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1 α ,25-dihydroxy vitamin D₃ induces nuclear matrix association of the 1 α ,25-dihydroxy vitamin D₃ receptor in osteoblasts independently of its ability to bind DNA

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Abstract

1 α ,25-dihydroxy vitamin D₃ (Vitamin D₃) has an important role during osteoblast differentiation as it directly modulates the expression of key bone-related genes. Vitamin D₃ binds to the Vitamin D₃ receptor (VDR), a member of the superfamily of nuclear receptors, which in turn interacts with transcriptional activators to target this regulatory complex to specific sequence elements within gene promoters. Increasing evidence demonstrates that the architectural organization of the genome and regulatory proteins within the eukaryotic nucleus support gene expression in a physiological manner. Previous reports indicated that the VDR exhibits a punctate nuclear distribution that is significantly enhanced in cells grown in the presence of Vitamin D₃. Here, we demonstrate that in osteoblastic cells, the VDR binds to the nuclear matrix in a vitamin D₃-dependent manner. This interaction of VDR with the nuclear matrix occurs rapidly after vitamin D₃ addition and does not require a functional VDR DNA binding domain. Importantly, nuclear matrix-bound VDR colocalizes with its transcriptional coactivator DRIP205/TRAP220/MED1 which is also matrix-bound. Together these results indicate that after ligand stimulation the VDR rapidly enters the nucleus and associates with the nuclear matrix preceding vitamin D₃-transcriptional upregulation.

Keywords

Vitamin D receptor; nuclear matrix; vitamin D-dependent transcription

Introduction

1 α ,25-dihydroxy vitamin D₃ (here called vitamin D₃) is a physiologically significant mediator of biological control. Vitamin D₃ has an important function in bone formation and resorption, as it directly regulates the expression of osteogenic genes during development and bone remodeling (Christakos et al. 2003; van Driel et al. 2004). Vitamin D₃ binds to the vitamin D₃ receptor (VDR), a member of the superfamily of nuclear receptors, which in the cell nucleus interacts with specific elements (VDRE: Vitamin D Responsive Elements)

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located within regulatory regions of target genes. This protein-DNA interaction requires the DNA binding domain (DBD) of the VDR which consists of two highly-conserved zinc fingers (Haussler et al. 1995; Rachez and Freedman, 2000). The VDR binds DNA as a heterodimer with the 9-cis-retinoic acid receptor RXR α (Ross et al. 1992; MacDonald et al. 1993; Staal et al. 1996). As in other nuclear receptors, binding of the ligand induces conformational changes in the C-terminal ligand-binding domain (LBD) of the VDR. These changes establish competency for VDR interaction with coactivators of the p160/SRC family and with the DRIP (VDR-interacting protein)/TRAP/Mediator complex (Rachez and Freedman, 2000; Montecino et al. 2007), through the DRIP205/TRAP220/MED1 (here called DRIP205) subunit (Rachez et al. 1999). The coactivator NCoA-62/Skip can also interact in a ligand-dependent manner with the VDR, although in a domain of VDR that is different from that of p160/SRC and DRIP205 (Barry et al. 2003). Moreover, NCoA-62/Skip can form a ternary complex with VDR and SRC-1 to cooperatively mediate VDR-dependent transcription (Zhang et al. 2001).

Increasing evidence indicates that the architectural organization of nucleic acids and regulatory proteins within the nucleus supports functional interrelationships between nuclear structure and gene expression. Hence, components of nuclear structure are functionally linked to the organization and intranuclear distribution of regulatory information therefore allowing its correct utilization (Zaidi et al. 2005; Parada et al. 2004). The components of higher order nuclear architecture, including nuclear pores (Iborra et al. 2000), the nuclear matrix and subnuclear domains, contribute to subnuclear distribution and activities of genes and regulatory factors (Zaidi et al. 2005; Parada et al. 2004). Compartmentalization of regulatory complexes is illustrated by focal organization of PML bodies (Dyck et al. 1994), Runx bodies (Zeng et al. 1998; Zaidi et al. 2001), the nucleolus (Olson et al. 2002), and chromosomes (Ma et al. 1999; Croft et al. 1999), as well as by the punctate intranuclear distribution of sites for replication (Cook, 1999), DNA repair (Scully and Livingston, 2000), transcription (Verschure et al. 1999), and the processing of gene transcripts (Misteli and Spector, 1999; Smith et al. 1999; Wagner et al. 2003).

Previous reports have demonstrated that in mammalian cell systems the VDR exhibits a nuclear punctate signal distribution that is significantly enhanced upon ligand stimulation (Barsony et al. 1997; Paredes et al. 2004). It has been proposed that this may be an indication that in these cells the VDR is interacting with components of the nuclear architecture (Montecino et al. 2007). Recently it has been shown that within the osteocalcin gene promoter context there is tight functional association between the Runx2, master transcriptional regulator of the osteoblastic phenotype, and the VDR (Paredes et al. 2004). Thus Runx2 and VDR are components of the same nuclear complexes and colocalize at punctate foci within the nucleus of osteoblastic cells. As Runx2 is strongly associated with the nuclear matrix in bone-derived cells (Zeng et al. 1998; Choi et al. 2001), the VDR-Runx2 interaction raises the possibility that Runx2 is contributing to the retention of the receptor at specific intranuclear domains where VDR-mediated transcriptional control occurs.

It has been reported that transcriptional coactivators are associated with the nuclear matrix fraction. GRIP/SRC2 is associated with specific subnuclear domains (Bauman et al. 2001) that can colocalize with either the glucocorticoid receptor (GR) or the estrogen receptor (ER) upon activation by dexamethasone or estradiol, respectively (Reviewed by Day et al. 1999). Similarly, SRC1 is associated with specific subnuclear domains in human breast carcinoma cells (MCF-7) and is bound to the nuclear matrix fraction in response to estrogen (Stenoien et al. 2000). In addition, NCoA-62/Skip is bound to the nuclear matrix in osteoblastic cells that respond to vitamin D₃ (Zhang et al. 2003), further indicating that

nuclear regulatory complexes including VDR may be associated with subnuclear domains where vitamin D₃-dependent transcriptional control occurs.

Together, these results indicate that there is a necessity to establish whether the VDR is bound to the nuclear architecture in osteoblastic cells, to define the contribution of the transcription factor Runx2 in this VDR-nuclear matrix association, and to determine whether this process is vitamin D₃-dependent. Here, we report that in osteoblastic cells the VDR is bound to the nuclear matrix fraction in a vitamin D₃-dependent manner. This VDR-nuclear matrix association occurs rapidly after addition of the ligand and is independent of the Runx2 transcription factor. A significant percentage of the nuclear matrix-bound VDR molecules colocalize with the transcriptional coactivator DRIP205. Importantly, the ability of the VDR to specifically recognize its DNA target sequence is not required to bind to the nuclear matrix. Based on these results, we propose that binding of the VDR to the nuclear matrix represents an early step during osteoblast responsiveness to vitamin D₃ which does not require competency of the receptor to interact with DNA target sequences.

Materials and Methods

Cell culture

ROS 17/2.8 rat osteoblastic cells were cultured in F12 (Invitrogen) media as previously described (Majeska et al. 1980). The media was supplemented with 5% fetal bovine serum (Hyclone). Saos-2 human osteoblastic cells were cultured in McCoy 5A (Sigma-Aldrich) media, supplemented with 10% fetal bovine serum. MCF-7 human breast cancer cells were cultured in DMEM/F12 (Hyclone) supplemented with 10% fetal bovine serum. Runx2-mouse osteoblastic cells were cultured as previously described (Javed et al. 2008). 100 U/ml of penicillin G (Invitrogen) and 100 µg/ml of streptomycin (Invitrogen) were added to each media. 293QB1 cells were cultured in DMEM (Invitrogen) supplemented with 5% fetal bovine serum and 25 µg/ml gentamycin (Invitrogen). 24 or 48 hours before hormone stimulation (vitamin D₃ or estradiol), the cells were incubated with the corresponding media supplemented with charcoal stripped serum (Hyclone). Primary rat osteoblasts (ROB) were isolated from the calvaria of 19 day rat embryos and grown as previously described (Owen et al. 1990).

Immunofluorescence microscopy

Cells were plated at a density of 5×10^4 per well on gelatin-coated cover slips. Proliferating osteoblastic cells were infected with GFP-VDR encoding adenoviruses (wild-type or mutants) for 24 hours and then treated with 10^{-8} M $1\alpha,25$ -dihydroxy vitamin D₃ (vitamin D₃) for 4 hours. Cells treated with vehicle were used as control. For immunofluorescence studies, cells were washed twice with ice-cold phosphate-buffered saline (PBS), fixed with 3.7% formaldehyde for 10 minutes on ice, and then permeabilized for 20 minutes with PBS containing 0.5% Triton X-100. Alternatively, cells were extracted for in situ nuclear matrix preparations as previously described (Javed et al. 2000). To detect endogenous VDR, DRIP205, Lamin B1 or overexpressed GFP-VDR proteins, cells were incubated overnight with a rabbit polyclonal antibody against VDR (dilution 1:100), a rabbit polyclonal antibody against DRIP205 (dilution 1:200) or a goat polyclonal antibody against Lamin B1 (dilution 1:500), respectively (Santa Cruz Biotechnology, Santa Cruz, CA). Also GFP-tagged VDR was detected with a mouse monoclonal antibody against GFP (dilution 1:1000) (Molecular Probes Inc). Fluorochrome-conjugated Alexa antibodies (Molecular Probes Inc) raised in donkey and directed against mouse, rabbit or goat IgG, were used as secondary antibodies (dilution 1:800). Cells were also stained with TOPRO-3 iodide (Molecular Probes Inc) to detect nuclear DNA. Coverslips were finally mounted on glass slides with VectaShield mounting medium (Vector Laboratories, Burlingame, CA) and analyzed using a Zeiss,

Pascal 5 axiovert 200 confocal microscope with LSM 5 3.2 image capture-analysis software for image acquisitions. The Metamorph imaging software was used to process the images.

Nuclear matrix preparations

After 24 hours of adenoviral infection Saos-2 cells were treated with 10^{-8} M $1\alpha,25$ dihydroxyvitamin D3 for 15, 30, and 45 minutes or 1, 2, 4 and 8 hours. MCF-7 cells were treated for 1 hour with 10^{-8} M β -estradiol. Cells were then subjected to biochemical fractionation using a protocol previously reported (Zaidi et al. 2001). Equivalent amounts of each fraction were then separated by electrophoresis using 6% and 10% SDS-PAGE. The separated proteins were then transferred to a nitrocellulose membrane and analyzed by Western blot. The different proteins were identified using the following antibodies: Anti SRC-1 clone 1135 (1:2000; Upstate Biotechnology, Rochester, NY), VDR C-20 (1:1000), Runx2 M-70 (1:1000), PKC MC5 (1:1000), Lamin B1 C-20 (1:1000), TRAP220/DRIP205 M-255 (1:1000), Brg1 G-7 (1:2000), p300 N-15 (1:500), estrogen receptor alpha (ER α) H-184 (1:1000) (Santa Cruz Biotechnology). The antibody against NcoA62/SKIP (1:4000) was generously provided by Dr. Paul MacDonald, Department of Pharmacology, Case Western Reserve University, Cleveland, OH.

Recombinant proteins, GST pull down assays and EMSA

The pGEX-VDRS51A plasmid encoding a mutated VDR (serine residue 51 mutated to alanine) was generated by site-directed mutagenesis of the pCMX-VDR plasmid (Umesono et al. 1991) using the primers 5'-cttcttcaggcgagccatgaagcggag-3' (forward) and 5'-ctccgcttcgatgctgcctgaagaag-3' (reverse). The pGEX-VDRS51G plasmid encoding a mutated version of VDR in which serine residue 51 is replaced by glycine was previously described (Paredes et al. 2004). The pGEX-VDRS208A plasmid encoding a mutated VDR where the serine residue 208 has been mutated to alanine, was generated by site-directed mutagenesis of the pCMX-VDR plasmid using the primers 5'-caatctggatctggctgaagaaga-3' (forward) and 5'-ctgaatcttctcactcagatccagattg-3' (reverse). The mutated VDR genes were amplified by PCR using the primers SVDRS Δ I (forward) and ASVDRN Δ I (reverse) previously described (Paredes et al. 2004). The PCR products were then cleaved with SalI and NotI and cloned into the pGEX5X1 vector (G. E. Healthcare). The fusion proteins GST-VDR, GST-VDR_{S51A}, GST-VDR_{S51G} and GST-VDR_{S208A} were obtained by expression in *Escherichia coli* BL21 as previously reported (Paredes et al. 2002). GST-pull down assays were performed as previously described (Sierra et al. 2003; Paredes et al. 2004). Precipitated VDR, Runx2 and DRIP205 proteins were detected by Western blot using specific antibodies. Binding of wild-type GST-VDR and mutant GST-VDR_{S51A}, GST-VDR_{S51G} and GST-VDR_{S208A} proteins to the rat osteocalcin (OC) VDRE was evaluated by EMSA as described before (Paredes et al. 2002).

Luciferase reporter assays

ROS17/2.8 cells grown in 24-well plates were transfected by the FUGENE 6 (Roche) method with 25 ng of the plasmid pOC-Luc encoding the rat osteocalcin gene promoter (1.1 Kb) driving the expression of the luciferase reporter gene (Sierra et al., 2003) and co-transfected with the vectors pCMX-VDRS51A and pCMX-VDRS51G. As a transfection control the pSV40-RL vector (1.25 μ g), encoding for Renilla Luciferase was also co-transfected. After 12 hours of transfection in media supplemented with 5% charcoal-stripped fetal bovine serum, the cells were treated with 10^{-8} M $1\alpha,25(\text{OH})_2\text{D}_3$ for 24 hours. The cells were then collected and assayed for Firefly and Renilla Luciferase activities (Promega, Madison, WI) in a luminometer.

Plasmids and Adenoviral vectors

To generate GFP-tagged versions of the wild-type and mutated VDR proteins, the corresponding VDR cDNAs were isolated from the pCMX-VDR vectors by cleavage with the restriction enzymes Asp718 and PmlI. These fragments were then subcloned into the Asp718 and SmaI sites of the pEGFP-C2 plasmid (Clontech, Takara Bio Company, Mountain View, CA). The GFP-fused VDR genes were isolated by cleavage with Eco47III and BamHI and subcloned into the SmaI and BglII sites of the adenoviral vector pDC316 (Microbix Inc. Ontario).

Adenoviral production

The recombinant adenoviral particles were produced using the AdMax™ system (Microbix Inc.). Briefly, 293QB1 cells (kindly donated by Dr. Paulette Conget, Universidad del Desarrollo, Santiago, Chile) were cotransfected with the pBHGlox(delta)E1,3Cre adenoviral genomic plasmid DNA and the pDC316-GFP-VDRs expression vectors. After 10 days, viral particles were collected from the media and the infection repeated several times until a highly-enriched viral fraction was obtained. Then, each adenoviral fraction was purified by the CsCl gradient centrifugation method (Csaky, 2001). The titer of the different virus fractions was estimated by the plate lysis method. Each viral fraction was also carefully evaluated in the different osteoblastic cells such that after the infections each VDR protein (wild-type and mutants) was expressed at comparable concentrations.

Results

VDR binds to the nuclear matrix fraction in a ligand-dependent manner

Previous reports have indicated that in different mammalian cell types VDR exhibits a nuclear punctate distribution that is significantly enhanced upon ligand stimulation (Barsony et al. 1997; Paredes et al. 2004). Also, VDR-related proteins of different molecular sizes were detected by Western blot using an anti VDR polyclonal antibody in nuclear matrix preparations isolated from rat and human genitourinary tissues (Nangia et al. 1998). These results raised the possibility that the VDR is specifically associated with components of the nuclear architecture in these cells and that this interaction may be ligand-dependent.

To address this hypothesis experimentally, we generated adenoviral vectors coding for wild-type or mutated forms of the VDR proteins fused to GFP. These adenoviral particles allow infection of a wide range of osteoblastic cells in culture with high efficiency (data not shown). Also, we developed conditions in which the infected cells express only moderate intracellular concentrations of the VDR-GFP fusion proteins. As shown in Figure 1, we find that the human osteoblastic cells Saos-2 (as well as other osteoblastic cell lines, but not shown) expressing VDR-GFP exhibit nuclear punctate patterns for VDR, most particularly when the cells are cultured in the presence of vitamin D₃ (Figure 1, compare left and right panels). The presence of this nuclear VDR punctate pattern is found whether the receptor is detected using anti VDR or anti GFP antibodies (Figure 1, upper and lower panels), as well as when GFP-VDR is detected directly in fixed cells (Figure 1, middle panel).

We next determined whether the receptor was associated with nuclear matrix preparations isolated from osteoblastic cell lines. In situ nuclear matrix preparations were isolated from Saos-2 cells infected with adenoviruses expressing GFP-VDR and cultured in the absence or presence of vitamin D₃ as described previously (Zaidi et al. 2001), and the interaction of GFP-VDR detected by immunocytochemistry. As shown in Figure 2A, we found that the VDR is retained in the nuclear matrix-intermediate filament fraction (NMIF) of osteoblastic cells exposed to vitamin D₃. In agreement with previous reports (Zhang et al. 2003), we do not detect significant association of VDR with the NMIF in cells that have been cultured in

the absence of the ligand (Figure 2A). The quality of our in situ NMIF preparations was confirmed by the absence of DNA staining (see Figure 2A, TOPRO staining) as well as by the presence of well established nuclear matrix-associated proteins (e. g., Lamin B1, see supplementary Figure S1).

We also used biochemical fractionation to assess the association of VDR with the nuclear matrix of osteoblastic cells (Bidwell et al. 1993; Merriman et al. 1994). We treated Saos-2 cell cultures with vitamin D₃ for different periods of time (from 15 minutes to 8 hours) and then isolated protein fractions according to the protocol described in Materials and Methods. The presence of VDR and other cellular proteins in the different nuclear fractions was determined by Western blot analyses using specific antibodies. In agreement with our immunocytochemistry analyses, VDR is not associated with the NMIF in cells grown in the absence of vitamin D₃ (Figure 2B, top panel). However, a short treatment with the ligand (e. g., 15 minutes) results in a significant enrichment of VDR in the nuclear matrix fraction (Figure 2B, top panel). Long treatments with vitamin D₃ (e. g., 8 hours) result in increased VDR protein bound to the NMIF. However, this increased interaction of VDR parallels a higher expression of the VDR protein in the osteoblastic cells (Figure 2B, upper panel see WC), confirming previous reports which indicate that long vitamin D₃ treatments induce higher expression of the VDR gene in osteoblastic cells (Arbour et al. 1993; Mahonen and Maenpaa, 1994; Zella et al. 2006).

Lamin B1 is a well-known component of the nuclear matrix fraction in eukaryotic cells (Stein et al. 2002). Accordingly, we detect Lamin B1 in all our NMIF and whole cell protein preparations either isolated from control or vitamin D₃-treated cells (Figure 2B, second panel). In contrast to VDR, the Lamin B1 protein is not found in other cellular fractions (CSK and DNase I) analyzed. Importantly, vitamin D₃ treatment neither alters the total Lamin B1 protein concentration nor its level of enrichment in the NMIF (Figure 2B). This result confirms the high quality of the biochemical extraction procedures and further supports the ligand-dependent association of VDR with the NMIF.

Several groups have shown that transcriptional enhancement mediated by the VDR involves the formation of nuclear complexes including transcription factors and coactivators, some of which interact with VDR in a ligand-dependent manner (reviewed in Rachez and Freedman, 2000; Montecino et al. 2007). Therefore, we next evaluated whether these co-regulators are also bound to our NMIF preparations and whether the presence of vitamin D₃ modulates their interaction with the nuclear matrix.

Brg1 is the catalytic subunit of the mammalian SWI/SNF chromatin-remodeling complex and has been shown to be localized in specific nuclear foci within mammalian cells (Stein et al. 2002). Brg1 has also been identified as a component of a multiprotein complex including VDR which is targeted to gene promoters (Kitagawa et al. 2003). We find that the Brg1 protein is partitioned among all of our osteoblastic cell protein fractions and that it is significantly enriched in the NMIF (Figure 2B, third panel). Importantly, this distribution pattern is not affected by treatment of the osteoblastic cells with vitamin D₃. The transcription coactivator p300 possesses histone acetyl transferase (HAT) activity and has been shown to be an important component of VDR-associated transcriptional regulatory complexes that modulate the expression of osteoblast-related genes in response to vitamin D₃ (Sierra et al. 2003; Paredes et al. 2004; Carvallo et al. 2008). Interestingly, we find that p300 is also enriched in the nuclear matrix fraction of osteoblastic cells, although in a vitamin D₃-independent manner (Figure 2B, fourth panel).

MacDonald and colleagues have recently described that the coactivator NCoA62/SKIP is targeted to the nuclear matrix fraction of osteoblastic cells where it colocalizes and interacts

with other known nuclear matrix components including mRNA splicing factors (Zhang et al. 2003). Our results not only confirm this previous finding but in addition establish that the association of NCoA62/SKIP with the NMIF is unaffected in cells stimulated with vitamin D₃ (Figure 2B, fifth panel).

It has been reported that in human breast cancer cells (MCF-7), estrogen treatment results in a rapid colocalization of the estrogen receptor (ER) and its coactivator SRC1 with the nuclear matrix fraction (Stenoien et al. 2000). Work from different groups indicates that in osteoblastic cells stimulation with vitamin D₃ induces a rapid and coordinated interaction of VDR/SRC1-containing complexes with target genes (Kim et al. 2005; Carvallo et al. 2008; Zhang et al. 2003). Therefore, we determined whether vitamin D₃-dependent transcriptional enhancement also promotes the retention of the SRC1 coactivator in the NMIF. As shown in Figure 2B (sixth panel), in the osteoblastic cells grown under control conditions (vehicle) SRC1 is only found enriched in the whole cell and soluble protein fractions (Figure 2B, sixth panel, see WC and CSK). Surprisingly, treatment with vitamin D₃ for either short or long periods of time (15 minutes to 8 hours) does not promote enrichment of SRC1 in the NMIF. It was important to demonstrate that this lack of SRC1 association with the nuclear matrix in osteoblastic cells is not due to minor differences between the biochemical fractionation procedure utilized here to obtain the NMIF and the approaches used previously (Stenoien et al. 2000). Hence, we applied our experimental procedure using MCF-7 cells and reproduced the findings reported by Mancini and collaborators (Stenoien et al. 2000). As shown in Figure 2C both ER α and SRC1 are found associated with the nuclear matrix fraction of MCF-7 cells grown in the presence of estradiol. Incubation of these same cells with either vehicle or vitamin D₃ does not result in significant interaction of ER or SRC1 with the NMIF (Figure 2C). Together, these results indicate that in osteoblastic cells vitamin D₃-induced association of VDR with the nuclear matrix does not involve stable recruitment of the SRC1 coactivator at this intranuclear compartment.

DRIP205 is a component of the DRIP/TRAP/Mediator complex which is a key regulator of RNA polymerase II activity (Kornberg, 2005). DRIP205 functions by connecting this complex with the ligand binding domain of several steroid receptors, including VDR (Rachez and Freedman, 2000), thus establishing a molecular bridge between the DNA-bound receptor and the basic transcriptional machinery. The DRIP205-VDR interaction is ligand-dependent and has been shown to be necessary during VDR-mediated transcriptional enhancement of several target genes in osteoblasts (Kim et al. 2005; Vaissanen et al. 2005; Carvallo et al. 2008). Interestingly, we find that DRIP205 is highly enriched in the nuclear matrix fraction of osteoblastic cells and that this association is vitamin D₃-independent (Figure 2B, seventh panel). Hence, treatment with the ligand does not alter the DRIP205-NMIF association. DRIP205 is also detected in the other cellular fractions analyzed (Figure 2B, seventh panel), suggesting that it may dynamically partition between the soluble and nuclear matrix subnuclear compartments.

The Runx2 transcription factor is a key regulator of skeletogenesis as it regulates the expression of several bone-related genes during osteoblast differentiation (Ducy et al. 1997; Banerjee et al. 1997; for a review see Lian et al. 2004). Runx2 is also bound to the nuclear matrix in bone-related cells and this association is required for Runx2 function in a physiological context (Zeng et al. 1998; Choi et al. 2001). As expected, we find that the Runx2 protein is highly enriched in our nuclear matrix preparations, and in agreement with previous reports (Bidwell et al. 1993; van Wijnen et al. 1993; Merriman et al. 1994) is also detected in the other cellular protein fractions analyzed (Figure 2B, eighth panel). These results not only further confirm the quality of our nuclear matrix biochemical preparations, but also demonstrate that binding of Runx2 to the NMIF is not modulated by vitamin D₃.

As a necessary control, we confirmed that other key cellular regulatory proteins that have been shown to participate in membrane to nucleus signaling pathways and in the modulation of nuclear receptor functions are not found associated with the NMIF. Thus, as shown Figure 2B (ninth panel) protein kinase PKC is mostly extracted as a soluble protein (CSK) and is not retained in our nuclear matrix preparations.

VDR and DRIP205 colocalize at the nuclear matrix fraction in osteoblasts treated with vitamin D₃

Because in osteoblastic cells exposed to vitamin D₃ both VDR and DRIP205 are found enriched in the nuclear matrix fraction (Figure 2B, first and seventh panels, respectively), it was important to determine whether these two proteins are colocalizing at the NMIF. As shown in Figure 3A, GST pull-down assays demonstrate that VDR and DRIP205 proteins interact only in the presence of vitamin D₃ (Rachez et al. 1999; Paredes et al. 2004). Similarly, we find that VDR and DRIP205 colocalize at discrete foci in the nuclei of osteoblastic cells (WC) treated with vitamin D₃ (Fig. 3B, upper panel). Moreover, it was determined that a significant fraction of the matrix-bound VDR colocalizes with DRIP205 in our in situ preparations of nuclear matrix (Figure 3B, lower panel). Together, these results indicate that both VDR and DRIP205 proteins can interact and form nuclear regulatory complexes within the context of the nuclear matrix in a ligand-dependent manner.

Association of VDR with the nuclear matrix in osteoblastic cells is independent of the nuclear matrix-bound Runx2 factor

The transcription factor Runx2 has been shown to serve as a scaffold for the assembly and organization of coregulatory proteins that mediate biochemical and architectural control of osteoblast-related gene activity (Lian et al. 2004; Zaidi et al. 2005). We have recently shown that within the osteocalcin gene promoter context there is a tight functional relationship between the Runx2 and the VDR-dependent pathways (Paredes et al. 2004). Runx2 and VDR are components of the same soluble nuclear complexes and colocalize at punctate foci within the nucleus of osteoblastic cells (Paredes et al. 2004). As shown in Figure 4A, Runx2 and VDR can interact directly by GST pull-down assays, either using recombinant bacterially-expressed purified Runx2 and VDR proteins (Figure 4A, left panel) or by precipitating Runx2 from nuclear extracts of ROS 17/2.8 osteoblastic cells using GST-VDR (Figure 4A, right panel). Runx2 is strongly bound to the nuclear matrix fraction in osteoblastic cells (Figure 2B), raising the possibility that the retention of VDR at the NMIF is mediated by Runx2. To experimentally address this possibility, we prepared in situ NMIF from Runx2-*null* osteoblastic cells isolated from Runx2-*null* mouse embryos (Bae et al. 2007). As shown in Figure 4B, the VDR is strongly concentrated in the nuclei of these cells after incubation with vitamin D₃, where it is distributed in discrete foci (Figure 4B, compare top left and right panels). In agreement with the human- and rat-derived osteoblastic cells previously analyzed, we find that VDR is bound to the nuclear matrix fraction of these mouse cells only after stimulation with vitamin D₃ (Figure 4B, compare left and right lower panels). These results indicate that the association of VDR with the NMIF is independent of the Runx2 transcription factor and further confirm that this VDR-NMIF interaction is ligand-dependent.

The association of VDR with the nuclear matrix does not require a functional DNA binding domain

It is well-established that ligand-bound VDR interacts with specific vitamin D₃ responsive elements (VDREs) located within regulatory regions of VDR target genes (Rachez and Freedman, 2000; Christakos et al. 2003; Montecino et al. 2007). These protein-DNA interactions require the VDR DNA binding domain (DBD) located at the N-terminal portion of the VDR protein, which is structured by two zinc-finger domains separated by a short

aminoacid sequence containing a highly conserved serine residue (Serine 51) (Haussler et al. 1995). It has been demonstrated that mutations in this VDR serine 51 residue result in a receptor molecule that is unable to bind DNA (e.g., mutation to glycine or to alanine) or to interact with the Runx2 transcription factor (e.g. to glycine) (Hsieh et al. 1993; Paredes et al. 2004). It was important to evaluate whether the ability of VDR to specifically recognize a VDRE was also relevant for its interaction with the nuclear matrix in osteoblastic cells. Therefore, we generated adenoviruses encoding GFP-tagged VDR isoforms carrying mutations of serine 51 to either glycine or alanine and determined the ability of these VDR proteins to interact with the nuclear matrix. As shown in Figure 5A, when rat-derived normal primary diploid osteoblasts are infected with the different VDR-coding adenoviruses, live cells show expression (detecting GFP by fluorescence microscopy) of wild-type (left panel), mutant VDR_{S51A} (middle panel), or VDR_{S51G} (right panel) GFP-fused VDR proteins. Both VDR_{WT} and VDR_{S51A} exhibit similar cellular distribution patterns (nuclei and cytoplasm) in cells grown in the absence of vitamin D₃ (Figure 5A, left and middle upper panels). Both molecules also become primarily nuclear after a short incubation with the ligand (Figure 5A, left and middle lower panels). Intriguingly, the VDR_{S51G} mutant protein exhibits a primarily nuclear localization, independent of whether the cells are exposed to vitamin D₃ (Figure 5A, left upper and lower panels).

In agreement with previous reports (Hsieh et al. 1993; Paredes et al. 2004) mutation of serine 51 prevents the VDR from binding to the osteocalcin VDRE in gel mobility shift assays (EMSA, Figure 5B) using GST-fused recombinant wild-type and mutant VDR proteins expressed in bacteria (Paredes et al. 2002 and see supplementary Figure S2). As a necessary control in this assay, we demonstrate that mutation of another serine residue (VDR_{S208A}) located towards the C-terminus of the VDR protein and distant from the DBD, does not interfere with dimerization of the VDR with recombinant RXR α and the subsequent binding of the VDR/RXR heterodimer to the OC VDRE probe (Figure 5B). Additionally, we show that both VDR_{S51A} and VDR_{S51G} mutant proteins inhibit vitamin D₃-dependent enhancement of the rat OC promoter activity when transiently co-expressed in rat-derived ROS17/2.8 osteoblastic cell lines (Figure 5C). Both mutant VDR proteins were expressed in osteoblastic cells at comparable concentrations, as determined by Western blot analyses (data not shown). This result confirms that both VDR mutant proteins function as dominant negative mutants as they can assemble regulatory complexes but are unable to recruit them to target promoters (Hsieh et al. 1993; Paredes et al. 2004).

Interestingly, the adenovirus-encoded VDR_{S51A} and VDR_{S51G} mutant proteins retain their ability to interact with the nuclear matrix of osteoblastic cells in a vitamin D₃-dependent manner (Figure 5D). Thus, both GFP-fused VDR mutant proteins are detected by immunocytochemistry as associated with the NMIF in human-derived osteoblastic cells that are grown in the presence of vitamin D₃ (Figure 5D). Similar to the wild-type VDR (Figure 3B) we find that both mutant VDR proteins colocalize with the coactivator DRIP205 in situ NMIF preparations of osteoblastic cells treated with vitamin D₃ (Figure 6). Together these results indicate that ligand-dependent interaction of VDR with the nuclear matrix is independent of the ability of this receptor to specifically bind to DNA and to regulate osteoblast-related gene transcription. Importantly, as the VDR_{S51G} mutant protein also requires vitamin D₃ in the media to become associated with the NMIF (Figure 5D, right panels) we propose that a primarily nuclear localization of the VDR is not sufficient to promote interaction of this receptor with the nuclear matrix in the absence of the ligand.

Discussion

Increasing evidence indicates that the architectural organization of the genome and regulatory proteins within the nucleus supports functional interrelationships between nuclear

structure and gene expression (Zaidi et al. 2005; Parada et al. 2004). Previous reports indicated that the VDR exhibits a punctate nuclear distribution that is significantly enhanced after exposure of cells to vitamin D₃ (Barsony et al. 1997; Paredes et al. 2004). Hence, it was proposed that this distribution may indicate an association of VDR with components of the nuclear architecture which could be relevant during vitamin D₃-dependent gene expression control (Montecino et al. 2007). Here, we demonstrate that in osteoblastic cells, the VDR binds to the nuclear matrix in a vitamin D₃-dependent manner. This VDR-NMIF interaction occurs rapidly after addition of vitamin D₃ and does not require a functional VDR DNA binding domain. A significant fraction of the NMIF-bound VDR molecules are found colocalizing with the transcriptional coactivator DRIP205, which has been shown to interact with the VDR in a highly dynamic ligand-dependent manner (Rachez and Freedman, 2000) to upregulate transcription of bone-related target genes (Paredes et al. 2004; Kim et al. 2005; Carvallo et al. 2008). DRIP205, as well as the previously described NcoA62/Skip coactivator (Zhang et al. 2003), is bound to the nuclear matrix of osteoblastic cells in the absence of vitamin D₃, indicating that the association of VDR and these transcriptional coactivators with components of the nuclear architecture is controlled through different mechanisms.

We find that the p160 family coactivator SRC-1 is not bound to the nuclear matrix fraction in osteoblastic cells, whether grown in the presence or absence of vitamin D₃. SRC-1 was shown previously to colocalize with the estrogen receptor (ER) at subnuclear compartments in the human breast cancer cell line MCF-7 incubated with estradiol (Stenoien et al. 2000). Here, we find that in these same MCF-7 cells SRC-1 and ER proteins become associated with the nuclear matrix fraction after a short treatment with estradiol. Together, these results indicate that the association of SRC-1 with subnuclear structural components may be a cell- and ligand-specific event.

Paschal and colleagues have reported that upon androgen stimulation the androgen receptor (AR) is transiently bound to a subnuclear compartment. This transient association appears to be facilitating protein-protein interactions between the receptor and coactivators localized at these subnuclear domains before ligand-induced transcription of target genes occurs (Black and Paschal, 2004). Interestingly, these authors find that specific mutations in the DBD that prevent the AR from interacting with its DNA element result in the arrest of AR at specific subnuclear foci and strong colocalization with transcriptional coactivators. This distribution pattern, however, was not found when other nuclear receptors were analyzed, including the glucocorticoid receptor, retinoic acid receptor and thyroid receptor. Hence, these results raise the possibility that different nuclear receptors may follow alternative subnuclear distribution pathways once activated by their cognate ligands. We find that single aminoacid mutations in the DBD of VDR (S51) that prevent direct DNA sequence-specific recognition and vitamin D₃-mediated transcriptional enhancement in osteoblastic cells, do not affect the ability of this receptor to interact with the nuclear matrix fraction in a ligand-dependent manner. Moreover, these mutations do not affect the ability of VDR to colocalize with the transcriptional coactivator DRIP205 within the nuclear matrix context. These results indicate that both vitamin D₃-mediated activities of VDR, nuclear matrix association and DNA binding-transcriptional activation, represent two independent and perhaps sequential, events occurring in osteoblastic cells exposed to the ligand.

It was previously shown that the VDR_{S51G} mutation introduces a high degree of flexibility within the two zinc-finger structure of the DBD which prevents binding to DNA and interaction with other transcriptional regulators (Hsieh et al. 1993; Haussler et al. 1995; Paredes et al. 2004). Similarly, this increased structural flexibility may be affecting the interaction of VDR with the nuclear export machinery, as this residue is located within a highly conserved sequence that has been shown to be responsible for the exit of other

nuclear receptors from the nucleus (Black et al. 2001; Prufer and Barsony, 2002; Saporita et al. 2003). Accordingly, we find that the VDR_{S51G} protein exhibits a robust nuclear localization in all the osteoblastic and non-osteoblastic (not shown) cells so far tested. Taking advantage of this mutant VDR isoform, we confirmed that nuclear localization of VDR is required but not sufficient for association with the nuclear matrix, as the VDR_{S51G} is not retained at the NMIF in the absence of ligand.

The mechanism for targeting the VDR to subnuclear compartments has not been defined. Unpublished results from our group indicate that there is a domain located within the C-terminal half of the VDR protein that is required for retention at the nuclear matrix in a ligand-dependent manner. In addition, it remains to be established whether specific signaling pathways, perhaps induced by vitamin D₃ through non-genomic actions (Norman et al. 2004) are also contributing to VDR subnuclear localization. It has been recently reported that in vitamin D₃-treated osteoblastic cells VDR accumulates in the nucleus due to a protein-protein interaction with the tumor suppressor BRCA1 (Deng et al. 2009). Hence, in osteoblastic cells in which BRCA1 expression has been depleted, vitamin D₃-dependent accumulation of VDR in the nucleus is significantly reduced. Interestingly, BRCA1 has been shown to be localized in subnuclear domains (Scully et al. 2000) and bound to the nuclear matrix (Huber and Chodosh, 2005), raising the possibility that it may be contributing to the targeting of the VDR to subnuclear structural components in vitamin D₃-treated cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. VDR exhibits a punctate distribution in osteoblastic cells

Human osteoblastic Saos-2 cells were plated on gelatin-coated coverslips and treated with 10^{-8} M vitamin D₃ ($1\alpha,25(\text{OH})_2\text{D}_3$) or vehicle (control) for 4 hours. Cells were fixed and processed for immunofluorescence analyses by confocal microscopy as described in Materials and Methods. The distribution of endogenous VDR was determined using an antibody against VDR (upper panel) and a secondary antibody conjugated with Alexa fluor-488. Alternatively, the cells were transduced with an adenovirus encoding for GFP-VDR and the distribution of GFP-tagged receptor determined by direct fluorescence of GFP (middle panel) or by immunofluorescence using an antibody against GFP and a secondary antibody conjugated with Alexa Fluor-488.

Figure 2. VDR is associated with the nuclear matrix in a ligand dependent manner

(A) Human osteoblastic Saos-2 cells were plated on gelatin-coated coverslips, transduced with an adenovirus encoding for GFP-VDR for 12 hours and then treated with 10^{-8} M vitamin D $_3$ (1 α ,25(OH) $_2$ D $_3$) or vehicle (control) for 4 hours. The cells were fixed (WC) or processed for an in situ preparation of the nuclear matrix-intermediate filament fraction (NMIF). The presence of GFP-VDR was detected by immunofluorescence and confocal microscopy analysis using an anti GFP antibody and a secondary antibody conjugated with Alexa Fluor-488. TOPRO-3 iodide (TOPRO) staining was utilized to evaluate DNA content (shown in squares at the bottom right of each panel). A phase-contrast image of each field (DIC) is also shown (see squares at the bottom left of each panel). (B) Saos-2 cells were treated with 10^{-8} M vitamin D $_3$ for 15, 30 or 45 minutes and 1, 2, 4 and 8 hours or with vehicle (–) for 8 hours and then subjected to biochemical fractionation as described in Materials and Methods. Equivalent amounts of protein from the whole cell (WC), cytoskeletal (including soluble nuclear proteins, CSK), DNaseI (after chromatin digestion), and nuclear matrix intermediate filament (NMIF) fractions were electrophoresed by SDS-PAGE and then analyzed by Western blot using specific antibodies against VDR, Lamin B1, Brg1, p300, NCoA62/SKIP, SRC-1, DRIP205, Runx2 and PKC β . (C) MCF-7 cells were treated for 1 hour with 10^{-8} M vitamin D $_3$ (1 α ,25(OH) $_2$ D $_3$), 17 β -estradiol (E $_2$) or vehicle (control) and biochemically processed as described above. The presence of SRC-1 and ER α in the different cellular fractions was determined by Western blot using specific antibodies.

Figure 3. VDR colocalizes with the coactivator DRIP205 in the context of the nuclear matrix
 (A) The ability of recombinant GST-VDR to interact in vitro with the DRIP205 coactivator present in nuclear extracts from Saos-2 cells is shown by GST pull-down assays. The presence of DRIP205 in the precipitated proteins was determined by Western blot. The different combinations of recombinant proteins and the presence of Vitamin D $_3$ (1 α , 25(OH) $_2$ D $_3$) are indicated at the top of the panel. Input corresponds to 10% of the total amount used in the assay. The position of a reference molecular mass marker is shown at the left of the blot. (B) Saos-2 cells were transduced with an adenovirus encoding GFP-VDR for 12 hours, treated with vitamin D $_3$ for 4 hours and then fixed (WC, upper panels) or processed to obtain an in situ preparation of the nuclear matrix-intermediate filament (NMIF, lower panels) fraction. The presence of GFP-VDR was detected by immunocytochemistry and confocal microscopy using an anti GFP antibody and a secondary antibody conjugated with Alexa Fluor-488. The presence of DRIP205 was determined using a specific antibody and a secondary antibody conjugated with Alexa Fluor-546. The merged images are presented at the right panel.

Figure 4. Association of VDR with the nuclear matrix is independent of the Runx2 transcription factor

(A) The ability of recombinant GST-VDR to interact in vitro with either recombinant His-Runx2 (left panel) or Runx2 present in nuclear extracts from ROS17/2.8 osteoblastic cells (right panel) is demonstrated by GST pull-down assays. Input corresponds to 10% of the total amount using in the interaction assay. The position of molecular mass markers is shown at the left of the blots. (B) The ability of GFP-VDR to interact with the nuclear matrix in Runx2-*null* mouse osteoblastic cells was determined as described in Materials and Methods. Runx2-*null* cells plated on gelatin-coated coverslips were transduced with an adenovirus encoding for GFP-VDR for 12 hours, treated with 10^{-8} M vitamin D₃ ($1\alpha, 25(\text{OH})_2\text{D}_3$) or vehicle (control) for 4 hours, and then fixed (WC, upper panels) or processed to obtain an in situ preparation of the nuclear matrix-intermediate filament (NMIF, lower panels) fraction. The presence of GFP-VDR was determined by immunofluorescence and confocal microscopy using an anti GFP antibody and a secondary antibody conjugated with Alexa Fluor-488. As a control, the simultaneous presence of Lamin B in the samples was determined using an anti Lamin B specific antibody and a secondary antibody conjugated with Alexa Fluor-546. TOPRO-3 iodide (TOPRO) staining was utilized to detect DNA content (squares at the bottom right of each panel). A phase contrast light image (DIC) is also shown at the bottom left of each panel.

Figure 5. Association of VDR with nuclear matrix is independent of its ability to interact with DNA

(A) Primary fetal rat osteoblast cultures were transduced with adenoviruses encoding GFP-VDR_{wt} (left panel), GFP-VDR_{S51A} (middle panel) or GFP-VDR_{S51G} (right panel) for 24 hours and then treated with 10^{-8} M vitamin D₃ ($1\alpha,25(\text{OH})_2\text{D}_3$) or vehicle (control) for 4 hours. The cellular distribution of the different GFP-VDR proteins was determined in live cells by directly detecting the fluorescence of the GFP moiety in a fluorescence microscope. (B) The ability of VDR_{wt}, VDR_{S51A}, VDR_{S51G} and VDR_{S208A} proteins to bind DNA was analyzed by EMSA. The different versions of the GST-fused VDR proteins were purified from bacteria and incubated in the presence of recombinant GST-RXR α with a ^{32}P -labeled probe that includes the rat osteocalcin (OC) VDRE. The migration of the free labeled probe and the VDR/RXR-DNA complexes is indicated at the right of the gel. * Indicates a non-specific protein-DNA complex. The combination of proteins analyzed in each lane is indicated at the top of the gel. (C) The inability of the mutated VDR_{S51A} and VDR_{S51G} proteins to mediate vitamin D₃-dependent upregulation of the OC promoter is analyzed by transient transfection experiments. ROS 17/2.8 cells were transfected with pOC-Luc and cotransfected with increasing amounts of pCMX-VDRS51A (upper panel) or pCMX-VDRS51G (lower panel) vectors for 12 hours and then treated with 10^{-8} M vitamin D₃ (black bars) or vehicle (white bars) for 24 hours. Firefly luciferase activity was measured and normalized to Renilla luciferase activity. The graph corresponds to a representative analysis of 4 independent experiments. Each bar represents the average of three measurements and the corresponding standard deviation ($n=3$ $p<0.01$). (D) The ability of the VDR mutant proteins to interact with the nuclear matrix is analyzed. Saos-2 cells were transduced with adenoviruses encoding GFP-VDR_{S51A} (left panel) or GFP-VDR_{S51G} (right panel) for 12 hours, treated for 4 hours with 10^{-8}M vitamin D₃ ($1\alpha,25(\text{OH})_2\text{D}_3$) or vehicle (control), and then fixed (WC, upper panels) or processed to obtain an in situ preparation of

the nuclear matrix-intermediate filament (NMIF, lower panels) fraction. The presence of the mutant VDR proteins and DNA was determined as described in figure legends 2 and 4.

Figure 6. DNA binding VDR mutants colocalize with the coactivator DRIP205 within the context of the nuclear matrix

Human osteoblastic Saos-2 cells were transduced with adenoviruses encoding GFP-VDR_{S51A} (upper panel) or GFP-VDR_{S51G} (lower panel) for 12 hours, treated with 10^{-8} M vitamin D₃ ($1\alpha,25(\text{OH})_2\text{D}_3$) for 4 hours and then processed for an in situ NMIF preparation. The presence of the mutant VDR and DRIP205 proteins was determined as described in figure legend 3. The merge of the images is shown at the right of each panel.