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1,25-Dihydroxyvitamin D3 Upregulates Functional CXCR4 Human Immunodeficiency Virus Type 1 Coreceptors in U937 Minus Clones: NF-κB-Independent Enhancement of Viral Replication

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U937 cell clones which sustain efficient or poor replication of human immunodeficiency virus type 1 (HIV-1) (referred to herein as plus clones and minus clones, respectively) have been previously described. 1,25-Dihydroxyvitamin D3 (vitamin D3) potently induced HIV-1 replication and proviral DNA accumulation in minus clones but not in plus clones. Vitamin D3 did not induce NF-κB activation but selectively upregulated CXCR4 expression in minus clones. The CXCR4 ligand stromal-cell derived factor-1 induced Ca^{2+} fluxes and inhibited both constitutive and vitamin D3-enhanced HIV replication in minus clones.

Chemokine receptors have been recently shown to act as human immunodeficiency (HIV) coreceptors together with CD4 for viral entry (10, 26). Understanding which physiological factors regulate HIV-coreceptor expression in $CD4^+$ cells is of great importance for developing strategies aimed at curtailing or preventing viral spreading in vivo.

The human promonocytic leukemia cell line U937 (34), which expresses both CD4 and CXCR4, is a well-known target for CXCR4-using (X4) T-cell-line-adapted (TCLA) strains of HIV type 1 (HIV-1) (1, 6, 12, 19, 23, 31). Efficient and inefficient patterns (referred to herein as plus patterns and minus patterns, respectively) of X4 virus replication have been described among U937 cellular clones by us (2, 13) and others (4, 18). Recently, the deficient ability of U937 minus clones (i.e., those demonstrating minus patterns) has been explained by the lack of fusogenic capacity for TCLA viruses in spite of good levels of CXCR4 expression (23).

1,25-Dihydroxyvitamin D3 (vitamin D3), a well-characterized differentiating agent for myelomonocytic cells (28), was previously reported to increase HIV expression in chronically infected U937 cells (21), including stimulated U1 cells (14). Plus or minus U937 cells (2×10^5/ml) were adsorbed with pelleted HIV-$1_{LAI/IIIB}$ propagated on the H9 T cell line (Advanced Biotechnology, Inc., Columbia, Md.) for 1 h at 37°C, washed, and seeded in duplicate cultures in the presence or absence of vitamin D3 in 48-well plastic plates. Culture supernatants were tested for Mg^{2+}-dependent reverse transcriptase (RT) activity (13). Strikingly, stimulation with vitamin D3 upregulated HIV replication in minus clones to levels comparable to those observed in parallel cultures of infected plus cells. In contrast, vitamin D3 did not modulate viral replication in U937 plus clones (Fig. 1).

Modulation of NF-κB activation by vitamin D3 has been documented in different cell types (9, 37). Therefore, time course experiments were performed (5, 13) with plus and minus clones stimulated with tumor necrosis factor alpha (TNF-α), phorbol 12,myristate-13,acetate (PMA), or vitamin D3. However, unlike TNF-α or PMA, vitamin D3 stimulation did not induce activation of NF-κB in either type of U937 clones, whether they were uninfected (Fig. 2) or infected with HIV-1 (data not shown).

Lack of fusogenic capacity of X4 HIV-1 in spite of functional CXCR4 receptors has been reported as a correlate of the so-called resistance of U937 minus clones to supporting viral replication (23). U937 minus cells showed mean fluorescence intensities for CXCR4 lower, although more stable, than those of plus clones, whereas a relative downmodulation of the chemokine receptor occurred in plus cells after 4 to 5 days of culture (Fig. 3A). In agreement with a previous study (23), CXCR4 was a functional chemokine receptor in U937 minus clones in that Ca^{2+} fluxes were promptly demonstrated after stimulation by its ligand stromal cell-derived factor-1 (SDF-1) (Fig. 3B).

Vitamin D3 has been shown to upregulate CXCR4 mRNA in the promyelocytic cell line HL-60 (22). Consistent with its effect on HIV replication, a selective upregulation of CXCR4 mRNA expression (not shown) and cell surface density was observed in vitamin D3-treated minus clones (Fig. 4A) but not in U937 plus cells (data not shown). In contrast, vitamin D3 did not affect CD4 surface expression in minus clones (data not shown).

Minus U937 cells were differentiated for 72 h with vitamin D3 and then infected with DNase-treated HIV-$1_{LAI/IIIB}$ previously propagated and titered on phytohemagglutinin-blasts (multiplicity of infection = 1). After virus adsorption for 1 h at 37°C, excess virus was removed by extensive washing. Accumulation of HIV-1 proviral DNA was quantified by real-time PCR (16) with an ABI Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, Calif.). The following primer pair set and probe in *gag* were used: forward primer, *for* (5′-ACA TCA AGC AGC CAT GCA AAT-3′); reverse primer, *rev* (5′-ATC TGG CCT GGT GCA ATA GG-3′); and probe, *probe* [5′(FAM) CAT CAA TGA GGA AGC TGC AGA ATG GGA TAG A (TAMRA)-3′]. Accelerated kinetics and higher levels of HIV proviral DNA accumulation were observed within a time frame compatible with a single round of

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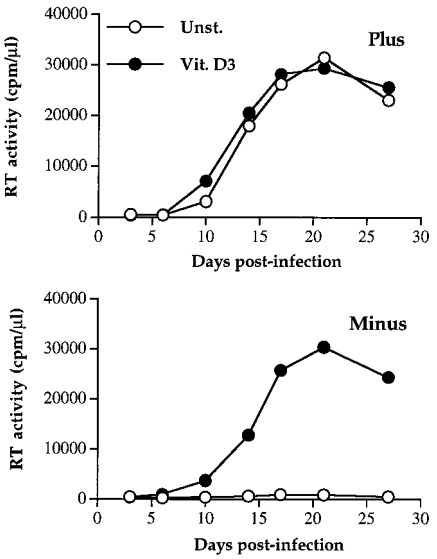

FIG. 1. Vitamin D3 selectively upregulates viral replication in U937 minus clones. One minus clone and one plus clone were infected with H9-derived HIV-$1_{LAI/IIIB}$ in the absence of vitamin D3 (unstimulated) [Unst.]) or presence of 10 nM vitamin D3 (Vit. D3). Mean values of duplicate cultures are shown. The data are representative of four independently performed experiments, including two additional minus clones and one plus clone. Low but detectable levels of viral replication were observed in minus clones (peak RT activity, 930 cpm/μl at day 17 postinfection).

HIV replication (12 to 24 h) in vitamin D3-stimulated cells compared to unstimulated cells (Fig. 4B).

In order to demonstrate that HIV-1 infection and spreading in U937 minus clones occurs in a CXCR4-dependent manner (3, 24), U937 minus clones were adsorbed with HIV-$1_{LAI/IIIB}$ (propagated on H9 cells) and then cultured either alone or in the presence of vitamin D3, SDF-1, or both agents. Unlike what was previously shown in a fusogenicity assay (23), SDF-1 strongly inhibited both constitutive and vitamin D3-enhanced HIV replication in U937 minus clones (Fig. 4C).

Vitamin D3 has been previously shown to exert both positive

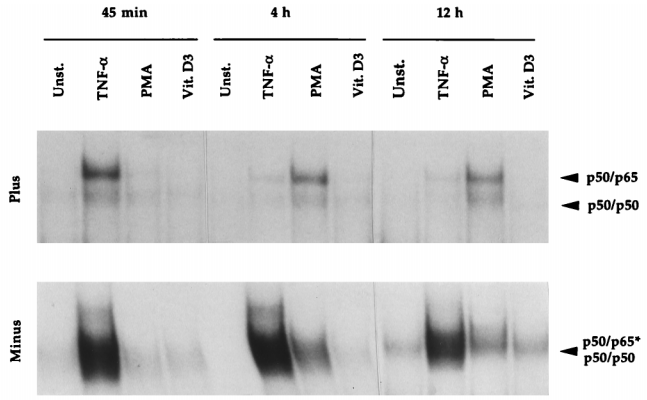

FIG. 2. Vitamin D3 does not induce NF-κB binding in U937 plus and minus cells. Electrophoretic mobility shift assays were performed with cell extracts from unstimulated (Unst.) and stimulated (TNF-α, 2 ng/ml; vitamin D3, 10 nM (Vit. D3); PMA, 10 nM) plus and minus cells at the indicated time points. Vitamin D3 failed to induce NF-κB activation also when cells were examined at a later time point (20 h). The NF-κB probe used and the molecular dissection of the NF-κB complexes from plus and minus clones have been previously described (5, 13). An asterisk indicates the truncated form of p65.

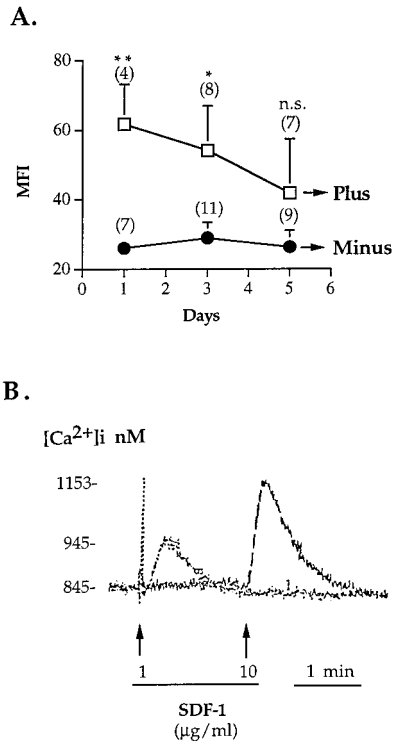

FIG. 3. Expression of functional CXCR4 on U937 minus cells. (A) Comparative analysis of constitutive CXCR4 expression in U937 plus and minus clones tested at different time points after staining with the 12G5 MAb (R&D Systems, Minneapolis, Minn.). Fluorescence-activated cell sorter analyses were performed with a FACScan instrument (Becton & Dickinson, San José, Calif.). The percentage of positive cells was always ≥95% in both types of clones. Results (mean fluorescence intensities [MFI]) are averages of independent determinations (*n*). Plus cell clones showed a higher constitutive expression of CXCR4 than minus cell clones (**, $P = 0.003$; *, $P = 0.055$; n.s., not significant [Student's *t* test]). Error bars show standard errors of the means. (B) SDF-1 (1 and 10 μg/ml) induces Ca^{2+} fluxes in U937 minus clones in a concentration-dependent manner. The analysis was performed on cells loaded with fura-2 (Calbiochem, San Diego, Calif.) (15) by using a Perkin-Elmer (Norwalk, Conn.) LS-5B fluorimeter.

and negative effects on primary monocytic cells or cell lines, including U937 cells (7, 14, 21, 25, 30, 32). However, at least in U937 minus clones, vitamin D3 restored the inefficient HIV replication without inducing either TNF-α secretion (not shown) or NF-κB activation.

Thus far, CXCR4 is the only chemokine receptor known to act as a coreceptor for TCLA HIV-1 (11). Little is known, however, of the physiologic regulation of CXCR4, although interleukin-4 has been recently shown to increase its expression (17). In our study, vitamin D3 selectively enhanced the density of CXCR4 receptors on the cell surface of minus clones but not plus clones. Consistent with the lower constitutive levels of CXCR4 expressed by minus clones compared to plus clones, this effect was responsible, at least in part, for the potent enhancement of HIV replication induced in U937 minus clones by vitamin D3 stimulation. These conclusions were supported by the kinetic quantitative analysis of proviral DNA synthesis. Of interest, Moriuchi et al. (23) have shown that both SDF-1 and the anti-CXCR4 12G5 monoclonal antibody (MAb) poorly inhibited HIV-1 Env-mediated fusion in one U937 plus clone, suggesting the existence of additional, though unidentified, entry cofactors (23). We have confirmed that SDF-1 minimally induced Ca^{2+} fluxes and poorly inhibited HIV replication in plus clones (2a). However, we here dem-

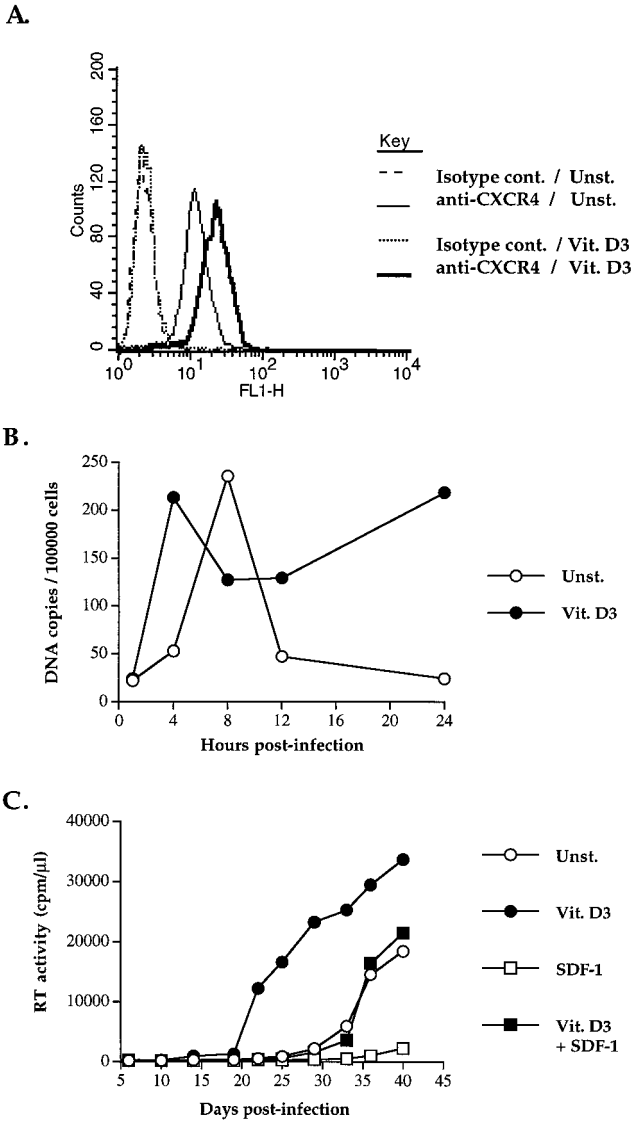

FIG. 4. Vitamin D3 enhances CXCR4 expression and HIV infection in U937 minus cells. (A) U937 minus clones either unstimulated (Unst.) or stimulated with 10 nM vitamin D3 (Vit. D3) for 96 h were analyzed for surface expression of CXCR4 by staining with the anti-CXCR4 MAb 12G5 or with isotype-matched control antibody (murine immunoglobulin G2a) (Isotype cont.). (B) U937 minus cells were prestimulated for 72 h with 10 nM vitamin D3 (Vit. D3) or medium alone (unstimulated [Unst.]) before infection with HIV-$1_{LAI/IIIB}$ grown and titered on phytohemagglutinin-blasts. The thermal cycling conditions were 50°C for 2 min, 95°C for 12 min, and 40 cycles of 95°C for 15 s and 65°C for 1 min. The DNA extracted from serially diluted chronically infected ACH-2 T cells, containing one proviral DNA copy per cell (27), distributed on a curve with a linear regression ($r \geq 0.98$) between 2 and 31,250 cells. A second wave of proviral DNA accumulation was evident in vitamin D3-stimulated cells but not in unstimulated cells, likely as a result of an accelerated spreading of infection. (C) SDF-1 inhibits both constitutive and vitamin D3-enhanced viral replication in U937 minus clones. U937 minus clones infected with HIV-$1_{LAI/IIIB}$ (propagated on H9 cells) were cultured with medium alone (unstimulated [unst.]), 10 nM vitamin D3 (Vit. D3), SDF-1 (1 μg/ml), and vitamin D3 plus SDF-1 (10 nM and 1 μg/ml, respectively). Mean RT activities of duplicate cultures (<15% intersample variability) from one of three independent experiments are shown.

onstrate that SDF-1 promptly triggered Ca^{2+} release and strongly inhibited both constitutive and vitamin D3-induced viral replication in U937 minus cell clones, emphasizing that functional coreceptor molecules, capable of acting as HIV coreceptors, are expressed on their cell surface. Finally, we cannot exclude the possibility that vitamin D3 is able to change the intracellular environment toward a more permissive state in terms of viral integration and/or spreading in the cell culture, although these effects unlikely encompass NF-κB-dependent viral transcription in U937 minus cells. However, in this regard, transcriptional control by enhancers outside of the viral promoter (35) or by Tat-associated kinase activity triggered upon differentiation of U937 cells (36) may play a role.

In conclusion, our findings provide a model for investigating the usage and modulation of the CXCR4 HIV coreceptor in differentiating monocytic cells. In this regard, infection of bone marrow-derived $CD34^+$ cells has been demonstrated in some very advanced AIDS patients but not in individuals at earlier stages of infection (33). Because acquisition of CXCR4 coreceptor usage, leading to a phenotypic switch from macrophage-tropic to T-cell-tropic strains, occurs during the advanced disease stages (8, 20, 29), it is conceivable that factors influencing the levels of CXCR4 expression on HIV target cells—as demonstrated here for vitamin D3—play a role in determining the ability of HIV to expand its cellular tropisms, potentially involving bone marrow-derived progenitor cells.

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