Tetraspanin CD81 Provides a Costimulatory Signal Resulting in Increased Human Immunodeficiency Virus Type 1 Gene Expression in Primary CD4⁺ T Lymphocytes through NF- κ B, NFAT, and AP-1 Transduction Pathways

Mélanie R. Tardif and Michel J. Tremblay*

Research Center in Infectious Diseases, CHUL Research Center, and Faculty of Medicine, Laval University, Quebec, Canada

Received 14 June 2004/Accepted 18 November 2004

The tetraspanin superfamily member CD81 has been shown to form microdomains in the plasma membrane and to participate in the recruitment of numerous adhesion molecules, receptors, and signaling proteins in the central zone of the immune synapse. Beside its structural role, CD81 also delivers a cosignal for T cells to trigger cytokine production and cellular proliferation, thus suggesting a key role in some fundamental biological functions. It has been shown that signaling events initiated through the T-cell receptor (TCR)/CD3 complex and the coactivator CD28 positively affect human immunodeficiency virus type 1 (HIV-1) gene expression, but no study had investigated the putative costimulatory activity of CD81 on HIV-1 transcriptional activity. We observed that CD81 engagement potentiates TCR/CD3-mediated signaling, resulting in an enhancement of HIV-1 transcription and de novo virus production in both established Jurkat cells and primary CD4⁺ T lymphocytes at a magnitude that approximates that with CD28. These observations were made by using transiently transfected plasmids (i.e., nonintegrated viral DNA) and fully competent viruses (i.e., stably integrated provirus). Moreover, the CD81-mediated enhancement of HIV-1 gene expression is linked with increased nuclear translocation of transcription factors known to positively regulate virus transcription, i.e., NF- κ B, NFAT, and AP-1. These findings suggest that engagement of CD81 decreases the signaling threshold required to initiate TCR/CD3-mediated induction of integrated HIV-1 proviral DNA in primary CD4⁺ T cells.

Human immunodeficiency virus type 1 (HIV-1) replication can be initiated following an antigen-specific major histocompatibility complex-restricted signal through the T-cell receptor (TCR)/CD3 complex (10, 42, 43). Virus transcription is further enhanced by a costimulatory signal usually induced by the coengagement of CD28 (11, 42). Ligation of both TCR/CD3 and CD28 triggers multiple signaling pathways, which converge in the activation of transcription factors such as nuclear factor κ B (NF- κ B), nuclear factor for activated T cells (NFAT), and activator protein-1 (AP-1). While CD28 is generally considered the primary receptor for delivering costimulatory signals to T lymphocytes, various studies have revealed that signaling transduction through other cell surface constituents can also cooperate with TCR/CD3-dependent biochemical events to more fully activate T cells. Such alternate costimulatory signals could involve members of the tetraspanin superfamily.

Tetraspanin proteins are made of four transmembrane domains and are widely expressed on different cell types, including epithelial cells and leukocytes. The tetraspanin family comprises several members including CD81, CD82, CD63, CD9, and CD151. These proteins modulate a number of biological effects such as cell-cell adhesion, proliferation, and differentiation (9, 40). Although the exact mechanism by which these

proteins modulate signal transduction pathways remains mostly undefined, it seems to be linked with the ability of tetraspanins to form various complexes with other membrane proteins. Indeed, tetraspanins interact with other tetraspanins and their associated partners to form a larger complex that behaves as a membrane microdomain (48). Thus, the organization of multiple proteins into a network appears to be one of the main roles of the tetraspanin proteins.

CD81 is a member of the tetraspanin family that is expressed on B cells, T cells, antigen-presenting cells (APCs), and some nonlymphoid tumors (26). CD81 has been proposed to play a key role during antigenic presentation, since it colocalizes with the TCR/CD3 complex in the supramolecular activation complex (30). The CD81 protein contributes to the formation of specialized microdomains in the plasma membrane, recruiting various adhesion molecules, receptors, and signaling proteins to the central zone of the immune synapse in both T lymphocytes and APCs (48). In addition, microarray analyses of the gene expression profiles of resting and activated Jurkat and human peripheral blood T cells have demonstrated that CD81 is upregulated upon T-cell activation, supporting the hypothesis that this tetraspanin plays a role in T-cell activation (29). Although the cytoplasmic tail of CD81 lacks the common signaling motif, it provides a costimulatory signal in T cells that enhances gamma interferon and tumor necrosis factor alpha (TNF- α) production and promotes thymocyte proliferation (26, 45). These properties seem to be associated with the capacity of CD81 to form complexes with cell surface proteins such as CD4, CD8 (21), β 1 integrins, and other tetraspanin

* Corresponding author. Mailing address: Laboratory of Human Immuno-Retrovirology, Research Center in Infectious Diseases, RC709, CHUL Research Center, 2705 Laurier Blvd., Quebec (QC), Canada G1V 4G2. Phone: (418) 654-2705. Fax: (418) 654-2212. E-mail: michel.j.tremblay@crchul.ulaval.ca.

proteins, as well as with signaling proteins such as protein kinase C and phosphatidylinositol-4-kinase (4, 19). Thus, CD81 may act as a linker protein that promotes and stabilizes the formation of signaling complexes, which are required for several biological responses.

The induction of HIV-1 transcription is tightly regulated by specific interactions of the viral transcriptional *trans*-activator Tat (15, 27), but cellular transcription factors also contribute actively to provirus transactivation. They do so by binding the *cis*-acting DNA sequences in the HIV-1 long terminal repeat (LTR). One of the main mediators of HIV-1 transcription is NF- κ B, which binds the two NF- κ B-binding motifs located in the enhancer sequence of the LTR promoter (31). The classic NF- κ B complex (p50/p65 heterodimer) is sequestered in the cytoplasm by interaction with its inhibitor (i.e., I κ B- α , - β , - ϵ , and - γ) (18). Upon cell activation, I κ B is phosphorylated, followed by ubiquitination and proteasome-mediated degradation, allowing translocation of NF- κ B into the nucleus. The rate of viral transcription in response to T-cell activation can also be modulated by other cellular transcription factors such as NFAT (12) and AP-1 (24). The latter is composed of Jun homodimers or Jun/Fos heterodimers. NFAT acts in synergy with NF- κ B on the enhancer region of the HIV-1 LTR to positively modulate viral transcription (25). Conversely, AP-1 binds the 5' region of the LTR, located upstream of the enhancer region (1). It has also been shown that AP-1 can cooperate with NF- κ B to activate the HIV-1 LTR through the dual NF- κ B-binding sites (47). Whereas NFAT translocation is regulated mainly by the phosphatase calcineurin, which is itself activated upon elevation of intracellular calcium (34), AP-1 activity is regulated by activation of c-Jun N-terminal kinase (JNK) and extracellular signal-related kinase (ERK) (23). Consequently, cytokines, mitogens, and T-cell activators triggering the activation of NF- κ B, NFAT, and/or AP-1 can contribute to HIV-1 provirus transactivation.

In this report, we demonstrate that engagement of cell surface human CD81 triggers HIV-1 replication independently of the TCR/CD3 signal in Jurkat T lymphoid cells. Moreover, CD81 and TCR/CD3 act in a concerted manner to enhance HIV-1 transcriptional activity in both Jurkat cells and purified human CD4⁺ T lymphocytes. These signaling events initiate signal transduction pathways converging toward activation of NF- κ B, NFAT, and AP-1, which positively modulate HIV-1 transcription and production of progeny virus.

(This work was performed by M.R.T. in partial fulfillment of the requirements for a Ph.D. in the Microbiology-Immunology Program, Faculty of Medicine, Laval University, Quebec, Canada, 2005.)

MATERIALS AND METHODS

Cells. Jurkat cells (clone E6.1) were obtained through the AIDS Repository Reagent Program (Germantown, Md.), whereas 293T cells were kindly provided by W. C. Greene (The J. Gladstone Institutes, San Francisco, Calif.). Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), while Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% FCS. Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by Ficoll-Hypaque gradient centrifugation, whereas CD4⁺ T cells were purified from freshly isolated PBMCs by immunomagnetic negative selection according to the manufacturer's instructions (Miltenyi Biotec, Auburn, Calif.). The purity of CD4⁺ T cells was determined by cytofluorometry analysis and was always greater

than 97%. Isolated PBMCs and purified CD4⁺ T cells were cultured for 2 days in RPMI 1640 medium supplemented with 10% FCS in the presence of phytohemagglutinin (PHA; 1 μ g/ml) and recombinant human interleukin-2 (IL-2) (50 U/ml).

Antibodies. The hybridoma cell line OKT3, which produces the anti-CD3 ζ monoclonal antibody, was obtained from the American Type Culture Collection (ATCC) (Manassas, Va.). A purified anti-CD28 antibody (clone 9.3) was kindly provided by J. A. Ledbetter (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, N.J.). The anti-CD81 antibody (clone 5A6) was a generous gift from S. Levy (Department of Medicine, Division of Oncology, Stanford University Medical Center, Stanford, Calif.) and has been described previously (35). Anti-I κ B α and anti-phospho-I κ B α were purchased from Cell Signaling (Beverly, Mass.), whereas anti-actin was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, Calif.). Hybridomas producing 183-H12-5C and 31-90-25, two antibodies recognizing different epitopes of the HIV-1 major viral core protein p24, were supplied by the AIDS Repository Reagent Program and ATCC, respectively. Antibodies obtained from these cells were purified by using mAbTrap protein G affinity columns according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, N.J.).

Vectors. pNL4-3 is a full-length infectious molecular clone of HIV-1, while pNL4-3 Luc⁺E⁻R⁺ produces Env-deficient HIV-1 reporter particles. These vectors were provided by the AIDS Repository Reagent Program. The pHCMV-G molecular construct codes for the broad-host-range vesicular stomatitis virus envelope glycoprotein G (VSV-G) under the control of the human cytomegalovirus promoter. To study HIV-1 transcriptional activity in Jurkat cells, we used the pLTRx-LUC plasmid, which contains a 722-bp fragment from HIV-1_{LAI} and the luciferase reporter gene. This plasmid was a kind gift from O. Schwartz (Unité d'Oncologie Virale, Institut Pasteur, Paris, France). The pCEP4Tat vector, which carries the HIV-1_{SF-2} *tat* gene, was provided by the AIDS Repository Reagent Program. The involvement of NF- κ B was studied with pLTR-Luc and pm κ BLTR-Luc, kind gifts from K. Calame (Columbia University, New York, N.Y.). These constructs contain the luciferase reporter gene under the control of wild-type and NF- κ B mutant HIV-1 HXB2 LTR. pNF- κ B-LUC contains five consensus NF- κ B sequences upstream of the luciferase reporter gene along with a minimal promoter (Stratagene). The induction of NFAT activity was studied with the pNFAT-LUC molecular construct, harboring the luciferase reporter gene under the control of the minimal IL-2 promoter that contains three tandem copies of the NFAT-binding site. This plasmid was generously provided by G. Crabtree (Howard Hughes Medical Institute, Stanford, Calif.).

Production of viral stocks. Viruses were produced by the calcium phosphate coprecipitation method in 293T cells as described previously (17). Briefly, 293T cells were transfected with pNL4-3 to produce HIV-1 particles or were cotransfected with pNL4-3 Luc⁺E⁻R⁺ and pHCMV-G to produce HIV-1-based viruses pseudotyped with VSV-G. Virus preparations were normalized for virion content by using an in-house enzymatic assay specific for the major viral protein p24. In this test, 183-H12-5C and 31-90-25 are used in combination to quantify p24 levels (6).

Cell transfection and stimulation. Jurkat cells were electroporated with the various vectors by using a Gene Pulser apparatus (Bio-Rad) as described previously (3). Briefly, at 40 h posttransfection, cells (10⁵) were plated in 96-well flat-bottom plates. In some experiments, cells were pretreated for 45 min before stimulation with subcytotoxic concentrations of the calcineurin inhibitor FK506 (Sigma), the proteasome inhibitor MG-132 (Sigma), the MEK inhibitor PD98059 (Calbiochem), or the JNK inhibitor SP600125 (Calbiochem). Where indicated, cells were treated with various combinations of anti-CD3 (clone OKT3; 0.025 to 0.5 μ g/ml), anti-CD28 (clone 9.3; 1 μ g/ml), and anti-CD81 (clone 5A6; 2 μ g/ml) antibodies, followed by cross-linking with a goat anti-mouse IgG (GAM; 10 μ g/ml) in a final volume of 100 μ l. Stimulations were performed at 37°C for the indicated times. Finally, cells were lysed, and luciferase activity (as expressed in relative light units [RLU]) was monitored as described previously (17). To evaluate the ability of MG-132 to inhibit proteasome activity and impair the degradation of phosphorylated I κ B α , Jurkat cells were pretreated with different concentrations of MG-132 for 30 min at 37°C. Cells were then stimulated with TNF- α (20 ng/ml) for 0, 5, 15, or 30 min at 37°C. Cells were next washed and lysed in sample buffer.

Virus infection. Jurkat cells and CD4⁺ T cells (10⁷ cells) were incubated with luciferase-encoding HIV-1 pseudotyped with VSV-G (350 ng of p24) at 37°C for 48 h before treatment with the tested antibodies. Cells were then washed with phosphate-buffered saline (PBS) and resuspended in 10 ml of complete culture medium, and 10⁵ cells were transferred to each well of 96-well flat-bottom tissue culture plates. Next, cells infected with luciferase-encoding viruses were stimulated as described above, and luciferase activity was analyzed at 8, 24, or 48 h posttreatment. To quantify viral production upon stimulation, CD4⁺ T lympho-

cytes were infected with fully infectious NL4-3 (10 ng of p24 per 10^5 cells) for 48 h at 37°C. After a wash, 10^5 cells were plated in 96-well flat-bottom plates precoated with goat anti-mouse IgG (20 µg/ml), anti-CD3 (clone OKT3 at 0.5 µg/ml), anti-CD28 (clone 9.3 at 2 µg/ml), and anti-CD81 (clone 5A6 at 4 µg/ml) used either alone or in various combinations. Cell-free supernatants were harvested at 48 and 96 h poststimulation and then frozen at -20°C until they were assayed for p24 contents.

Fluorescence-activated cell sorter analysis. To monitor CD81 expression on Jurkat and PHA-IL-2-treated CD4⁺ T lymphocytes, cells were incubated with anti-CD81 (5A6) or an isotype-matched irrelevant control antibody for 30 min at 4°C. To evaluate whether HIV-1 infection can down-regulate surface expression of CD81 on CD4⁺ T cells, cells were infected or not with NL4-3 for 3 days before they were labeled with anti-CD81. Cells were next washed with ice-cold PBS and then incubated with an R-phycoerythrin-conjugated goat anti-mouse secondary antibody for 30 min at 4°C. After two washes, cells were fixed in 2% paraformaldehyde and analyzed by cell sorting (Epics ELITE ESP; Coulter Electronics, Burlington, Ontario, Canada).

Electrophoresis and Western blotting. Samples were boiled for 7 min before being loaded onto sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) gels. Proteins were then transferred to Immobilon membranes (Millipore Corporation, Bedford, Mass.). Immunoblotting was performed first with anti-phospho-IκBα. Next, the membrane was stripped and blotted with anti-IκBα. To measure the amount of protein loaded in the gel, the membrane was stripped again and immunoblotted with anti-actin. Proteins were detected with the enhanced chemiluminescence (ECL) reagent (Pierce), followed by exposure to X-Omat film.

Nuclear extracts and EMSA. Primary CD4⁺ T lymphocytes (7×10^6) that had been starved of IL-2 for 16 h were either left untreated or stimulated for 6 h at 37°C with various combinations of 5A6 (2 µg/ml), 9.3 (1 µg/ml), and OKT3 (0.25 µg/ml) in the presence of GAM (10 µg/ml) in a final volume of 1 ml. Cells were then washed twice with ice-cold PBS, and nuclear extracts were prepared according to a previously reported protocol (3). Electrophoretic mobility shift assays (EMSA) were performed by incubating 10 µg of nuclear proteins with 20 µl of 1× binding buffer [100 mM HEPES (pH 7.9), 40% glycerol, 10% Ficoll, 250 mM KCl, 10 mM dithiothreitol, 5 mM EDTA, 250 mM NaCl, 2 µg of poly(dI-dC), 10 µg of nuclease-free bovine serum albumin fraction V] containing 0.8 ng of a γ -³²P-labeled double-stranded DNA oligonucleotide for 20 min at room temperature. The enhancer region (-107 to -77) from HIV-1 strain NL4-3 (5'-CAAGGGACTTTCGCTGGGGACTTTCAGGG-3'), the distal NFAT-binding site from the murine IL-2 promoter (5'-TCGAGCCAAAGAGGAA AATTGTTTCATG-3'), the consensus NF-κB-binding site (5'-ATGTGAGG GGACTTTCAGGC-3'), the consensus AP-1 binding site (5'-CGCTTGAT GACTCAGCCGGA-3'), the consensus SP1 binding site (5'-ATTCGATCGG GCGGGGCGAG-3'), and the consensus binding site for Oct-2A (5'-GGAG TATCCAGCTCCGTAGCATGCAAATCCTCTGG-3') (used as a control for nonspecific competition) were utilized as probes and/or for competition assays. The DNA-protein complexes were resolved from free-labeled DNA by electrophoresis in native 4% (wt/vol) polyacrylamide gels. The gels were subsequently dried and exposed to Kodak X-ray film. Cold competition assays were carried out by adding a 100-fold molar excess of an unlabeled double-stranded DNA oligonucleotide simultaneously with the labeled probe. Supershift assays were performed by preincubation of nuclear extracts with 1 µg of an antibody against NF-κB p50 (Santa Cruz) or NF-κB p65 (kindly given by Nancy Rice, National Cancer Institute, Frederick, Md.) for 30 min on ice prior to addition of the binding buffer containing the labeled probe. Laser densitometry was performed with an Alpha Imager 2000 digital imaging and analysis system (Alpha Innotech Corporation, San Leandro, Calif.).

Statistical analysis. Results are expressed as means ± standard deviations (SD) of triplicate samples. The statistical significance of differences between groups was determined by analysis of variance. Calculations were made with Microsoft Excel. *P* values of <0.05 were considered statistically significant.

RESULTS

TCR/CD3-dependent induction of HIV-1 transcription of unintegrated and integrated proviral DNA is augmented upon CD81 ligation, both in the absence and in the presence of Tat. It was previously demonstrated that CD81 delivers a costimulatory signal upon engagement of the TCR/CD3 complex that results in phosphorylation of specific signaling proteins in Jurkat cells and PBMCs (39, 45). Since HIV-1 transcription is

intimately linked to the cellular activation state, we monitored the impact of CD81-mediated signaling events on HIV-1 LTR transcriptional activity. To this end, Jurkat cells were first transiently transfected with pLTRx-LUC, a vector containing the luciferase gene placed under the control of the complete HIV-1 LTR region. As shown in Fig. 1A, engagement of the tetraspanin CD81 alone by use of monoclonal antibody 5A6 gives rise to a signaling cascade that weakly augments HIV-1 LTR activity (2.2-fold increase). Interestingly, coengagement of CD81 and the TCR/CD3 complex resulted in a higher induction of HIV-1 LTR-driven reporter gene activity. A comparable induction of virus transcription was obtained following coengagement of TCR/CD3 and the well-known costimulatory molecule CD28 (by OKT3-9.3 treatment). Furthermore, cross-linking of CD81 and the TCR/CD3 complex in the presence of CD28 resulted in a more potent induction of HIV-1 LTR-oriented gene expression (7.3-fold increase). Given that saturating concentrations of anti-CD81 and anti-CD28 antibodies were used in such studies (2 µg/ml for 5A6 and 1 µg/ml for 9.3), it can be proposed that CD81 and CD28 use distinct signaling pathways in Jurkat cells, leading to an additive effect on HIV-1 transcription. Moreover, the CD81-dependent activating effect is further augmented upon addition of a suboptimal concentration (0.025 µg/ml) of the anti-CD3 antibody OKT3 (Fig. 1B), suggesting that engagement of CD81 reduces the activation threshold, resulting in a more potent up-regulation of HIV-1 LTR activity in Jurkat cells in response to anti-CD3. These findings demonstrate that CD81 efficiently enhances TCR/CD3-mediated HIV-1 LTR transcription.

HIV-1 transcription is known to be markedly enhanced by the virus-encoded transactivating protein Tat (27). We wanted to determine whether Tat could mask any costimulatory effect mediated by CD81 and/or modify the intracellular biochemical events associated with such a costimulatory molecule. To this end, a similar experimental strategy was applied except that Jurkat cells were also cotransfected with a Tat-encoding vector (pCEP4-Tat). As expected, the presence of Tat leads to a significant (36-fold) increase in HIV-1 LTR-driven luciferase activity. More importantly, Tat further amplifies HIV-1 transcription in the presence of the different inducers tested (Fig. 1C). For example, engagement of CD81 produces a 4.1-fold increase, whereas a 20-fold increase is obtained upon coligation of CD81 and TCR/CD3. Coengagement of CD81, TCR/CD3, and CD28 further enhances HIV-1 transcriptional activity (34.6-fold increase).

Integration into the host chromosome is an essential step of the HIV-1 life cycle. The provirus is coated with histones and nonhistone proteins, thus forming a nucleosome. The cellular factors required for transcription of integrated viral genetic material (proviral DNA) may thus differ from those required for activation of unintegrated viral DNA (a transiently transfected vector) (14, 33). In order to take this fact into account, Jurkat cells were infected with HIV-1 reporter particles pseudotyped with the VSV-G envelope protein. Transcription of integrated HIV-1 is again increased by the anti-CD81 antibody 5A6 when used alone, and coligation of CD81 and TCR/CD3 results in an additive effect (Fig. 1D). The observed up-regulatory effect on transcription of integrated viral genetic material is linked with CD81-mediated signal transduction events and is not attributable to a difference in virus infectivity,

FIG. 1. The tetraspanin CD81 cooperates with TCR/CD3 to enhance HIV-1 transcriptional activity in Jurkat cells. Jurkat cells either transiently transfected with pLTRx-LUC (A and B), cotransfected with pLTRx-LUC and pCEP4-Tat (C), or infected with reporter HIV-1 pseudotyped with VSV-G (D) were either left untreated or incubated for 8 (A, B, and C) or 24 (D) h with the indicated cross-linked antibodies in various combinations (5A6 at 2 μg/ml; OKT3 at 0.25 μg/ml; 9.3 at 1 μg/ml). Cells were next lysed in order to assess HIV-1 LTR-encoded reporter gene activity. Results are shown as the increase over the activity of samples treated with GAM (used as negative controls) and are calculated from the mean $\pm$ SD for three different lysed cell samples in the same experimental setting. The mean luciferase counts of control cells were 2.2 (A), 10.5 (B), 80.5 (C), and 3.4 (D) RLU. Results are representative of three independent experiments. Asterisks indicate significant differences between cells stimulated with OKT3–5A6, OKT3–9.3, or OKT3–9.3–5A6 and cells treated with OKT3 alone (*, $P < 0.05$; **, $P < 0.02$).

since pseudotyped virus can achieve only a single round of infection.

The tetraspanin CD81 cooperates with TCR/CD3 to more fully activate transcription of integrated HIV-1 and enhance the production of de novo virus particles in primary CD4⁺ T lymphocytes. To more closely approximate in vivo situations, experiments were also performed in primary CD4⁺ T lymphocytes, a cell type considered a major target and reservoir of HIV-1. Flow cytometric analyses revealed that high levels of CD81 are expressed on both Jurkat cells and primary CD4⁺ T lymphocytes (Fig. 2A). These primary human cells were infected with recombinant luciferase-encoding HIV-1 particles that had been pseudotyped with the VSV-G envelope and were next subjected to the stimuli at 48 h postinfection. In contrast to what is seen in the established Jurkat cell line, engagement of CD81 alone is not sufficient per se to achieve HIV-1 transcription in CD4⁺ T cells. However, costimulation of CD81 and TCR/CD3 results in a >10-fold increase in virus gene expression (Fig. 2B). It should be noted that coligation of CD81 and TCR/CD3 is a less potent activator of HIV-1 tran-

scription in CD4⁺ T lymphocytes than coengagement of CD28 and TCR/CD3. Moreover, engagement of CD81, CD28, and the TCR/CD3 complex does not mediate a more important increase in HIV-1 transcription than coligation of CD28 and TCR/CD3, an observation different from that for Jurkat cells.

The HIV-1 replicative cycle is complex and is under the influence of various viral and cellular proteins. Our next series of investigations was thus performed with fully competent viruses (i.e., the prototypic X4-utilizing virus strain NL4-3), which were used to inoculate mitogen-stimulated CD4⁺ T lymphocytes. Forty-eight hours postinfection, HIV-1-infected cells were incubated in plates coated with the anti-CD81, anti-CD28, and/or anti-TCR/CD3 antibody, alone or in various combinations. Virus release was assessed by measuring p24 levels in cell-free culture supernatants at 96 h after treatment with the tested antibodies. The data shown in Fig. 2C indicate that coengagement of CD81 and the TCR/CD3 complex mediates higher virus production than ligation of TCR/CD3 alone, thus confirming the costimulatory capacity of the tetraspanin CD81 with regard to HIV-1 biology. Surprisingly,

FIG. 2. CD81 acts as a costimulatory molecule to enhance TCR/CD3-mediated induction of HIV-1 transcription and virus production in primary CD4⁺ T lymphocytes. (A) Flow cytometric analyses of CD81 surface expression on Jurkat cells and purified CD4⁺ T lymphocytes after staining with an isotype-matched control antibody (dotted lines) or the anti-CD81 antibody 5A6 (continuous lines). (B) Primary CD4⁺ T cells were inoculated with VSV-G-pseudotyped reporter viruses and were either left untreated or treated for 48 h with cross-linked 5A6 (2 μ g/ml), OKT3 (0.25 μ g/ml), and/or 9.3 (1 μ g/ml). Cells were next lysed to assess HIV-1 LTR-encoded reporter gene activity. The mean luciferase activity in control cells was 10 RLU. (C) CD4⁺ T lymphocytes were infected with fully competent HIV-1 (strain NL4-3) and were either left untreated or treated with the listed cross-linked antibodies. Cell-free supernatants were harvested 96 h poststimulation, and p24 contents were measured by an enzymatic assay. Results are calculated from the mean $\pm$ SD of three different cell samples in the same experimental setting. Results are representative of three independent experiments. Asterisks indicate significant differences between cells stimulated with OKT3–5A6, OKT3–9.3, or OKT3–9.3–5A6 and cells treated with OKT3 only (*, $P < 0.05$; **, $P < 0.01$).

there is no direct correlation between CD28- and TCR/CD3-mediated induction of HIV-1 transcription and virus production (compare Fig. 2B and C). It is noteworthy that CD81 surface expression is not affected either in Jurkat cells or in primary CD4⁺ T cells upon HIV-1 infection (data not shown). Our results demonstrate that CD81 provides a costimulatory signal comparable to that mediated by CD28, resulting in an augmentation of TCR/CD3-mediated HIV-1 production in primary CD4⁺ T cells.

The costimulating property of the tetraspanin CD81 is linked with its capacity to mediate nuclear translocation of

various transcription factors that can bind to the HIV-1 enhancer region. We scrutinized the mechanism(s) through which the CD81-dependent biochemical events can amplify TCR/CD3-mediated induction of HIV-1 gene expression. In an attempt to define whether the observed up-regulating effect of CD81 on virus gene expression is due to an effect on transcription factors that can bind to the viral DNA regulatory sequences, we performed EMSA with a labeled probe containing the minimal enhancer region of the HIV-1 LTR (positions –107 to –77 relative to the transcription initiation site). Nuclear extracts from primary CD4⁺ T lymphocytes costimulated

FIG. 3. CD81-mediated costimulatory capacity enhances nuclear translocation of transcription factors that can bind to the HIV-1 enhancer region. Primary CD4⁺ T lymphocytes were incubated for 6 h either with goat anti-mouse IgG, used as a negative control (Ctrl), or with the indicated antibodies (5A6 at 2 μ g/ml; OKT3 at 0.25 μ g/ml) before nuclear extraction. Nuclear proteins were then incubated with a labeled HIV-1 enhancer probe (A) or the labeled negative internal control SP1 (B), and the complexes were resolved on a native 4% acrylamide gel. The specificities of the migrating complexes were verified by competition with a specific (HIV-1 enhancer [Cold probe]) or nonspecific (Oct-2A probe) probe. The intensities of the bands were quantified with an Alpha Imager 2000 digital imaging and analysis system. Results are representative of three independent experiments. Arrows on the left indicate the specific migrating complexes.

by CD81 and the TCR/CD3 complex contained more HIV-1 enhancer-bound proteins than samples subjected to ligation of TCR/CD3 alone (Fig. 3A). This is convincingly demonstrated when the intensities of the complexes are quantified with an Alpha Imager 200 digital imaging and analysis system. The specificities of the complexes were established by competition with a 100-fold excess of a specific (cold HIV-1 enhancer) or nonspecific (Oct-2A) probe. The intensities of the bands were comparable when a negative internal control (SP1) was used, confirming that the amounts of the extracts used were the same and that the results observed were specific (Fig. 3B). These data indicate that the signaling cascade triggered by CD81, when combined with TCR/CD3 ligation, increases the translocation of transcription factors into the nucleus, where it can bind to the HIV-1 enhancer domain.

NF- κ B is activated upon costimulation of CD81 and TCR/CD3, and this event strongly contributes to enhanced HIV-1 transcription in both Jurkat cells and primary CD4⁺ T cells. NF- κ B is recognized as a powerful activator of HIV-1 transcription. Therefore, we next studied the possible CD81-mediated induction of NF- κ B by transient transfection of Jurkat cells with pNF- κ B-Luc, a vector containing multiple tandem copies of NF- κ B-binding sites. As depicted in Fig. 4A, treatment of these cells with antibody 5A6 results in activation of NF- κ B to a level comparable to that with TCR/CD3 engagement. This induction is further amplified upon cross-linking of CD81 and TCR/CD3. The observed CD81- and TCR/CD3-dependent induction of HIV-1 LTR activity was abolished when a plasmid containing a mutated NF- κ B-binding site (pLTRm κ B-Luc) was used instead (Fig. 4B). Next, Jurkat cells were inoculated with VSV-G-pseudotyped HIV-1 particles and were treated with a subcytotoxic dose of MG-132 (1 or 10 μ M) before antibody-mediated engagement of the cell surface com-

ponents studied. MG-132 is a proteasome inhibitor that blocks the degradation of phosphorylated I κ B α and consequently inhibits NF- κ B translocation from the cytosol to the nucleus. The capacity of MG-132 to impair the degradation of phosphorylated I κ B α upon TNF- α stimulation is shown in Fig. 4C. The data in Fig. 4D confirm the importance of NF- κ B in the enhancement of transcription of the integrated HIV-1 genome that is seen upon engagement of cell surface CD81 and TCR/CD3. However, experiments performed with CD4⁺ T lymphocytes revealed that, as was the case for Jurkat cells, the CD81-triggered enhancement of the TCR/CD3-dependent effect on virus transcription is not completely abolished by MG-132 (Fig. 4E), even at the highest concentration tested (10 μ M [data not shown]). This suggests that another transcription factor(s) might be activated once CD81 and TCR/CD3 are cross-linked on the surfaces of primary CD4⁺ T cells. Mobility shift assays performed with an NF- κ B-specific labeled probe provide additional evidence that the anti-CD3-dependent nuclear translocation of NF- κ B is augmented upon engagement of CD81 in CD4⁺ T lymphocytes (Fig. 5A). Experiments conducted with the cold NF- κ B oligonucleotides and the negative internal control SP1 (Fig. 5B) confirmed the specificities of the retarded complexes and the fact that similar amounts of nuclear extracts were loaded. Comparable observations were made when the labeled HIV-1 enhancer region was used as a probe (Fig. 5C). It should be noted that the complex specific for NF- κ B was supershifted when nuclear extracts were pretreated with a polyclonal anti-p50 or anti-p65 antibody.

Coligation of CD81 and the TCR/CD3 complex also mediates an NFAT-dependent increase in HIV-1 transcriptional activity. Considering that NFAT represents another transcription factor known to positively affect HIV-1 LTR-driven gene expression, we assessed whether engagement of the tet-

FIG. 4. CD81-directed costimulatory signal leads to augmented induction of NF- κ B resulting in increased HIV-1 transcription in Jurkat and CD4⁺ T cells. Jurkat cells were transiently transfected with pNF- κ B-LUC (A) or were transfected with pLTR-LUC or pm κ B-LTR-LUC (B). In some instances, Jurkat cells (D) or primary CD4⁺ T lymphocytes (E) were used as targets for pseudotyped reporter HIV-1 particles. Jurkat and CD4⁺ T cells that had been inoculated with reporter HIV-1 particles pseudotyped with VSV-G envelope proteins were either left untreated or pretreated for 1 h with the proteasome inhibitor MG-132 at the indicated concentrations. Next, cells were either left untreated or treated for 8 (A, B, and D) or 24 (E) h with the listed cross-linked antibodies (5A6 at 2 μ g/ml; OKT3 at 0.25 μ g/ml; 9.3 at 1 μ g/ml). Finally, cells were lysed to assess HIV-1 LTR-encoded reporter gene activity. Results are shown as the increase over the activity in samples treated with GAM (used as negative controls) and are calculated from the mean $\pm$ SD of three different lysed cell samples in the same experimental setting. The mean luciferase counts of control cells were 13 RLU in panel A, 19 (LTR-Luc) and 2.2 (m κ BLTR-Luc) RLU in panel B, 61 (without MG-132) and 223 (1 μ M MG-132) RLU in panel D, and 10 (without MG-132), 10.7 (0.1 μ M MG-132), and 4.3 (1 μ M MG-132) RLU in panel E. Results are representative of three independent experiments. Asterisks indicate significant differences between cells treated with MG-132 and untreated cells (*, $P < 0.05$). (C) Untreated or MG-132-treated Jurkat cells were stimulated with TNF- α (20 ng/ml) for the indicated times (in minutes) and then lysed in sample buffer. Samples were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

raspanin CD81 can modulate NFAT activity by using Jurkat cells transfected with pNFAT-LUC, a vector bearing multiple NFAT-binding sites. Engagement of CD81 alone does not trigger a signal leading to NFAT activation, but it strongly increases the TCR/CD3-mediated nuclear translocation of NFAT (Fig. 6A). To assess the participation of NFAT in the enhanced virus transcription observed upon engagement of CD81 and TCR/CD3, the Jurkat cells that were inoculated with pseudotyped reporter viruses were treated with FK506 prior to stimulation. The molecular mechanism of action of FK506 in T cells has been well defined and involves inhibition of calcineurin phosphatase activity, an event that is critical for the nuclear translocation of NFAT. This immunosuppressant caused a dose-dependent diminution of CD81- and TCR/CD3-

mediated induction of transcription of the integrated HIV-1 genome in both Jurkat cells (Fig. 6B) and primary CD4⁺ T cells (Fig. 6C). Our data are perfectly in line with previous work showing the cooperative effect of CD81 with TCR/CD3 in mediating an NFAT-dependent increase in cytokine production (39, 45). The capacity of the tetraspanin CD81 to enhance TCR/CD3-mediated induction of NFAT in primary CD4⁺ T cells was next tested by EMSA. Engagement of the TCR/CD3 complex leads to a weak signal specific for NFAT (Fig. 7A). However, the intensity of the migrating NFAT complex is significantly increased when CD81 is also engaged. The specificity of the signal is confirmed by use of an unlabeled specific (NFAT) and a nonspecific (Oct-2A) probe, as well as by the negative internal control SP1 (Fig. 7B).

FIG. 5. Nuclear translocation of NF- κ B is augmented upon coligation of CD81 and the OKT3/CD3 complex in primary CD4⁺ T lymphocytes. (A and B) Primary CD4⁺ T lymphocytes were incubated for 6 h either with GAM, used as a negative control (Ctrl), or with the indicated antibodies (5A6 at 2 μ g/ml; OKT3 at 0.25 μ g/ml) before nuclear extraction. Nuclear proteins were then incubated with a labeled NF- κ B-specific probe (A) or the labeled negative internal control SP1 (B), and the complexes were resolved on a native 4% acrylamide gel. The intensities of the bands were quantified with an Alpha Imager 2000 digital imaging and analysis system. (C) The labeled HIV-1 enhancer region was also used as a probe, and the resolved complexes were supershifted with anti-p50 or anti-p65 antibodies. The specificities of the migrating complexes were verified by competition with a specific (NF- κ B specific [A] or HIV-1 enhancer [C]) or nonspecific (Oct-2A) probe. Results are representative of three independent experiments. Arrows indicate the antibody-mediated migrating and supershifted complexes.

The costimulating ability of CD81 that leads to increased TCR/CD3-mediated virus transcription in CD4⁺ T cells requires AP-1 stimulation via ERK1/2 and JNK. Previous studies have shown that AP-1 can positively regulate HIV-1 through the AP-1 binding sites located in the HIV-1 5' LTR region. It has also been reported that ERK1 and -2 (ERK1/2) mitogen-activated protein kinase-dependent AP-1 activation leads to the formation of AP-1/NF- κ B complexes, resulting in enhancement of HIV-1 transcriptional activity (47). ERK1/2 activation induces *c-fos* expression and contributes to *c-jun* gene induction, whereas phosphorylation of *c-jun*, which is required for optimal AP-1 DNA binding activity, is mediated by JNK (22, 23). These observations, coupled with our findings with the NFAT multimer reporter gene that is derived from the IL-2 promoter and consists of composite NFAT/AP-1 sites (Fig. 6A), prompted us to investigate the involvement of the AP-1 transcription factor. The data in Fig. 8A demonstrate that coligation of CD81 and TCR/CD3 in CD4⁺ T cells results in a significant increase in the intensities of AP-1 binding complexes over that seen with engagement of the TCR/CD3 com-

plex alone, whereas the intensities of the bands remained unchanged with the negative internal control SP1 (Fig. 8B). The involvement of ERK1/2 was tested by treating CD4⁺ T lymphocytes infected with pseudotyped HIV-1 reporter viruses with PD098059, an inhibitor of ERK1/2, before incubation with the tested antibodies. As shown in Fig. 8C, the ERK1/2-mediated signaling pathways are required for enhancement of virus gene expression in response to the coengagement of CD81 and TCR/CD3. Comparable results were obtained when the pharmaceutical agent SP600125, which impairs the ability of JNK to phosphorylate *c-jun*, was used instead (Fig. 8D).

DISCUSSION

There is a paucity of data concerning the ability of tetraspanin proteins to initiate and/or potentiate signal transduction events in primary T lymphocytes. It is known that CD81, CD82, CD9, and CD53 can provide a cosignal when coupled with TCR/CD3 engagement to produce increased levels of IL-2 as well as other cytokines in the established Jurkat cell

FIG. 6. The cosignal provided by CD81 further enhances TCR/CD3-mediated induction of NFAT, an event that leads to increased HIV-1 transcription in Jurkat and primary CD4⁺ T cells. Jurkat cells were transiently transfected with pNFAT-LUC (A) or infected with VSV-G-pseudotyped reporter virus (B). In some experiments, primary CD4⁺ T lymphocytes were used as targets for pseudotyped reporter HIV-1 particles (C). Jurkat and CD4⁺ T cells inoculated with reporter HIV-1 particles pseudotyped with VSV-G envelope proteins were either left untreated or pretreated for 1 h with FK506 at the indicated concentrations. Next, cells were either left untreated or treated for 8 (A and B) or 24 (C) h with the indicated cross-linked antibodies (5A6 at 2 μ g/ml; OKT3 at 0.25 μ g/ml; 9.3 at 1 μ g/ml). Finally, cells were lysed to assess HIV-1 LTR-encoded reporter gene activity. Results are shown as the increase over the activity in samples treated with goat-anti-mouse IgG (used as negative controls) and are calculated from the mean $\pm$ SD of three different lysed cell samples in the same experimental setting. The mean luciferase counts of control cells were 0.5 RLU in panel A, 7.5 (0 and 0.2 ng of FK506/ml) and 7.9 (2 ng of FK506/ml) RLU in panel B, and 10 (0 and 0.2 ng of FK506/ml) and 9 (2 ng of FK506/ml) RLU in panel C. Results are representative of three independent experiments. Asterisks indicate significant differences between cells treated with FK506 and untreated cells (*, $P < 0.05$; **, $P < 0.005$).

line and in murine T cells (45), but the signaling pathways involved in these biological effects have not yet been fully elucidated. Moreover, except for the hepatitis C virus (HCV) envelope protein E2 (16), no physiological ligand has so far been identified for CD81. Nevertheless, studies performed with antibodies specific for CD81 have clearly demonstrated the potential of this membrane protein to amplify the signaling cascades in response to TCR/CD3 engagement in both Jurkat cells and PBMCs, resulting in NFAT-dependent cytokine production. It has also been reported that binding of HCV E2 protein to CD81 triggers a costimulatory signal for human CD4⁺ T lymphocytes through the activation of p56^{lck} (39). Since both HCV E2 and an anti-CD81 antibody can deliver a costimulatory signal through this tetraspanin and activate CD4⁺ T cells, we were interested in studying whether engagement of CD81 can modulate HIV-1 transcription in this cell subset.

Induction of HIV-1 gene expression in CD4⁺ T lymphocytes is influenced by several stimuli. However, the exact mechanisms by which costimulatory molecules other than CD28 control viral transcription are poorly known. In this report, we show for the first time that the tetraspanin CD81 acts as a coactivator of HIV-1 transcription upon a concomitant ligation of the TCR/CD3 complex to enhance virus gene expression in both Jurkat cells and primary CD4⁺ T cells. Our results also indicate that the CD81-directed enhancement of HIV-1 production requires the involvement of several cellular transcription factors.

Homotypic adhesion is one of the most apparent biological effects induced very early upon engagement of CD81 in the absence of other stimulation in Jurkat and primary CD4⁺ T cells. T-cell adhesion has already been proposed to provide the first signal, resulting in a lowering of the threshold required for initiation of TCR/CD3 signaling (2, 37). This phenomenon seems to be connected to an increase in the calcium content of intracellular stores and in the amount of phosphatidylinositol-4,5-bisphosphate in the plasma membrane (37, 44). The induction of an intracellular calcium flux in response to CD81 cross-linking in T cells has already been described, suggesting that CD81 engagement can prime such cells for activation of certain signaling pathways (39). Interestingly, our experiments using Jurkat cells have shown that ligation of CD81 alone can activate HIV-1 transcriptional activity through NF- κ B activation, indicating that signaling events can occur via CD81 in CD4-expressing T-lymphoid cells. Nevertheless, the engagement of CD81 is not sufficient by itself to induce HIV-1 transcription in primary CD4⁺ T cells. It can be proposed that variations in the signal transducer(s) and/or signaling complex(es) might be responsible for the differences in CD81-mediated activation of HIV-1 transcription between primary CD4⁺ T cells and Jurkat cells. This observation is reminiscent of the reported biochemical defects in Jurkat cells. For example, Jurkat cells carry a defect in expression of the D3 phosphoinositide phosphatase PTEN, which leads to constitutive activation of the phosphoinositide 3-kinase (PI3K) signaling pathway. By virtue of the cardinal role played by PI3K in several biological responses, the constitutive PI3K activation results in the presence of a number of signaling proteins recruited at the plasma membrane as well as in the persistence of ERK1/2 and JNK activation (38). Since numerous biochemical

FIG. 7. CD81 cooperates with TCR/CD3 to increase the translocation of NFAT to the nucleus, where it can bind to the HIV-1 enhancer region. Primary CD4⁺ T lymphocytes were incubated for 6 h either with GAM, used as a negative control (Ctrl), or with the indicated antibodies (5A6 at 2 μ g/ml; OKT3 at 0.25 μ g/ml) before nuclear extraction. Nuclear proteins were then incubated with a labeled NFAT-specific probe (A) or the labeled negative internal control SP1 (B), and the complexes were resolved on a native 4% acrylamide gel. The intensities of the bands were quantified with an Alpha Imager 2000 digital imaging and analysis system. Results are representative of three independent experiments.

transducers are already located close to the plasma membrane, waiting to transmit the signal toward the nucleus, the signal mediated through membrane receptors is faster and intensified. This might help to explain how the engagement of CD81 alone can induce HIV-1 transcription through an NF- κ B-dependent pathway in Jurkat cells but not in CD4⁺ T lymphocytes, which require additional signals (e.g., via the TCR/CD3 complex).

An increase in TCR/CD3-dependent HIV-1 transcriptional activity is observed in Jurkat cells upon engagement of CD81. The magnitude of the costimulatory effect mediated by CD81 is comparable to that of CD28, a well-recognized potent costimulatory molecule. Moreover, the effects of both types of costimulation are additive, suggesting that signaling events triggered by each coactivator might be different in Jurkat cells, as previously proposed (45). In contrast, experiments performed with primary CD4⁺ T cells demonstrate that CD28 delivers a stronger cosignal than does CD81, as determined by measuring induction of HIV-1 gene expression. However, production of HIV-1 particles is equally up-regulated upon coligation of TCR/CD3 and either CD81 or CD28 in CD4⁺ T lymphocytes.

We demonstrate for the first time that engagement of CD81 results in enhancement of NF- κ B-, NFAT-, and AP-1 DNA-binding activities in response to TCR/CD3 stimulation in primary CD4⁺ T lymphocytes. By use of NF- κ B- and NFAT-regulated reporter gene constructs and specific chemical agents, we provide evidence that the costimulating activity of CD81 implicates degradation of I κ B α and activation of the calmodulin/calcineurin pathway. In addition, we found that coligation of CD81 led to an augmentation of AP-1 nuclear translocation, an event that was also accompanied by increased phosphorylation of ERK1/2 (data not shown). These results

confirm previous observations that the CD81-induced cosignal favors the formation of an efficient AP-1 complex, formed by *c-jun* and *c-fos*, which is regulated at least in part by ERK1/2 and JNK (23). These findings suggest that ligation of the tetraspanin CD81 might play a role in the formation and stabilization of the signaling complex which may assist TCR/CD3-mediated biochemical events in the activation of downstream transcription factors. Furthermore, we found that the CD81- and TCR/CD3-mediated increase in HIV-1 transcription was more severely decreased by the proteasome inhibitor MG-132 than TCR/CD3-dependent virus gene expression. Since MG-132 has been also shown to activate AP-1 (32, 46), it can be postulated that the negative impact of this compound on viral transcription is partially compensated for by a possible AP-1-directed up-regulatory effect on the HIV-1 LTR domain. As for AP-1, this transcription factor has been reported to activate HIV-1 expression through binding sites located both in the modulatory region and in the nontranslated leader sequence (1, 8, 36). Moreover, it was reported that the *c-fos/c-jun* complex cooperates with NF- κ B to promote HIV-1 transcription through the NF- κ B-binding region (47). While treatment of cells with an ERK1/2 or JNK inhibitor (PD098059 or SP600125, respectively) affects HIV-1 transcriptional activity in response to the costimulating property of CD81, the effect of AP-1 through its own binding sites or in cooperation with NF- κ B on the HIV-1 LTR remains to be determined.

One of the unique features of the tetraspanins is their ability to form a network of multimolecular complexes, the "tetraspanin web." Indeed, it has been shown that these transmembrane proteins are physically associated with a wide variety of partner proteins such as signaling enzymes, several immunoglobulin superfamily proteins, proteoglycans, complement regulatory proteins, integrins, growth factors, growth factor recep-

FIG. 8. Coengagement of CD81 and TCR/CD3 leads to increased AP-1 DNA-binding activity and HIV-1 transcription in CD4⁺ T cells through ERK1/2- and JNK-dependent pathways. (A and B) Primary CD4⁺ T lymphocytes were incubated for 6 h either with GAM, used as a negative control (Ctrl), or with the indicated cross-linked antibodies (5A6 at 2 μ g/ml; OKT3 at 0.25 μ g/ml) before nuclear extraction. Nuclear proteins were then incubated with a labeled specific AP-1 probe (A) or the labeled negative internal control SP1 (B), and the complexes were resolved on a native 4% acrylamide gel. Arrows indicate the antibody-mediated migrating complexes. The intensities of the bands were quantified with an Alpha Imager 2000 digital imaging and analysis system. The specificities of the migrating complexes were verified by competition with a specific (AP-1 specific) or nonspecific (Oct-2A) probe. Results are representative of two independent experiments. (C and D) In some experiments, primary CD4⁺ T cells were infected with VSV-G-pseudotyped reporter virus and were either left untreated or pretreated for 1 h with the ERK inhibitor PD098059 (40 μ M) (C) or the JNK inhibitor SP600125 (40 μ M) (D). Next, cells were either left untreated or treated for 24 h with the listed cross-linked antibodies (5A6 at 2 μ g/ml; OKT3 at 0.25 μ g/ml; 9.3 at 1 μ g/ml). Finally, cells were lysed to assess HIV-1 LTR-encoded reporter gene activity. Results are shown as the increase over the activity in samples treated with GAM (used as negative controls) and are calculated from the mean $\pm$ SD of three different lysed cell samples in the same experimental setting. The mean luciferase activity in control cells was 1.2 RLU for untreated cells, 1.3 RLU for PD098059-treated cells, and 0.6 RLU for SP600125-treated cells. Results are representative of three independent experiments. Asterisks indicate significant differences between cells treated with FK506 or SP600125 and untreated cells (*, $P < 0.05$; **, $P < 0.02$).

tors, and even other tetraspanins (5). The various molecular interactions between tetraspanins and intracellular signaling molecules have been proposed to influence the efficiency of the signal transmitted toward the nucleus. Some members of the tetraspanin family have previously been shown to activate cells

by increasing tyrosine phosphorylation and cytoskeleton remodeling (13, 49). While there is little evidence indicating that CD81 can generate signaling events by itself in primary T lymphocytes, it is clear that it might favor the recruitment and formation of downstream signaling complexes. Signaling path-

ways activated upon engagement of the TCR/CD3 complex initiate phosphorylation of numerous tyrosine kinases, assembly of signaling complexes, and a cascade of biochemical events that converge to generate cellular responses. Costimulation usually provides an intensification of signal transduction orchestrated by TCR/CD3 engagement, but it also triggers its own signaling events to generate other responses. It can be proposed that engagement of CD81 increases and stabilizes the scaffolding of signaling complexes, a phenomenon that might extend signal transmission and promote HIV-1 production in primary CD4⁺ T lymphocytes in the absence of CD28 stimulation. This scenario is conceivable considering that the virus-encoded regulatory Nef protein has been reported to down-regulate CD28 cell surface expression, resulting in impairment of TCR/CD28-initiated signaling (41). It should be noted that surface expression of CD81 is not modulated upon HIV-1 infection of Jurkat cells and CD4⁺ T lymphocytes. The precise biochemical transducers and signal transduction pathways that are responsible for the CD81 costimulating property leading to induction of HIV-1 transcriptional activity remain to be elucidated.

Although we demonstrate that CD81 costimulation enhances virus production in cells acutely infected with HIV-1, additional studies indicate that coengagement of CD81 and TCR/CD3 is not sufficient to drive virus production in latently infected cells. Indeed, treatment of PBMCs from three asymptomatic HIV-1-infected persons with cross-linked anti-CD3 and anti-CD81 does not promote expression of Tat mRNA as monitored by real-time PCR (data not shown). This early-expressed viral gene was tested because it is a good indicator of latent HIV-1 reactivation (28). Our inability to drive HIV-1 gene expression in PBMCs from HIV-1-infected individuals is not surprising when one considers that activation of latent virus in primary human cells is a complex phenomenon. For example, although activation of NF- κ B is known to be critical for achieving viral reactivation, all pathways that stimulate NF- κ B do not necessarily reactivate latent virus (7). Moreover, several inducers that trigger HIV-1 reactivation from latency in T-cell lines are less potent when tested in primary human T cells. This is exemplified by the previous demonstration that only 1% of resting CD4⁺ T cells containing HIV-1 DNA were induced to transcribe viral genes following activation with stimuli that mediate global T-cell activation, i.e., PHA used in the presence of irradiated allogeneic PBMCs in culture medium supplemented with IL-2 (20).

Altogether, our findings suggest that engagement of the tetraspanin CD81 decreases the signaling threshold required to initiate TCR/CD3-mediated induction of HIV-1 gene expression in primary CD4⁺ T lymphocytes. The clustering of CD81 in the contact area of the immune synapse in both T lymphocytes and APCs is in favor of this hypothesis (30). Considering that CD81 has been described as a putative receptor for HCV and that HCV-HIV-1 coinfections are frequent, it can be proposed that CD4⁺ T cells acutely infected with HIV-1 might be subjected to signaling effects produced by the binding of HCV particles to the cell surface, a process leading ultimately to a diminution of the threshold necessary to achieve TCR/CD3-mediated induction of virus gene expression. Experiments to assess this possibility are currently under way.

ACKNOWLEDGMENTS

We are grateful to S. Méthot for editorial assistance.

This work has been rendered possible through financial assistance from the Canadian Institutes of Health Research (CIHR) HIV/AIDS Program to M.J.T. (grant HOP-15575). M.R.T. holds a CIHR Doctoral Award, while M.J.T. is the recipient of the Canada Research Chair in Human Immuno-Retrovirology (Tier 1 level).

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