Activation of the human immunodeficiency virus type I long terminal repeat by 1 α ,25-dihydroxyvitamin D3

Julián Nevado¹, Stephan P Tenbaum², Ana Isabel Castillo, Aurora Sánchez-Pacheco and Ana Aranda

Instituto de Investigaciones Biomédicas (CSIC)-Universidad Autónoma de Madrid, Arturo Duperier 4, 28029 Madrid, Spain, ¹Unidad de Investigación, Hospital Universitario de Getafe, Getafe, 28905 Madrid, Spain and ²Centro Nacional de Investigaciones Oncológicas (CNIO), 28029 Madrid, Spain

(Requests for offprints should be addressed to A Aranda; Email: aaranda@iib.uam.es)

Abstract

The genetic predisposition of the host and the virus is the most important determinant for prediction of the course of human immunodeficiency virus type I (HIV-1) viral infection and acquired immune deficiency syndrome (AIDS) progression. Transcription from the HIV-1 long terminal repeat (LTR) is a crucial step for viral replication. Here, we describe a stimulatory role of the vitamin D receptor (VDR) on HIV-1 LTR transactivation. Transient transfections reveal that VDR activates the LTR in HeLa, U937, and Cos-1 cells in a ligand-dependent manner. 1 α ,25-Dihydroxyvitamin D3 (vitD3) promotes activation of a minimal LTR construct (from nucleotides –35 to +89), lacking a previously described hormone response element that binds several nuclear receptors. NF- κ B (nuclear factor-kappa B) and Sp1-binding sites, which are responsible for most basal LTR activity in HeLa cells, are also dispensable for vitD3-dependent HIV-1 transcription. Although the tat response element is not required for VDR-mediated *HIV-1* gene expression, the viral protein Tat acts in a synergistic manner with the receptor to stimulate LTR activity. Furthermore, our data also show cooperation of the receptor with various cellular coactivators for HIV-1 transactivation by vitD3. Paradoxically, mutations in the VDR ligand-dependent transcriptional activation function-2 that abrogate vitD3-dependent stimulation through classical vitamin D response elements, do not reduce vitD3-mediated LTR transactivation. Furthermore, point mutations in the DNA-binding domain that abolish receptor binding to consensus DNA sequences do not affect ligand-dependent HIV-1 stimulation. These results show that VDR activates the HIV-1 LTR through different mechanisms, including non-classical nuclear receptor transcriptional actions that may ensure viral transcription under different physiological scenarios.

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Introduction

Human immunodeficiency virus type I (HIV-1) is responsible for the development of AIDS. It has been suggested that viral replication may involve complex interactions among chromatin-associated pro-viral DNA, cellular transcription factors, and viral proteins (VPs; El Kharroubi *et al.* 1998). The transcription of the viral genome is directed by the long terminal repeat (LTR) sequences. Numerous studies have identified essential DNA elements within the LTR, located between nucleotides –454 and +184, as well as many host and VPs for the transcriptional activation of the LTR *in vitro*, and in cultured cells (Pereira *et al.* 2000, Rohr *et al.* 2003). The involvement of multiple host factors in HIV-1 transcription suggests that HIV-1 propagation and AIDS progression are likely to be influenced by physiological conditions that affect the availability and function of host factors. So far, the genetic predisposition of the host and the virus is the most important determinant for prediction and understanding the course of HIV infection and

progression to AIDS. At this respect, previous studies have revealed that ligands of nuclear receptors such as thyroid hormones (T3) may have a role in AIDS development (LoPresti *et al.* 1989). In fact, data from our laboratory, among others, have demonstrated a role for T3 and retinoic acid (RA), other nuclear receptor ligands, on HIV-1 LTR regulation, demonstrating the binding of RA receptors (RARs) and T3 receptors (TRs) to different LTR regions (Desai-Yajnik & Samuels 1993, Sawaya *et al.* 1996, Xu *et al.* 1996, Recio *et al.* 2000, Hsia & Shi 2002). A very recent study has also shown stimulation of HIV-1 transactivation by 17 β -estradiol via the nuclear estrogen receptor (ER)- α , augmenting Sp-1 DNA binding and transcriptional activity (Katagiri *et al.* 2006).

Interestingly, other studies had shown that several variations at the locus coding for the vitamin D receptor (VDR) are associated with susceptibility and progression of several immune diseases, including AIDS (Barber *et al.* 2001, Nieto *et al.* 2004). VDRs are members of the superfamily of nuclear receptors, which act as ligand-inducible transcription factors by

binding generally as heterodimers with the retinoid X receptor (RXR) to hormone responsive elements (HRE), normally located in the regulatory regions of their target genes (Mangelsdorf *et al.* 1995). In the case of VDR, the most common response elements (VDREs) are composed of direct repeats of the sequence AGG/TTCA spaced by three nucleotides (DR3). The receptors bind the HREs through a DNA-binding domain (DBD) or region C, which contains two zinc fingers. Upon ligand binding, the receptors undergo conformational changes that facilitate recruitment of coactivators responsible for gene activation (for review see Aranda & Pascual 2001).

In the present study, we aimed to investigate the control of the HIV-1 LTR activity by VDR in HeLa cells, and eventually to unmask the elements involved in this regulation. The data obtained strongly support the view that VDR and its ligand may play a role in HIV-1 transactivation through several classical and non-classical nuclear receptor transactivation mechanisms.

Materials and methods

Plasmids

Different reporter plasmids used in this study have been previously described elsewhere: –453HIV-luc contains HIV-1 LTR sequences from –452 to +80 bp fused to luciferase, and in –453ΔNF-κBHIV-luc the GCG motif of both NF-κB sites located at –104/–76 was mutated to TCT (Devary *et al.* 1993). Deletion mutants extending from –453 to nucleotides –104, –76, and –28 of the HIV-1 LTR, and –453ΔHREHIV-luc in which a nuclear receptor-binding site is mutated, have been previously described (Recio & Aranda 1997, Recio *et al.* 2000). HIV LTR Tat response element (ΔTAR)-luc (pXp1LTRΔTAR) has been described in (Gomez-Gonzalo *et al.* 2001). The 4×VDRE-TK-Luc reporter plasmid bears four copies of DR3 type VDRE from the rat *ANF* gene promoter (Herdick *et al.* 2000). The expression vector for Tat (1–86; LRG286), a gift from Dr Gaudreau, has been cloned in pcNeo plasmid containing the cytomegalovirus promoter (CMV). Expression vectors for steroid receptor coactivator-1 (SRC-1; Henttu *et al.* 1997), CBP/p300 (Chakrabarti *et al.* 1996), and DRIP205 (Rachez *et al.* 1999), were previously described. Glutathione *S*-transferase (GST)-SRC-1 contains the receptor interacting domain from residues 570 to 780. Human VDR and RXRα, its truncated activation function (AF)-2 and VDRΔ1-111 forms, and the VDR-mutants K246A, E417A, and E420Q have been also described (Jiménez-Lara & Aranda 1999a,b).

To construct the DBD VDR mutants C24G, K45E, and C24G/K45E, site directed mutagenesis was performed

by PCR with Pfu Turbo DNA polymerase (Stratagene) using hVDR (pSG5) or GST-VDR (pGEx) plasmid as templates, and the following oligonucleotides: sense, 5'-GTGCCCCGGATCGGCGGGGTGTGTGGA-3' (for C24G), 5'-TGTGAAGGCTGCCAAGGCTCTTTCAGG-3' (for K45E), containing the desired nucleotides changes, as well as the corresponding antisense oligonucleotides. The 5'-TGTGAAGGCTGCCAAGGCTCTTTCAGG-3' oligonucleotide was used for constructing C24G/K45E using C24G mutant as a template. Mutations were confirmed by direct DNA sequencing.

Cell cultures and transfections

HeLa and Cos-1 cells were obtained from the American Type Cell Culture (ATCC) and are maintained in DMEM containing 10% fetal calf serum. U-937 cells were kindly provided by Dr S Alemany. For transfection experiments, cells were plated in six-well dishes at a density of $1-2 \times 10^6$ cells/well. Transfections were performed with 1.5 μl/μg DNA mixture of commercial liposomes which is composed of DDAB (SIGMA D2779) and DOPE (SIGMA P1223) at a 1:1 ratio. In all experiments, the amount of DNA was kept constant by addition of the required amount of 'empty' vectors. Mixtures were incubated for 20–30 min at room temperature, added to the six-well plates and incubation was continued for other 12 h. Cells were washed again and maintained in DMEM serum-free medium before treatment with vitD3, or its analogs. After 24 h, cells were harvested and lysed by freezing and thawing once in 1× Reporter Lysis Buffer (Promega). The obtained extracts were then centrifuged for 20 s at 12 000 g, and used to assay luciferase activity following the manufacturer's specifications (Promega). Luciferase activity was corrected by the amount of protein of the different extracts. Triplicates of each condition were considered for analysis, and in all cases each experiment was repeated at least three times. Data presented are the mean ± s.d. values obtained.

GST-pull-down experiments

To assay protein–protein interactions *in vitro*, recombinant GST-fusion proteins were expressed in the bacterial strain BL21 (DE3), and purified using standard techniques. Pull-down assays were performed with 5 μl *in vitro* translated [³⁵S]methionine-labeled VDR or the same amounts of its truncated forms (TNT-T7, Quick Transcription/Translation System, Promega) in GST-interaction buffer (25 mM HEPES, pH 7.9; 50 mM KCl; 6% glycerol; 5 mM MgCl₂; 1 mM dithiothreitol; 0.05% Triton X-100; 1 mM phenylmethylsulfonyl fluoride; 5 mM EDTA). These proteins were

incubated with the fusion protein GST-SRC-1 or with the same amount of GST alone, immobilized in glutathione-sepharose beads (Amersham, Pharmacia Biotech) as previously described (Jiménez-Lara & Aranda 1999a). When indicated, 1 μ M vitD3 was included in the binding reaction. The bound proteins were analyzed by SDS-PAGE and autoradiography.

Gel mobility shift assays

Electrophoretic mobility shift assays with a consensus VDRE were performed using 2.5 μ l each of *in vitro* translated RXR and VDR or its mutants in the presence and absence of recombinant SRC-1 as previously described (Jiménez-Lara & Aranda 1999b). The VDRE oligonucleotide used was 5'-AGCTCAGGTCAAG-GAGGTCAG-3'. Gel retardation assays were also performed with the oligonucleotide 5'-A TAT AAG CAG CTG CTT TTT GCC TGT ACT GGG TCT CTC TGG TTA GAC CAG ATC TGA GCC-3', containing sequences from -28

to +30 of the HIV-1 LTR and 100 ng recombinant RXR and/or VDR or its mutants. When indicated vitD3 (1 μ M) was included in the binding reactions.

Statistics analysis

The statistical analysis was carried out using the Stat-View statistics program (Abacus Concepts, Inc., Berkeley, CA, USA). Differences in the mean or in the variance were evaluated using the factorial ANOVA, followed by Fisher's protected least significance test with the level of significance chosen at $P \leq 0.05$.

Results

VDR is able to transactivate the HIV-1 LTR in the presence of vitD3

Figure 1A shows transactivation of the HIV-1 LTR (-453HIV-luc) in the presence and absence of VDR

Figure 1 Vitamin D3 stimulates HIV-1 LTR transcription. HeLa cells (A, C, and D) and U937 cells (B) were transfected with the reporter plasmid -453HIV-LUC, which contains HIV-1 LTR sequences from -453 to +80 fused to the luciferase gene. (A and B) VDR-mediated HIV promoter activity in response to 100 nM vitamin D3 (vitD3) for 48 h. (C) HIV promoter activity in response to treatment with increasing concentrations of vitD3 for 48 h. (D). HIV promoter activity in cells treated for 48 h with a 100 nM concentration of vitD3 or the vitamin D analogs ZK161422 and ZK159222. Data of luciferase activity were expressed as either fold induction over the values obtained in Et-OH-treated control cells in the absence of VDR (A and B) or RLUs (C and D). * $P \leq 0.05$ versus EtOH-treated cells.

and its ligand in HeLa cells. Incubation of VDR-expressing cells with 100 nM vitD3 was able to induce HIV-1 LTR transcription about fourfold ($P \leq 0.05$), while almost no induction was observed in the absence of ligand (1.50 ± 0.25 -fold induction over non-VDR-transfected cells; Fig. 1A). In order to distinguish whether VDR-mediated LTR transactivation was a cell-type specific effect, we further analyzed this effect in human U937 cells, which are putative hematopoietic target cells of the HIV virus *in vivo*. Stimulation by vitD3 was similar in range to that observed in HeLa cells (about 4.5-fold, Fig. 1B). Closely similar results were obtained in transiently transfected Cos-1 cells (data not shown). All these data together indicate that liganded VDR is able to induce transcription from the HIV-1 LTR in different cell types.

Further transient transfection experiments of HeLa cells with the 453HIV-luc reporter in presence of increasing concentrations of vitD3 (0–1000 nM) showed that the vitamin stimulated the HIV-1 LTR in a dose-dependent manner, with a half-maximal luciferase induction obtained with approximately 75 nM vitD3 (Fig. 1C). In addition, the synthetic vitamin D agonist ZK161422 at 100 nM was as potent as vitD3 (Fig. 1D), and is also a good putative candidate to activate the HIV-1 LTR. In contrast, the same concentration of the vitD3 analog ZK159222 that binds VDR with high affinity but shows a partial vitD3 antagonistic activity, barely increased HIV-1 LTR transactivation (Fig. 1D).

Transactivation of HIV-1 LTR truncated forms by vitD3

A series of 5' deletion constructs derived from –453 HIV-luc, as well as several mutated constructs, were used to define the elements responsible for basal and VDR-stimulated HIV-1 LTR expression in HeLa cells (Fig. 2). A mutant of –453HIV-luc (–453 Δ HRE), affecting a previously described HRE that binds multiple nuclear receptors, including VDR (Ladías 1994), showed a slightly decreased basal activity versus the wild-type promoter, but interestingly stimulation by vitD3 (100 nM) was at least as high as that produced on the wild-type promoter (3.96 ± 0.56 -fold induction over control cells). In addition, removal of the LTR sequences from –453 to –104 bp, which deletes among others the HRE, increased the basal activity of this LTR construct (around 1.5–1.7-fold over basal), and still showed a significant response to the hormone as compared with the –453HIV-luc reporter plasmid (4.10 ± 0.70 -fold induction). These results suggest that elements different from the HRE contribute to VDR-mediated HIV-1 LTR activation in HeLa cells.

The core HIV-1 LTR promoter also contains two NF- κ B-binding sites (at nucleotides –104 to –81), and Fig. 2 shows that mutations of these motifs reduced very

significantly basal luciferase levels (up to 2–3-fold), as well as vitD3-mediated activation (from 3.20 ± 0.17 - to 2.10 ± 0.16 -fold induction). However, the induction by vitD3 observed in the absence of these motifs was still statistically significant. Accordingly, a shorter LTR-based construct extending to –76 bp, in which both NF- κ B-binding sites were deleted, showed a pattern of basal and vitD3-induced activation that was similar to that observed with the mutant construct. These results confirm that these elements have an important role for basal HIV-1 LTR activity in these cells, and suggest that although NF- κ B-binding sites may play a minor role HIV-1 LTR transactivation by VDR, additional elements must participate in this stimulation.

Related to that, and in close proximity to the NF- κ B sites, the HIV-1 LTR also contains three binding sites for the transcription factor Sp1 (from nucleotide –76 to –46). Indeed, a cooperative interaction between both transcription factors, NF- κ B and Sp1, has been shown to be required for viral activation (Perkins *et al.* 1993). Thus, we also analyzed the deletion of the LTR to –28 bp, which eliminates the three Sp1 sites. This mutation essentially abolished basal promoter activity, although vitD3 was still able to transactivate the –28 HIV-luc construct (Fig. 2), showing that Sp-1 sites are not required for VDR-dependent HIV-1 LTR stimulation. In addition, VDR expression did not affect activity of the plasmid backbone, showing that the effect of vitD3 is mediated by this minimal HIV-1 LTR fragment.

The VP Tat is critical for HIV transcriptional activation, and this effect depends on its binding to the TAR. We transfected HeLa cells with a mutated LTR reporter lacking the TAR element (–453 Δ TAR), and analyzed the putative effect of VDR on activation of this construct. Figure 2 also shows that although the basal activity was reduced significantly, vitD3-induced transactivation of this construct was similar to that obtained with the wild-type promoter, showing that such effect appears to be independent of the TAR element. The finding that this construct, as well as the –28HIV-luc construct, are responsive to vitD3 treatment, suggests that the –28 to 30 bp LTR region is sufficient to mediate the receptor effects.

Since VPs appear to cooperate with cellular transcription factors to induce HIV-1 transcription, we also examined whether a functional interaction between Tat and VDR to stimulate LTR activity occurred. For this purpose, a native form of Tat was cotransfected with VDR and the –28HIV-luc promoter construct harboring the TAR element, and activity was measured in the absence and presence of 100 nM vitD3 (Fig. 3A). Expression of Tat caused a strong stimulation of reporter activity, and interestingly, in the presence of the VP the LTR was activated by vitD3 even in the absence of transfected VDR, showing that Tat was able

Figure 2 Elements of the HIV-1 LTR mediating regulation by vitD3 in HeLa cells. Constructs extending to -453, -104, -76, and -28, as well as different constructs in which the HRE element, NF- κ B-binding sites or TAR element have been mutated in the context of -453 plasmid, were cotransfected with a VDR expression plasmid into HeLa cells. Luciferase activity was determined in EtOH-treated cells and cells treated with 100 nM vitD3 for 48 h. Luciferase activity is expressed as RLUs. * $P \leq 0.05$ vitD3 versus EtOH-treated cells.

to sensitize the cells allowing a ligand-dependent response through the endogenous receptor. The unliganded receptor was able to synergistically enhance activation by Tat, although in the presence of vitD3 further transactivation was not found indicating saturation of the system. The synergistic effect between the unoccupied receptor and Tat was also observed with the -28HIV-luc LTR reporter construct. However, the hormone increased the cooperative activation of this minimal reporter construct by these two proteins (Fig. 3B). In addition, the cooperation of VDR and

Tat to activate transcription of this minimal reporter construct was not restricted to HeLa cells, since it was also observed in U937 cells, which, as indicated, could be a natural target for HIV infection (Fig. 3B).

Influence of coactivators on HIV-1 LTR stimulation by VDR

To assess a possible role of nuclear receptor coactivators in regulation of HIV gene expression by VDR, we cotransfected the HIV-1 LTR luciferase reporter, and

Figure 3 Synergism of Tat with VDR to stimulate the HIV-1 LTR. (A) HeLa cells were transfected with the -453HIV-Luc reporter in the absence and presence of VDR and/or 100 ng of an expression vector encoding the viral activator Tat (pCMV-Tat). Reporter activity was determined after 48 h in EtOH-treated cells and cells treated with 50 nM vitD3. * $P \leq 0.05$ vitD3 versus EtOH-treated cells; # $P \leq 0.05$ versus VDR-transfected cells in the absence of vitD3. (B) HeLa and U937 cells were transiently cotransfected with the -28HIV LTR reporter and VDR alone or in combination with 50 and 100 ng pCMV-Tat. Luciferase activity was determined in cells incubated with or without 100 nM vitD3 for 48 h. Luciferase activity is expressed as fold induction over the values obtained in the absence of VDR.

the receptor expression plasmid, with expression vectors for different known coactivators for VDR, such as (SRC-1), CREB-binding protein (CBP/p300) or vitamin D-interacting protein 205 (DRIP 205). In all the cases studied, the presence of the coactivator increased VDR-induced HIV-1 LTR transactivation in the absence of ligand (around 2.5-fold). In addition, such a cooperative effect was stronger in the presence of vitD3 (Fig. 4A). Interestingly, the synergistic effect between VDR and

either SRC-1 or CBP/p300 was only observed on the complete HIV-1 LTR reporter construct. On several truncated forms of the HIV-LTR, such as -104, -77, Δ HRE or Δ NF- κ B, VDR was not able to cooperate with the coactivators (data not shown), although it was still able to promote a very significant LTR transactivation in a ligand-dependent manner (see Fig. 2).

VDR binds DNA preferentially as a heterodimer with the RXR, and we have recently demonstrated that the

Figure 4 Influence of nuclear receptor coactivators on the HIV-1 response to vitD3. (A) HeLa cells were cotransfected with -453HIVLuc and VDR expression plasmid alone or in combination with an expression vector for the coactivators SRC-1, CBP/p300, or DRIP205. Luciferase activity was determined in EtOH-treated cells and in cells treated for 48 h with 100 nM vitD3. (B). VDR acts as a heterodimer with RXR in HIV-1 LTR stimulation. HeLa cells were transfected with -453HIVLuc and VDR expression plasmid alone or in combination with an expression vector for the coactivator DRIP205. Luciferase activity was determined in EtOH-treated cells and cells treated for 48 h with 100 nM vitD3 and 1 μ M 9-cis alone or in combination. * $P \leq 0.05$ versus EtOH-treated cells. # $P \leq 0.05$ versus VDR-transfected cells in the presence of vitD3.

VDR/RXR heterodimer is able to recruit coactivators in response to both vitamin D and the RXR ligand 9-cis-RA, and that both ligands can cooperate to stimulate transcription (Sánchez-Martínez *et al.* 2006). In fact, Fig. 4B shows that 9-cis-RA (1 μ M) was also able to promote transactivation of the HIV-1 LTR in a

synergistic manner with vitD3. Furthermore, 9-cis-RA alone was also able to trigger a cooperative effect between unliganded VDR and DRIP-205 coactivator (Fig. 4B). The presence of vitD3 in the last scenario squelched such cooperative behavior (Fig. 4B). All these data together suggest a role for the VDR/RXR

heterodimer and its respective ligands in the response of the HIV-1 LTR to vitD3.

Transactivation of the HIV-1 LTR by VDR mutants

An autonomous AF-2 domain, which is located at the C-terminal helix 12 of the ligand-binding domain, together with residues in helices 3, 4, and 5 form a hydrophobic groove that accommodates the coactivators (Gronemeyer *et al.* 2004). Furthermore, a conserved glutamic acid in helix 12 (E420) and an invariable lysine (K246) in helix 3 of VDR contact directly with the coactivators and form a charge clamp that stabilizes coactivator binding (Vanhooke *et al.* 2004). Accordingly, mutations in these residues render a VDR unable to mediate vitamin D-dependent transactivation (Jiménez-Lara & Aranda 1999a, Sánchez-Martínez *et al.* 2006). Since the obtained results indicated participation of coactivator molecules in the activation mechanism, we analyzed whether the VDR AF-2 domain plays a role in HIV-1 LTR-induced activation by vitD3. To this end, HeLa cells were cotransfected with -453HIV-luc and expression vectors for a truncated VDR lacking AF-2 domain, as well as the VDR mutants E420Q and K246A. Figure 5A shows that, surprisingly, these mutations did not eliminate vitD3-mediated HIV-1 LTR transactivation. Paradoxically, in some experiments, HIV-1 LTR-induced transcription by these AF-2 VDR mutants seemed to be even higher than that caused by wild-type VDR (around fivefold for K246A and E420Q mutants, Fig. 5A). Western blot analysis of whole HeLa cell lysates revealed nearly equivalent expression of the VDR mutants examined in this assay (data not shown).

Gel retardation assays were performed to confirm that the AF-2 mutants that stimulate HIV-1 LTR promoter activity are indeed unable to recruit coactivators. Whereas, SRC-1 was recruited to the DNA bound VDR/RXR heterodimer in a vitD3-dependent manner, neither the receptor lacking the AF-2 domain nor the E420Q or K246A mutant was able to bind SRC-1 (Fig. 5C). As shown in Fig. 5D, GST pull-down experiments confirmed that the Δ AF-2 mutant was not able to recruit SRC-1 (lanes 5–8), and that both the E420Q and K246A VDR mutants showed a strongly reduced ability to bind the coactivator (Fig. 5D, lanes 9–16). Thus, surprisingly, VDR mutants that are unable to bind to coactivators are able to strongly induce HIV-1 LTR transactivation.

To investigate whether VDR binding to DNA is necessary for HIV-1 LTR induction, we took advantage of several mutations that affect the DBD. A truncated receptor lacking 111 N-terminal aminoacids, i.e. the DBD and the A/B region that in VDR is only composed of a few residues (Jiménez-Lara & Aranda 1999b), was not able to promote HIV-1 LTR transactivation in the

presence of vitD3 (Fig. 5B). Thus, we further evaluated whether VDR binding to a consensus VDRE was essential for vitD3-mediated LTR transactivation. Consequently, we performed site-directed mutagenesis to create three different DBD mutants affecting the first zinc finger: C24G, K45E, and the double mutant C24G/K45E. Figure 5B shows that, surprisingly, these three VDR mutants were still able to transactivate the HIV-1 LTR in a hormone-dependent manner with a similar potency to the wild-type VDR.

Gel retardation experiments testing the ability of those mutants to bind DNA and to coactivators showed that these three DBD mutants had a different behavior. While the C24G mutant was still able to bind to a consensus VDRE, K45E, and the double mutant were not (Fig. 5C), although all of them showed a strong ligand-dependent HIV-1 LTR transactivation. On the other hand, *in vitro* GST pull-down assays confirmed such differences among the different DBD-mutants. C24G was similar to wild-type VDR, and was still able to recruit the coactivator SRC-1 (Fig. 5D), confirming the results obtained in the gel retardation assays (Fig. 5C). The K45E mutant that did not bind DNA (Fig. 5C) was still able to recruit SRC-1, although to a lesser extent than wild-type VDR or the C24G mutant (Fig. 5D). Unexpectedly, the double mutant (C24G/K45E) not only did not bind the VDRE, but also was unable to recruit the coactivator (Fig. 5D). In addition to that, neither the DBD mutants nor the AF-2-mutants were able to synergize with either SRC-1 or CBP coactivators (data not shown). These data suggest that although an intact DBD is not essential for VDR-mediated transactivation of the HIV-1 LTR, this domain may have a role on coactivators recruitment to the LTR. Tethering of a DBD defective VDR to the promoter may happen through interaction with other DNA-binding transcription factors.

In order to confirm that these untypical results regarding transactivation of the HIV-1 LTR by the different VDR mutants used in this analysis are actually HIV-1 LTR promoter specific effects, we compared the ability of wild-type VDR and its different mutants to transactivate a classical VDRE promoter construct (4 $\times$ VDRE-luc). Figure 5E shows that, as expected, the AF-2-related mutants (Δ AF-2, K246A, and E420Q) were not able to activate such promoter construct. In addition, wild-type VDR and the C24G mutant, whose behavior seemed to be similar to wild-type VDR in different aspects (Fig. 5C and D), but not the DBD mutants K45E and C24G/K45E were able to transactivate this reporter construct (Fig. 5F). These results are in accordance with a requirement for canonical conditions to activate through a VDRE element, and support the idea that the VDR DBD and AF-2 domains are essential to transactivate in such scenario, but not for VDR-dependent stimulation of the HIV-1 LTR.

Figure 5 Influence of several VDR mutants on HIV-1 promoter activity. HeLa cells were transfected with 5 µg of either -453HIVLuc (A and B) or 4×VDRE-TKLuc (E and F) reporter plasmids in combination with an empty expression vector (-), or expression vectors for wt VDR, or for the VDR mutants ΔAF-2, K246A, E420Q, Δ1-111, C24G, K45E and C24G/K45E. Luciferase activity was determined in EtOH-treated cells and in cells treated for 48 h with 100 nM vitamin D3 (vitD3), and is expressed as fold induction over EtOH-treated cells transfected with an empty expression vector. * $P \leq 0.05$ versus EtOH-treated cells. (C) Analysis of DNA binding by VDR mutants. Gel retardation assays were performed with 2.5 µl of each *in vitro* translated RXR and VDR, or VDR-truncated forms, in combination with recombinant SRC-1 in the presence or absence of a saturating concentration of vitD3 (1 µM). HD and HD-CoA are the mobility bands corresponding to the VDR/RXR heterodimer and the SRC-1-VDR/RXR complexes, respectively. (D) *In vitro* interaction between VDR (and different VDR mutants) with SRC-1 was analyzed by GST pull-down, which was performed with 5 µl *in vitro* translated L³⁵S methionine-labeled receptors. Where indicated, 1 µM vitamin D was included in the binding reaction. The bound proteins were analyzed by SDS-PAGE and autoradiography. The input lanes represent 10% of the protein in the binding assay.

That the K45E receptor mutant that does not stimulate a consensus VDRE is able to activate not only the complete LTR, but also the different truncated and mutated HIV-1 LTR reporters is shown in Fig. 6A. This mutant was as potent as the wild-type VDR to transactivate HIV-1 constructs extending to -104 and -76 bp, as well as the HRE, NF- κ B and TAR mutants in the context of -453 HIV-luc. In addition, the influence of the DBD and the AF-2 VDR mutants on vitD₃-dependent activation of the -104 HIV-luc construct was also examined. As illustrated in Fig. 6B, the different mutants were able to increase reporter activity in a ligand-dependent manner, showing that the VDR AF-2 and DBD domains are also dispensable for transactivation of this shorter LTR construct. Furthermore, the activity of the minimal reporter construct extending only to -28 bp was also stimulated by these mutants in a ligand-dependent manner (Fig. 6B), demonstrating again that proximal promoter sequences are sufficient to mediate induction of LTR transcription by vitD₃.

To analyze whether mutations in the VDR DBD could abolish binding to a consensus VDRE without affecting binding to the HIV-1 promoter, additional gel retardation assays were performed with an oligonucleotide spanning the -28 to $+30$ bp LTR fragment. As shown in Fig. 7, wild-type VDR bound this sequence as a heterodimer with RXR both in the absence and presence of vitD₃, whereas no monomeric or homodimeric VDR binding to this LTR region was observed. In contrast, neither VDR K45E nor C24G/K45E were able to bind to this element.

The finding that VDR DBD mutants cannot bind the LTR but enhance its activity suggests that transactivation by these receptors is mediated by interaction with other factors. To analyze whether these mutants could still cooperate with Tat, their influence on stimulation of -453 HIV-luc was also assessed (Fig. 8). In cells transfected with a small amount of Tat vector alone (10 ng), there was little stimulation of reporter activity and again a vitD₃-

Figure 6 Stimulation of several HIV-1 LTR constructs by VDR mutants. (A) HIV-1 constructs extending to -453 , -104 , and -76 , as well as constructs in which the HRE, NF- κ B, or TAR elements have been mutated in the context of -453 plasmid, were cotransfected with either wild-type VDR (Wt) or the VDR DBD mutant K45E into HeLa cells. Luciferase activity was determined in EtOH-treated cells and in cells treated with 100 nM vitD₃ for 48 h and is expressed as RLUs. * $P \leq 0.05$ vitD₃ versus EtOH-treated cells. (B) The HIV-1 LTR plasmids extending to nucleotides -104 and -28 were cotransfected with either VDR wild-type or the VDR mutants indicated, and reporter activity determined as in (A). Results are expressed as fold induction of vitD₃ over EtOH-treated cells for each receptor. * $P \leq 0.05$ versus EtOH-treated cells.

Figure 7 Binding of VDR mutants to proximal LTR sequences. Gel retardation assays were performed with a labeled oligo nucleotide encompassing the -28 to +30 HIV-1 LTR region. This LTR fragment was incubated with wild-type VDR and the DBD mutants K45E and C24G/K45E in the presence and absence of RXR. When indicated, 1 μ M vitD3 was present in the binding assay.

dependent response in the absence of transfected VDR was observed. In addition, whereas the wild-type receptor in the absence of ligand was able to cooperate with Tat and did not show a further response to vitD3, the unoccupied VDR K45E and K45E/C24G did not cooperate with the VP, although they showed a normal vitD3-dependent response in the absence of Tat.

Discussion

Our previous findings showed the participation of nuclear receptors, transcription factors and coactivators in the stimulation of *HIV* gene expression by RA in neuronal cells (Recio *et al.* 2000), demonstrating that both host and VPs cooperate to induce RA-mediated transcription of HIV. In addition, it has been also suggested a role for the T3 in HIV-1 LTR regulation, revealing the existence of binding sites for the T3 receptor (T3R) in different regions of the LTR sequence (Sawaya *et al.* 1996, Xu *et al.* 1996, Recio *et al.* 2000, Hsia & Shi 2002). In this sense, previous studies have revealed that different hormones, such as T3, may have a role in AIDS development (LoPresti *et al.* 1989), and a therapeutic use of retinoic antagonists has been already proposed to repress viral replication by inhibiting retinoid-dependent transcription (Lee *et al.* 1994, 1996, Hanley *et al.* 2004).

In this work, we have analyzed the activation of the HIV-1 LTR by another nuclear receptor, VDR. Our results clearly show that VDR is also a potent HIV-1 LTR transactivator in a vitD3-dependent manner in HeLa cells. However, in order to give a more physiological significance to HIV-1 LTR transactivation by VDR, and to exclude a cell-type specific effect, we also analyzed the role of VDR on the LTR in U937 cells, as a model of mononuclear cells, which are a HIV-1 virus target. Our data show VDR-dependent activation of the HIV-1 LTR in this cell type, supporting a possible role for this

Figure 8 The VDR mutants in the DNA-binding domain do not cooperate with Tat. HeLa cells were transfected with the -453HIV-luc promoter construct, 10 ng pCMV-Tat and expression vectors for wild-type VDR and the DBD mutants K45E and C24G/K45E. Luciferase activity was determined after 48 h incubation with EtOH or vitD3 and is expressed as RLUs. * $P \leq 0.05$ vitD3 versus EtOH-treated cells.

receptor in HIV-LTR-mediated transactivation and therefore on the progress of AIDS.

New receptor chemical modulators are valuable tools for studying the molecular mechanisms of VDR signaling and could also be good candidates to modulate HIV-1 LTR activity and therefore viral transcription and replication. Some of these compounds show a clear agonistic activity and are at least as strong as the natural ligand. This is the case for ZK16422 (Castillo *et al.* 2006) that also stimulated the LTR. In contrast, compounds such as ZK159222, which binds VDR with high affinity but only shows a residual agonistic activity at high concentrations, can inhibit vitD3-dependent transactivation (Castillo *et al.* 2006), and might therefore be useful to repress viral transcription in response to the endogenous ligand.

Trying to unmask the regulatory regions involved in HIV-1 LTR transactivation by VDR, we used a series of deletion mutants. An HRE that can bind multiple nuclear receptors, including VDR (Ladías 1994) was expected to be responsible for vitD3-dependent stimulation. However, our data reveal that when the HRE was deleted a full transactivation of the LTR was still observed. We have previously observed a similar result for RA-mediated stimulation of HIV-1 LTR in neuronal cells (Recio *et al.* 2000). Surprisingly, the HRE seemed to be necessary for cooperation between VDR and the SRC-1 coactivator. On the other hand, a truncated HIV LTR form, in which sequences from -453 to -104 bp were deleted, showed increased transcription by the unoccupied VDR, without affecting vitD3-mediated LTR activation, and suggesting that a putative repressive element could be located within this region. All these results together suggest that other downstream-located sequences (different from the HRE) are involved in ligand-dependent stimulation of *HIV* gene expression. Among these possible elements, NF- κ B-binding sites were good candidates to be involved in VDR-dependent transcription of HIV-1 LTR. However, our data do not demonstrate an important role for these sites. In contrast, NF- κ B sites seemed to be responsible for most basal activity of the LTR in HeLa cells. In a further dissection analysis of HIV-1 LTR, we also examined a role for the binding sites for the transcription factor Sp1, which seems to play (together with NF- κ B transcription factors), a crucial role in *HIV-1* gene expression (Perkins *et al.* 1993, Jones & Peterlin 1994). We have previously shown that RA was not able to promote activation of a minimal LTR construct lacking the Sp1 sites (-28 HIV-luc; Recio *et al.* 2000). In addition, Sp1 appears to mediate HIV-1 LTR induction by other nuclear receptor (ER α ; Katagiri *et al.* 2006), and binding of TR to both NF- κ B and Sp1 sites that appear to be implicated in regulation of HIV-1 LTR activity by this receptor has been reported (Desai-Yajnik & Samuels 1993). However, our data also exclude a

major role for the Sp1 motifs in VDR-dependent HIV-1 stimulation because the -28 HIV-luc minimal construct was still able to respond to vitD3.

Previous findings from our laboratory documented the existence of unrecognized nuclear receptor-binding sites in HIV-1 core promoter sequences located both upstream and downstream of the TATA box. Sequences contained between nucleotides -35 and $+89$ of the HIV-1 LTR can bind VDR-RXR heterodimers (Recio *et al.* 2000). In contrast with NF- κ B or Sp1 LTR motifs, which are rather selective for TR-RXR, these proximal elements can also bind RAR-RXR and VDR-RXR (Recio *et al.* 2000). In this study, we have shown that the HIV-1 LTR region comprised between nucleotides -28 and $+30$ bind VDR-RXR heterodimers, whereas neither monomers nor homodimers show high affinity binding to this element. The existence of various nuclear receptor-binding sites, both in the modulatory and in the proximal promoter region, may point at the importance of signaling by these transcription factors in HIV transactivation, which may ensure transcriptional regulation of the virus by different mechanisms even after an eventual mutation of the canonical receptor-binding site (HRE).

The synergism obtained between VDR and the viral Tat protein, both in HeLa and U937 cells, constitutes another example of the cooperation between host and VPs to induce HIV-1 transcription. A similar cooperative effect between Tat and RAR has been previously described by us (Recio *et al.* 2000). Tat is a potent transactivator of *HIV-LTR* gene expression and replication, which basically acts through a direct interaction with a RNA sequence, called TAR element, located between oligonucleotides $+4$ and $+44$, although several studies suggest that Tat is also able to activate gene expression in the absence of TAR (Taylor *et al.* 1992, Dal Monte *et al.* 1997, Yang *et al.* 1997; and Nevado *et al.* unpublished observations). Although the TAR element is not required for VDR-mediated *HIV-LTR* gene expression, cooperation of Tat with the receptor could facilitate targeting of coactivators and the transcriptional machinery to the promoter and the synergistic activation. Tat seems to be able to interact *in vitro* with many proteins, including basic transcriptional machinery components (Jones & Peterlin 1994), and other nuclear receptors such as TR (Desai-Yajnik *et al.* 1995). Therefore, a protein-protein interaction of Tat with VDR could be involved in the cooperation observed.

Our findings also reveal that VDR domains essential for vitD3-mediated transactivation of a VDRE-containing promoter, do not seem to be required for HIV-LTR transactivation by VDR in HeLa cells. Thus, point mutations in the DBD that abolish receptor binding to a consensus VDRE as well as VDRE-mediated transcription do not affect LTR activation by vitD3. In

contrast, deletion of the DBD abolishes induction, showing the importance of this domain for activation. Surprisingly, our data show that DBD mutations can affect not only DNA binding but also association with SRC-1, suggesting an additional role for this domain in VDR-mediated transcription through recruitment coactivators of the p160 family. On the other hand, VDR mutants lacking the AF-2 domain or point mutations affecting critical residues in helices 3 and 12 that are inactive to stimulate transcription through a classical VDRE, are still able to confer vitD3-dependent HIV-1 LTR induction. All these data together seem to point, among other mechanisms, at putative protein–protein interactions to drive VDR-mediated LTR transactivation in a ligand-dependent manner. Interestingly, the DBD mutants that can mediate vitD3-dependent HIV-1 LTR transcription are unable to cooperate with Tat.

Our results show that several nuclear receptor coactivators cause a significant increase in the induction of LTR transcription by both the unoccupied and ligand-bound receptor. Some of these coactivators, such as the p160 protein SRC-1 or CBP possess histone acetyltransferase activity (HAT) that cause chromatin decompaction, thus facilitating transcription (Aranda & Pascual 2001, Gronemeyer *et al.* 2004, Perissi & Rosenfeld 2005), while DRIP205, a component of the mediator complex, functions to recruit the RNApol II holoenzyme to the target promoter (Rachez *et al.* 1998, 1999). Although the AF-2 VDR mutants are able to stimulate the LTR, they have lost the ability to synergize with SRC-1 or CBP. The finding that the VDR AF-2 domain is not required for vitD3-mediated transactivation of the LTR, suggest that recruitment by other promoter-bound transcription factors could be involved in the stimulatory effect of the coactivators. In agreement with this hypothesis, CPB/p300 also acts as a coactivator for NF- κ B (Perkins *et al.* 1997) and Tat (Hottiger & Nabel 1998, 2000). The interaction of Tat with HAT complexes and their strong relevance in HIV-1 LTR activation has been established (Benkirane *et al.* 1998, Marzio *et al.* 1998, Rachez *et al.* 1998, Ott *et al.* 1999, Dorr *et al.* 2002, Kino *et al.* 2002, Mujtaba *et al.* 2002, Mahmoudi *et al.* 2006). In addition, Tat itself is subject to modification by acetyltransferases that regulate its activity (Ott *et al.* 1999, Kino *et al.* 2002, Mahmoudi *et al.* 2006). On the other hand, it has been described that p160 coactivators function as coactivators of the HIV-1 LTR. The p160 proteins appear to be tethered to the LTR via association with NF- κ B, allowing full activation of this promoter by Tat (Kino *et al.* 2002). Whether or not the different receptor mutants can be recruited *in vivo* to the LTR either directly or through protein–protein interactions with other factors remains to be analyzed in future studies.

In addition to Tat, other VPs such as VPR are thought to influence not only viral replication but also

numerous host cell functions (Kino & Pavlakis 2004). Transcription factors such as Sp1 or the glucocorticoid receptor (GR) and coactivators such as CBP/p300 also interact with VPR, which has been shown to act as a coactivator for GR (Kino *et al.* 1999). Therefore, it is possible that this important HIV-1 regulatory molecule could also have a function on LTR activation by VDR.

In conclusion, our data point to the importance of VDR signaling in HIV transactivation, which can occur through ‘classical’ and ‘non-classical’ mechanisms. All together seem to support the participation of receptors and their agonist ligands, transcription factors, cellular coactivators, and VPs in the stimulation of *HIV* gene expression by vitD3, although additional efforts will be necessary to reveal their specific role in such a process. Because the LTR represents the main regulatory region for viral replication, our present findings suggest that the vitamin D receptor may play a role during the infectious process and in the progress of AIDS in patients.

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