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Human vitamin D receptor is selectively phosphorylated by protein kinase C on serine 51, a residue crucial to its trans-activation function

(1,25-dihydroxyvitamin D_3/steroid hormone receptors/site-directed mutagenesis/tumor promoter)

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ABSTRACT The vitamin D receptor (VDR) is known to be a phosphoprotein and inspection of the deduced amino acid sequence of human VDR (hVDR) reveals the conservation of three potential sites of phosphorylation by protein kinase C (PKC)—namely, Ser-51, Ser-119, and Ser-125. Immunoprecipitated extracts derived from a rat osteoblast-like osteosarcoma cell line that contains the VDR in high copy number were incubated with the α, β, and γ isozymes of PKC, and VDR proved to be an effective substrate for PKC-β, *in vitro*. When hVDR cDNAs containing single, double, and triple mutations of Ser-51, Ser-119, and Ser-125 were expressed in CV-1 monkey kidney cells, immunoprecipitated and phosphorylated by PKC-β, *in vitro*, the mutation of Ser-51 selectively abolished phosphorylation. Furthermore, when transfected CV-1 cells were treated with phorbol 12-myristate 13-acetate, a PKC activator, phosphorylation of wild-type hVDR was enhanced, whereas that of the Ser-51 mutant hVDR was unaffected. Therefore, Ser-51 is the site of hVDR phosphorylation by PKC, both *in vitro* and *in vivo*. To evaluate the functional role of Ser-51 and its potential phosphorylation, hVDR-mediated transcription was tested using cotransfection with expression plasmids and a reporter gene that contained a vitamin D response element. Mutation of Ser-51 markedly inhibited transcriptional activation by the vitamin D hormone, suggesting that phosphorylation of Ser-51 by PKC could play a significant role in vitamin D-dependent transcriptional activation. Therefore, the present results link the PKC signal transduction pathway of growth regulation and tumor promotion to the phosphorylation and function of VDR.

The vitamin D receptor (VDR) is classified as a member of the steroid/thyroid hormone receptor superfamily of proteins by virtue of amino acid homologies within two separate domains (1–5). The N-terminal domain is configured into two zinc-coordinated fingers responsible for DNA recognition and binding, whereas the C-terminal domain binds the 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] hormone. By analogy to the other members of the steroid/thyroid hormone receptor superfamily, 1,25$(OH)_2D_3$ acts by binding to the VDR, whereupon this activated hormone–receptor complex associates with recently identified response elements (6, 7) to alter transcription of genes such as osteocalcin. The molecular mechanism of this transcriptional alteration, especially the role of posttranslational modifications, is currently a topic of considerable interest.

Several members of the steroid/thyroid hormone receptor superfamily are known to be phosphorylated, including the progesterone receptor (8), the glucocorticoid receptor (9), the thyroid hormone receptor (10), the estrogen receptor (11), and the VDR (3, 12, 13). However, the functional significance of steroid hormone receptor phosphorylation is unclear. Studies utilizing the mouse (3) and chicken (13) VDR have demonstrated that the phosphorylation of this receptor, *in vivo*, is a rapid event that is enhanced in the presence of 1,25$(OH)_2D_3$. Phosphoamino acid analysis performed on the mouse VDR has shown that, at least in this species, the predominant phosphorylated residue is serine (3). One attractive possibility is that, like the cAMP-responsive-element binding protein (14) and yeast transcriptional activator ADR1 (15), phosphorylation of VDR on a serine residue or residues is an important step in the signal transduction of 1,25$(OH)_2D_3$-dependent gene regulation. However, to date, no specific phosphorylation site(s) in VDR has been identified and the kinases that phosphorylate this receptor remain uncharacterized.

Based upon the deduced amino acid sequence, there are three potential protein kinase C (PKC) phosphorylation sites in the VDR. PKC is a family of at least six different isozymes, α, β, γ, δ, ε, and ζ, which are derived from alternate splicing of RNA transcripts and from multiple genes (16). Although most activated PKC is found at the cell membrane, a fraction of PKC has been found associated with the nucleus (17). Accordingly, PKC has been shown to phosphorylate a number of nuclear proteins involved in gene expression, including topoisomerase I (18) and the c-*erbA*α-encoded thyroid hormone receptor (10). Given the three potential PKC sites in the VDR, as well as the fact that 1,25$(OH)_2D_3$ has been demonstrated to transcriptionally regulate the expression of PKC-α and PKC-β in HL-60 leukemia cells (19), we were interested in the possibility that the VDR might be a substrate for one of the PKC isozymes.

MATERIALS AND METHODS

Materials. *Escherichia coli* CJ236 and *E. coli* XL1-blue were used as host strains for site-directed mutagenesis experiments (20). *In vitro* mutagenesis kits were purchased from Bio-Rad. [γ-^{32}P]ATP, [^{32}P]orthophosphate, and [^{35}S]methionine were obtained from NEN Radiochemical. ^{125}I-labeled protein A was purchased from ICN. Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma. Monoclonal antibodies (mAbs) 9A7γ and 4A5γ were purified from media produced by hybridoma cells (21).

Purification of PKC. Purification of PKC from mouse brain was carried out as described (22, 23). Briefly, sequential DEAE-cellulose, Mono Q, and hydroxyapatite FPLC chro-

Abbreviations: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; PKC, protein kinase C; PKA, protein kinase A; VDR, 1,25-dihydroxyvitamin D_3 receptor; hVDR, human VDR; VDRE, vitamin D response element; PMA, phorbol 12-myristate 13-acetate; hGH, human growth hormone; mAb, monoclonal antibody.

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matography was utilized to resolve the three major isozymes of PKC. Fractions containing these three PKC peaks (23) corresponding to type I (PKC-γ), type II (PKC-β), and type III (PKC-α) enzymes were pooled separately, desalted, and concentrated with a Minicon B15 and stored at −75°C.

Site-Directed Mutagenesis. Uracil-containing, single-stranded recombinant pSG5hVDR DNA was prepared according to the protocol of Kunkel *et al.* (20). Uracil-containing single-stranded DNA was mixed with phosphorylated mutagenic oligonucleotides and required enzymes. The mixtures were incubated at 37°C for 90 min to synthesize the second mutated strand. The resulting double-stranded pSG5hVDR was used to transform *E. coli* XL1-blue competent cells. Colonies were selected at random and directly sequenced to screen for mutants.

Transfection of Cells. CV-1 monkey kidney epithelial cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. Rat osteosarcoma (ROS 17/2.8) cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 5% fetal bovine serum and 5% newborn calf serum as well as antibiotics. Transfections were performed using a typical calcium phosphate-DNA coprecipitation method employing 20 μg of each expression plasmid. The calcium phosphate precipitates were removed from CV-1 cell cultures by washing the cells twice with the appropriate serum-free medium. The cells were subsequently cultured in fresh serum-containing medium for 48 hr.

Immunoblotting and Immunoprecipitation of Human VDR (hVDR). Transfected CV-1 cells were lysed directly in final sample buffer and 40 μg of cellular protein was run on 5–15% gradient SDS/polyacrylamide gels. Immunoblotting of hVDR was performed as described (7). For immunoprecipitations, transfected CV-1 and ROS 17/2.8 cells were lysed in immunoprecipitation lysis buffer (10 mM Tris·HCl, pH 7.4/1 mM EDTA/0.3 mM $ZnSO_4$/0.3 M KCl/0.5% Triton X-100) and incubated with mAb 4A5γ linked to agarose beads as described (24).

***In Vitro* Phosphorylation Reactions.** PKC reaction buffer contained 20 mM Tris·HCl (pH 7.5), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 25 μg of phosphatidylserine per ml, and 100 ng of PMA per ml. The phosphorylation reaction was initiated by addition of 30 μCi of [γ-^{32}P]ATP (1 Ci = 37 GBq) and PKC (1 unit) and the incubation was carried out at 30°C for 30 min. The reaction was terminated by addition of sample buffer and heating to 100°C for 3 min.

Whole Cell Labeling. CV-1 cells were pretreated for 24 hr with 10 nM 1,25$(OH)_2D_3$, washed with phosphate-free medium; then fresh phosphate-free medium including [^{32}P]-orthophosphate (0.5 mCi per plate) was added and the cells were incubated for 4 hr. The [^{32}P]orthophosphate-labeled cells were treated for 30 min with PMA (100 nM). Cells were lysed, immunoprecipitated, and analyzed by electrophoresis on a 10% SDS/polyacrylamide gel.

Cotransfection and Transcription Assay. Transcriptional activity was measured in CV-1 cells cotransfected with the pSG5 hVDR expression plasmids (7.5 μg) and the vitamin D response element (VDRE) containing reporter plasmid $(CT4)^4$TKGH (5 μg) (7). Cells were treated for 48 hr following transfection with either 10 nM 1,25$(OH)_2D_3$ or ethanol as a control. Medium was assayed by radioimmunoassay for the expression of human growth hormone (hGH) using a kit from Nichols Institute Diagnostics (San Juan Capistrano, CA), and cells were harvested for immunoblot analysis.

RESULTS

Sequence Analysis of hVDR and Identification of PKC Consensus Sites. We analyzed the deduced amino acid sequence of hVDR (1, 3) to determine whether the reported PKC consensus motif RXXSXR (25) or RXXXXXRXS (26) was present in the receptor. Ser-51, Ser-119, and Ser-125 were identified as the three most probable sites of PKC phosphorylation. The position of the Ser-51 residue partially matches the RXXSXR motif and conforms to the general property of PKC sites being flanked by N- and C-terminal basic residues, whereas Ser-119 and Ser-125 residues match the RXXXXXRXS motif except that lysine replaces arginine in each case. All three of these serine residues are located in the N-terminal domain of the hVDR molecule and are highly conserved in the deduced VDR amino acid sequences published to date—namely, that of the human (1), rat (2), and partial chicken sequence (27).

Phosphorylation of Rat VDR by Isozymes of PKC, *in Vitro*. To investigate whether VDR is a substrate for PKC, we prepared a VDR-containing immunocomplex from ROS 17/2.8 cells, which contain a high level of the VDR. This potential substrate was isolated by immunoprecipitation of ROS 17/2.8 cellular lysates with the anti-VDR mAb 4A5γ linked to agarose beads. The immunoprecipitates were incubated in the presence of [γ-^{32}P]ATP with the α, β, or γ isozymes of PKC. The results in Fig. 1 show that the β isozyme of PKC significantly phosphorylated the immunoprecipitated rat VDR, whereas PKC-α and PKC-γ exhibited little or no activity in phosphorylating VDR. This phospho-VDR species (arrow, lanes 6 and 7) may also be augmented in the presence of 1,25$(OH)_2D_3$ and is the major specific phosphorylated protein in that it is uniquely competitively inhibited by excess free VDR mAb (lane 8). Therefore, we concluded that rat VDR is a substrate for selective phosphorylation by PKC-β, *in vitro*.

Cloning, Site-Directed Mutagenesis, and Expression of hVDR. A 2-kilobase (kb) DNA fragment of the hVDR cDNA was inserted into an *Eco*RI site of the expression vector pSG5 (28). The orientation of the insert was verified by DNA sequence analysis, and this recombinant plasmid was named pSG5hVDR (Fig. 2*A*). To examine whether Ser-51, Ser-119, and Ser-125 residues were indeed the residues phosphorylated by PKC, a series of mutants were constructed by oligonucleotide-directed mutagenesis. Ser-51 (AGC), Ser-119 (AGT), and Ser-125 (TCT) were changed to glycine (GGC), glycine (GGT), and alanine (GCT), respectively. In addition, the double mutant Ser-51/Ser-119, and the triple mutant Ser-51/Ser-119/Ser-125 were also

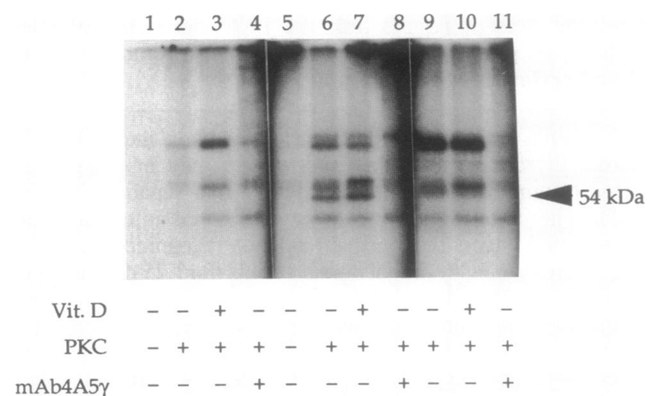

FIG. 1. Analysis of VDR phosphorylation by PKC-α, PKC-β, and PKC-γ isozymes, *in vitro*. Rat VDR was immunoprecipitated from ROS 17/2.8 cells and incubated under *in vitro* phosphorylation conditions in the absence or presence of 10 nM 1,25$(OH)_2D_3$ (± Vit. D) along with PKC-α (lanes 1–4), PKC-β (lanes 5–8), or PKC-γ (lanes 9–11). Excess free mAb 4A5γ was included in the original immunoprecipitations of the reactions analyzed in lanes 4, 8, and 11 to identify the specifically immunoprecipitated rat VDR substrate by its competition under these conditions. The arrowhead indicates the apparent molecular mass (54 kDa) of rat VDR as determined by molecular weight markers. Other bands represent abundant proteins that are phosphorylated and contaminate the immunoprecipitation.

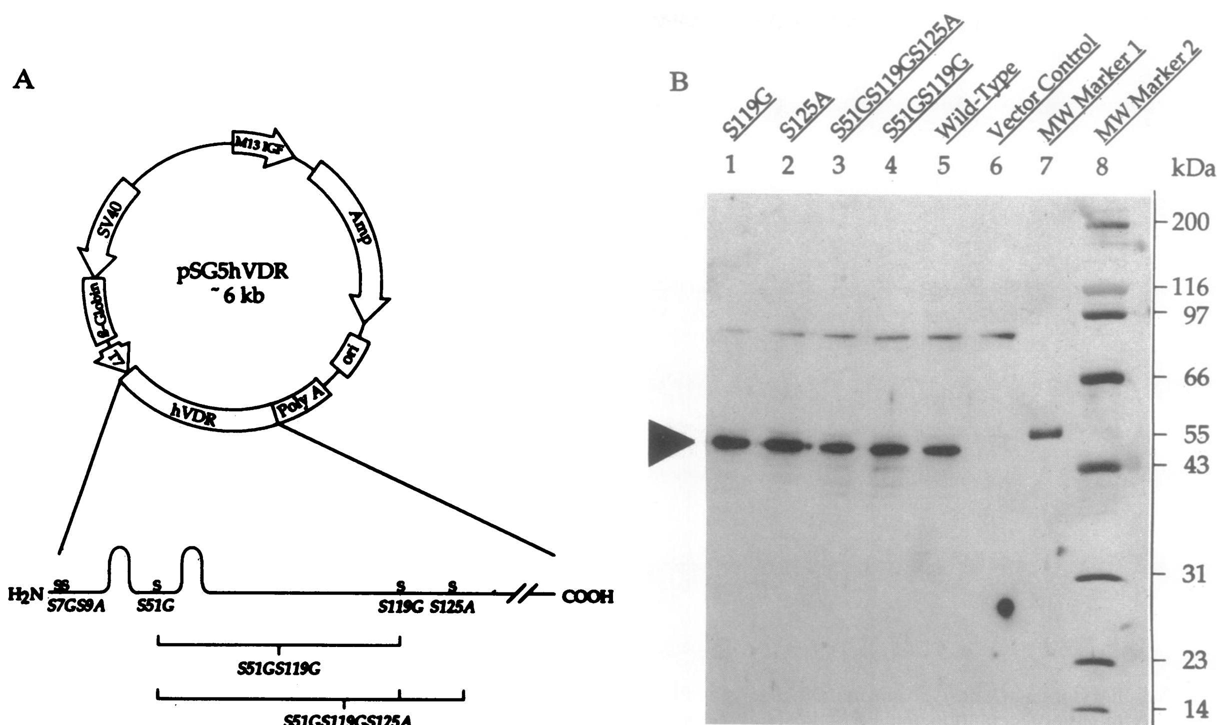

FIG. 2. Schematic representation of hVDR mutants engineered into a shuttle vector and immunoblot determination of their expression. (*A*) Diagram of the expression vector pSG5hVDR constructs for *in vitro* mutagenesis of the hVDR gene. Numbers indicate the positions of serine residues that were mutated to glycine/alanine residue(s) in hVDR. The two zinc fingers of the DNA-binding domain of hVDR are depicted schematically. SV40, simian virus 40; Amp, ampicillin. (*B*) Analysis of the expression of wild-type and mutant hVDR constructs in transfected CV-1 cells by Western blots. Equivalent amounts of total protein (40 μg) were loaded in each lane. Lane 6, expression vector control that contains no hVDR cDNA insert; lane 7, glutamate dehydrogenase (55 kDa) molecular weight marker; lane 8, range of molecular weight standards. hVDR is indicated by the arrowhead.

constructed. To evaluate the possibility that *any* alteration in the N-terminal region of hVDR would result in a generalized inhibition of phosphorylation, we engineered another double mutant that altered serines 7 and 9 to glycine and alanine, respectively. These hVDR mutants were denoted as S51G, S119G, S125A, S51GS119G, S7GS9A, and S51GS119GS125A, and their general position is depicted in a schematic diagram of hVDR in the lower part of Fig. 2*A*. After the DNA sequence of each mutant was confirmed, they were transfected individually into CV-1 cells to examine their expression via immunoblot analysis. As is illustrated in Fig. 2*B*, the expression efficiency was virtually equivalent among the wild-type hVDR and the various mutants.

Identification of the Site(s) in hVDR Phosphorylated by PKC-β, *in Vitro*. We next tested wild-type hVDR for phosphorylation by PKC-β, *in vitro*. To localize the putative serine residue(s) involved in PKC-β-mediated phosphorylation, single, double, and triple mutants of these residues were evaluated. As shown in Fig. 3*A*, the single mutant S51G, the double mutant S51GS119G, and the triple mutant S51GS119-GS125A completely abolished PKC-β-catalyzed phosphorylation compared with the significant phosphorylation observed in wild-type hVDR and in the control S7GS9A double mutant. In a second experiment (Fig. 3*B*), single mutants S119G and S125A were effectively phosphorylated by PKC-β, whereas S51G was not phosphorylated. In this latter gel (Fig. 3*B*), a wild-type hVDR lane was included (not shown) and each band corresponding to phosphorylated hVDR was cut from the gel and assayed for ^{32}P. The cpm obtained from wild-type, S119G, and S125A hVDR were similar (data not shown). Thus it appears that Ser-51 is the sole target of PKC-β-mediated phosphorylation in hVDR.

Phosphorylation of hVDR in Intact Cells. To investigate the PKC-catalyzed phosphorylation of hVDR *in vivo*, we assessed hVDR phosphorylation levels in 1,25(OH)$_2$D$_3$-treated CV-1 cells after exposure to PMA, a PKC activator. CV-1 cells were transfected with wild-type or mutant hVDR cDNA, preincubated for 4 hr with [^{32}P]orthophosphate, and exposed to 100 nM PMA for 30 min. The cells were lysed, and hVDR was precipitated with 4A5γ-agarose followed by SDS/PAGE analysis. Cells treated with PMA exhibited an increase in ^{32}P incorporation into wild-type hVDR (Fig. 4, compare lanes 6 and 5), whereas PMA had no effect on the mutant S51G hVDR (lanes 7 and 8). Note that in intact cells, hVDR is phosphorylated in the absence of PMA (Fig. 4, lanes 5 and 7), apparently by other endogenous protein kinases. The [^{32}P]orthophosphate-labeled hVDR (lanes 5–8) comigrated with [^{35}S]methionine-labeled hVDR (lanes 1–4), thereby verifying the identity of the specific hVDR band and demonstrating similar expression of wild-type and S51G hVDR. These observations strongly suggest that PMA-induced phosphorylation of hVDR *in vivo*, like the phosphorylation of hVDR *in vitro* by PKC-β, is localized to Ser-51.

Potential Functional Significance of PKC Phosphorylation. Cotransfection experiments were performed to investigate the functional role of PKC phosphorylation. CV-1 cells, which do not express appreciable levels of endogenous hVDR, were transfected with the pSG5hVDR expression vector and a reporter plasmid possessing a VDRE (7). A comparison of transcriptional activity of the wild-type and various mutant hVDRs is shown in Fig. 5*A*. The results indicate that the Ser-51 mutants, including S51G, S51GS119-G, and S51GS119GS125A, elicited 60%, 88%, and 92% inhibition of transcriptional activity, respectively. Much smaller, but significant, alteration of trans-activation occurred with the S119G and S125A mutants, whereas the S7GS9A mutant displayed the same level of transcriptional activity as the wild-type hVDR. These findings suggest that PKC-mediated phosphorylation of Ser-51 may play an im-

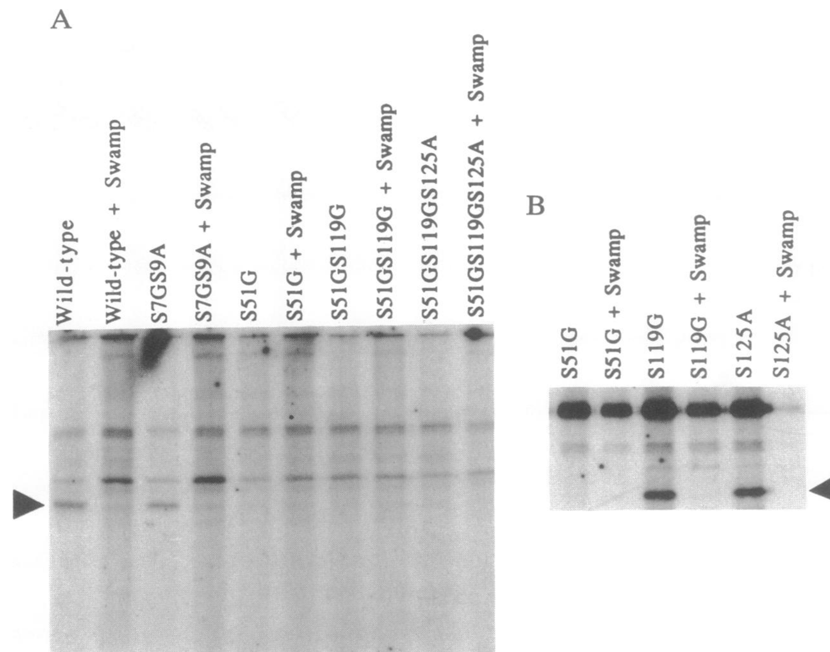

FIG. 3. Phosphorylation of wild-type and mutant hVDRs by PKC-β, *in vitro*. See text for the experimental conditions for expression of hVDR in CV-1 cells, its immunoprecipitation, and PKC phosphorylation. Data shown in *A* and *B* were obtained under identical experimental conditions but were exposed to film for different lengths of time. The arrowheads indicate the position of hVDR.

portant role in the transcriptional activation function of hVDR, although mutation of Ser-119 and Ser-125 also affects hVDR activity, possibly by altering the conformation C-terminal to the zinc fingers. The observed transcriptional inhibition was not a consequence of a decrease in mutant hVDR expression, as indicated by immunoblot analysis (Fig. 5*B*).

DISCUSSION

In this study, we have discovered that hVDR is a substrate for PKC-β and demonstrated that Ser-51 is the major PKC phosphorylation site in hVDR *in vitro* and *in vivo*. The FRRS[51]MKRK recognition motif for PKC-β in hVDR does not match exactly with any published PKC consensus sequence, but it resembles other known Ser/Thr PKC sites in the interleukin 2 receptor (29) and in desmin (30) that are flanked by basic amino acids (31). In the case of c-*erbAα*-encoded thyroid hormone receptor, the reported PKC phosphorylation sites are Ser-28 and Ser-29 in the sequence KRHKRKS[28]S[29]QCLVK (10). This phosphorylated region of the thyroid hormone receptor possesses a similar sequence to that found in the vicinity of Ser-51 of hVDR. These observations suggest that a nested set of positively charged residues in juxtaposition to a serine is a preferred recognition motif for PKC in steroid/thyroid receptors. Moreover, it should be emphasized that Ser-51 along with flanking basic residues are conserved only in a discrete subfamily of steroid/thyroid receptors, including the thyroid hormone receptor, the retinoic acid receptor, and the estrogen receptor (5).

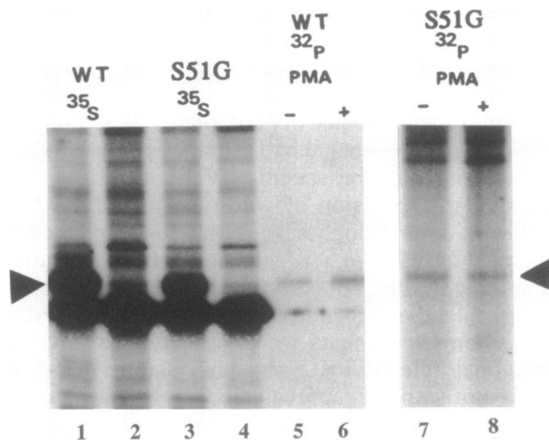

FIG. 4. Phosphorylation of the hVDR by PKC-β, *in vivo*. Lanes 1–4, CV-1 cells were transfected with hVDR constructs as shown and labeled with 500 μCi of [^{35}S]methionine for 4 hr, lysed, and subjected to immunoprecipitation. Immunoprecipitations in lanes 2 and 4 were competitively inhibited with excess free 4A5γ antibody and resulted in the expected diminution of the hVDR band. Lanes 5–8, CV-1 cells were transfected with wild-type (WT) or mutant hVDR as indicated and labeled with [^{32}P]orthophosphate for 4 hr followed by treatment with (lanes 6 and 8) and without (lanes 5 and 7) 100 nM PMA for 30 min prior to harvesting cells. Arrowheads indicate the migration position of hVDR on the SDS/polyacrylamide gel.

Comparison of the consensus motifs of PKC—i.e., RXXSXR—and of protein kinase A (PKA)—namely, RRXSX (25)—reveals that they contain a similar but not identical recognition sequence. This similarity has functional consequences in the cases of the thyroid hormone receptor (10), desmin (30), and the γ and δ subunits of the acetylcholine receptor (32), where PKC and PKA phosphorylate these proteins at a common site. We tested this possibility in the case of hVDR by incubating immunoprecipitated hVDR with PKA. The results (data not shown) showed clearly that the triple mutant S51GS119GS125A and wild-type hVDR can be equally phosphorylated by PKA. Therefore Ser-51 of hVDR is not a PKA phosphorylation target and it appears that the presence of a unique pattern of basic residues surrounding Ser-51 distinguishes it as an exclusive site for phosphorylation by PKC.

From the data presented in Fig. 4, it is evident that hVDR is phosphorylated by endogenous protein kinases other than PKC. Recent results (24) indicate that hVDR may be phosphorylated by casein kinase II at the N-terminal border of the $1,25(OH)_2D_3$-binding domain. It is therefore likely that hVDR is subjected to multisite phosphorylation, *in vivo*, and one could speculate that each class of phosphorylation could modulate different functions of VDR, such as hormone binding, DNA binding, trans-activation, desensitization, etc.

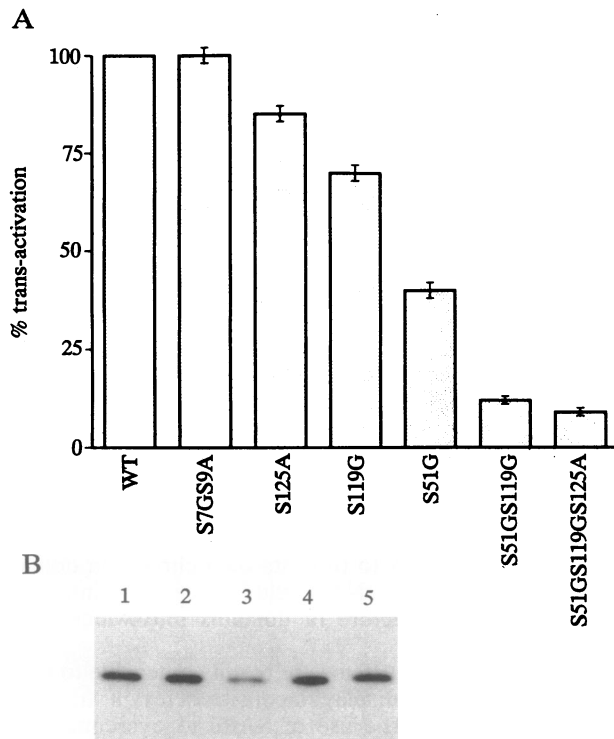

FIG. 5. Comparison of $1,25(OH)_2D_3$-induced transcriptional activity as measured by cotransfection of various mutated hVDRs with a VDRE-TKGH reporter construct. (*A*) Assays were performed as detailed in the text, and the trans-activation in the presence of wild-type (WT) hVDR [an ≈10-fold effect of $1,25(OH)_2D_3$] was set at 100%. The results are representative of at least five separate experiments with the standard deviations shown in the histogram. (*B*) Expression of wild-type and mutant hVDRs determined by immunoblotting. Lanes 1–5 represent the following hVDR constructs: wild-type, S51G, S119G, S51GS119G, and S51GS119GS125A, respectively.

Preliminary experiments have examined the potential role of PKC phosphorylation of hVDR by testing the functional consequences of the mutation of Ser-51. The mutant proteins we generated are expressed normally (Figs. 2*B* and 5*B*) and bind $1,25(OH)_2D_3$ with similar kinetics compared with the wild-type hVDR (data not shown). It may be of significance that Ser-51 lies in a position between the two zinc fingers that constitute the DNA-binding region of the steroid receptors. Determination of the ability of various mutant hVDRs to bind to the VDRE by VDRE-affinity chromatography awaits further investigation. Preliminary results indicate that the hVDR mutant S51G binds to the VDRE and elutes only slightly earlier than the wild-type hVDR in a KCl gradient (J.-C.H., M.A.G., C.M.T., and M.R.H., unpublished results). The major effect of mutation of Ser-51 and apparent loss of its capacity for PKC-β-catalyzed phosphorylation therefore appears to be a compromising of the trans-activation ability of hVDR (Fig. 5*A*). This indicates that the phosphorylation of Ser-51 could be an important step in the control of transcription that is effected by the $1,25(OH)_2D_3$–VDR complex.

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1. Baker, A. R., McDonnell, D. P., Hughes, M., Crisp, T. M., Mangelsdorf, D. J., Haussler, M. R., Pike, J. W., Shine, J. & O'Malley, B. W. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3294–3298.
2. Burmester, J. K., Wiese, R. J., Maeda, N. & DeLuca, H. F. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 9499–9502.
3. Haussler, M. R., Mangelsdorf, D. J., Komm, B. S., Terpening, C. M., Yamaoka, K., Allegretto, E. A., Baker, A. R., Shine, J., McDonnell, D. P., Hughes, M., Weigel, N. L., O'Malley, B. W. & Pike, J. W. (1988) *Rec. Prog. Horm. Res.* **44**, 263–305.
4. Evans, R. M. (1988) *Science* **240**, 889–895.
5. Beato, M. (1989) *Cell* **56**, 335–344.
6. Kerner, S. A., Scott, R. A. & Pike, J. W. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4455–4459.
7. Terpening, C. M., Haussler, C. A., Jurutka, P. W., Galligan, M. A., Komm, B. S. & Haussler, M. R. (1991) *Mol. Endocrinol.* **5**, 373–385.
8. Logeat, F., Le Cunff, M., Pamphile, R. & Milgrom, E. (1985) *Biochem. Biophys. Res. Commun.* **131**, 421–427.
9. Singh, V. B. & Moudgil, V. K. (1985) *J. Biol. Chem.* **260**, 3684–3690.
10. Goldberg, Y., Glineur, C., Gesquiere, J.-C., Ricouart, A., Sap, J., Vennstrom, B. & Ghysdael, J. (1988) *EMBO J.* **7**, 2425–2433.
11. Migliaccio, A., Rotondi, A. & Auricchio, F. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5921–5925.
12. Pike, J. W. & Sleator, N. M. (1985) *Biochem. Biophys. Res. Commun.* **131**, 378–385.
13. Brown, T. A. & DeLuca, H. F. (1990) *J. Biol. Chem.* **265**, 10025–10029.
14. Gonzalez, G. A. & Montminy, M. R. (1989) *Cell* **59**, 675–680.
15. Cherry, J. R., Johnson, T. R., Dollard, C., Shuster, J. R. & Denis, C. L. (1989) *Cell* **56**, 409–419.
16. Kikkawa, U., Kishimoto, A. & Nishizuka, Y. (1989) *Annu. Rev. Biochem.* **58**, 31–44.
17. Shimizu, Y. & Shimizu, N. (1989) *Somat. Cell Mol. Genet.* **15**, 321–329.
18. Samuels, D. S., Shimizu, Y. & Shimizu, N. (1989) *FEBS Lett.* **259**, 57–60.
19. Obeid, L. M., Okazaki, T., Karolak, L. A. & Hannun, Y. A. (1990) *J. Biol. Chem.* **265**, 2370–2374.
20. Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382.
21. Pike, J. W., Marion, S. L., Donaldson, C. A. & Haussler, M. R. (1983) *J. Biol. Chem.* **258**, 1289–1296.
22. Jeng, A. Y., Sharkey, N. A. & Blumberg, P. M. (1986) *Cancer Res.* **46**, 1966–1971.
23. Kikkawa, U., Ono, Y., Ogita, K., Fujii, T., Asaoka, Y., Sekiguchi, K., Kosaka, Y., Igarashi, K. & Nishizuka, Y. (1987) *FEBS Lett.* **5**, 1137–1146.
24. Jones, B. B., Jurutka, P. W., Haussler, C. A., Haussler, M. R. & Whitfield, G. K. (1991) *Mol. Endocrinol.* **5**, 1137–1146.
25. Kemp, B. E. & Pearson, R. B. (1990) *Trends Biochem. Sci.* **15**, 342–346.
26. Gettys, T. W. & Corbin, J. W. (1989) in *Receptor Phosphorylation*, ed. Moudgil, V. K. (CRC, Boca Raton, FL), pp. 39–88.
27. Haussler, M. R., Terpening, C. M., Jurutka, P. W., Meyer, J., Schulman, B. A., Haussler, C. A., Whitfield, G. K. & Komm, B. S. (1988) in *Progress in Endocrinology 1988*, eds. Imura, H., Shizume, K. & Yoshida, S. (Excerpta Med., Amsterdam), Vol. 1, pp. 763–770.
28. Green, S., Issemann, I. & Sheer, E. (1988) *Nucleic Acids Res.* **16**, 369.
29. Gallis, B., Lewis, A., Wignall, J., Alpert, A., Mochizuki, D. Y., Cosman, D., Hopp, T. & Urdal, D. (1986) *J. Biol. Chem.* **261**, 5075–5080.
30. Kitamura, S., Ando, S., Shibata, M., Tanabe, K., Sato, C. & Inagaki, M. (1989) *J. Biol. Chem.* **264**, 5674–5678.
31. House, C., Wettenhall, R. E. H. & Kemp, B. E. (1987) *J. Biol. Chem.* **262**, 772–777.
32. Safran, A., Provenzano, C., Sagi-Eisenberg, R. & Fuchs, S. (1990) *Biochemistry* **29**, 6730–6734.