Published in final edited form as:

J Steroid Biochem Mol Biol. 2007 March ; 103(3-5): 425–429. doi:10.1016/j.jsbmb.2006.12.021.

PHOSPHORYLATION AT SERINE 208 OF THE 1 α ,25-DIHYDROXY VITAMIN D3 RECEPTOR MODULATES THE INTERACTION WITH TRANSCRIPTIONAL COACTIVATORS

Gloria Arriagada^{1,*}, Roberto Paredes^{1,*}, Juan Olate¹, Andre van Wijnen², Jane B. Lian², Gary S. Stein², Janet L. Stein, Sergio Onate³, and Martin Montecino^{1,#}

¹Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Biológicas, Universidad de Concepción, Concepción, Chile

²Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA 01655

³Department of Urologic Oncology, Roswell Park Cancer Center, Buffalo, NY 14263.

Abstract

Upon ligand binding the 1 α ,25-dihydroxy vitamin D3 receptor (VDR) undergoes a conformational change that allows interaction with coactivator proteins including p160/SRC family members and the multimeric DRIP complex through the DRIP205 subunit. Casein kinase II (CKII) phosphorylates VDR both *in vitro* and *in vivo* at serine 208 within the hinge domain. This phosphorylation does not affect the ability of VDR to bind DNA, but increases its ability to transactivate target promoters. Here, we have analyzed whether phosphorylation of VDR by CKII modulates the ability of VDR to interact with coactivators *in vitro*. We find that both mutation of serine 208 to aspartic acid (VDRS208D) or phosphorylation of VDR by CKII enhance the interaction of VDR with DRIP205 in the presence of 1 α ,25-dihydroxy vitamin D3. We also find that the mutation VDRS208D neither affects the ability of this protein to bind DNA nor to interact with SRC-1 and RXR α . Together, our results indicate that phosphorylation of VDR at serine 208 contributes to modulate the affinity of VDR for the DRIP complex and therefore may have a role *in vivo* regulating VDR-mediated transcriptional enhancement.

Keywords

1 α ; 25-dihydroxy vitamin D3 receptor; Coactivators; Transcription

Introduction

1 α ,25-dihydroxy vitamin D3 modulates transcription after interacting with a specific cellular receptor (VDR), which is a member of the superfamily of nuclear receptors that recognize specific regulatory elements within the promoter region of target genes [1,2]. Upon ligand binding, VDR suffers a conformational change at the C-terminal ligand binding domain

© 2006 Elsevier Ltd. All rights reserved.

[#]To whom correspondence should be addressed: Dr. Martin Montecino, Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Biológicas, Universidad de Concepción, Casilla 160-C, Concepción, Chile. mmonteci@udec.cl.

*Both authors equally contributed to this work

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

(LBD) that allows recognition of this domain by coactivators of the p160/SRC family, an event that is critical for transcriptional activation [1,2]. p160/SRC coactivators contain intrinsic histone acetyl transferase (HAT) activity. Therefore, protein complexes containing HAT activity are recruited to target gene promoters by nuclear receptors in a ligand-dependent manner. Once associated with these promoters the HAT activity can mediate the chromatin remodeling events that accompany steroid hormone-regulated transcriptional enhancement [3].

The multisubunit DRIP (VDR-Interacting Protein) complex also binds to VDR in response to $1\alpha,25$ -dihydroxy vitamin D₃ [1,2]. This interaction requires the LBD of VDR and occurs in much the same manner as for the p160/SRC coactivators, resulting also in transcriptional enhancement. DRIP interacts with nuclear receptors through a single subunit called DRIP205, which anchors the rest of the DRIP complex to the LBD of VDR. Several subunits found in the DRIP complex are also part of the Mediator complex [4], which interacts with the C-terminal domain (CTD) of the RNA polymerase II complex, therefore forming the holoenzyme complex. Hence, it has been proposed that DRIP205 may be functioning as a coactivator by directly connecting VDR and the basal transcriptional machinery [1].

Recent reports indicate that p160/SRC and DRIP205 coactivators are recruited to hormone-regulated promoters in a sequential and cyclical manner, exhibiting high exchange rates [5,6,7,8,9]. However, the molecular mechanisms that control this rapid and sequential recruitment have not been assessed. Previous reports have shown that phosphorylation reactions regulate the transactivation potential of VDR in osteoblastic cells [10,11]. Here, we evaluate whether phosphorylation of human VDR on serine 208 modulates the ability of this receptor to interact with the coactivators SRC-1 and DRIP205. We find that phosphorylation of this residue *in vitro* by CKII enhances the interaction between VDR and DRIP205 without affecting the interaction with SRC-1.

Materials and Methods

Production of GST fusion proteins and *in vitro* protein phosphorylation

The pGEX-VDRS208D vector coding for a mutated version of VDR in which serine 208 has been mutated to aspartic acid, was generated by site direct mutagenesis of the pCDNA-VDR plasmid [12] using the primers 5'-caatctggatctggatgaagaagattcag-3' (forward) and 5'-ctgaatcttctcatccagatccagattg-3' (reverse). The mutated VDR gene was then cleaved with EcoRI and NotI and cloned into the pGEX5X3 vector (Pharmacia Biotech, Uppsala, Sweden). The fusion protein glutathione-S-transferase GST-VDR and GST-VDRS208D were obtained by expression in *Escherichia coli* BL21 as previously reported [12]. His tag-fused Casein Kinase II subunit alpha (CKII α) was produced in bacteria by expressing pT7HX-His-CKII α plasmid (kindly donated by Dr. Jorge Allende) and purified through Ni⁺⁺ NTA affinity chromatography (Novagen, Darmstadt, Germany) under manufacture's directions. 40 pmol of GST-fusion proteins were immobilized in 20 μ l of glutathione sepharose resin (Pharmacia Biotech, Uppsala, Sweden) and phosphorylated *in vitro* with 2 pmol of purified CKII α . The reaction was performed in 20 μ l of Reaction Buffer (150 mM KCl; 0.5 mM DTT; 5 mM MgCl₂; 20 mM Tris-HCl pH=7.4) supplemented with 100 μ M ATP (Calbiochem, La Jolla, CA) for 30 min at 30°C. The resin was then washed several times with 1 mL of Reaction Buffer and the GST-fusion proteins eluted as described before [12].

GST-pull down assay

GST pull-down assays were carried out as describe before [13]. Co-precipitated VDR, RXR α , SRC-1 and DRIP205 proteins were detected by Western blotting using specific antibodies [C-20 for VDR, D-20 for RXR α , M-255 for DRIP205 (Santa Cruz Biotechnology, Santa Cruz, CA) and clone 1135 for SRC-1 (Upstate Biotechnology, Lake Placid, NY)].

EMSA

Binding of GST-VDR and GST-VDRS208D to the osteocalcin (OC) VDRE was evaluated by EMSA as described before [14].

Results and Discussion

Previous reports indicated that phosphorylation reactions play an important role in the ability of VDR to upregulate transcription in a ligand-dependent manner [10,11]. It has been shown that human VDR can be phosphorylated in the serine residue 208 by the protein kinase CKII and that this modification increases the ability of this receptor to enhance transcription in response to 1 α ,25-dihydroxy vitamin D3 [15 and data not shown]. Therefore, we assessed whether phosphorylation in this serine residue 208 contributes to the cellular mechanisms that regulate the interaction of VDR with transcriptional coactivators.

We began our studies by evaluating the ability of bacterially produced VDR to bind coactivators *in vitro* that are present in nuclear extracts isolated from ROS 17/2.8 osteoblastic cells by GST-pull down assays. Recombinant full length VDR (GST-VDR, Figure 1A) or truncated forms of this protein where the C-terminal (GST-VDR Δ 111, Figure 1A) or N-terminal (GST-VDR Δ 1-111, Figure 1A) domains have been deleted were produced in bacteria as reported earlier [12]. Figure 1B shows that GST-VDR binds to RXR α , SRC-1, and DRIP205 proteins only in the presence of 1 α ,25-dihydroxy vitamin D3 (Figure 1B, compare lanes 2 and 3). As expected, these ligand-dependent interactions require an intact C-terminal LBD of VDR, as the GST-VDR Δ 1-111 mutant receptor protein, which lacks the N-terminal region of VDR, was capable of recruiting SRC-1 and DRIP205 only in the presence of 1 α ,25-dihydroxy vitamin D3 (Figure 1B, lanes 6 and 7). Accordingly, the GST-VDR Δ 111 mutant receptor, which lacks the LBD was unable to precipitate SRC-1 and DRIP205 in either the presence or absence of 1 α ,25-dihydroxy vitamin D3 (Figure 1B, lanes 2 and 3). Interestingly, we find that the GST-VDR Δ 1-111 mutant receptor protein consistently binds to both coactivators with higher affinity than GST-VDR, which contains the full-length VDR protein (Figure 1B, compare lanes 3 and 7). This result indicates that the LBD domain of VDR can function as an independent domain and does not require the DNA binding domain or the short N-terminal AF-1 region to recruit coactivators. In addition, both truncated VDR forms bind poorly to RXR α (Figure 1B, compare lanes 3, 5 and 7), confirming previous reports indicating that at least two domains of VDR are required for efficient ligand-dependent association with RXR α [16].

We next determined whether phosphorylation of VDR *in vitro* by CKII, changes the ability of this receptor to interact with coactivators SRC-1 and DRIP205 by GST-pull down assays. As shown in Figure 2B, GST-VDR interacts with both RXR α and DRIP205 in a 1 α ,25-dihydroxy vitamin D3-dependent manner (Figure 2B, compare lanes 3 and 4). Interestingly, when VDR is phosphorylated by CKII (data not shown) the interaction with DRIP205 is markedly increased (Figure 2B, compare lanes 4 and 6). This increased interaction is specific as the binding between phosphorylated VDR and its heterodimer partner RXR α remains similar to the level exhibited by unphosphorylated VDR (Figure 2B, middle panel, compare lanes 4 and 6). Similarly, this increased VDR-DRIP205 interaction was not due to

the presence of the GST moiety, as GST neither interferes with VDR-coactivator interactions (Figure 2B, lane 2) nor is phosphorylated under our experimental conditions (data not shown).

In contrast to DRIP205, the $1\alpha,25$ -dihydroxy vitamin D₃-dependent association of VDR with SRC-1 is neither enhanced nor down-regulated after phosphorylation by CKII (Figure 2C, compare lanes 3 and 4). Together, these results indicate that phosphorylation of VDR by CKII specifically increases the affinity of this receptor for the coactivator DRIP205.

To further demonstrate that a phosphorylated serine 208 residue in VDR is required for an increased interaction with DRIP205, we mutated this residue to aspartic acid (GST-VDRS208D, see Figure 3A), therefore mimicking the presence of a negatively charged phosphate group in this position [17]. This aminoacid change does not affect the ability of the VDRS208D protein to recognize its specific DNA target sequence (Figure 3). Thus, EMSA analyses show that the bacterially-produced GST-VDRS208D protein binds to the rat osteocalcin (OC) VDRE with an affinity equivalent to that of wild-type GSTVDR (Figure 3B, compare lanes 2 and 3). Interestingly, GST-pull down analyses demonstrate that the VDRS208D protein interacts with the DRIP205 coactivator with markedly higher affinity (Figure 3C, upper panel, compare lanes 3 and 4). This increased interaction is specific as the $1\alpha,25$ -dihydroxy vitamin D₃-dependent association of VDRS208D protein with SRC-1 and RXR α remains unaffected (Figure 3B, compare lanes 3 and 4).

Taken together, our results indicate that phosphorylation on serine residue 208 can modulate the affinity of VDR for the DRIP complex. Therefore, this covalent modification may have a role *in vivo* by regulating $1\alpha,25$ -dihydroxy vitamin D₃-mediated transcriptional enhancement. The results are in agreement with previous reports indicating the relevance of phosphorylation reactions in $1\alpha,25$ -dihydroxy vitamin D₃-dependent transcriptional regulation [10,11]. Moreover, these results offer a molecular explanation for how phosphorylation may directly influence the ability of VDR to alternatively interact with p160/SRC and DRIP205 coactivators during transcriptional upregulation of $1\alpha,25$ -dihydroxy vitamin D₃-responsive genes.

Acknowledgments

The authors thank Dr. Fernando Cruzat for the interesting discussions and critical reading of this manuscript. We also thank Dr. Jorge Allende for generously providing the pT7HX-His-CKII α plasmid. This work has been supported by grants from CONICYT-PBCT ACT-044 (to M.M) and NIH PO1 AR48818 (to G.S.S). G.A. was supported by fellowships from CONICYT and MECESUP-RED UCH0115. R.P. was supported by fellowships from CONICYT, MECESUP and FUNDACION ANDES.

References

1. Rachez C, Freedman L. Mechanisms of gene regulation by vitamin D(3) receptor: a network of coactivator interactions. *Gene*. 2000; 246:9–21. [PubMed: 10767523]
2. Christakos S, Dhawan P, Liu Y, Peng X, Porta A. New insights into the mechanisms of vitamin D action. *J. Cell. Biochem*. 2003; 88:695–705. [PubMed: 12577303]
3. Narlikar G, Fan H, Kingston R. Cooperation between complexes that regulate chromatin structure and transcription. *Cell*. 2002; 108:475–487. [PubMed: 11909519]
4. Kornberg R. Mediator and the mechanism of transcriptional activation. *Trends. Biochem. Sci*. 2005; 30:235–239. [PubMed: 15896740]
5. Shang Y, Hu X, DiRenzo J, Lazar M, Brown M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell*. 2000; 103:843–852. [PubMed: 11136970]

6. Sharma D, Fondell J. Ordered recruitment of histone acetyltransferases and the TRAP/Mediator complex to thyroid hormone-responsive promoters in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 2002; 99:7934–7939. [PubMed: 12034878]
7. Burakov D, Crofts L, Chang C, Freedman L. Reciprocal recruitment of DRIP/mediator and p160 coactivator complexes in vivo by estrogen receptor. *J. Biol. Chem.* 2002; 277:14359–14362. [PubMed: 11893728]
8. Metivier R, Penot G, Hubner M, Reid G, Brand H, Kos M, Gannon F. Estrogen receptor- α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell.* 2003; 115:751–763. [PubMed: 14675539]
9. Kim S, Shevde N, Pike J. 1,25-Dihydroxyvitamin D₃ stimulates cyclic vitamin D receptor/retinoid X receptor DNA-binding, co-activator recruitment, and histone acetylation in intact osteoblasts. *J. Bone. Miner. Res.* 2005; 20:305–317. [PubMed: 15647825]
10. Barletta F, Freedman LP, Christakos S. Enhancement of VDR-mediated transcription by phosphorylation: correlation with increased interaction between the VDR and DRIP205, a subunit of the VDR-interacting protein coactivator complex. *Mol. Endocrinol.* 2002; 16:301–314. [PubMed: 11818502]
11. Jurutka PW, Hsieh JC, Nakajima S, Haussler CA, Whitfield GK, Haussler MR. Human vitamin D receptor phosphorylation by casein kinase II at Ser-208 potentiates transcriptional activation. *Proc. Natl. Acad. Sci. U. S. A.* 1996; 93:3519–3524. [PubMed: 8622969]
12. Paredes R, Arriagada G, Cruzat F, Villagra A, Olate J, Zaidi K, van Wijnen A, Lian J, Stein G, Stein J, Montecino M. Bone-specific transcription factor Runx2 interacts with the 1 α ,25-dihydroxyvitamin D₃ receptor to up-regulate rat osteocalcin gene expression in osteoblastic cells. *Mol. Cell. Biol.* 2004; 24:8847–8861. [PubMed: 15456860]
13. Sierra J, Villagra A, Paredes R, Cruzat F, Gutierrez S, Arriagada J, Javed G, Olate J, Imschenetzky M, van Wijnen A, Lian J, Stein G, Stein J, Montecino M. Regulation of the bone-specific osteocalcin gene by p300 requires Runx2/Cbfa1 and the Vitamin D₃ receptor but not p300 intrinsic histone acetyltransferase activity. *Mol. Cell. Biol.* 2003; 23:3339–3351. [PubMed: 12697832]
14. Paredes R, Gutierrez J, Gutierrez S, Allison L, Puchi M, Imschenetzky M, van Wijnen A, Lian J, Stein G, Stein J, Montecino M. Interaction of the 1 α ,25-dihydroxyvitamin D₃ receptor at the distal promoter region of the bone-specific osteocalcin gene requires nucleosomal remodeling. *Biochem. J.* 2002; 363:667–676. [PubMed: 11964167]
15. Jurutka PW, Hsieh JC, MacDonald PM, Terpening CM, Haussler CA, Haussler MR, Whitfield GK. Phosphorylation of serine 208 in the human vitamin D receptor. The predominant amino acid phosphorylated by casein kinase II, in vitro, and identification as a significant phosphorylation site in intact cells. *J. Biol. Chem.* 1993; 268:6791–6799. [PubMed: 8384219]
16. Hsieh JC, Jurutka PW, Selznick SH, Reeder MC, Haussler CA, Whitfield GK, Haussler MR. The T-box near the zinc fingers of the human vitamin D receptor is required for heterodimeric DNA binding and transactivation. *Biochem. Biophys. Res. Commun.* 1995; 215:1–7. [PubMed: 7575575]
17. Hsieh JC, Jurutka PW, Nakajima S, Galligan MA, Haussler CA, Shimizu Y, Shimizu N, Whitfield GK, Haussler MR. Phosphorylation of the human vitamin D receptor by protein kinase C. Biochemical and functional evaluation of the serine 51 recognition site. *J. Biol. Chem.* 1993; 268:15118–15126. [PubMed: 8392065]

Figure 1. Bacterially produced GST-VDR proteins bind SRC-1 and DRIP205 coactivators in a $1\alpha,25$ -dihydroxivitamin D3-dependent manner

A) Schematic representation of the GST-VDR, GST-VDR Δ 1-111 and GST-VDR Δ 111 proteins utilized in the GST-pull down experiments. The DNA binding domain is indicated in black and the ligand-binding domain is represented as an open bar. 150 μ g of nuclear extracts (N.E.) from ROS 17/2.8 cells was incubated with either GST-VDR, GST-VDR Δ 1-111 or GST-VDR Δ 111 proteins (1.5 μ g each) bound to glutathione-Sepharose beads. Binding reactions were carried out in the presence (+) or absence (-) of $1\alpha,25$ -dihydroxivitamin D3 [$1\alpha,25(\text{OH})_2\text{D}_3$]. The precipitated proteins were resolved by SDS-PAGE and revealed by Western blotting using specific antibodies against GST (lower panel), RXR α (third panel), SRC-1 (second panel) and DRIP205 (upper panel). The combination of proteins analyzed in each binding reaction is indicated at the top of the blots. The positions of molecular mass markers are shown to the left of the blots.

Figure 2. Phosphorylation of VDR by CKII enhances interaction with coactivator DRIP205
 A) Schematic representation of the GST-fusion VDR protein (GST-VDR) utilized in these GST-pull down experiments. The phosphorylatable serine residue at position 208 is also indicated. B) 1.5 μ g of GST-VDR or *in vitro* phosphorylated GST-VDR by CK2 α (see Materials and Methods) bound to 20 μ L of glutathione sepharose resin were incubated with 150 μ g of ROS 17/2.8 nuclear extracts (N.E.). The co-precipitated proteins were analyzed by Western blot using specific antibodies against DRIP205, RXR α , and VDR. C) GST-VDR-pull down assays to evaluate the interaction VDR-SRC-1. Binding reactions were carried out in the presence (+) or absence (-) of 1 α ,25-dihydroxvitamin D3 [1 α ,25(OH) $_2$ D $_3$]. The combination of proteins analyzed in each binding reaction is indicated at the top of the blots. The positions of molecular mass markers are shown to the left of the blots.

Figure 3. Replacing serine residue 208 of VDR by aspartic acid results in enhanced VDR-DRIP205 interaction

A) Schematic representation of the bacterially-produced GST-VDR and GST-VDRS208D proteins evaluated in these experiments. B) Electro Mobility Shift Assay (EMSA) to evaluate the ability of the GST-VDR and GST-VDRS208D proteins to bind the OC VDRE. The combination of proteins included in each binding reaction is explained at the top of gel and the migration of the bound and unbound DNA is marked at the right of the gel. C) 1.5 μ g of GST-VDR or GST-VDRS208D proteins bound to 20 μ L of glutathione sepharose resin was incubated with 150 μ g of ROS 17/2.8 nuclear extracts. The co-precipitated proteins were analyzed by Western blot with specific antibodies. Binding reactions were carried out in the presence (+) or absence (–) of $1\alpha,25$ -dihydroxivitamin D3 [$1\alpha,25(\text{OH})_2\text{D}_3$]. The combination of proteins analyzed in each binding reaction is indicated at the top of the blots. The positions of molecular mass markers are shown to the left of the blots.