*. In this report, we utilize an expression vector to produce recombinant human VDR (hVDR) in 1,25-dihydroxyvitamin D_3-treated COS-1 cells. Immunoprecipitation of the phosphorylated hVDR followed by gel purification and phosphoamino acid analysis revealed modification exclusively on one or more serine residues, consistent with previous studies of the VDR in other species. To identify the region of phosphorylation, immunoprecipitated and gel-purified hVDR from COS-1 cells was first mixed with purified hVDR isolated to homogeneity from *Saccharomyces cerevisiae* and then digested with trypsin or V8 protease, and the peptides were resolved on HPLC. The single phosphate-containing peptides were recovered and subjected to amino acid sequence analysis, revealing the modification to reside in a region extending from residue 171 to residue 206 common to both the tryptic- and the V8 protease-derived peptides. Sequential cleavage of similar VDR mixtures using trypsin and then CNBr, α-chymotrypsin, or thermolysin demonstrated an amino-terminal boundary of the phosphorylated peptide at 202. Selective manual Edman degradation of phosphorylated peptides beginning at 171, 195, and 200 revealed phosphate release only at serine 205. This peptide contained an average of 8-fold less radioactive phosphate in the absence of prior treatment of the culture cells with $1,25(OH)_2D_3$. Site-directed modification of VDR serine 205 to alanine, aspartate, or glutamate each led to fully functional proteins when assessed in a transactivation assay using several VDRE-linked natural promoters. Unexpectedly, evaluation of the serine 205 to alanine hVDR mutant revealed that this protein continued to be phosphorylated in a hormone-dependent manner on an alternative site. These studies show directly that hVDR serine residue 205, a consensus site for casein kinase II, is modified *in vivo* in response to hormone.

The vitamin D_3 receptor (VDR) is a member of a superfamily of genes which encode intracellular receptors for the sex and adrenal steroids, thyroid hormone, and metabolically active forms of vitamins A and D (Evans, 1988; O'Malley, 1990; Truss & Beato, 1993). The members of this family are structurally and functionally related through striking amino acid homologies, particularly those found in the DNA and hormone binding regions of the proteins (Evans, 1988). The DNA binding domain comprises two α-helical zinc fingers which interact directly in the major groove of a hexanucleotide hormone responsive element (HRE) halfsite (Hard et al., 1990; Schwabe et al., 1990, 1993; Luisi et al., 1991; Lee et al., 1993). While there may be some exceptions (Wilson et al., 1992), the transcriptionally active receptor unit generally comprises either two identical receptor monomers such as those found in the estrogen receptor (Tsai et al., 1990; Kumar & Chambon, 1988; Fawell et al., 1990; DeMarzo et al., 1991) or nonidentical monomers such as those found in heterodimers of VDR/nuclear accessory factor (NAF) (Sone et al., 1991; Liao et al., 1990), VDR/retinoid X receptor (RXR) (Kliewer et al., 1992), or retinoic acid receptor (RAR)/RXR (Kliewer et al., 1992; Zhang et al., 1992). Holoreceptor formation on specific DNA is mediated by several distinct dimerization regions. Two weak surfaces are located in the DNA binding domain within the loop of the second zinc finger, one that facilitates symmetrical homodimer formation (Schwabe et al., 1990; Kumar & Chambon, 1988) and the other that mediates asymmetric heterodimer formation (Perlmann et al., 1993). A more complex and extensive dimerization surface common to all of the receptors is located within the carboxy-terminal ligand binding domain (Glass et al., 1989, 1990; Forman et al., 1989; Fawell et al., 1990). Interaction between two monomers mediated by this region can occur in the absence of DNA and is essential for high-affinity DNA binding. In response to activation by their cognate ligands, these receptors interact with specific hormone responsive elements (HREs) located adjacent to regulatable gene promoters (Beato, 1989; Umesono et al., 1991). Following their association with DNA, receptor dimers interact directly or indirectly (via comodulators) with the transcriptional initiation complex to positively or negatively regulate transcription (Ing et al., 1992; Klein-Hitpass et al., 1990).

Many transcription factors are phosphoproteins (Jackson et al., 1990; Jackson, 1992; Hunter & Karin, 1992; Bohman, 1990). This modification has been implicated in such diverse control processes as DNA binding capacity (Boyle et al., 1991a; Lin et al., 1992), transcriptional activation or repression (Wagner et al., 1992; Jackson et al., 1990), and cytoplasmic to nuclear trafficking (Gilmore, 1991; Schmitz et al., 1991). The steroid receptor family members are also targets of phosphorylation, including those that mediate estrogen,

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progesterone, glucocorticoid, thyroid hormone, retinoic acid, and vitamin D action (Moudgil, 1990; Orti et al., 1992). The bulk of these receptor phosphorylation sites are on serine or threonine residues, many of which occur in response to occupation by hormone (Orti et al., 1992). Several phosphorylated residues have been pinpointed within the chicken progesterone receptor (PR) (Denner et al., 1990; Weigel et al., 1992). Despite these significant advances in our identification of phosphorylation sites within steroid receptor proteins, as well as the link between ligand binding and phosphorylation, distinct functional roles for this modification on specific receptors remain largely speculative.

Hormone-dependent phosphorylation has been observed within the VDR from several species. This includes an initial report using cultured mouse 3T6 fibroblasts wherein phosphorylation in response to 1,25$(OH)_2D_3$ resulted in retarded migration of the receptor protein during denaturing gel electrophoresis (Pike & Sleator, 1985). Phosphorylation has also been shown to occur on chicken and porcine VDRs (Brown & DeLuca, 1990, 1991). Phosphorylation within the porcine VDR has been mapped via domain-specific antibodies to a large portion of a region lying between the DNA and ligand binding domains (Brown & DeLuca, 1991). Finally, recombinant human VDR also has been shown to undergo a similar hormone-specific phosphorylation, although this modification uniquely does not result in altered electrophoretic migration under denaturing conditions (McDonnell et al., 1989). These latter studies did not identify the nature of the phosphoamino acid, nor did they map the modification to a specific residue.

More recently, it has been reported that recombinant human VDR (hVDR) in COS-7 cells undergoes phosphorylation at several serine sites, one located within the DNA binding domain at residue 48 (Hsieh et al., 1991, 1993) [residue 1 within the hVDR in this paper is equivalent to residue 4 of the hVDR sequence reported by Baker et al. (1988)], and one located immediately amino terminal to the ligand binding region at residue 205 (Jones et al., 1991; Jurutka et al., 1993a). One or more cAMP-dependent protein kinase sites between residues 130 and 198 also have been suggested (Jurutka et al., 1993b). Serine 205 represents a consensus site for casein kinase II (CKII) (Marin et al., 1986), forming the basis for an approach largely *in vitro* in nature wherein Jurutka et al. (1993a) concluded that phosphorylation at this site was at best weakly modulated by hormone. A similar approach was taken for the additional site wherein modification of residue 48 by protein kinase C (PKC) was shown to result in loss of DNA binding (Hseih et al., 1993). Despite the suggestion that signals via the PKC pathway converge negatively on VDR activity, it is clear that the effects of phorbol esters and other inducers of PKC action on 1,25$(OH)_2D_3$-induced gene expression are predominantly positive (Chen et al., 1993; van Leeuwen et al., 1992; Krishnan & Feldman, 1992). In addition, phorbol esters are known to upregulate receptor mRNA and protein levels, also leading to an enhancement of biological response (van Leeuwen et al., 1992). Irrespective of the conclusions of these recent efforts to define VDR phosphorylation events, each is compromised by the lack of direct evidence that the modifications identified occur within the normal VDR in an intact cellular context.

In this report, we utilized an expression vector to direct synthesis of the human VDR in COS-1 cells and employed immunoprecipitation with an anti-VDR monoclonal antibody together with gel electrophoresis to isolate the phosphorylated receptor. We mixed this latter material with highly purified recombinant VDR derived from yeast, subjected the mixture to single or sequential enzymatic digestions, and purified the peptides by chromatographic means. Phosphate-release studies using manual Edman degradation coupled with direct amino acid sequence analysis of the isolated peptides allowed us to identify the residue(s) modified by phosphorylation in intact cells. We report that this modification in the hVDR occurs at serine 205 and is strongly dependent upon the presence of hormone. Interestingly, evaluation of hVDR altered at this site through site-directed mutagenesis revealed alternative hormone-modulated phosphorylation at a nearby residue. Full transactivation capacity of these mutants was also observed when cotransfected with a reporter gene linked to a vitamin D-responsive promoter. Thus, the role of this hormone-dependent modification remains currently undefined.

MATERIALS AND METHODS

Reagents. Trypsin was obtained from Worthington Biochemical Corp., Freehold, NJ. Restriction enzymes and proteases α-chymotrypsin, Asp-N, V8 protease (Glu-C), and thermolysin were obtained from Boehringer Mannheim, Indianapolis, IN. CNBr, trifluoroacetic acid (TFA), and phenyl isothiocyanate (PITC) were obtained from Pierce Chemicals, Rockford, IL. HPLC was conducted on Vydac 15 cm × 21 mm C4 reverse-phase columns (Vydac, Hesperia, CA). Water and acetonitrile were Optima grade from Fisher Scientific, Tustin, CA. [^{32}P]Orthophosphate and [^{35}S]-methionine were from New England Nuclear, Boston, MA. 1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] was from Duphar, da Weesp, the Netherlands. [^{3}H]-1,25$(OH)_2D_3$ (180 Ci/mmol) was from Amersham International, Arlington Heights, IL. [^{125}I]Protein A (30 Ci/mmol) was from ICN, Irvine, CA.

Plasmids. The pAV-hVDR and pAV-hVDR (Δ 100-111) expression vectors for hVDR and an hVDR internally deleted for the anti-VDR monoclonal epitope have been previously described (McDonnell et al., 1989; Sone et al., 1991). pRSV-hVDR was also utilized to express the wild-type hVDR (Berger et al., 1992). The reporter plasmid phOC-1300-luc contains 1300 nucleotides of the human osteocalcin gene promoter 5′ to the start site of transcription (Kerner et al., 1989). The reporter plasmid ph24OH-6000-luc contains 6 kb of the human 25-hydroxyvitamin D_3-24 hydroxylase promoter (S. A. Kerner and J. W. Pike, manuscript in preparation). Both were cloned into the luciferase reporter pL-luc (Berger et al., 1992; Tzukerman et al., 1994). pTK-VDRE(3)-luc contains three copies of the human osteocalcin gene activator protein 1 (AP-1)/vitamin D response element (VDRE) composite sequence (Ozono et al., 1990) fused to the *Herpes simplex* thymidine kinase gene promoter in pL-luc. The pCH110 β-galactosidase internal standard was from Pharmacia, Piscataway, NJ, and has been previously described (Berger et al., 1992; Tzukerman et al., 1994).

Cultured Cells. CV-1 African green monkey kidney fibroblasts, COS-1 simian virus 40-transformed monkey kidney fibroblasts, and HepG2 human hepatoma cells were obtained from the American Type Culture Collection, Rockville, MD, and grown as described previously (Berger et al., 1992; Ozono et al., 1990; Vegeto et al., 1993).

Transfection and Metabolic Labeling of hVDR. COS-1 cells were cultured in Dulbecco's modifed Eagle's medium (DMEM) (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine calf serum (FBS) and utilized for both hVDR expression and metabolic labeling studies. The cells were plated 24 h prior to [(2-diethylamino)ethyl]dextran (DEAE-dextran) transfection at a density of 1.8 × 10^6 cells/

10-cm dish. Serum-free DMEM (3.0 mL) containing 20 μg of intact pAV-hVDR expression plasmid and 12 μL of a 50 mg/mL DEAE-dextran stock, also in serum-free DMEM, was applied to the COS-1 monolayer and incubated at 37 °C for 2–3 h. The medium was then removed, and the cells were shocked for 30 s with 5% dimethyl sulfoxide (DMSO) in serum-free DMEM. The monolayers were rinsed twice with 5 mL of DMEM containing 10% FBS and then incubated in the same medium at 37 °C for 40 h. Following this incubation, the monolayers were rinsed twice with 5 mL of phosphate-free or methionine-free DMEM containing 10% FBS that had been exhaustively dialyzed against 0.15 M NaCl. The cells were starved in this medium for 2 h, rinsed with an additional 5 mL of phosphate- or methionine-free medium, and then placed in 2.5 mL of medium containing [^{35}S]-methionine or [^{32}P]orthophosphate at a concentration of 500 μCi/mL or 1 mCi/mL, respectively. Following a 2-h incubation at 37 °C, 0.4 mM 1,25(OH)$_2$D$_3$ was added, whereupon the cells were gently rocked for an additional 4 h at 37 °C. The medium was removed, and the cells were washed twice with ice-cold PBS and lysed on the plate in 1 mL of ice-cold H buffer (50 mM potassium phosphate, pH 7.6, 10 mM sodium molybdate, 50 mM sodium fluoride, 5 mM sodium metavanadate, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 300 mM KCl) plus an additional 0.0125% deionized Triton X 100. The crude cell extract was centrifuged at 14000*g* for 20 min at 0 °C, and the supernatant was used for immunopurification of radiolabeled VDR.

Transcriptional Cotransfection Assays. For transcriptional response studies, HepG2 cells were transfected with pAV-hVDR expression vector; the reporter plasmid phOC-1300-luc, ph24OH-6000-luc, or pTK-VDRE(3)-luc; and a β-galactosidase standardization plasmid as previously described (Berger et al., 1992). Cells were lysed on the plates, and both β-galactosidase and luciferase activities were assessed (Berger et al., 1992). All luciferase data was normalized to β-galactosidase activity.

Expression and Purification of hVDR in Yeast. The yeast strain BJ3505 (MATa pep4::HIS3 prb1–Δ1.6R his 3 lys2-208 trp-1Δ101 ura3-52) was a recipient host for YEpV1. YEpV1 contains the human VDR cDNA cloned into the *Afl*II/*Kpn*I site of the yeast vector YEp46 (TRP1, 2 μm, Amp^R) (Sone et al., 1990). The result is an in-frame fusion with the yeast ubiquitin gene sequence that produces a ubiquitin–VDR fusion protein under the control of the copper-inducible CUP1 promoter. An endogenous enzyme within the yeast strain cleaves the VDR–ubiquitin fusion protein thus liberating free hVDR. The crude VDR from yeast was purified as previously described (Sone et al., 1990). Purity was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) at approximately 90–95%.

Immunopurification. Immunoaffinity resins were prepared using CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's directions using purified anti-VDR antibodies 4A5γ or 9A7γ (Pike et al., 1987). Monoclonal antibodies were purified as described earlier (Pike, 1984), dissolved in 0.1 M NaHCO$_3$ containing 0.5 M NaCl, and mixed with activated resin (1 mg of antibody/mL resin) in the same buffer at 4 °C overnight. The resin was washed, and the remaining reactive groups were blocked with 0.2 M glycine, pH 8.0, for 2 h at room temperature. Uncoupled antibody was removed by several sequential washes with 0.1 M acetate, pH 4.0, 0.5 M NaCl, and coupling buffer. The resin was then resuspended and stored as a 50% slurry in PBS containing 0.02% sodium azide. One-tenth volume of 4A5- or 9A7-Sepharose was added to cellular extracts and incubated for 12 h at 4 °C. The resin was collected and sequentially washed and sedimented with 1 mL of the following: H buffer, H buffer containing 0.25% Tween-20, H buffer containing 1 M sucrose, and finally TKD-0 (10 mM Tris-HCl, pH 7.6, and 5 mM dithiothreitol). The immunoprecipitated protein was extracted from the immunoaffinity resin three times at 100 °C with 100 μL of standard Laemmli sample loading buffer lacking β-mercaptoethanol. The samples were then electrophoresed through a 4% polyacrylamide stacking gel and a 10% resolving gel. The absence of reducing agent allowed resolution of the VDR from unreduced antibody heavy and light chains. The VDR was located through autoradiography of the wet gel, and the bands were excised. Radioactivity in the samples was determined by Cerenkov radiation.

Elution of VDR Protein from Polyacrylamide Gels. Procedures involving elution, precipitation, and oxidation of the VDR protein were modified from Boyle et al. (1991b). The band of interest was excised from SDS–PAGE wet gels using the autoradiogram as a reference. The gel was slurried using a disposable Kontes tissue homogenizer in 50 mM ammonium bicarbonate. The slurry was made 0.1% with sodium dodecyl sulfate (SDS) and 5% with β-mercaptoethanol and heated at 100 °C for 5 min. The tube containing the slurry was then rotated overnight at 37 °C. The mixture was centrifuged, and the supernatant was removed for Cerenkov counting. This process was repeated three times or until typically >90% of the radioactive protein was eluted from the gel. After the addition of carrier protein (pure hVDR), the supernatant was brought to 15–20% with trichloroacetic acid (TCA) for 1 h on ice, and the precipitate was pelleted in a microfuge and washed with −20 °C acetone. The pellet was resuspended in 50 μL of ice-cold performic acid (900 μL of 88% (v/v) formic acid and 100 μL of 33% (v/v) hydrogen peroxide) and oxidized for 1 h on ice. This reaction mixture was then diluted with 0.5 mL of ice-cold deionized H$_2$O, frozen at −70 °C, and lyophilyzed.

Phosphoamino Acid Analysis. Phosphoamino acid analysis was performed on the eluted human VDR protein exactly as in Boyle et al. (1991b). Samples were resuspended in boiling 6 N HCl (Pierce) and hydrolyzed at 100 °C for 1 h. The samples were lyophilized to dryness and then resuspended in pH 1.9 buffer (2.5% formic acid (88%) and 7.8% glacial acetic acid in water) which contained phosphoamino acid standards (15:1, v/v) from 1 mg/mL stocks (phosphoserine, phosphothreonine, and phosphotyrosine; Sigma Chemical Co., St. Louis, MO). The samples were loaded onto thin-layer cellulose plates (Whatman, Maidstone, U.K.) and electrophoresed at 1.5 kV for 20 min. The plate was allowed to dry and then remoistened for subsequent electrophoresis at pH 3.5 and 1.3 kV for 16 min. The standards were visualized with ninhydrin (0.25% in acetone) and baked at 65 °C for 15 min. The plate was then subjected to autoradiography to detect ^{32}P-labeled amino acids.

VDR Endoproteolysis. Enzyme and chemical digestions of the human VDR were modifications of the procedure of Allen (1989). Immunopurified, gel-eluted hVDR was co-precipitated with hVDR carrier protein (95% pure hVDR) using 15–20% TCA on ice for at least 1 h. The precipitated protein was treated with performic acid, oxidized, lyophilized as above, and then dissolved with sonication in 25 μL of 8 M urea in 50 mM ammonium bicarbonate (pH 8.0). The mixture was diluted to 2 M urea (100 μL total volume) with 50 mM ammonium bicarbonate and incubated with L-1-(*p*-tosylamine)-2-phenylethyl chloromethylketone-treated (TPCK-

treated) trypsin or other enzymes (5% by weight of carrier VDR protein) at 37 °C or room temperature overnight. The peptide mixture was then desalted and purified by manual injection over reverse-phase HPLC (see below). TPCK-trypsin was prepared immediately before use as a stock solution of 1 mg/mL in 0.1 mM HCl. Digestions with V8 protease (Glu-C, Boehringer) or CNBr were modified from the above procedure to include either dilution to 1 M urea or exclusion of the oxidation step, respectively.

HPLC Separation of Peptides. Peptides were chromatographed on 21 mm × 15 cm Vydac C4 reverse-phase columns of 5-μm particle size using a Waters Model 625 LC chromatograph. Solvent A contained 0.1% trifluoroacetic acid (TFA) in water (Optima, Fisher), and solvent B contained 0.1% TFA in acetonitrile (Optima, Fisher). Peptides were manually injected in a volume of 100 μL at a flow rate of 0.25 mL/min for an initial minute in 98% solvent A and 2% solvent B (v/v). The flow rate was progressively increased to 0.5 mL/min over the second minute and then held constant until minute 5. A gradient increase in solvent B of 0.75% per minute was then inititated and maintained until minute 82.3 when solvent B reached 60%. Following 5 min of elution at this concentration of solvent B, the flow was returned to initial conditions. The column was brought to 90% solvent B at the termination of several chromatographic runs. No UV_{214nm}-absorbing material was evident during this process, indicating that peptides that could feasibly contain undetected phosphate were not retained on the column during the analyses. Fractions (0.25 mL) were usually collected every 30 s for 70 min, and Cerenkov radiation was measured. Enzymatic digestions of VDR protein derived from cellular samples treated with or without 1,25(OH)$_2$D$_3$ were evaluated by manually injecting equivalent amounts of radioactivity on the HPLC.

Protein Sequencing and Manual Edman Degradation. Peptide sequence determinations were carried out on lyophilized chromatographic fractions using an Applied Biosystems Model 477A or 473A protein sequencer. Samples were resolubilized in 50% CH_3CN and 1% TFA in water and applied to Biobrene-conditioned glass fiber filters. Manual Edman degradation procedures were direct modifications from Sullivan and Wong (1991).

Two-Dimensional Thin-Layer Electrophoresis and Chromatography. Two-Dimensional thin-layer electrophoresis and chromatography (TLE/C) was performed with modifications from Boyle et al. (1991b). Peptide fractions were recovered from HPLC purification, endoproteolyzed, and lyophilized. The samples were resuspended in pH 3.5 buffer (5% glacial acetic acid and 0.5% pyridine in water) and applied to thin-layer cellulose plates. First-dimension electrophoresis at pH 3.5 was programmed as follows: 250 V for 2 min, 500 V for 2 min, and 1000 V for 21 min. The plate was allowed to dry and then subjected to liquid chromatography in the second dimension using chromatography buffer (37.5% *n*-butanol, 25% pyridine, and 7.5% acetic acid in water).

Site-Directed Mutagenesis. The hVDR cDNA was introduced into the *Eco*RI site of M13 mp18 as previously outlined (Sone et al., 1990) and subjected to oligonucleotide-directed point mutagenesis as described by Kunkel et al. (1987). Mutant VDR cDNAs were selected through standard DNA sequencing methodologies.

General Analyses. The method of 40% alkaline PAGE gels described by West et al. (1984) was utilized to examine small peptides. Gels were usually electrophoresed at 5 mA for approximately 12 h. Following electrophoresis, the gels were dried down and subjected to autoradiography. Immunoblots were carried out as follows: proteins were transferred following SDS–PAGE to nitrocellulose and subjected to western blot analysis as previously described (Pike et al., 1987). 1,25(OH)$_2$D$_3$ binding assays were carried out as documented in Baker et al. (1989). DNA–cellulose chromatography was also performed as outlined in an earlier paper (McDonnell et al., 1989).

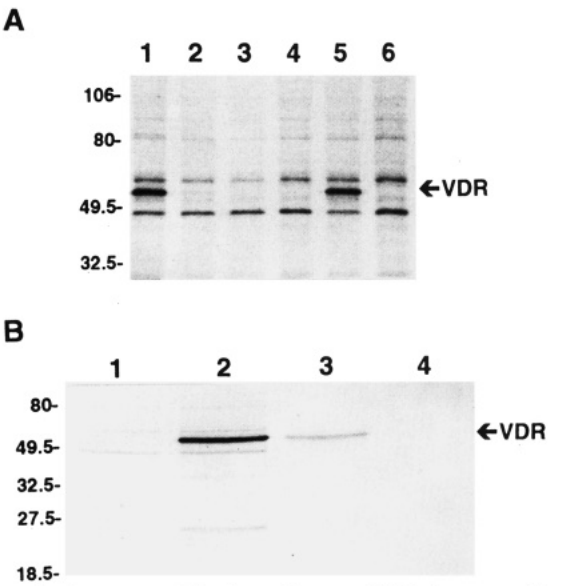

FIGURE 1: Immunopurification of human VDR from transfected and radiolabeled COS-1 cells. Panel A: Selectivity of the immunoprecipitation procedure. Purified anti-VDR monoclonal antibody (4A5) immobilized on Sepharose was used to immunoprecipitate hVDR from [^{35}S]methionine-labeled COS-1 extracts. Two days prior to extract preparation, COS-1 cells were transfected with the following expression plasmids: pAV-hVDR (lanes 1 and 2), p91023b control plasmid (lane 3), pAV-hVDR (Δ100-111) (lane 4), pRSV-hVDR (lane 5), and pRSV control vector (lane 6). The immunoprecipitation reaction mixture in lane 2 contained soluble 4A5 antibody in 100-fold excess over immobilized antibody as a competition test. [^{3}H]-1,25-dihydroxyvitamin D$_3$-binding activity confirmed positive expression of the hVDR or hVDR(Δ100-111) in COS-1 extracts subsequently analyzed in lanes 1, 2, 4, and 5. Panel B: Demonstration that 1,25-dihydroxyvitamin D$_3$-treated human VDR is a phosphoprotein. As in panel A, COS-1 cells were transfected with p91023b control vector (lanes 1 and 4) or pAV-hVDR (lane 2 and 3). Cells were radiolabeled with [^{35}S]methionine (lanes 1 and 2) or with [^{32}P]-orthophosphate (lanes 3 and 4), and extracts were subjected to immunoprecipitation and 10% SDS–PAGE.

RESULTS

Phosphorylation of the Human VDR Occurs on Serine(s). Previous studies have shown that endogenous VDR from mouse, chick, and pig is phosphorylated in response to physiological concentrations of 1,25(OH)$_2$D$_3$ hormone (Pike & Sleator, 1985; Brown & DeLuca, 1990, 1991). In addition, recombinant human VDR, synthesized in COS-1 cells following transfection of a hVDR expression vector, was also shown to be phosphorylated in a hormone-modulated manner (McDonnell et al., 1989). In order to ascertain whether this modification was directed toward one or more serine residues, as noted earlier in both mouse (Haussler et al., 1988) and pig (Brown & DeLuca, 1991) studies, we transfected the pAV-hVDR expression vector into COS-1 cells and cultured the cells in methionine- or phosphate-depleted media containing 1,25(OH)$_2$D$_3$ and either [^{35}S]methionine or [^{32}P]orthophosphate. Extracts were prepared as described in Materials and Methods, and the hVDR was immunoprecipitated by the extensively validated methodology in Figure 1A. The immunoprecipitation specificity allowed for purification by band

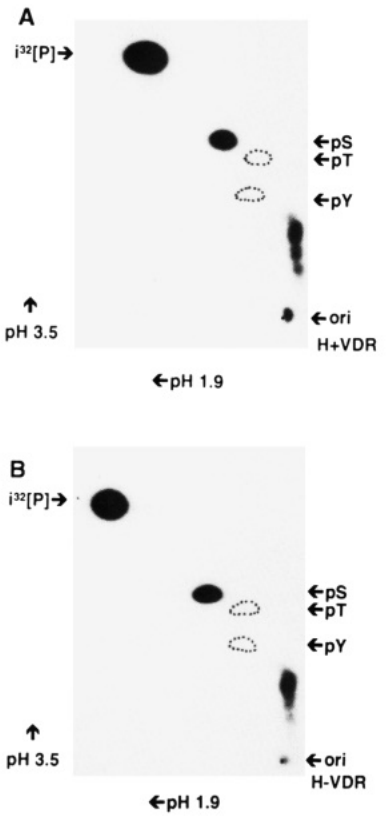

FIGURE 2: Phosphoamino acid analysis of human VDR. COS-1 cells transfected with hVDR cDNA were grown in the presence (panel A) or absence (panel B) of 1,25-dihydroxyvitamin D_3. Extracts were prepared from these cells and human VDR was immunopurified and subjected to phosphoamino acid analysis as described in Materials and Methods. The arrows indicate electrophoresis toward the anode at each pH. The positions of phosphoamino acid standards are as indicated: pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine; ori, sample origin for electrophoresis; i^{32}[P], inorganic [^{32}P]-orthophosphate.

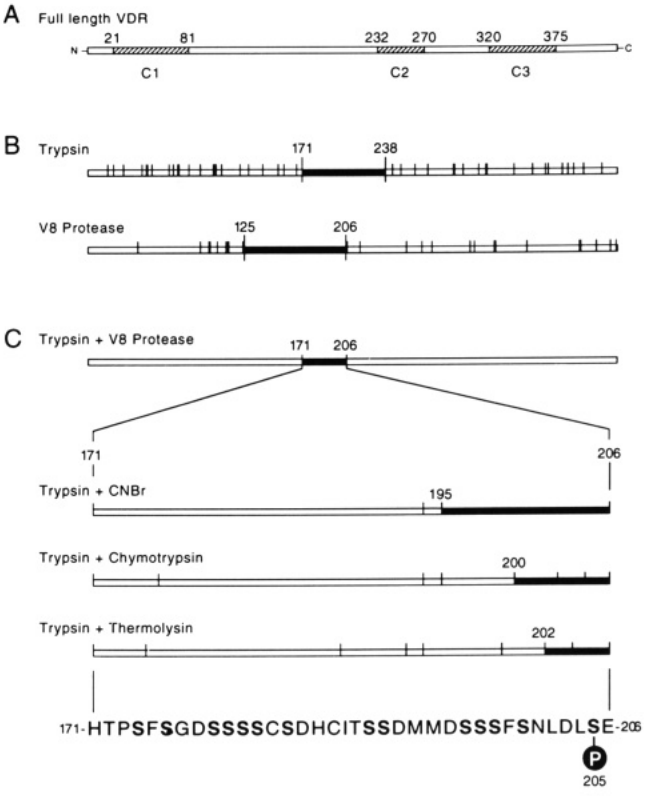

FIGURE 3: Schematic diagram of human VDR. Panel A: Full-length human 1,25-dihydroxyvitamin D_3 receptor depicted with shaded regions of amino acid identity to the steroid receptor superfamily. Region C1 is the portion of the hVDR involved in DNA binding, and C2 and C3 represent two additional regions of homology within the steroid receptor gene family located within the ligand binding domain. Panel B: Full-length hVDR with sites of trypsin and V8 protease cleavage depicted by vertical tick marks. The filled-in portions of the bars schematically represent the specific peptides obtained by N-terminal sequencing of the radioactive fractions in Figure 4, panels B and D. Proteolysis of full-length hVDR with trypsin yielded a radioactive peptide from 171 to 238. Proteolysis of full-length hVDR with V8 protease yielded a peptide from 125 to 206. The peptides revealed from protein sequencing procedures exactly matched peptides predicted by the sequence specificity of each protease. Panel C: Sequential cleavages of the trypsin peptide 171–238 with V8 protease, CNBr, α-chymotrypsin, and thermolysin, followed by protein sequencing of the radioactive fractions. The radioactive fractions of full-length hVDR trypsinization were pooled (see Figure 4, panel B) and, after each secondary proteolysis, were repurified, and the radioactive fraction was sequenced. The filled area of each bar schematically represents the N-terminal sequence of a radioactive peptide derived from each sequential cleavage protocol. Sequencing the trypsin + V8 protease fraction revealed a peptide beginning at residue 125; trypsin + CNBr sequencing revealed a peptide beginning at residue 195; trypsin + chymotrypsin sequencing revealed a peptide beginning at residue 200; and trypsin + thermolysin sequencing revealed a peptide beginning at residue 202. The results from protein sequencing procedures exactly matched peptides predicted by the sequence specificity of each cleavage protocol.

excision and elution. As indicated in Figure 1B, the presence of [^{32}P]phosphate on a comigrating band (Figure 1B, lane 3) confirmed the hVDR as a phosphoprotein consistent with our previous studies (McDonnell et al., 1989). Phosphate label, although substantially reduced, was also noted when the experiment was carried out in the absence of $1,25(OH)_2D_3$, suggesting that the hVDR undergoes a constitutive modification (data not shown). As observed in Figure 2, phosphoamino acid analysis of immunoprecipitated hVDR derived from identical experiments carried out in both the presence and the absence of $1,25(OH)_2D_3$ revealed that the modified amino acid(s) was (were) serine. While a reduction in phosphate was observed on the immunoprecipitated hVDR in the absence of $1,25(OH)_2D_3$ treatment, no attempts were made to quantitate the differences at this stage. These studies verify our previous results (McDonnell et al., 1989) as well as those of others (Pike & Sleator, 1985; Jones et al., 1991; Jurutka et al., 1993a,b; Hsieh et al., 1991, 1993) which suggest that the hVDR is subject to phosphorylation in both the absence and the presence of $1,25(OH)_2D_3$. Further, our results demonstrate for the first time that the hVDR is modified on one or more serine residues.

One or More Phosphorylation Sites on the hVDR Map to a Peptide Containing Residues 171–206. We set out to identify the site(s) of phosphorylation on the hVDR using a protein mapping strategy. This procedure involved enzymatic digestion of the receptor, subsequent HPLC isolation of the peptides, direct amino acid sequence analysis of the isolated peptides, and simultaneous [^{32}P]phosphoserine release using manual Edman degradation techniques. As a source of substrate, we utilized a mixture of COS-1-derived hVDR phosphoprotein and VDR from YEpV1-transformed yeast, purified to homogeneity as previously documented (Sone et al., 1990). Since our initial experiments had shown that phosphoserine was exclusively present in hVDR, we postulated that the release of [^{32}P]phosphoserine from an isolated peptide during manual Edman degradation would align directly with a serine residue generated from the sequencing information obtained from carrier VDR. This alignment could be judged relative to the receptor sequence, whose organizational map is depicted in Figure 3A.

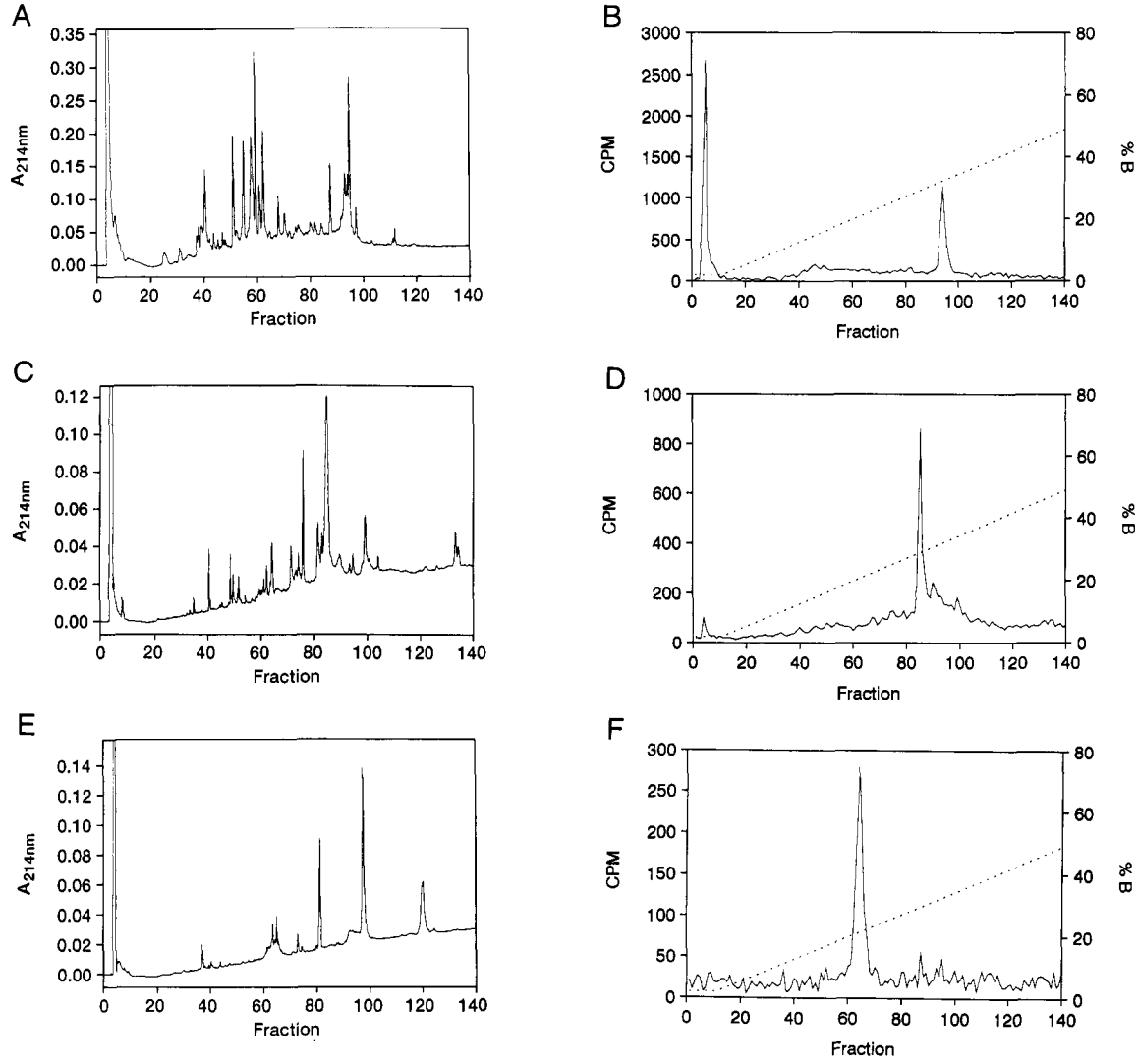

FIGURE 4: Reverse-phase HPLC purification of [^{32}P]hVDR peptides resulting from endoproteolysis. Panels A and B represent the UV and Cerenkov traces, respectively, of hVDR peptides from trypsin proteolysis and purification as in Materials and Methods. Panels C and D represent the UV and Cerenkov traces, respectively, of hVDR peptides from V8 proteolysis and purification. From panels A and B, fractions 92 thru 97 of the trypsin digestion were pooled and subjected to V8 proteolysis. Panels E and F represent the UV and Cerenkov traces, respectively, of this double digestion of full-length hVDR with trypsin followed by V8 protease. The radioactive fractions from each purification were dried down and sequenced. The UV peak at fraction 97 in panel E is V8 protease enzyme.

We initially utilized the endoproteases trypsin (calf intestine) and V8 protease (*Staphylococcus aureus*) to exhaustively digest the [^{32}P]VDR mixture and analyzed the resultant products on a C4 reverse-phase column by HPLC. As observed in panels A and B of Figure 4, digestion with 5% (w/w) trypsin resulted in the production of a number of UV-absorbing peptides, only one of which contained labeled phosphate. The radiolabeled peak was collected and subjected to protein sequence analysis. The results, as documented in Table 1, revealed a tryptic peptide beginning with histidine (residue 171 of the VDR) and identical in sequence to the receptor for an additional nine cycles. The start site for sequencing was immediately C-terminal to an arginine, thus confirming the specificity of the VDR cleavage with this particular enzyme. No peptides were detected in the column fall-through by alkaline gel analysis, indicating complete retention of all radiolabelled receptor fragments. The VDR protein was similarly subjected to V8 protease digestion and chromatography. Digestion of the hVDR mixture with this protease also resulted in several UV-absorbing peptides (Figure 4C) as well as a single peak of associated radioactivity (Figure 4D). Accordingly, sequence analysis of the radioactive peak revealed a peptide beginning at glutamine (residue 125 of the VDR) and also identical to the VDR for an additional nine cycles (see Table 1). The start site for sequencing was immediately C-terminal to a glutamate residue, a V8 protease recognition site. As illustrated in Figure 3B, these two mutually exclusive methods of enzymatic cleavage identify an overlapping pair of peptides which contain phosphoserine(s) (Figure 2) and which map to a region located between the DNA binding domain (region C1) and the N-terminal boundary of the ligand binding domain (region C2) (Figure 3A). Consistent with this interpretation, use of the tryptic hVDR fragment from residue 171 to residue 238 for sequential digestion with V8 protease resulted in a shortened phosphorylated peptide whose sequence began at residue 171 of hVDR (Table 1) but whose elution time (Figure 4F) was earlier than that of the substrate peptide generated by trypsin cleavage alone (compare with Figure 4B). Thus, as illustrated in Figure 3C, we propose that the peptide that contains the phosphoserine(s) of interest exhibits an N-terminal boundary represented by a trypsin cleavage site at histidine 171 and a C-terminal boundary represented by a V8 protease digestion site at glutamate residue 206. This region is 36 amino acids in length, of which strikingly 14 (39%) are serine residues.

Table 1: hVDR Peptide Sequence Analysis[a]

VDR cleavage protocol	elution fraction	cpm of peak		sequencing cycle 1	2	3	4	5	6	7	8	9	10
trypsin	94	3144	amino acid	H	T	P	S	F	S	G	D	S	S
			VDR position	171	172	173	174	175	176	177	178	179	180
			pmol	9.41	10.9	16.2	4.0	18.9	3.9	17.9	8.2	3.1	3.5
V8 protease	83	1230	amino acid	Q	Q	R	I	I	A	I	L	L	D
			VDR position	125	126	127	128	129	130	131	132	133	134
			pmol	30.7	32.7	19.5	34.1	34.2	36.2	32.0	30.0	30.4	16.7
trypsin + V8 protease	64	280	amino acid	H	T	P	S	F	S	G	D	S	S
			VDR position	171	172	173	174	175	176	177	178	179	180
			pmol	5.6	5.4	8.3	2.4	9.6	2.6	8.4	2.6	1.5	2.1
trypsin + CNBr	78	507	amino acid	D	S	S	S	F	S	N	L	D	L
			VDR position	195	196	197	198	199	200	201	202	203	204
			pmol	1.4	0.8	1.0	0.6	1.9	0.4	0.4	1.0	1.0	1.0
	42	110	amino acid	H	T	P	S	F	S	G	D	S	S
			VDR position	171	172	173	174	175	176	177	178	179	180
			pmol	7.6	15.4	14.5	5.0	21.0	4.4	20.0	9.4	4.7	4.0
trypsin + chymotrypsin	53	2405	amino acid	S	N	L	D	L	S	E	E	D	S
			VDR position	200	201	202	203	204	205	206	207	208	209
			pmol	23.7	16.1	26.3	16.1	17.5	4.4	9.1	9.4	6.7	2.3
	47	864	amino acid	H	T	P	S	F	S	G	D	S	S
			VDR position	171	172	173	174	175	176	177	178	179	180
			pmol	3.3	2.4	6.1	1.0	4.8	1.5	4.8	1.6	0.8	1.0
trypsin + thermolysin	27	336	amino acid	L	D	L	S	E	E	D	S	D	D
			VDR position	202	203	204	205	206	207	208	209	210	211
			pmol	42.3	14.1	19.6	12.0	9.6	10.0	8.7	5.4	7.5	8.7

[a] The protocol column describes each particular cleavage protocol. The elution fraction column lists the times of labeled peptide retention during HPLC purification. cpm of peak is an integration of total peptide cpm. The sequencing cycle rows list the amino acid identity in each cycle, followed by each residue's position within the hVDR protein sequence and the quantity in picomoles for the amino acid in each respective sequencing cycle.

Phosphorylation of the hVDR Maps to Residues 202–206. With the above information, we elected to further map the location of the phosphoserine(s) utilizing a sequential enzymatic digestion strategy. We first employed trypsin to generate a peptide whose N-terminal coordinate was residue 171 and whose putative C-terminal coordinate was residue 238 (see Figure 3B), and purified this fragment chromatographically. We then subjected this peptide to further digestion using CNBr, α-chymotrypsin, or thermolysin; resolved the phosphorylated peaks from substrate peptide by reverse-phase HPLC; and then sequenced the resulting peptides. Each cleavage protocol required optimization in order to efficiently convert the substrate peak (171–238) into new, uniquely eluting peaks (data not shown). As determined from the sequencing data summarized in Table 1, secondary digestion with CNBr, α-chymotrypsin, and thermolysin resulted in phosphopeptides with amino termini beginning at residues 195, 200, and 202, respectively. These results, as illustrated schematically in Figure 3C, suggest that one or more phosphoserine residues are located between residues 202 and 206. On the basis of these data together with the additional data presented in Table 1 and generated using sequential digestion with trypsin and V8 protease, we propose that the serine that undergoes modification is located between residues 202 and 206. Importantly, additional peaks of radioactivity were observed following secondary cleavage with CNBr and α-chymotrypsin. Sequence analysis of each of these peaks revealed start sites at residue 171, as seen in Table 1, consistent with the substrate peptide. The earlier elution times of these peaks, however, supported cleavage at an unidentified carboxy-terminal site. While both observations are consistent with our view that the phosphoserine lies C-terminal to residue 202, neither excludes the possibility that one or more additional sites N-terminal to residue 202 might exist.

Serine 205 Is Phosphorylated within the Human VDR. We used manual Edman degradation of the three peptide cleavage products to precisely localize the serine residue(s) which was (were) modified by [^{32}P]phosphate. Edman degradation was performed on the isolated peptides beginning at residue 171 (trypsin digestion), 195 (sequential trypsin and CNBr digestion), or 200 (sequential trypsin and chymotrypsin digestion) so as to examine each of the 14 serine residues located from residue 171 to 206 (see Figure 3C). The results are depicted in Figure 5, where it is clear that a major release of [^{32}P]phosphoserine occurred only at the Edman release cycle that hydrolyzes the last serine in this 36 amino acid peptide, residue 205 (Figure 5C). From these results, we suggest that, under the conditions of the experiments conducted here, the hVDR appears to be phosphorylated in intact cultured COS-1 cells on a single serine, residue 205. Additional sites within the peptide from 171 to 238 would represent only a small percentage of the total radioactivity incorporated and were undetected by ^{32}P release. Equally important, we find virtually no evidence for modification at any sites either amino terminal or carboxy terminal to this 36 amino acid peptide on the VDR molecule. We cannot, however, discount the possibility of additional sites undetectable by either our current approach or our level of sensitivity, a result of expression in other cell lines or under different conditions of cell growth.

1,25(OH)$_2$D$_3$ Modulates hVDR Phosphorylation. Previous experiments have demonstrated that, like that of endogenous VDR in mouse (Pike & Sleator, 1985), pig, and chicken cells (Brown & DeLuca, 1990, 1991), the phosphorylation state of recombinant hVDR synthesized in COS-1 cells is modulated by 1,25(OH)$_2$D$_3$ (McDonnell et al., 1989). These studies, however, have been limited to a comparison of the level of the VDR phosphorylation evident in autoradiograms following SDS–PAGE. Considering that the single phosphoserine that we identified was derived from 1,25(OH)$_2$D$_3$-treated cells, we determined whether there was a reduction of incorporated phosphate in the absence of hormone and whether constitutive levels of ^{32}P mapped to the same VDR region. We labeled hVDR-transfected cells with [^{32}P]orthophosphate in the presence or absence of 1,25(OH)$_2$D$_3$, immunoprecipitated and gel-purified the hVDR from each group, and subjected the

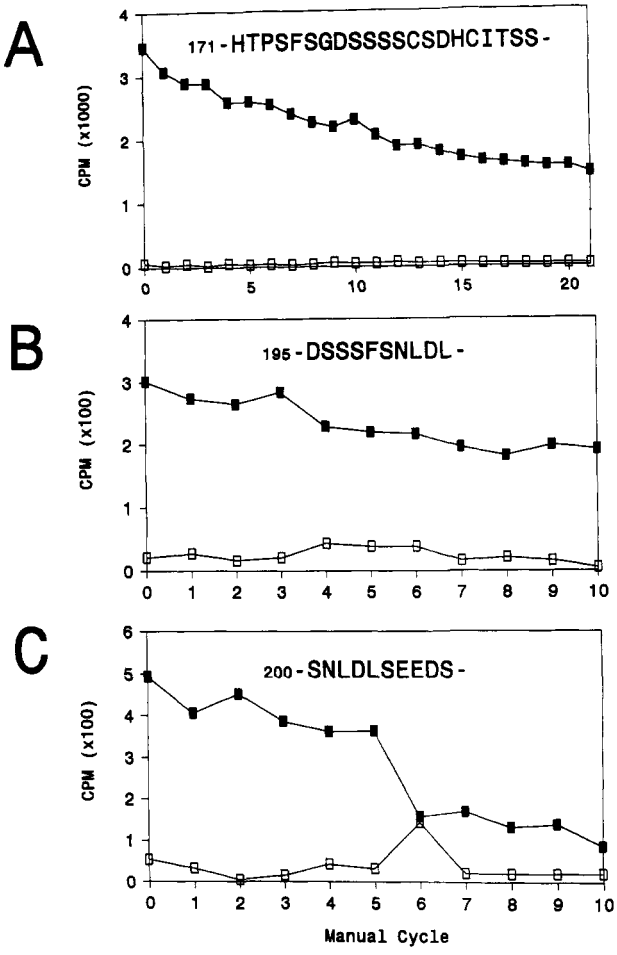

FIGURE 5: Manual Edman degradation of the hVDR phosphorylated peptide. Peptides were analyzed for the release of [^{32}P]phosphoserine over the entire 36 amino acids mapped by the boundaries of the trypsin site at residue 171 and the V8 protease site at residue 206. The radioactive peptides were immobilized on a filter (■) and analyzed for the release of [^{32}P]phosphoserine into the acid wash (□) during manual Edman degradation. Panel A: hVDR trypsin peptide beginning at residue 171. Panel B: hVDR trypsin + CNBr peptide beginning at residue 195. Panel C: hVDR trypsin + chymotrypsin peptide beginning at residue 200. Each experiment was repeated with identical results.

hVDR to proteolysis with trypsin or V8 protease. As illustrated in Figure 6A (trypsin) and Figure 6B (V8 protease), reverse-phase HPLC of the resultant phosphopeptides reveals that the absence of hormone treatment leads to a reduction in phosphate on peptide 171–238 and peptide 125–206 as compared to hormonally treated controls. Integration of the trypsin and V8 protease peaks demonstrates an 8-fold average reduction in [^{32}P]orthophosphate incorporated into these peptides. Due to a potential upregulation of the hVDR following treatment with hormone (Santiso-Mere et al., 1993; Arbour et al., 1993; Weise et al., 1992; McDonnell et al., 1987), we evaluated receptor levels produced in these experiments by western blot analysis and via [^{35}S]methionine pulse/chase analysis. No significant differences were observed after densitometric analysis of the resultant data, probably because of the relatively short-term nature of the hormonal treatment (data not shown). Low levels of [^{32}P]phosphoserine in the VDR in the absence of hormone hindered further mapping of the unoccupied hVDR phosphorylation site. Thus, we have not mapped the constitutively phosphorylated site. Clearly, however, a substantial increase in phosphorylation occurs on the VDR in the presence of 1,25(OH)$_2$D$_3$, and that increase is limited under our conditions exclusively to serine 205.

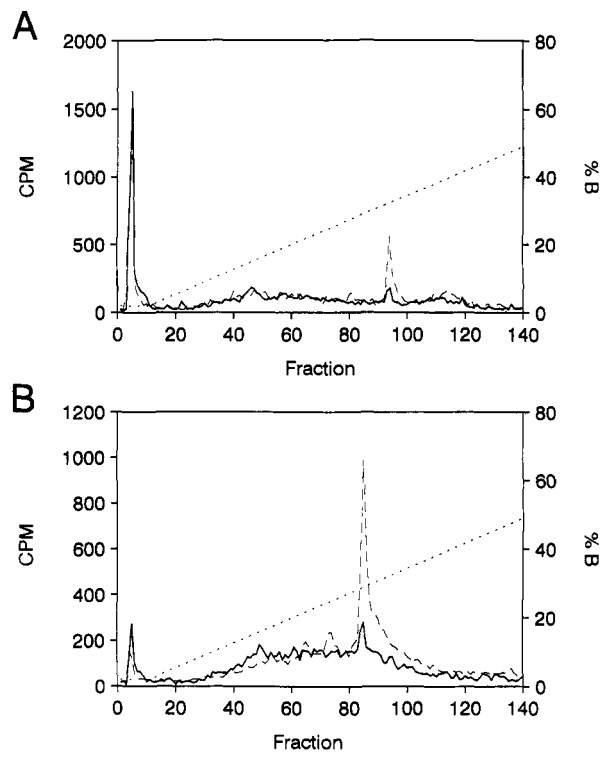

FIGURE 6: Hormone modulation of serine 205. COS-1 cells were transfected with pAV-hVDR and then incubated with [^{32}P]orthophosphate in the absence or presence of 1,25(OH)$_2$D$_3$ as outlined in Materials and Methods. hVDR was immunoprecipitated and subjected to enzymatic cleavage with either trypsin (panel A) or V8 protease (panel B), and the peptides were separated by HPLC. The solid lines in both panels represent the Cerenkov traces of the radioactive peptides from the unoccupied hVDR, the dashed lines indicate the peptides from occupied hVDR, and the dotted lines represent the linear gradient. Integration of these peaks indicates an average 8-fold reduction of peptide radiolabeling in the absence of 1,25(OH)$_2$D$_3$.

Transcriptional Activity of the hVDR Containing the S205 Mutation Is Not Compromised. To examine the possible role of phosphorylation on hVDR function, we utilized oligonucleotide-directed mutagenesis to induce mutation of serine 205 to alanine, aspartic acid, or glutamic acid within the hVDR. We then transfected each of these receptor expression vectors together with VDRE-containing promoter plasmids into various cell lines and assessed 1,25(OH)$_2$D$_3$ response. Western blot detection of the receptors expressed from these plasmids in COS-1 cells revealed equivalent levels of protein expression (data not shown). As observed in Figure 7, the wild-type hVDR and each of the mutant receptors were fully capable of directing reporter gene transcription in a hormone-dependent manner in human HepG2 cells. We also observed identical results using the CV-1 fibroblast cell line as a recipient host (data not shown). Whether directed by the human osteocalcin gene promoter (Figure 7A), the human 25-hydroxyvitamin D$_3$-24-hydroxylase gene promoter (Figure 7C), or the synthetic VDRE–TK fusion gene (Figure 7B), transcriptional activity was normal. Importantly, while some differences in the overall efficacy of the various receptors are seen in Figure 7, repeated experiments failed to demonstrate any consistent trend. Finally, the positive transcriptional response seen in these studies implied strongly that each mutant receptor bound specific DNA as well as the hormonal ligand with normal affinity.

Alternative Phosphorylation of hVDR Occurs N-Terminally to Residue 205. In view of the above observations, we examined the ability of the hVDR to undergo phosphorylation

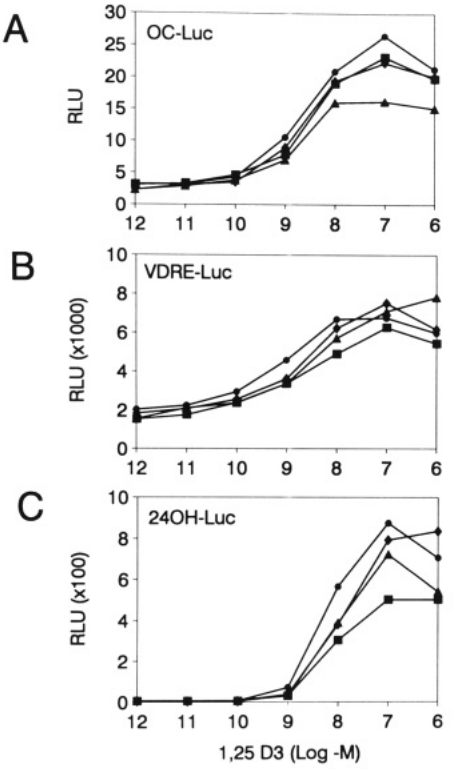

FIGURE 7: Transactivation capacity of the wild-type and serine 205 mutant hVDRs. HepG2 cells were cotransfected with the indicated combinations of hVDR expression vectors, standardization plasmids, and reporter plasmids, treated with increasing concentrations of 1,-25$(OH)_2D_3$, and assessed for β-galactosidase and luciferase activities as indicated in Materials and Methods: Activities of wild-type hVDR (●), S205A-hVDR (♦), S205D-hVDR (▲), and S205E-hVDR (■) on phOC1300-luc reporter (panel A), pTK-VDRE(3)-luc (panel B), or ph24OH 6000-luc (panel C). Relative light units (RLU) represent luciferase activity normalized for β-galactosidase activity. Each point represents the average of a triplicate assessment where variation is less than 10%. Each transfection experiment was independently carried out three or four times.

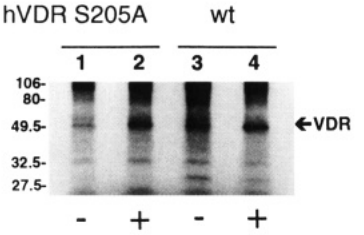

FIGURE 8: Analysis of S205A hVDR for hormone-modulated phosphorylation. COS-1 cells were transfected (lanes 1 and 2 with pAV-S205A hVDR, lanes 3 and 4 with pAV-hVDR) and radiolabeled ([^{32}P]orthophosphate) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 1,25-dihydroxyvitamin D_3. The extracts of these cells were subjected to immunopurification and 10% SDS–PAGE.

following mutation at serine 205 to alanine. COS-1 cells transfected with normal or mutant DNA were incubated with [^{32}P]orthophosphate in the absence or presence of 1,25-$(OH)_2D_3$, and the VDRs were immunoprecipitated and evaluated by SDS–PAGE. As is clearly evident in Figure 8, both the normal and the mutant VDRs were labeled with phosphate. Perhaps more importantly, the level of phosphorylation was increased in both during treatment with 1,25-$(OH)_2D_3$. This unanticipated observation suggests that the mutant VDR is capable of being modified at an alternative site adjacent to serine 205. Indeed, the region surrounding residue 205 is rich in serines and contains multiple casein kinase II consensus sites (Marin et al., 1986; Jurutka et al., 1993a). We extracted the phosphorylated wild-type and

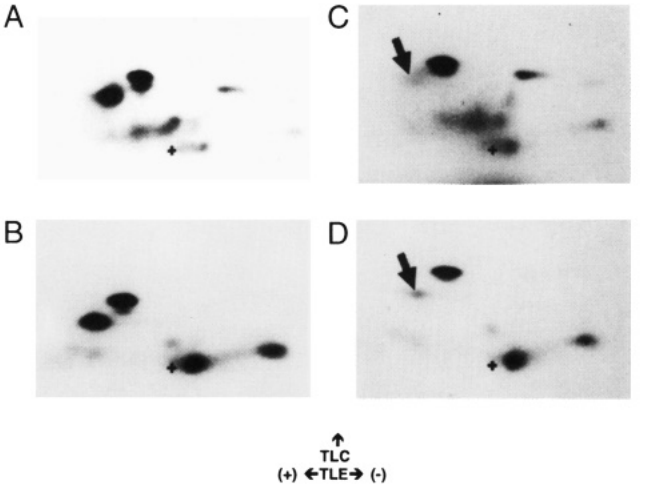

FIGURE 9: Two-dimensional TLE/C analysis of phosphopeptides derived from secondary Asp-N cleavage of S205A and wild-type hVDRs. Wild-type (panels A and B) and S205A hVDR peptides (panels C and D) were analyzed by Asp-N endoproteolysis and separated using 2D TLE/C. The hVDR substrates for the Asp-N cleavages were the trypsin peptide 171–238 (panels A and C) and the V8 protease peptide 125–206 (panels B and D). The origin is indicated by a plus (+). The directions of TLC and TLE are indicated at the bottom of the figure.

mutant VDRs, digested them with either trypsin or V8 protease, and isolated the radiolabeled peptide fragments chromatographically. Both the tryptic- and V8 protease-derived fragments from the normal and mutant receptors, respectively, eluted in identical fractions as before, supporting the presence of an alternatively phosphorylated serine on the mutant hVDR between the previously mapped residues 171 and 206 (data not shown). In view of this finding, we isolated both the tryptic- and V8 protease-derived fragments from each of the receptors and subjected them to cleavage with Asp-N, an enzyme capable of cleaving at the N-terminal side of aspartate residues. We utilized two-dimensional TLE/C to fingerprint the results of these cleavages since initial efforts to proteolyze the receptor in this region with Asp-N led routinely to partial digestion products (patterns complicated by the proximity of aspartic acid and cysteic acid cleavage sites, the latter an analog of Asp-N specificity) (data not shown). As is apparent in Figure 9, panels A and B, the wild-type hVDR exhibits radiolabeling on two major peptides. One or both are the result of partial cleavage, and both clearly contain serine 205. The radiolabeling patterns seen in Figure 9, panels C and D, for the hVDR mutant S-205A, however, are obviously different. The two peptides originally observed in panels A and B and which in the mutant VDR now contain alanine instead of serine are no longer labeled (one of the positions is indicated by arrows in Figure 9, panels C and D). Correspondingly, a new peptide, similar but not identical in migration to the original two peptides on the basis of an internal marker, now contains ^{32}P. This new peptide must contain the alternatively modified residue. Although these data do not identify the site of alternative modification, they are consistent with a more amino terminal location for the site on peptide 171–206. Regardless of the location, they provide unequivocal evidence that the S205A mutant VDR has undergone an alternative phosphorylation event.

DISCUSSION

Our results suggest that hVDR synthesized in COS-1 cells in the presence of 1,25$(OH)_2D_3$ is subject to a single phosphorylation event on serine 205. In the absence of

hormone, an 8-fold reduction in phosphate is observed. While it is likely that $1,25(OH)_2D_3$ upregulates the level of phosphorylation on serine 205, we have not mapped the site in the absence of ligand. Thus, an alternative possibility is that serine 205 is uniquely modified in the presence of hormone and a separate site, located within residues 171–206, contains residual or hormone-independent phosphate. If the latter is true, however, that site is not detected in our mapping studies following $1,25(OH)_2D_3$ treatment. Assessment of the transcriptional activation properties of several mutant hVDRs containing changes of serine 205 to alanine, aspartic acid, or glutamic acid revealed activities identical to that of the wild-type protein on several promoters. These results suggest that serine 205 does not play a role in the transactivation properties of the hVDR. However, this conclusion is compromised by the surprising observation that the mutant receptor remained subject to a hormone-dependent phosphorylation at an alternative site to serine 205 within fragment 171–200. These experiments suggest that the wild-type hVDR is indeed modified by phosphorylation in response to hormone, although the functional role of this covalent change remains unknown.

We utilized phospholabeling of $1,25(OH)_2D_3$-treated COS-1 cells transfected with an hVDR expression vector together with a peptide-mapping approach to identify directly the site of hormone-modulated phosphorylation on the hVDR. Expression of recombinant hVDR in a cultured cell line was necessary to overcome the exceedingly low levels of endogenous receptor normally present in tissues and cell lines (Haussler et al., 1981). One caveat to this approach is the possibility that high levels of receptor in each transfected cell (Baker et al., 1988) might saturate the hVDR kinase, or result in aberrant phosphorylation. There are a number of speculative reasons why this may not be a problem in our experiments. Indeed, we do not yet know what proportion of the hVDR expressed in COS-1 cells is modified by phosphorylation or the stoichiometry of the modification. However, the fact that the hVDR is phosphorylated in a hormone-dependent manner identical to that seen for endogenous receptor in other cell lines (Pike & Sleator, 1985; Brown & DeLuca, 1990, 1991) provides the best evidence that this event mirrors a normal process and reflects a real physiological event. While this methodology produced sufficient phosphorylated hVDR to determine that one or more serine residues were modified, in initial experiments even these techniques of overexpression were incapable of producing sufficient amounts of hVDR for enzymatic digestion and direct peptide sequence analysis. As a result, we purified hVDR to homogeneity from a transformed *S. cerevisiae* strain (Sone et al., 1990), mixed this material with the immunoprecipitated and gel-purified phosphoprotein, and enzymatically digested the combination under denaturing conditions. Following chromatography, radiolabeled peaks were harvested and evaluated by amino acid sequence analysis and manual Edman degradation. An essential feature of this approach is the necessity for coelution of the mammalian phosphopeptides and the yeast-derived carrier hVDR peptides. We do not yet know whether the hVDR produced in yeast is phosphorylated at serine 205 or on any other serine residue. It is clear, however, that the peptides did coelute, either because the relevant peptides were identically phosphorylated or because the large size of the identified peptides minimized potential dissimilarities in migration. Irrespective of the reason, coelution of the two sources of VDR peptides was systematically supported by six different strategies of both primary and sequential primary and secondary digestion of the mixtures. The likelihood of sequential coelution of two peptides following four sequential independent digestions is exceedingly small. We believe that the approach we have taken, the first of its kind with regard to the hVDR, provides definitive evidence that the hVDR is singularly modified on serine 205.

Manual Edman degradation of material from three separate digestion protocols allowed us to localize precisely phosphoserine 205 within the hVDR. With regard to the tryptic fragment beginning at 171, the extent of the radioactivity in this peptide allowed 21 cycles of systematic degradation, enabling us to confirm the lack of phosphorylation on serines up to and including residues 190 and 191. Likewise, degradation of the trypsin/CNBr-derived peptide beginning at residue 195 eliminated phosphorylation of serines at 196, 197, 198, and 200. Finally, trypsin cleavage followed by chymotrypsin cleavage resulted in a peptide beginning at residue 200 as determined by amino acid sequence analysis. Edman degradation of this fragment clearly and consistently yielded release of [^{32}P]phosphoserine only at cycle 6, corresponding to serine 205. These findings are each consistent with our earlier prediction that only the region of the hVDR between residues 202 and 206 contained [^{32}P] phosphate (Figure 3C).

Previous reports by Jones et al. (1991) and Jurutka et al. (1993a), the latter utilizing almost identical expression methods in related COS-7 cells, have implied that serine 205 in the hVDR is modified by phosphorylation *in vivo*. The studies are based upon the observation that mutagenesis of the site from serine to glycine led to a 40% reduction of phosphorylation in the hVDR when normalized for protein expression. These data, as well as the additional observation that the mutant continued to exhibit a 2-fold increase in phosphorylation following hormone treatment, led the authors to conclude that serine 205 was not hormone-modulated (Jurutka et al., 1993a) and that one or more additional sites other than serine 205 were phosphorylated. While considerable *in vitro* data involving the use of purified casein kinase II was presented to support this conclusion, the relevance of these data to their *in vivo* observations is unclear. Our data suggest an alternative explanation for these authors' experiments. First, we suggest that the phosphorylation state of serine 205 is indeed modulated by hormone. The addition of substantially greater amounts of hormone in our experiments may provide a clue. The amount of receptor being expressed in either COS-1 or COS-7 cells (both SV-40 large T antigen-transformed CV-1 cells) following transfection is high; it is possible that 10 nM $1,25(OH)_2D_3$ in these authors' experiments might have been insufficient to occupy all expressed VDR, particularly if the ligand undergoes significant metabolism. Second, we suggest on the basis of peptide mapping that serine 205 appears to be the only amino acid modified in this region of the VDR. The presence of hormone-inducible phosphate on the mutant receptor utilized by Jurutka et al. (1993a) may well come from phosphorylation on an alternative site, a site perhaps made available because of slight structural perturbations of the hVDR which arise from a serine to glycine change or because secondary sites are available for modification. This possibility is exactly what we report here and suggests that extreme caution should be utilized with regard to conclusions drawn solely from mutagenesis-based experiments.

Why is an alternative site on the VDR phosphorylated in our experiments? Alternative sites of phosphorylation, particularly in mutant proteins, have been well documented for

both the tyrosine and the serine/threonine class of sites. Indeed, modification at these alternative sites has been shown to produce functional proteins. We can only speculate here that because this region of the hVDR is exceedingly rich in serine clusters, slight changes in structure induced through a mutation might expose unique sites appropriate for similar modification and function. Alternatively, sufficiently similar sites are available matching the CKII consensus to perhaps obviate the need for absolute site specificity. A final possibility is that the alternatively modulated site represents an authentic secondary site simply not observed under our conditions of specific activity. These latter two possibilities are supported by the observation that while this small region of the hVDR is also highly serine-rich in other mammalian VDRs such as in rat (Burmester et al., 1988), human serine 205 is not conserved. This observation has been used to argue that modification at this site is not crucial for function (Jurutka et al., 1993a). Our data would suggest that an alternative site within the rat sequence might serve a similar functional role. We would therefore speculate that the alternative site might be conserved in both rat and human as well as perhaps chicken. Human serine 200, for example, fulfills both of these criteria in the other species (McDonnell et al., 1987; Baker et al., 1988; Burmester et al., 1988) and represents a CKII consensus site similar to that of serine 205 as well.

Is there evidence for additional sites of modification distal to the hinge region comprising residues 171–238 of the hVDR *in vivo*? Hsieh and colleagues (Hsieh et al., 1991, 1993) propose that serine 48, located between the DNA-binding zinc fingers of the hVDR, is also phosphorylated. They suggest that the modification represents negative regulation by PKC. Like their previous studies, these conclusions are also based upon mutational analysis of the hVDR. Our peptide-mapping approach using COS-1 cells does not support additional sites of modification on the hVDR. Thus, we would argue that this modification may initiate abnormal events that lead to a reduction in phosphorylation at serine 205. In support of this, while mutation of serine 48 to glycine (Hsieh et al., 1993) resulted in a receptor that was not transcriptionally active, its conversion to alanine produced a fully functional receptor. Jurutka et al. (1993b) also suggest that a site between residues 130 and 198 is modified and represents a mechanism for negative regulation by cAMP-dependent protein kinase. Clearly, our data do not support this possibility in the normal hVDR. Finally, recent studies have shown that okadaic acid, cAMP, and dopamine activate transfection-derived VDR in cultured cells in the absence of $1,25(OH)_2D_3$ (Power et al., 1991; Darwish et al., 1993). The exact nature of these agents' effects on the VDR remains to be elucidated.

The potential role of phosphorylation in hVDR function must await identification of the alternative site of modification so that a double mutation can be created and tested. This approach, however, runs the risk that yet an additional site may be modified or that structural changes as a result of mutation will ensue that will compromise function and complicate the interpretation. Unfortunately, the site of modification does not provide an obvious clue. The serine clusters in which serine 205 is located are positioned approximately 30 amino acids N-terminal to a conserved region within the steroid receptor gene family [see Figure 3A and O'Malley (1990)]. This region represents the N-terminal boundary of a domain that includes not only the ligand binding function but dimerization and transactivation functions as well. Whether the phosphorylated amino acid within the hVDR will play a role in modifying these functions will have to be clarified.

We have mapped the hormone-modulated site of phosphorylation on the hVDR to serine 205. This site appears in a region between the DNA and ligand binding domains that is rich in serine residues, several of which match the CKII consensus. The hVDR undergoes alternative phosphorylation on an adjacent serine site in a serine 205 to alanine mutant and is functionally indistinguishable from the native receptor. This finding prevents current assessment of the role of phosphorylation in the transcriptional activity of the VDR.

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