Human vitamin D receptor phosphorylation by casein kinase II at Ser-208 potentiates transcriptional activation

(1,25-dihydroxyvitamin D₃/control of transcription/steroid, retinoid, and thyroid hormone receptors/vitamin D responsive element/rat osteocalcin gene)

PETER W. JURUTKA, J.-C. HSIEH, SHIGEO NAKAJIMA*, CAROL A. HAUSSLER, G. KERR WHITFIELD, AND MARK R. HAUSSLERT

Department of Biochemistry, College of Medicine, The University of Arizona, Tucson, AZ 85724

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ABSTRACT The potential functional significance of human 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] receptor (hVDR) phosphorylation at Ser-208 was evaluated by cotransfecting COS-7 kidney cells with hVDR constructs and the catalytic subunit of human casein kinase II (CK-II). Under these conditions, hVDR is intensely phosphorylated in a reaction that depends on both CK-II and the presence of Ser-208. The resulting hyperphosphorylated receptor is altered in its kinetics for binding the 1,25(OH)₂D₃ ligand, its partitioning into the nucleus, and its ability to associate with a vitamin D responsive element. Replacement of Ser-208 with glycine or alanine indicates that phosphorylation of hVDR at Ser-208 is not obligatory for 1,25(OH)₂D₃ action, but coexpression of wild-type hVDR and CK-II elicits a dose-dependent enhancement of 1,25(OH)₂D₃-stimulated transcription of a vitamin D responsive element reporter construct. This enhancement by CK-II is abolished by mutating Ser-208 to glycine or alanine and does not occur with glucocorticoid receptor-mediated transcription. Therefore, phosphorylation of hVDR by CK-II at Ser-208 specifically modulates its transcriptional capacity, suggesting that this covalent modification alters the conformation of VDR to potentiate its interaction with the machinery for DNA transcription.

Protein phosphorylation is recognized as a significant mechanism for the regulation of a number of cellular processes and metabolic pathways (1), and this covalent modification can also control the activity of certain receptor proteins (2). Members of the steroid/thyroid hormone receptor superfamily that have been shown to exist as phosphoproteins include the progesterone receptor, glucocorticoid receptor (GR), thyroid hormone receptor, estrogen receptor, androgen receptor (reviewed in ref. 3), as well as the 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] receptor (VDR) (4–6). Biochemical analysis has revealed that a majority of the sites phosphorylated in these receptors are serine residues. Based on kinase consensus recognition sequences and in vitro assays, several kinases have been implicated in the phosphorylation of nuclear receptors, including proline-directed kinase, calcium-calmodulin-dependent kinase, DNA-dependent protein kinase, protein kinase A, and protein kinase C (reviewed in ref. 7). Another kinase of potential significance in terms of steroid hormone receptor phosphorylation is casein kinase II (CK-II). This latter enzyme, a predominantly nuclear-localized kinase (8), is present in a wide variety of eukaryotic cells (9) and is known to phosphorylate nuclear oncogene products and a number of proteins involved in gene expression such as RNA polymerases I and II, c-myc, c-myb, and max (10). CK-II has also been implicated in the phosphorylation of steroid/thyroid hormone receptors including the thyroid hormone receptor (11), estrogen receptor (12), and progesterone receptor (13). We have previously reported that the human VDR (hVDR) is phosphorylated by CK-II in vitro at Ser-208, a CK-II consensus recognition site, and that this residue represents the predominant endogenous hVDR phosphorylation site in transfected COS-7 cells (14). Using a similar approach of hVDR overexpression in COS-1 cells, Hilliard et al. (15) used phosphopeptide mapping to identify Ser-208* as the major residue phosphorylated in a 1,25(OH)₂D₃-dependent fashion.

Functional studies utilizing other steroid hormone and retinoid receptors have implicated phosphorylation in the regulation of DNA binding (16), hormone binding (17), nuclear localization (18), and transcriptional activation (19, 20). However, recent probing of the significance of five to seven GR phosphorylation sites in or near the N-terminal transactivation domain, one of which is a CK-II consensus site, reveals that they are not absolutely essential for stimulation of transcription by the hormonal ligand (21, 22). Thus, the mechanistic implications of steroid hormone receptor phosphorylation remain unclear, and direct evidence for modulation of receptor activity by phosphorylation of these proteins at a specific residue(s) by a particular kinase has been difficult to obtain. This paper presents an analysis of the functional consequences of CK-II-mediated phosphorylation of hVDR.

MATERIALS AND METHODS

Transfection of COS-7 Cells and Phosphorylation of hVDR. COS-7 monkey kidney epithelial cells were transfected with 1–15 μg of pSG5-hVDR1/3 (wild type) or mutant hVDRs and/or 0.05–1.5 μg of pSG5-CK-IIα (containing the cDNA encoding the catalytic α subunit of human CK-II; a gift from H. Meisner, University of Massachusetts, Worcester) using the calcium phosphate-DNA coprecipitation technique. For phosphorylation experiments, COS-7 cells (5 × 10⁶ cells/60-mm plate) were cotransfected with 15 μg of pSG5-hVDR1/3 or the pSG5-S208G mutant receptor and either 1.5 μg of pSG5-CK-IIα or pTZ18U carrier DNA. Forty-eight hours posttransfection, the cells were washed twice with phosphate-free Dulbecco’s modified Eagle’s medium (GIBCO), then the same medium supplemented with 2% dialyzed fetal bovine serum and 0.5 mM (1 Ci = 37 GBq) [³²P]orthophosphate (New England Nuclear; carrier-free, 8500–9120 Ci/mmol) was added to each

Abbreviations: GR, glucocorticoid receptor; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; VDR, 1,25(OH)₂D₃ receptor; hVDR, human VDR; CK-II, casein kinase II; VDRE, vitamin D responsive element; GRe, glucocorticoid responsive element.

*Present address: Department of Environmental Medicine, Research Institute, Osaka Medical Center for Maternal and Child Health, Osaka 590-02, Japan.

†To whom reprint requests should be addressed.

‡Hilliard et al. (15) referred to Ser-208 as Ser-205 because they began numbering hVDR from Met-4 instead of Met-1 (GenBank accession no. J03258).

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plate followed by a 30-min orthophosphate preincubation at 37°C. 1,25(OH)₂D₃ (10 nM) was then added to the culture medium and the cells were incubated for an additional 1.5 h at 37°C. Cells were washed twice with Tris-buffered saline (10 mM Tris-HCl, pH 7.4/150 mM NaCl/3 mM KCl) before adding 1.0 ml KETZD-0.3 buffer (0.3 M KCl/10 mM Tris-HCl, pH 7.4/1 mM EDTA/0.3 mM ZnCl₂/5 mM dithiothreitol) containing 0.5% Triton X-100 and supplemented with phosphatase inhibitors (20 mM sodium fluoride/10 mM sodium molybdate/100 μM sodium ortho-avanadate) and protease inhibitors (2 μg/ml aprotinin/0.5 μg/ml leupeptin/50 μg/ml trypsin). Lysates were clarified by centrifugation for 15 min at 16,000 × g at 4°C followed by immunoprecipitation essentially as described (6).

**Assay of hVDR Transcriptional Activity.** Transcriptional activity of the hVDR was measured in COS-7 cells (5 × 10⁵ cells/60-mm plate) which had been transfected with 5 μg of pSG5-hVDR1/3 or mutant hVDREs and either 0.05–0.5 μg of pSG5-CK-IIα or pTZ18U carrier DNA and a reporter vector (5 μg/plate) containing four copies of the vitamin D responsive element (VDRE) linked upstream of the thymidine kinase core promoter driving the expression of the human growth hormone gene (23). Also used was a mouse GR (24) expression plasmid (pSG5-mGR) and a reporter vector containing the glucocorticoid responsive element (GRE) derived from the long terminal repeat of the mouse mammary tumor virus (25) linked upstream of the thymidine kinase promoter driving the expression of the human growth hormone gene. Following transfection, cells were treated for 24 h with 10⁻⁸ M 1,25(OH)₂D₃, 10⁻⁶ M dexamethasone, or ethanol vehicle. Medium was then assayed for human growth hormone by RIA using a kit from Nichols Institute Diagnostics (San Juan Capistrano, CA).

**Gel Mobility Shift Assay.** COS-7 cells (1 × 10⁶ cells/60-mm plate) were cotransfected with 15.0 μg of pSG5-hVDR1/3 or pSG5-S208G and either 1.5 μg of pSG5-CK-IIα or pTZ18U carrier DNA. Forty-eight hours posttransfection the cells were lysed and gel mobility shift analyses were carried out as described (26).

**Isolation of hVDR from Cytosplasmic and Nuclear Components and Immunoblotting of Extracts from Transfected COS-7 Cells.** COS-7 cells (1.5 × 10⁶ cells/100-mm plate) were cotransfected with 10 μg of pSG5-hVDR1/3 or the pSG5-S208G mutant hVDR and either 1.0 μg of pSG5-CK-IIα or pTZ18U carrier DNA. Forty-eight hours posttransfection the cells were treated with 100 nM of 1,25(OH)₂D₃ for 1.5 h at 37°C. Cytosolic and nuclear extracts were then prepared as described (26) and monitored for hVDR by immunoblotting using the 9A7γ monoclonal anti-VDR antibody (27).

**1,25(OH)₂D₃ Ligand Binding Assay.** COS-7 cells (8 × 10⁵ cells/60-mm plate) were cotransfected with 1.0 μg of pSG5-hVDR1/3 or pSG5-S208G and either 0.10 μg of pSG5-CK-IIα or pTZ18U carrier DNA. Forty-eight hours posttransfection cellular extracts were assayed for 1,25(OH)₂D₃ ligand binding as described (26).

**RESULTS**

**Phosphorylation of hVDR in COS-7 Cells Overexpressing CK-II.** As can be seen in Fig. 1, in the absence of transfected CK-II, the wild-type, transfected hVDR exists as a minimally phosphorylated protein in COS-7 cells (lanes 1 and 2). Mutation of Ser-208 to glycine (S208G) results in an ~60% reduction in the basal level of phosphorylation of the receptor as determined by quantitative densitometric scanning of the 3²P-VDR images (compare lanes 1 and 2 with lanes 5 and 6). Coexpression of CK-II enhances the phosphorylation of the wild-type hVDR by ~20-fold (lanes 3 and 4); in contrast, phosphorylation of the Ser-208 mutant is elevated only 2–3 fold (lanes 7 and 8) and does not approach that of wild-type receptor. Because immunoblot analysis showed that the level of S208G expression was equivalent to that of the wild-type hVDR (data not shown; see also Fig. 2D), the experiment illustrated in Fig. 1 demonstrates that the hVDR can be phosphorylated by CK-II in intact cells, predominantly at Ser-208.

**Transcriptional Activity of hVDR Phosphorylated by CK-II in Transfected COS-7 Cells.** In the absence of CK-II coexpression, the addition of the 1,25(OH)₂D₃ ligand in VDR-coexpressed cells results in a 30- to 35-fold enhancement of VDRE-dependent transcription (Fig. 2A, Right), with no effect using the control (~VDRE) reporter plasmid (Fig. 2A, Left). The transfection of increasing amounts of the CK-II catalytic subunit resulted in a dose-dependent stimulation in transcriptional activation by wild-type hVDR in response to 1,25(OH)₂D₃ (from ~30-fold to >50-fold; Fig. 2C, WT) without increasing the level of hVDR expression as monitored by immunoblotting (Fig. 2D). In control experiments, the activity of the thymidine kinase core promoter, without the VDRE, was not affected significantly by transfection of even the highest amount of CK-II (Fig. 2B), thus indicating that the effect of CK-II is mediated through the VDRE and that CK-II does not enhance basal promoter activity. Importantly, the transcriptional activation capacity of the S208G mutant (Fig. 2C, S208G) does not increase in response to escalating levels of cotransfected CK-II. Similar results are obtained with a Ser-208 to alanine (S208A) mutant (data not shown). In the absence of CK-II, there is a slight (20%) reduction in transcription activation by 1,25(OH)₂D₃ in the S208G mutant compared to the wild-type hVDR (Fig. 2C), whereas the transcriptional activity in response to 1,25(OH)₂D₃ of the S208A mutant was actually increased about 10% relative to the wild-type receptor (data not shown). Hilliard et al. (15) also demonstrated that the alanine-substituted mutant was entirely competent in transcriptional activation. Taken together, the above observations indicate that, while endogenous phosphorylation of Ser-208 is not required for VDR-mediated transcription per se, there exists a qualitative correlation between exogenous (cotransfected) CK-II-catalyzed hVDR phosphor-
ylation of Ser-208 (Fig. 1) and CK-II-mediated enhancement of hVDR transcriptional activity (Fig. 2C).

Two final control experiments were performed to probe the VDR residue and receptor specificity of CK-II-augmented transactivation. First, transactivation was tested by using a hVDR mutant in which a minimally phosphorylated CK-II consensus residue in the immediate vicinity of Ser-208, namely Ser-203, was altered to an alanine (S203A). Fig. 2C illustrates that in this case 1,25(OH)_{2}D_{3}-stimulated transcription is elevated in response to increasing expression of CK-II similarly to that of the wild-type hVDR. Second, as depicted in Fig. 3, in the absence of CK-II coexpression, the addition of dexamethasone to GR cotransfected cells results in a 16- to 22-fold enhancement of GRE-dependent transcription (Fig. 3A), but increasing amounts of transfected CK-II have no significant effect on either dexamethasone-induced transcriptional activation (Fig. 3C) or on basal thymidine kinase promoter activity (Fig. 3B). Thus, the CK-II-mediated stimulation of transcriptional activation appears to be confined to Ser-208 and specific for the VDR.

**DNA-Binding Activity of hVDR Phosphorylated by CK-II in Transfected COS-7 Cells.** Incubation of the rat osteocalcin VDRE and increasing amounts of extract from cells transfected with either the wild-type or S208G mutant hVDR leads to a corresponding and equivalent increase of the VDR–VDRE complex (Fig. 4, compare lanes 2 and 3 with lanes 7 and 8). In a control reaction, preincubation of the wild-type-containing extract with anti-VDR monoclonal antibody inhibits the shifted complex, confirming that this shift is dependent on the presence of VDR (lane 4). When extracts from cells transfected with either the wild-type or S208G mutant hVDR and the catalytic subunit of CK-II are used, the extent of

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**FIG. 2.** 1,25(OH)_{2}D_{3}-mediated transcriptional activation of a VDRE-linked reporter gene in COS-7 cells cotransfected with CK-II and wild-type or mutant hVDR. (A) COS-7 cells were cotransfected with 5 μg each of wild-type hVDR expression plasmid and either a reporter vector containing four copies of the VDRE linked upstream of the human growth hormone gene (right side of panel) or a control TKGH vector without the VDRE insert (left side of panel). (B) Effect of CK-II on VDRE-independent (basal) transcription. A wild-type hVDR-encoding vector was coexpressed with CK-II and the TKGH reporter plasmid without the VDRE. The basal transcription measured in the absence of CK-II was set at 100%. (C) Effect of CK-II on 1,25(OH)_{2}D_{3}-mediated transcriptional activation. The hVDR or indicated mutant (top of panel) was coexpressed with increasing amounts of CK-II in the presence of a VDRE-containing TKGH reporter plasmid. The 1,25(OH)_{2}D_{3}-stimulated transcriptional activation measured in the absence of CK-II was set at 100%. All values are the average (±SD) of three independent experiments, with triplicate samples in each of the various treatment groups in the three sets of data. The difference in transcriptional activation measured in the absence and presence of CK-II was statistically significant (at least P < 0.01) for all levels of CK-II. (D) Western blot analysis of hVDR and the S208G mutant under the conditions tested in C.
complex formation is similar for both the wild-type and S208G receptors (Fig. 4, compare lanes 5 and 6 with lanes 9 and 10) and, importantly, the VDRE-binding activity of the wild-type receptor is not significantly affected by CK-II-catalyzed phosphorylation (compare lanes 2 and 3 with lanes 5 and 6). Therefore, CK-II-catalyzed phosphorylation of hVDR does not affect its DNA binding activity and, because the retarded complex shown in Fig. 4 consists of a VDRE-bound heterodimer of VDR and an endogenous retinoid X receptor (24), the heterodimerization function of hVDR is likewise not influenced by overexpression of CK-II.

**1,25(OH)$_2$D$_3$ Hormone Binding and Subcellular Distribution of hVDR Phosphorylated by CK-II in Transfected COS-7 Cells.** Whole cell extracts from COS-7 cells transfected with either the wild-type or S208G mutant receptor were incubated with increasing concentrations of radiolabeled 1,25(OH)$_2$H$_2$D$_3$ for 6–8 h at 4°C to obtain saturation ligand binding data. Transformation of these data by Scatchard analysis revealed an apparent dissociation constant (Kd) for the wild-type receptor and S208G mutant of 0.36 nM (Fig. 5A) and 0.40 nM (Fig. 5B), respectively. In a parallel set of experiments, data from extracts of cells cotransfected with either the wild-type or S208G mutant hVDR and CK-II yielded Kd values of 0.52 nM (Fig. 5C) and 0.55 nM (Fig. 5D), respectively. These results demonstrate that neither phosphorylation of hVDR by CK-II nor alteration of the Ser-208 residue appreciably influences the affinity of the receptor for the 1,25(OH)$_2$D$_3$ hormone in vitro.

Finally, the subcellular partitioning of the receptor was examined. Quantitative densitometric scanning of the images depicted in Fig. 5E revealed that the specific activity of the wild-type receptor was ~2.3-fold greater in the nuclear compartment relative to the cytosolic activity (compare lanes 6 and 8). In the case of the S208G mutant, the specific activity was 1.7-fold greater in the nucleus relative to the specific activity in the cytosol (compare lanes 2 and 4). Most relevant to the present study, the coexpression of CK-II did not alter the specific activity of either the wild-type (compare lanes 5 and 6; lanes 7 and 8) or S208G receptors (compare lanes 1 and 2 and lanes 3 and 4) in either subcellular compartment.

**DISCUSSION**

Ser-208 of hVDR has been revealed as the major residue phosphorylated by CK-II in vitro and as the predominant endogenous receptor phosphorylation site in transfected COS-7 cells (14). Furthermore, phosphopeptide mapping studies of hVDR expressed in COS-1 cells point to Ser-208 as the primary phosphorylated amino acid in response to supraphysiologic doses of the 1,25(OH)$_2$D$_3$ hormone (15). In the present report, we have shown that expression of the catalytic subunit of human CK-II in transfected cells leads to a dramatic stimulation of hVDR phosphorylation at Ser-208. Whereas the DNA- and hormone-binding, as well as nuclear localization functions of hVDR appeared unaffected by CK-II-catalyzed phosphorylation, the 1,25(OH)$_2$D$_3$-mediated transcriptional activation capacity of the receptor was enhanced by this posttranslational modification. Although results...
from point mutagenesis still must be interpreted with caution, these observations indicate that phosphorylation at Ser-208 may stimulate the ability of hVDR to recruit a transcription factor(s) necessary for 1,25(OH)\(_2\)D\(_3\)-stimulated gene activation. Whether the region immediately surrounding Ser-208 is involved in such an interaction, or whether CK-II-catalyzed phosphorylation at Ser-208 affects a more distal region of hVDR via conformational changes, is not clear at present. Recently, the VDR has been reported to interact physically and functionally with the basal transcription factor TFIIB (28, 29) and a region of hVDR reported to participate in this association (residues 123–257) includes Ser-208 (28). CK-II-mediated phosphorylation of hVDR could perhaps facilitate or optimize such an interaction. However, mutation of Ser-208 to aspartate or glutamate does not result in the enhancement of hVDR transcriptional activity (15). Thus, similar to phosphorylation of the cAMP response element binding protein (30), endowing hVDR with a constitutive negative charge may not be sufficient to generate the functional conformational change putatively elicited by phosphorylation.

In addition to hVDR, other members of the steroid/thyroid hormone receptor superfamily are known to be phosphorylated by CK-II, although the exact cellular consequences of this event have not been elucidated. For example, a major site of thyroid hormone receptor phosphorylation in transfected COS-1 cells is Ser-12, a residue that was shown to be phosphorylated by CK-II \textit{in vitro}. Interestingly, Ser-12 is deleted in the gag/thyroid hormone receptor oncogenic fusion protein known as v-erbA and it has been suggested that the absence of Ser-12 may play a role in the oncogenic activation of v-erbA (11). Similarly, the human progesterone receptor is phosphorylated by CK-II \textit{in vitro} at Ser-81, a residue unique to the B isoform of the receptor, thus raising the possibility of selective modulation of progesterone receptor activity via phosphorylation (13). Finally, analogous to hVDR, Arnold et al. (12) have reported that Ser-167 is the major estrogen-enhanced phosphorylation site in the human estrogen receptor, both in MCF-7 cells and when incubated with CK-II \textit{in vitro}. In contrast to nuclear receptors, specific biological activities of other transcription factors have been shown to be modulated by CK-II-catalyzed phosphorylation. For example, the interaction of serum response factor with its DNA binding element is increased after phosphorylation of the protein at Ser-83 and -85 by CK-II (31). Moreover, CK-II-elicted stimulation of transcriptional activity has been reported for PU.1, a transcription factor whose expression is restricted to macrophages and B cells and may be important in the regulation of immunoglobulin genes (32).

The VDR is also known to be phosphorylated at Ser-51 by protein kinase C both \textit{in vitro} and \textit{in vivo}, resulting in a decrease in the specific interaction between the receptor and the VDREs, yet mutation of this serine to alanine does not affect DNA binding (27). Similarly, replacement of Ser-208 with alanine or glycine does not markedly attenuate 1,25(OH)\(_2\)D\(_3\)-mediated transcriptional activation in transfected COS-7 cells. This result suggests that the modest decrease in phosphorylation elicited by alteration of Ser-208 (Fig. 1) is not sufficient to generate detectable parallel changes in transcriptional activity apparently because of low endogenous levels of CK-II. Only under conditions of elevated CK-II activity, as has been suggested to occur in cells exposed to mitogenic stimuli or during certain phases of the cell cycle (10), or by increasing CK-II through supplemental coexpression of the enzyme (Fig. 2C), can a difference in the transcriptional capacity of the Ser-208 mutant VDR be revealed. We envisage two populations of liganded VDR, one that is hypophosphorylated at Ser-208 yet still active in transcriptional enhancement, and a superactive, hyperphosphorylated form that is even more effective at cooperatively recruiting coactivators and/or basal transcription factors. Therefore, taken together, these observations indicate that posttranslational modification of Ser-208 or -51 is not an obligatory switch for VDR function; rather, these phosphorylations represent both positive (CK-II) and negative (protein kinase C) modulatory mechanisms that apparently govern receptor activity under appropriate cellular conditions. Thus, the composite activity of hVDR may depend on the convergence of integrated inputs from several signal transduction pathways, including CK-II and protein kinase C, through “cross-talk” facilitated by phosphorylation.

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