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The Importance of Nuclear Import in Protection of the Vitamin D Receptor from Polyubiquitination and Proteasome-Mediated Degradation

Sara Peleg* and Cuong V. Nguyen

Department of Endocrine Neoplasia and Hormonal Disorders, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

Others and we previously showed that the vitamin D receptor (VDR) is subject to degradation by the 26S proteasome and that treatment with 1,25-dihydroxyvitamin D_3 ($1,25D_3$) inhibited this degradation. In the present study, we found that in osteoblasts, but not in intestinal epithelial cells, the VDR was susceptible to degradation by the 26S proteasome. The subcellular site for degradation of the VDR in osteoblasts is the cytoplasm and the site for ligand-dependent protection of the VDR from the 26S proteasome is the chromatin. These direct relationships between nuclear localization and protection of the VDR from 26S proteasome degradation led us to hypothesize that the unoccupied cytoplasmic VDR is a substrate for polyubiquitination, which targets VDR for degradation by the 26S proteasome, and that nuclear localization has the ability to protect the VDR from polyubiquitination and degradation. To test these hypotheses, we used Cos-1 cells transfected with human VDR and histidine-tagged ubiquitin expression vectors. We found that unoccupied VDR was polyubiquitinated and that $1,25D_3$ inhibited this modification. Mutations in the nuclear localization signal of VDR (R49W/R50G and K53Q/R54G/K55E) or in the dimerization interface of VDR with retinoid X receptor (M383G/Q385A) abolished the ability of $1,25D_3$ to protect the VDR from polyubiquitination, although these mutations had no effect on the ligand-binding activity of VDR. Therefore, we concluded that in some cellular environments unoccupied cytoplasmic VDR is susceptible to polyubiquitination and proteasome degradation and that ligand-dependent heterodimerization and nuclear localization protect the VDR from these modifications. J. Cell. Biochem. 110: 926–934, 2010.

KEY WORDS: VITAMIN D RECEPTOR; OSTEOBLASTS; INTESTINE; POLYUBIQUITINATION; 26S PROTEASOME; NUCLEAR LOCALIZATION

The actions of the hormone 1,25-dihydroxyvitamin D_3 ($1,25D_3$) are transduced through the vitamin D receptor (VDR), a transcription factor that regulates gene expression by binding to specific DNA response elements usually localized in the promoters of target genes [Carlberg and Polly, 1998; Haussler et al., 1998; Rachez and Freedman, 2000]. Ligand binding is associated with conformational changes in VDR protein that facilitate its nuclear import, interaction with dimerization partners (retinoid X receptors, RXRs), binding to DNA, and interaction with co-regulators of transcription, including p160 co-activators and the VDR-interacting protein (DRIP 205) complex [Rachez and Freedman, 2000; Lee et al., 2001; Shaffer and Gewirth, 2004]. Although ligand binding is a critical step in transcriptional activation of the VDR, cellular factors that control the amount of VDR (through synthesis and degradation), the nature of the nuclear localization (import/export) machineries, and the relative abundance and turnover of co-regulators (ratio of transcription co-activators to co-repressors) are important components that determine the transcriptional potency and efficacy of nuclear receptors, including the VDR in distinct cellular environments [Shang and Brown, 2002; Oda et al., 2007].

Our studies of mechanisms that contribute to cell-selective actions of vitamin D analogs have revealed that this cell selectivity may be attributed to distinct differences in nuclear import machineries and the mechanisms that regulate the abundance of VDR protein in two cellular environments: intestinal epithelial cells (IEC, represented by the colon carcinoma cell line, Caco-2) and osteoblasts (represented by the human fetal osteoblast line, hFOB) [Peleg et al., 2003; Ismail et al., 2004]. In Caco-2 cells, nuclear localization of the VDR was both ligand-independent and ligand-dependent, and the abundance of VDR was not affected by ligand

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*Correspondence to: Dr. Sara Peleg, Department of Endocrine Neoplasia and Hormonal Disorders, Unit 1461, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030.
E-mail: speleg@mdanderson.org

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treatment. In contrast, in hFOB cells, nuclear localization of the VDR was primarily ligand-dependent, and ligand treatment caused a rapid increase in the abundance of VDR. These differences were further underscored by confocal microscopy which measured, in live cells, nuclear import/export of unoccupied and ligand-occupied VDR in these two cell types [Klopot et al., 2007]. Additional studies of VDR properties in several osteoblastic cell lines [Masuyama and MacDonald, 1998; Jaaskelainen et al., 2000] revealed that the abundance of VDR was strongly upregulated by 26S proteasome inhibitors, whereas the VDR in Caco-2 cells responded poorly to such inhibitor [Ismail et al., 2004]. We hypothesized that there must be a cause-and-effect relationship between nuclear localization of the VDR and its susceptibility to 26S proteasome degradation and that in cellular environments such as IECs where the VDR is engaged by the nuclear import machinery even in the absence of a ligand, it may become resistant to 26S proteasome degradation; consequently its cellular abundance is constitutively high.

Although several osteoblastic cell lines were used in the aforementioned studies, only one IEC line was used as a prototype for these putative distinct mechanisms in our previous studies. Therefore, we had to determine whether we could extend these observations to other IECs, and so, to substantiate the presence of cell type-specific mechanisms for regulating nuclear import and susceptibility of VDR to proteasome degradation. Furthermore, because degradation by the 26S proteasome depends on poly-ubiquitination of the protein substrate, in most but not all cases [Coux et al., 1996], we asked whether susceptibility of VDR to proteasome degradation also depends on its polyubiquitination. Because we observed that in osteoblasts, ligand binding protected the VDR from degradation, we wished to determine if $1,25D_3$ does so by modulating ubiquitination of the VDR or by preventing access of the 26S proteasome to the VDR. Finally, we directly analyzed the relationship between nuclear localization and ubiquitination/26S proteasome degradation of the VDR. The experiments in this study demonstrated that the properties of VDR in Caco-2 cells are similar in other IECs, whereas its properties in hFOB cells are similar in other osteoblast cells. Furthermore, our experiments clearly showed that the VDR is a substrate for polyubiquitination and that the nuclear import machinery has the ability to protect it from this modification. Taken together, these results may explain the differential sensitivity of the intestines and skeleton to the VDR-mediated transcriptional activity of $1,25D_3$ [Suda et al., 2003].

MATERIALS AND METHODS

MATERIALS

The human osteoblast line, hFOB; human colon carcinoma cell lines, Caco-2, HT29, and LS174; and monkey kidney cell line Cos-1 were obtained from the American Type Culture Collection (Manassas, VA). The histidine-tagged ubiquitin expression vector was a gift from Dr. Tetsu Kamitani (The University of Texas M.D. Anderson Cancer Center, Houston, TX), The nuclear localization VDR mutants (R49W/R50G and K53Q/R54G/K55E) were a gift from Dr. Hsieh and Dr. Haussler [Hsieh et al., 1998]. $1,25D_3$ was a gift from Dr. Milan Uskokovic (Roche, Palo-Alto, CA). MG132 (Z-Leu-Leu-Leu-H) was purchased from Peptides International, Inc. (Osaka, Japan). VDR antibodies (MA1-710) were obtained from Affinity Bioreagents, Inc. (Golden, CO), and Ni^{++}-NTA agarose beads were from Qiagen (Valencia, CA).

CELL CULTURE

hFOB cells were grown under T-antigen expression-permissive conditions (33°C; 400 μg/ml G418) in F12/Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and were passed up to 23–25 times at 80% confluency. ROS 17/2.8 cells were grown in F12/DMEM and 10% FCS. Primary mouse osteoblasts (mOSB) were isolated from calvarias of 1- to 2-day-old mice as described previously [Yang et al., 2001] and grown for 48 h in α-minimal essential medium and 10% FCS before plating for the experiments described below. All of the intestinal cell lines were maintained in DMEM (high glucose) and 10% FCS. Fresh duodenal tissue specimens were prepared by dissecting 2 cm from the proximal small intestine of 2- to 3-month-old mice, slitting the lumen longitudinally, and rinsing the specimens twice in warm phosphate buffered saline (PBS) and then once in warm DMEM without serum. The dissected duodenal loops were used immediately in the experiments described below.

VDR ABUNDANCE AND STABILITY

To assess the effect of the proteasome inhibitor MG132 and $1,25D_3$ on the abundance of VDR, cells were plated in 10 cm Petri dishes in the indicated medium containing 10% FCS and grown until 2 days after confluency. On that day, the medium was removed, and fresh medium containing 10% serum with or without 10^{-7} M $1,25D_3$ and with or without 10 μM MG132 was added. Fresh duodenal loops were incubated in 60 mm Petri dishes in DMEM and 10% FCS with or without the indicated amount of $1,25D_3$, and duodenal mucosa specimens were harvested by scraping the lumen with a glass coverslip 1 h later. The effect of $1,25D_3$ on VDR abundance in the cultured cells was assessed after 1 h of incubation (unless otherwise indicated). The effect of MG132 on VDR abundance was assessed after 24 h of incubation.

To determine the approximate half-life of unoccupied and ligand-occupied VDR, the culture media containing hFOB, Caco-2, LS174, and HT29 cells were replaced with fresh media, and the cells were treated for 24, 3, and 1 h before harvesting with or without $1,25D_3$ (10^{-7} M) and with or without cycloheximide (10 μg/ml). This amount of cylcoheximide inhibited more than 90% of ^{35}S-methionine incorporation into trichloroacetic acid-precipitated proteins.

WHOLE-CELL AND TISSUE EXTRACTION

Upon completion of the incubations, the culture medium was aspirated, and each culture dish was washed with 15 ml of warm PBS. The cells were then scraped into 10 ml of ice-cold PBS and transferred to 15 ml tubes. The tubes were centrifuged at 800*g* for 10 min at 4°C; the PBS was decanted; cell pellets were resuspended in 500 μl of cold PBS, transferred to 1.5 ml tubes, centrifuged at 1,000*g* for 3 min, and the PBS was again decanted. An extraction buffer (0.4 M NaCl, 2 mM dithiothreitol, 25% glycerol, 20 mM HEPES, pH 7.4, 1.5 mM $MgCl_2$, 0.25 mM ethylene diamine tetra acetic acid (EDTA), 5 mM phenyl methyl sulfonyl fluoride, and 1×

complete protease inhibitor cocktail (Roche, Indianapolis, IN) was added to the cell pellet at a ratio of 2:1, and the cells were homogenized using 10 strokes with a Teflon pestle. The tubes containing cell homogenates were rocked for 1 h at 4°C and then centrifuged at 12,000*g* for 10 min at 4°C. Supernatants containing whole cell extracts were transferred to fresh tubes and stored at −80°C until used for further analysis. A similar procedure was used for preparation of homogenates and whole tissue extracts from duodenal mucosa. Aliquots containing equal amounts of proteins (10–50 μg) were denatured by heating in Laemmeli buffer and analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting with anti-VDR antibodies.

SUBCELLULAR FRACTIONATION

To assess the effect of ligand on subcellular localization of VDR, sequential extractions of the cells were performed to obtain the soluble cellular proteins (namely, cytosol) followed by extraction of chromatin-associated proteins, as described previously [Ismail et al., 2004]. Briefly, each of the cell lines (2 days after confluence) were scraped into ice-cold PBS and centrifuged for 5 min at 4°C at 800*g*. The cell pellets (80 μl each) were suspended by adding 300 μl of cytoskeletal buffer (10 mM PIPES, pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, and 1× EDTA-free protease inhibitor cocktail). The cell suspensions were incubated on ice for 10 min and then centrifuged at 6,000*g* for 20 s. The supernatants (cytosolic fractions) were then transferred to another set of tubes. Next, the pellets were resuspended in 300 μl of chromatin extraction buffer (10 mM PIPES, pH 7.0, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 1× EDTA-free protease inhibitor cocktail, and 400 IU of ribonuclease-free deoxyribonuclease I; Roche), and the tubes were rocked at room temperature for 30 min. Freshly prepared ammonium sulfate was added to the pellets at a final concentration of 250 mM, and the incubation was continued for 10 min at room temperature. The tubes were then centrifuged at 6,000*g* for 5 min, and the supernatants (chromatin fractions) were collected. Aliquots of the cytosolic and chromatin fractions were boiled for 5 min in Laemmeli buffer and then analyzed by using 12% SDS–PAGE and Western blotting with VDR antibodies.

POLYUBIQUITINATION ASSAYS

To determine whether VDR is a substrate for ubiquitination and how ligand binding or mutations regulate ubiquitination, wild-type, or mutant (AF-2 deletion mutants Δ410–427 and Δ403–427, the heterodimerization mutant M383G/Q385A, and nuclear localization mutants R49W/R50G and K53Q/R54G/K55E) [Liu et al., 1997; Hsieh et al., 1998; Liu et al., 2001] human VDR expression plasmids (50–100 ng per 10 cm dish) were transfected with or without histidine-tagged ubiquitin expression vector (0.5–1.0 μg per 10 cm dish) into Cos-1 cells using the DEAE dextran method as described previously [Liu et al., 1997]. Twenty-four hours after transfection, the cells were treated with $1,25D_3$ with or without 10 μM MG132. Twenty-four hours later, the cells were washed twice in cold PBS and lysed in 4 ml of guanidine HCl solution (6 M guanidine–HCl, 0.1 M NaH_2PO_4/Na_2HPO_4, pH 8, and 5 mM imidazole). Each cell lysate was incubated on a rocking platform with 100 μl of 50% slurry of Ni++-NTA agarose for 4 h at 4°C to capture histidine-tagged proteins. The cell lysates were then decanted after centrifugation at 800*g* for 2 min, and the agarose beads were washed once with 4 ml of the guanidine-containing lysis buffer and then three times with 4 ml each of a buffer containing 50 mM NaH_2PO_4/Na_2HPO_4, pH 8, 0.3 M NaCl, and 20 mM imidazole. Proteins bound to the beads were then extracted by boiling in 100 μl of Laemmeli buffer, and the denatured proteins were stored at −80°C for further analysis using SDS–PAGE and Western blotting with anti-VDR antibodies to detect polyubiquitinated VDR.

PULL-DOWN ASSAYS

To determine the effects mutations of the VDR had on its ability to fold in a conformation that permits ligand-dependent interaction with RXR (integrity of the dimerization interface) or with p160 co-regulators (integrity of the AF-2 core function), we prepared ^{35}S-labeled synthetic VDR by using the transcription and translation-coupled transcription/translation system (Promega, Madison, WI) and a plasmid (pGEM-4) containing either WT or mutant (M383G/Q385A, R49W/R50G, or K53Q/R54G/K55E) hVDR cDNA.

To determine the efficacy of $1,25D_3$ in inducing interaction of VDR with RXR or with glucocorticoid receptor-interacting protein (GRIP), we used glutathione-S-transferase (GST)-pull-down assays [Liu et al., 2001]. Each pull-down reaction containing 11 μl of PBSDP buffer (1 mM dithiothreitol and 10 mM phenylmethylsulfonyl fluoride in PBS), 3 μl of ^{35}S-labeled VDR, and 1 μl of ethanol or $1,25D_3$ (in ethanol) was incubated at ambient temperature for 15 min. Next, 3–5 μg of purified GST fusion protein and 20 μl of glutathione-sepharose beads (equilibrated in PBSDP buffer) were added to the reaction mixture, and the volume was brought up to 100 μl with the addition of NETND buffer (20 mM Tris–HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, and 1 mM dithiothreitol). The mixtures were incubated at 4°C for 1 h, and the beads were washed once with NETND buffer and twice with PBSDP buffer. The bound proteins were eluted from the beads by boiling in Laemmeli buffer for 3 min and analyzed using SDS–PAGE and autoradiography.

RESULTS

DIFFERENTIAL REGULATION OF THE ABUNDANCE OF VDR IN INTESTINAL CELLS AND OSTEOBLASTS

Previous reports described the ability of $1,25D_3$ to increase the abundance of VDR in osteoblasts and keratinocytes, and researchers postulated that this upregulation was primarily a result of protection from degradation by the 26S proteasome [Li et al., 1999; Jaaskelainen et al., 2000]. To determine whether this action of $1,25D_3$ is cell-type specific, we compared the effects of short-term treatment with $1,25D_3$ on VDR abundance in several osteoblast and IEC lines (Fig. 1A). We found that this treatment induced rapid accumulation of the VDR in all three osteoblastic lines tested (hFOB, MG63, ROS 17/2.8) and primary mOSBs but had no effect on the abundance of VDR in the IEC lines tested (Caco-2, HT29, and LS174) or in mouse duodenal mucosa. To determine whether the lack of response of IECs to $1,25D_3$ resulted from resistance to 26S

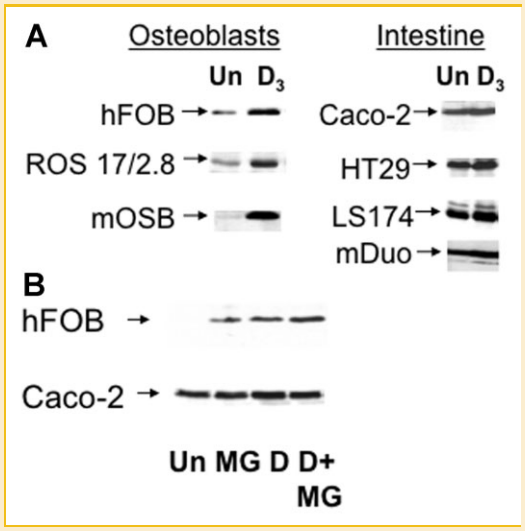

Fig. 1. Differential regulation of the abundance of VDR in osteoblasts and intestinal cells. A: VDR abundance was detected using Western blotting of whole cell extracts prepared from several osteoblast cell lines (hFOB, ROS 17/2.8, primary mOSBs) and IECs (Caco-2, LS174, HT29, fresh mouse duodenal mucosa [mDuo]) 1 h after treatment with vehicle (Un) or $1,25D_3$ (D_3 10^{-7} M). B: VDR abundance detected using Western blotting of whole cell extracts prepared from osteoblasts (hFOB) and intestinal cells (Caco-2) treated for 24 h with vehicle (Un; dimethyl sulfoxide), the proteasome inhibitor MG132 (MG; 10 μM), $1,25D_3$ (D; 10^{-7} M), or a combination of MG132 and $1,25D_3$ (D + MG).

proteasome degradation, we compared the susceptibility of the unoccupied VDR to 26S proteasome degradation in hFOB and Caco-2 cells (Fig. 1B). We found that in hFOB cells, treatment with the proteasome inhibitor MG132 dramatically increased its abundance, as been described for the response of VDR to treatment with proteasome inhibitors in other osteoblast-like cells (MG63 and ROS 17/2.8) [Masuyama and MacDonald, 1998; Jaaskelainen et al., 2000]. In contrast, the abundance of unoccupied VDR in Caco-2 cells did not change in response to MG132 treatment. These results confirmed that in these IECs, unoccupied VDR is resistant to 26S proteasome degradation whereas in osteoblasts, unoccupied VDR is susceptible to 26S proteasome degradation.

To determine whether changes in the abundance of VDR in osteoblasts were dependent on or independent of new protein synthesis, hFOB cells were treated with 10 μg/ml cycloheximide in the absence or presence of $1,25D_3$ for 1, 3, and 24 h. This experiment revealed that in these cells the approximate half-life of unoccupied VDR was less than 1 h (Fig. 2A), whereas ligand treatment increased the half-life to more than 3 h (Fig. 2B). Comparing increases in VDR abundance in ligand-treated cells in the absence and presence of cycloheximide (Fig. 2B,C) suggested that an increase in VDR abundance that occurred between 1 and 3 h was independent of new protein synthesis, whereas an increase in VDR abundance that occurred after 3 h, was mainly dependent on new protein synthesis. Taken together, these results suggested that an increase in VDR abundance in osteoblasts by treatment with $1,25D_3$ is subject to two regulatory mechanisms: protein stabilization and transcription.

To determine whether incubation with $1,25D_3$ had any effect at all on VDR stability in IECs, we repeated the experiments described above using Caco-2, HT29, and LS174 cells. Interestingly, we found that in all of these cells the half-life of either unoccupied or $1,25D_3$-occupied VDR was more than 3 h (similar to that of ligand-occupied VDR in osteoblasts), suggesting that $1,25D_3$ neither stabilize the VDR nor protect it from 26S proteasome degradation in IECs.

DIFFERENT MECHANISMS FOR NUCLEAR LOCALIZATION OF VDR IN OSTEOBLASTS/FIBROBLASTS AND INTESTINAL CELLS

Previous studies in our laboratory showed that unoccupied VDR in osteoblasts was localized primarily in the cytoplasm and that ligand treatment induced nuclear localization associated with an increase in the abundance of VDR (as described above) [Ismail et al., 2004]. In contrast, in Caco-2 cells, unoccupied VDR was localized in both the cytoplasm and chromatin, and treatment with $1,25D_3$ caused further accumulation of VDR in the chromatin. Therefore, in osteoblasts, the VDR apparently is subject to a ligand-dependent nuclear import mechanism, whereas in IECs, it is subject to both ligand-independent

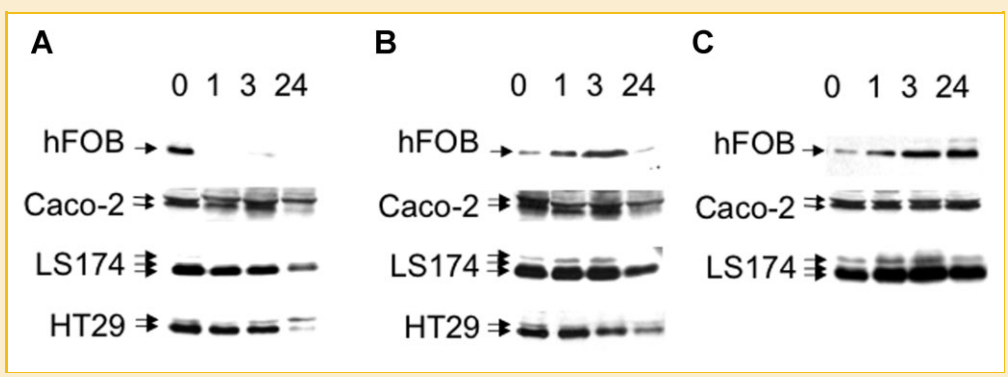

Fig. 2. Effect of the cell type and $1,25D_3$ on the half-life of VDR. A: VDR abundance was assessed using Western blotting of whole cell extracts prepared from vehicle-treated cells in the presence of 10 μg/ml cycloheximide (CHX) after incubation of osteoblasts (hFOB) and intestinal cells (Caco-2, HT29, and LS174) for 0, 1, 3, or 24 h. B: VDR abundance was assessed in cells co-treated with $1,25D_3$ (10^{-7} M) and cycloheximide as described in (A) for 0, 1, 3, or 24 h. C: VDR abundance was assessed in hFOB, Caco-2, and LS174 cells after treatment with $1,25D_3$ (10^{-7} M), for 0, 1, 3, or 24 h.

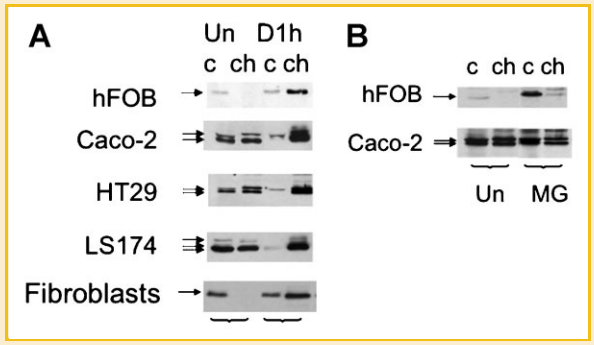

Fig. 3. Cell type-specific use of ligand-independent and -dependent nuclear import machineries. A: Subcellular fractions (c, cytosol; ch, chromatin) were prepared from osteoblasts (hFOB), fibroblasts, or intestinal cells (Caco-2, HT29, and LS174) 1 h after treatment with vehicle (U) or $1,25D_3$ (D; 10^{-7} M). SDS–PAGE and Western blotting were performed to assess VDR abundance. B: Subcellular fractions were prepared from osteoblasts (hFOB) and intestinal cells (Caco-2) 24 h after treatment with vehicle (Un) or MG132 (MG; 10 μM). SDS–PAGE and Western blotting were performed to assess VDR abundance.

and -dependent nuclear import mechanisms. To further substantiate the presence of distinct mechanisms for nuclear localization in osteoblasts/stroma and IECs, we analyzed subcellular localization of VDR in additional IECs (HT29 and LS174) and primary human fibroblasts (Fig. 3A). We found that in all of the IECs, unoccupied VDR was localized in both the cytosol and chromatin, and ligand treatment induced depletion of the VDR from the cytosol and increase of it in the chromatin, confirming the presence of both ligand-independent and -dependent nuclear import mechanisms of VDR in IECs. In contrast, in fibroblasts, as in osteoblasts, unoccupied VDR was localized only in the cytosol, and accumulation of VDR protein in the chromatin was strictly ligand-dependent. Taken together, these results substantiated the cause-and-effect relationship between nuclear localization of the VDR and its protection from 26S proteasome degradation and suggested that degradation of the VDR by the 26S proteasome occurs in the cytoplasm, whereas nuclear localization of VDR, either ligand-dependent or -independent has the potential to protect the VDR from degradation by the 26S proteasome.

To determine whether degradation of the VDR by the 26S proteasome indeed occurs in the cytoplasm, we repeated the subcellular fractionation of hFOB cells with or without treatment with the proteasome inhibitor MG132 (Fig. 3B). We found that this treatment caused accumulation of the unoccupied VDR protein exclusively in the cytosol, thus proving that the cytosol is the site of VDR degradation by the proteasome. In the same experiment, treatment with $1,25D_3$ alone, again caused an increase in VDR abundance, but only in the chromatin, whereas combined treatment with MG132 and $1,25D_3$ induced accumulation of VDR in both the cytosol and chromatin (data not shown). As expected, treatment with MG132 did not change the abundance of VDR in either the cytosol or chromatin in Caco-2 cells (Fig. 3B), further substantiating that cytoplasmic VDR in these cells must be resistant to 26S proteasome degradation. Thus, we propose that in the absence of ligand-independent nuclear import machinery, possibly regulated by importin 4 [Miyauchi et al., 2005], the VDR is susceptible to degradation by the 26S proteasome in the cytoplasm and that nuclear localization of VDR (ligand-dependent or -independent) is sufficient to protect the VDR from this degradation.

EVIDENCE THAT VDR IS A SUBSTRATE FOR POLYUBIQUITINATION

Because polyubiquitination is a common mechanism of targeting proteins for degradation by the 26S proteasome, we utilized an assay system to determine whether human VDR (hVDR) is subject to polyubiquitination and to map the amino acid residues necessary for polyubiquitination. To that end, we transfected Cos-1 cells with an hVDR expression plasmid and a histidine-tagged ubiquitin expression vector. We purified ubiquitinated VDR using affinity chromatography with Ni++-NTA agarose beads, which capture histidine-tagged proteins. We performed SDS–PAGE and Western blotting with anti-VDR antibodies to examine the proteins eluted from these beads for the presence of polyubiquitinated VDR. Using these assays, we found that the wild-type hVDR was a substrate for polyubiquitination (Fig. 4A). Because studies have shown that polyubiquitination of transcription factors is localized in transcription activation domains and that recruitment of the 26S proteasome component SUG1 to the VDR depends on the presence of activation function 2 (AF-2) core residues (helix 12 residues) [Masuyama and MacDonald, 1998], we sought to determine whether sequences important for VDR ubiquitination were localized at or near AF-2 core residues. We found that deletion of amino acid residues 410–427 (entire AF-2 core residues) had no effect on ubiquitination of VDR. However, deletion of amino acid residues 403–427 diminished

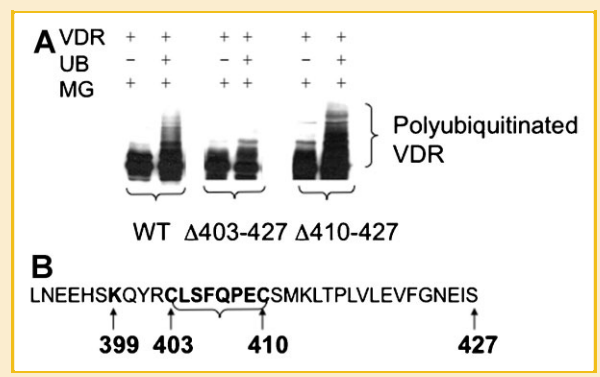

Fig. 4. Mapping of amino acid residues required for polyubiquitination of the VDR. A: Plasmids expressing WT or C-terminal deletion mutants (Δ410–427 and Δ403–427) of hVDR were transfected into Cos-1 cells with or without histidine-tagged ubiquitin expression vector (UB). Twenty-four hours later, the cells were treated with 10 μM proteasome inhibitor MG132 (MG)' and 48 h after transfection, the cells were lysed in guanidine–HCl buffer, and histidine-tagged proteins were absorbed by affinity purification with Ni++-NTA agarose beads. Unmodified and polyubiquitinated VDRs were detected using Western blotting with anti-VDR antibodies in samples eluted from the beads and separated using SDS–PAGE. Of note is the significant amount of unubiquitinated VDR that was eluted from the beads. B: Amino acid sequence of the VDR C-terminus. Highlighted are the seven amino acid residues necessary for polyubiquitination of the VDR and the lysine residue immediately upstream of this region.

polyubiquitination of VDR (Fig. 4A). These results suggested that the sequences required for polyubiquitination of VDR are localized between amino acid residues 403 and 410. Because these residues do not resemble PEST (a proline, glutamic acid, serine-, and threonine-rich consensus motif for ubiquitination-promoting phosphorylation) or contain a lysine residue (which is required for covalent ligation of ubiquitin by E3 ligase) (Fig. 4B) [Rogers et al., 1986; Ciechanover, 1994], neither phosphorylation nor polyubiquitination are likely to occur between residues 403 and 410. However, because a lysine residue is localized immediately upstream from amino acid 403 (K399), it is possible that the amino acid sequences downstream from this residue are necessary to maintain a conformation that exposes residue K399 to an E3 ligase.

EFFECT OF LIGAND BINDING AND NUCLEAR LOCALIZATION ON POLYUBIQUITINATION OF VDR

To determine whether ligand protection of the VDR from 26S proteasome degradation is a result of inhibition of the upstream event, polyubiquitination, we repeated the experiments described above with and without treatment of transfected Cos-1 cells with $1,25D_3$ (Fig. 5). We found that treatment with $1,25D_3$ inhibited polyubiquitination of the VDR in a dose-dependent manner, thus providing evidence that ligand protection of the VDR from 26S proteasome degradation (as described previously in osteoblasts) is mediated, at least in part, by inhibition of polyubiquitination.

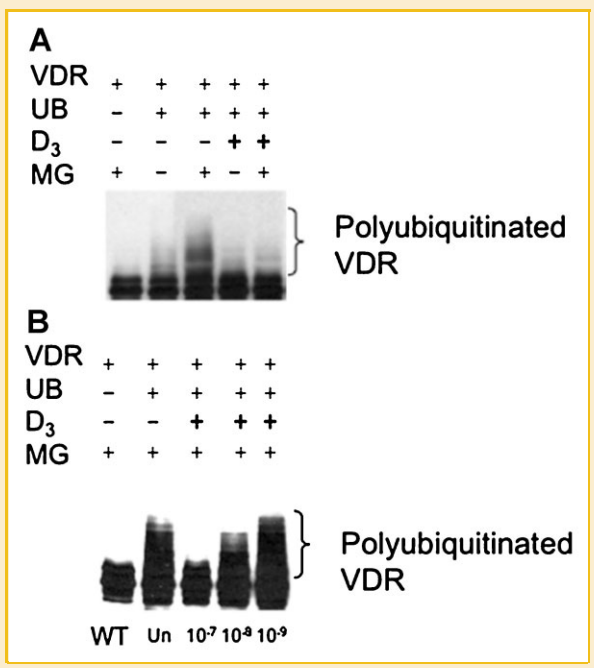

Fig. 5. Inhibition of polyubiquitination of VDR by treatment with $1,25D_3$. A: Polyubiquitination of VDR was assessed in Cos-1 cells transfected with the WT VDR and the histidine-tagged ubiquitin expression vectors (UB). The transfected cells were treated with or without MG132 (MG; 10 μM), $1,25D_3$ (D_3; 10^{-7} M), or a combination of the two. Extraction of cells and detection of immunoreactive VDR species were performed as described in Materials and Methods Section and in Fig. 4. B: Effect of the concentration of ligand on inhibition of VDR polyubiquitination. The experiment was performed with transfected Cos-1 cells as described above in (A), and the cells were treated with vehicle of with the indicated doses (10^{-9}–10^{-7} M) of $1,25D_3$ in the presence of MG132 for 24 h before they were lysed.

Several studies have documented that an early step in transcriptional activation of the VDR by its ligand is nuclear import [Yasmin et al., 2005]. Because our results thus far have strongly suggested that there is a cause-and-effect relationship between nuclear localization and protection of the VDR from 26S proteasome degradation, we first sought to determine whether the disruption of nuclear localization signal of VDR has any effect on its polyubiquitination or on the ligand ability to protect the VDR from this modification. Using two nuclear localization mutant VDRs (R49W/R50G and K53Q/R54G/K55E) [Hsieh et al., 1998], we found that loss of nuclear localization signal had no effect on the ligand-dependent functions of the VDR's LBD (interaction with GRIP, an AF-2 function, and interaction with RXR–LBD, a DNA-independent heterodimerization function; Fig. 6A,B) [Liu et al., 2001]. However, these mutations abolished the ability of $1,25D_3$ to protect the VDR from polyubiquitination (Fig. 6C). Because these mutations (substitutions of amino acid residues localized at the DNA-binding domain) had no effect on ligand binding (as reported previously) or on the ability of the VDR to form a conformation supporting the ligand-dependent functions described above, we speculate that ligand inhibition of polyubiquitination of VDR is not a result of conformational changes in the LBD, but probably a result of the ability of $1,25D_3$ to induce nuclear import of VDR.

Because RXR has been shown to be important for nuclear import of VDR (although there is a debate about whether it takes part in ligand-independent or -dependent nuclear import) [Prüfer and Barsony, 2002; Yasmin et al., 2005], we analyzed disruption of VDR interaction with RXR to determine whether it affects polyubiquitination of VDR. Our results showed that amino acid substitutions that disrupted ligand-dependent interaction of VDR with RXR (LBD mutations in heptad 9, M383G/Q385A; Fig. 7A) [Liu et al., 2001] did not change the polyubiquitination of unoccupied VDR (Fig. 7B). Furthermore, binding of $1,25D_3$ to the heterodimerization mutant VDR failed to inhibit polyubiquitination of it. Because this mutant VDR also has an intact ligand-binding activity [Liu et al., 2001] and intact ability to interact with p160 co-activators in a ligand-dependent fashion (Fig. 7A), we concluded that its inability to respond to $1,25D_3$ in our ubiquitination assays did not result from loss of these functions. Instead, failure of the VDR mutant M383G/Q385A to heterodimerize with RXR and be delivered to the nucleus is likely the reason for loss of protection from polyubiquitination induced by $1,25D_3$.

DISCUSSION

In the present study, we identified for the first time the functional features of the VDR which are required for regulation of its polyubiquitination (i.e., C-terminus amino acids 403–410) and for its protection from proteasome-mediated degradation (heterodimerization and nuclear localization functions). These findings stemmed from incremental evidence for biochemical differences of VDR in two cell types: osteoblasts and IECs. These differences can be

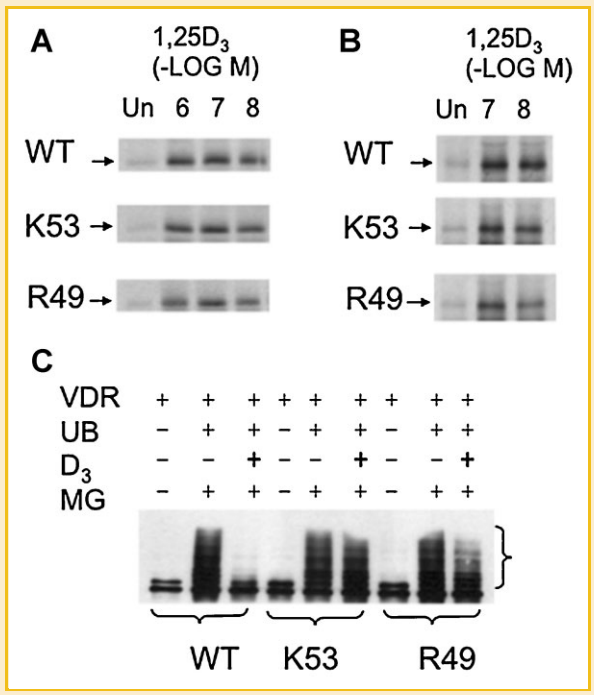

Fig. 6. The importance of nuclear localization signal in ligand-dependent protection of the VDR from polyubiquitination. A: Effect of nuclear localization mutations on ligand-dependent interaction of ^{35}S-VDR with GST–GRIP (AF-2 function). ^{35}S-VDRs (WT or mutants) were synthesized in vitro and then incubated with or without 1,25D_3, and GST–GRIP, and the complexes were absorbed onto glutathione beads as described in Materials and Methods Section. The bound VDR was eluted and analyzed using SDS–PAGE and autoradiography of the dried gels. B: Effect of nuclear localization mutations on ligand-dependent interaction of ^{35}S-VDR with GST–RXR–LBD (heterodimerization function). ^{35}S-VDRs (WT or mutants) were synthesized in vitro, and then incubated with or without 1,25D_3 and GST–RXR–LBD, and the complexes were absorbed onto glutathione beads as described in Materials and Methods Section. The bound VDR was eluted and analyzed using SDS–PAGE and autoradiography of the dried gels. C: Cos-1 cells were transfected with WT or nuclear localization mutant VDR expression vectors and with a histidine-tagged ubiquitin expression vector (UB). The cells were treated with vehicle or 1,25D_3 (D_3; 10^{-7} M) in the presence of MG132 (MG; 10 μM) for 24 h and then harvested and lysed as described above. SDS–PAGE and Western blotting with anti-VDR antibodies were performed to assess polyubiquitinated VDR. K53, nuclear localization mutant K53Q/R54G/K55E; R49, nuclear localization mutant R49W/R50G.

summarized as follows: resistance of unoccupied VDR to 26S proteasome degradation in IECs versus susceptibility of unoccupied (cytosolic) VDR to proteasome degradation in osteoblasts; significantly longer half-life of the unoccupied VDR in IECs (>3 h) than in osteoblasts (<1 h); and the ability of unoccupied VDR in IECs to use nuclear import machinery as opposed to VDR in osteoblasts, which localizes in the chromatin only in a ligand-dependent fashion. This latter property has been recently confirmed through an independent study, which used confocal microscopy and living cells to demonstrate a ligand-dependent nuclear localization of recombinant green fluorescent protein (GFP)–VDR fusion protein in osteoblast-like cells, ROS17/2.8, as opposed to ligand-independent and -dependent nuclear localization of recombinant GFP–VDR fusion protein in the colon carcinoma cell line, Caco-2 [Klopot et al., 2007].

Since degradation of unoccupied VDR in osteoblasts occurs in the cytosol but not in the chromatin and the increase in the half-life of ligand-treated VDR in these cells was associated with accumulation of VDR protein in the chromatin, we hypothesized that the subcellular localization of the VDR dominates its susceptibility to degradation by the 26S proteasome. We substantiated this hypothesis by demonstrating that recombinant VDR in Cos-1 cells was subject to polyubiquitination and that ligand binding protected it from this modification, but only as long as its nuclear localization signal and ability to heterodimerize with RXR (a proven nuclear chaperone of VDR) [Prüfer and Barsony, 2002; Yasmin et al., 2005] were intact. Ligand binding alone does not protect the VDR from polyubiquitination as is evidenced from the efficient polyubiquitination of 1,25D_3-treated VDRs with mutations at the nuclear localization or heterodimerization domains, because these mutants have intact ligand-binding activity. However, interaction with RXR and binding to the chromatin are prerequisites for protection of the VDR from polyubiquitination, and eventually, cytoplasmic degradation by the 26S proteasome. In general, these experiments reproduced a previous report on the polyubiquitination of VDR in keratinocytes and the ability of 1,25D_3 to inhibit this modification [Li et al., 1999]. However, our experiments extend these earlier findings, providing a structure–function relationship for both polyubiquitination and the mechanisms by which the ligand attenuates this modification.

What are the outcomes of the differences in the biochemical properties of the VDR in IECs and osteoblasts? The first difference appears to be a greater abundance of VDR protein in IECs. Although we cannot exclude the possibility that the higher abundance of VDR in IECs is also caused by higher level of VDR mRNA, the contribution of the long half-life of VDR to accumulation of VDR protein in IECs cannot be overruled. Do the built-in receptor pools in IECs contribute to the mode of transcription activation of target genes when compared with the slower, ligand-dependent accumulation of VDR protein in osteoblasts? Recent published reports on the dynamics of VDR recruitment to promoters of target genes in cultured cells demonstrate that the time course for maximal recruitment of the 1,25D_3–VDR complex to the promoter of the CYP24 gene in osteoblasts is 3 h, whereas the time course for maximal recruitment of the VDR to the promoter of this gene in IECs is only 30 min [Kim et al., 2005; Meyer et al., 2006]. Furthermore, the ED_{50} for maximal induction of CYP24 in osteoblasts is greater than 100 nM, whereas in IECs it is 10 nM [Yamamoto et al., 2003; Meyer et al., 2006]. Interestingly, experiments with another target gene for VDR in IECs (TRPV6) also revealed rapid ligand-dependent recruitment (30 min) of the VDR to three vitamin D response elements on the TRPV6 promoter, but more importantly, there is evidence of ligand-independent presence of VDR and RXR on other vitamin D response elements of the TRPV6 promoter [Meyer et al., 2006]. This raises the possibility that the ligand-independent nuclear import machinery in IECs includes RXR, which our studies have shown to be necessary for protection of the VDR from polyubiquitination. Furthermore, these studies underscore the probable constitutive availability of the VDR to

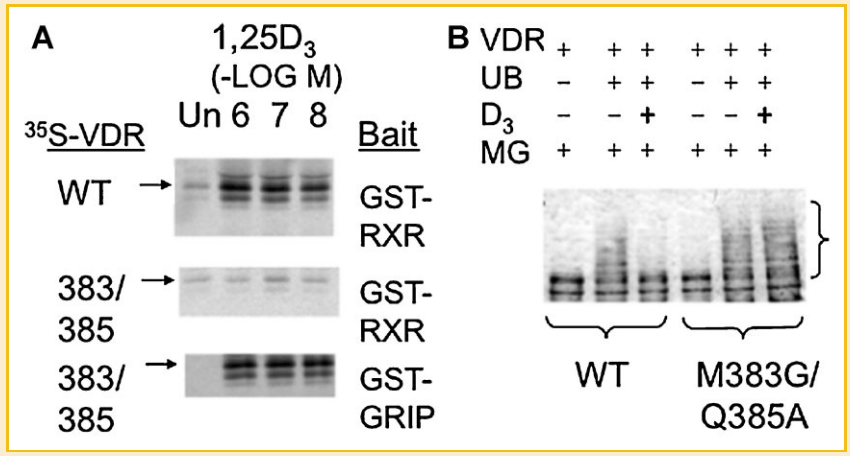

Fig. 7. The importance of heptad 9 (the DNA-independent heterodimerization interface) in ligand-dependent protection of the VDR from polyubiquitination. A: Effect of the heterodimerization (heptad 9) mutation on ligand-dependent interaction of ^{35}S-VDR with GST-RXR-LBD (heterodimerization) and GST-GRIP (AF-2 function). ^{35}S-VDRs (WT or mutants) were synthesized in vitro, and then incubated with or without 1,25D_3(10^{-6}–10^{-8} M). The VDR was then incubated with wither GST-GRIP or GST-RXR (baits), and the complexes were absorbed onto glutathione beads as described in Materials and Methods Section. The bound VDR was eluted and analyzed using SDS-PAGE and autoradiography of the dried gels. B: Cos-1 cells were transfected with WT or heterodimerization mutant VDR expression vectors (M383G/Q385A) and with a histidine-tagged ubiquitin expression vector (UB). The cells were treated with vehicle or 1,25D_3 (D_3; 10^{-7} M) in the presence of MG132 (MG) for 24 h and then harvested and lysed as described above. SDS-PAGE and Western blotting with anti-VDR antibodies were performed to assess polyubiquitinated VDR.

execute transcriptional responses in IECs, which is absent from osteoblasts.

Although we performed all of our experiments with cultured IECs and osteoblasts, they may reflect the distinct biological needs for VDR action in the intestinal tract and skeleton. In the gastrointestinal tract VDR actions in epithelial cells are well defined and confined to regulating mineral absorption and possibly maintaining barrier integrity [Johnson and Kumar, 1994; Bouillon et al., 2003; Kong et al., 2008]. In contrast, in the skeleton, VDR actions are more complex, depend on cross-talk with many other effectors, and are associated with tissue remodeling [Bar-Shavit et al., 1983; Owen et al., 1991; van Leeuwen et al., 2001]. Therefore, the contribution of skeletal VDR to maintaining calcium and phosphorus homeostasis may be secondary to that of the VDR in the gastrointestinal tract, as been recently demonstrated by Xue and Fleet [2009]. Another potential proof of a differential contribution was provided by Suda et al. [2003], which demonstrated that the sensitivity of the gastrointestinal tract to the calcium-absorbing activity of 1,25D_3 is 10–25 times greater than the sensitivity of the skeleton to the resorbing activity of 1,25D_3.

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