Engagement of CD43 Enhances Human Immunodeficiency Virus Type 1 Transcriptional Activity and Virus Production That Is Induced upon TCR/CD3 Stimulation*

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Human immunodeficiency virus type 1 (HIV-1) transcriptional activity is regulated by several cytokines and T cell activators. CD43 (sialophorin) is a sial glycoprotein expressed on the surface of a wide variety of blood cells including T lymphocytes. Several studies have shown that CD43 ligation induces proliferation and activation of human T lymphocytes. We were thus interested in defining whether CD43-mediated signaling events can modulate the life cycle of HIV-1. We demonstrate here that CD43 cross-linking potentiates HIV-1 promoter-driven activity and virus production that is seen following the engagement of the T-cell receptor (TCR)-CD3 complex. This effect is independent of the CD28 co-stimulatory molecule and is mediated by both NF-κB and NFAT transcription factors. A number of signal transducers known to be involved in the TCR/CD3-dependent signal transduction pathway, including p56lck, p36linker for activation of T-cells (LAT), and SLP-76, as well as capacitative entry of calcium, are crucial for the noticed CD43 co-stimulatory effect. Calcium mobilization studies indicate that a syn-ergy is occurring between CD43- and TCR/CD3-mediated signaling events leading to an augmented calcium release. These data suggest that CD43 can be seen as a co-stimulatory cell surface constituent that can modu-late HIV-1 expression in T lymphocytes.

Replication of human immunodeficiency virus type-1 (HIV-1) is regulated by several cytokines and T cell activators via transcriptional regulation through the long terminal repeat (LTR) promoter and enhancer sequences. The nuclear factor κ-B (NF-κB) is playing a cardinal role in virus transcription via the two tandem conserved NF-κB-binding motifs that are located within the enhancer sequence (1). More recently, the implication of the NFAT family of transcription factors in HIV-1 LTR activity has been suggested (2), although contradictory results are still questioning the role of the different NFAT members.

The regulatory domain of HIV-1 can be activated in vivo following engagement of the antigen-specific TCR-CD3 complex, an event that can be mimicked in vitro in established T-cell lines using some specific anti-CD3 monoclonal antibodies. It has been shown that antibody-mediated signaling through the TCR-CD3 complex activates HIV-1 transcription and co-engagement of CD28 further augmented virus gene expression (3, 4). Interestingly, ligation of CD28 alone is sufficient to induce HIV-1 transcription and replication both in Jurkat cells (5) and in naturally infected leukocytes (6). Considering the complex interplay between T-cell signaling events and HIV-1 replication, it is of prime importance to identify other cell surface constituents that are likely to affect HIV-1 transcription. An increasing number of accessory cell surface molecules are involved in up-regulation of T-cell activation. Among them, CD43 (sialophorin, leukosialin, or gpL115) is a constitutively phosphorylated 115-kDa sialglycoprotein expressed in a wide variety of blood cells including lymphocytes, monocytes, neutrophils, and platelets. It is considered as the most abundant membrane protein of T lymphocytes. On T-cells, CD43 is differently glycosylated in two major isoforms, i.e. a 113–123-kDa product, mainly present on resting CD4+ T cells, and a 125–135-kDa form expressed mostly on resting CD8 lymphocytes. Previous work has shown that this isoform is up-regulated following activation of both CD4-positive and CD8-positive T-cells (7). CD43 has been involved in the selection and maturation of thymocytes and in the migration, adhe-sion, and activation of mature T-cells. Four natural ligands have been identified for CD43, namely ICAM-1 (CD54), Galvec-tin 1, major histocompatibility complex-1, and sialoadhesin (Siglec-1). However, there is no direct evidence of how the inter-action of CD43 with these ligands regulates T-cell function (8–11).

Numerous reports document a role for CD43 in T-cell signaling. For example, CD43 ligation by monoclonal antibodies has been reported to increase proliferation of activated T-cells and to enhance antigen-specific activation of T-cells, resulting in secretion of IL-2 and expression of both CD69 and CD40L (12–15). Such CD43-mediated effects are independent from the CD28 receptor (16, 17) and require the intracellular domain of CD43, which is hyperphosphorylated during T-cell activation. Further studies revealed that CD43 is functionally coupled to the phospholipase C/phosphoinositides signaling pathway, resulting in translocation of protein kinase C to the membrane.

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CD43-mediated signaling cascade include activation of several transcription factors such as activator protein-1, NFAT, and NF-kB (22). Besides its co-stimulatory potential, a negative regulatory role in T-cell activation was proposed for CD43 based on the observation that CD43-deficient mice are hyper-responsive following both in vivo and in vitro activation (23). However, in another study, the absence of CD43 did not alter T-cell development and responsiveness (24). CD43 has been shown to induce apoptosis in human T-cell lines (25) and, paradoxically, a high level of CD43 expression can protect T-cell hybridomas from activation-induced cell death (26). Further studies are thus warranted to document the exact contribution of CD43 in T-cell functions.

More relevant to the present work, persons infected with HIV-1 make autoantibodies that bind to CD43 on normal thymic lymphocytes (27). Moreover, an altered glycosylation pattern of CD43 is observed on the surface of HIV-1-infected CEM cells and also on peripheral T lymphocytes from patients infected with HIV-1 (28, 29). These findings along with the previously reported implication of CD43 in T-cell signaling and the intimate link between T-cell activation and HIV-1 transcription (30) led us to scrutinize the effect of CD43 ligation on the regulatory elements of HIV-1. In the present study, we provide evidence indicating that, although CD43-mediated signal transduction events are weak inducers of virus transcription, co-ligation of CD43 with the TCR-CD3 complex markedly augmented both HIV-1LTR-driven gene activity and virus gene expression. This CD43-dependent co-stimulus was independent of CD28 and promoted nuclear translocation of both NF-kB and NFAT transcription factors. Several intracellular second messengers known to participate to the TCR/CD3 signaling cascade were found to be important for the CD43 co-stimulating ability, therefore suggesting that stimulation via CD43 could act by lowering the threshold for T-cell activation that is seen upon the engagement of the TCR-CD3 complex.

**EXPERIMENTAL PROCEDURES**

**Cells Used in This Study**—The cell lines used in this work include parental Jurkat (clone E6.1), IGV, JCAM1.6, JCAM2.1, J14-V-29, J14-76-11, CJ, CJ 5.13, CJ 1.1, and LuSIV. Jurkat is considered as a model cell line for the study of T-cell signaling machinery (31), whereas the IGV T-cell line is a Jurkat derivative that harbors two stably integrated CVs expressing vector (32). JCAM1.6 and JCAM2 are Jurkat derivatives that are deficient in p56lck and p32LAT expression, respectively (33, 34). The J14-V-29 cell line is also derived from Jurkat and lacks expression of the T cell specific adaptor SAPL-76 that has been reintroduced using an expression vector, thus creating the J14-76-11 clone (35). The CJ cell lines have been derived from Jurkat using a toxic gene under the control of the NFAT transcription factor (36). The CJ parental cell line (B-cell/T-cell hybrid) and carries the luciferase reporter construct. LuSIV cell lines have, respectively, 40 and less than 10% of the wild-type capacitative entry of calcium following stimulation. The reporter LuSIV cell line, which is prototypic T-tropic isolate of HIV-1 (37). The mitogen-activated protein kinase pathway has been demonstrated to be involved in CD43-dependent interleukin-2 gene expression (20). Also, CD43 cross-linking on the T-cell surface induces interaction between CD43 and Fyn leading to Fyn tyrosine phosphorylation and signal propagation (21). Downstream events of the CD43-mediated signaling cascade include activation of several transcription factors such as activator protein-1, NFAT, and NF-kB (22).

**Vectors and Antibodies**—In our studies we have used pLTR-LUC and pmBCLTR-LUC that have been kindly provided by Dr. K. Calame (Columbia University, New York). These molecular constructs contain the luciferase reporter gene under the control of wild-type (GGGACTT- TCC) or NF-kB-mutated (GTCCTTTCCT) HIV-1 LTR (64) to +78) from HIV-1LAI placed in front of the luciferase reporter gene (39) and was kindly given by Dr. O. Schwartz (Unité d'oncologie virale, Institut Pasteur, France). The pEB-TATTA-LUC vector contains the minimal HIV-1 5′ region and a TATA box placed upstream of the luciferase reporter gene (40) (from Dr. W. C. Greene, The J. Gladstone Institutes, San Francisco, CA). The following reagents were obtained through the National Institutes of Health AIDS repository reagent program: pNL4-3, a full-length infectious molecular clone of HIV-1 (a prototypic T-tropic isolate of HIV-1) (41), and pCEP4-Tat, a plasmid that contains the HIV-1 3′LTR, Tat gene ligated to the pCEP4 CMV-based expression vector (42). The dominant negative IκBα expressing vector pCMV-IκBα S32A/S36A has been described previously (40). pNFRAT LUC is a mutant of the luciferase reporter gene placed downstream of the minimal NF-2 promoter that carries three copies of the NFAT-binding site (kindly provided by Dr. G. Crabtree, Howard Hughes Medical Institute, Stanford, CA) (43). pNF-kB-LUC contains five consensus NF-kB binding sequences placed upstream of the luciferase gene along with a minimal promoter (Stratagene). The expression vector for p56lck, pEFNef LCK-wt, as well as the pEFNef-empty vector have already been described (44) and were kindly gifts from Dr. C. Couture (Lady Davis Institute, Montreal). The expression vector for p32LAT, pCDNA3.1 LAT, was generously provided by Dr. A. Weiss (University of California, San Francisco, CA) (34). The luciferase-containing pNL4-3-LUC-E-R+ construct was generously provided by Dr. N. R. Lando (The Salk Institute for Biological Studies, La Jolla, CA). The pHCMV-G expressing the broad host-range vesicular stomatitis virus envelope glycoprotein G (VSV-G) from the human cytomegalovirus promoter has been described previously (45). The hybridoma cell line that produces the anti-CD3 OKT3 monoclonal antibody (specific for the β-chain of the CD3 complex) was obtained from the American Type Culture Collection (Manassas, VA). Purified anti-CD28 antibody (clone 9.3) was a generous gift from Dr. J. A. Ledbetter (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). The anti-CD43 antibodies reacting with different epitopes were used in this study: L10, which reacts with both sialylated and desialylated CD43 (15), was purchased from Caltag (Burlingame, CA), whereas MEM-59, which is directed against a sialic acid-dependent epitope (47), is a kind gift from Dr. V. Horejsk (University of Prague, Czech Republic). Purified goat IgG antibody was obtained from Jackson ImmunoResearch (West Grove, PA). Rabbit antisera raised against peptides from NFAT1 (38) and the p50 and p65 subunits of NF-kB were kindly supplied by Dr. N. Rice (NCI-Frederick, National Institutes of Health, Frederick, MD). Polyclonal anti-NFATc (NFAT2) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Transient Transfection and Cell Stimulation**—Cells were electroporated at room temperature using a gene pulser I apparatus (Bio-Rad) (960 microfarads, 250 V). Cells were concentrated at 37.5 × 10⁶/ml in RPMI medium. Cells (400-μl aliquots) were electroporated either with 5 μg of the reporter construct DNA alone or, in the case of reconstitution experiments, with 5 μg of reporter construct DNA and 0, 10 or 20 μg of the expression plasmid. The total DNA amount for the reconstitution experiments was maintained constant at 25 μg using the empty vector. To minimize variations in plasmid transfection efficiencies, cells were transfected in bulk and were separated into various treatment groups at a density of 10⁶ cells/well (100 μl) in 96-well flat-bottom plates at 36 h post-transfection. For studies using the pharmacological inhibitor FK506 (Sigma), cells were resuspended in fresh cell culture medium at 1 × 10⁶ cells/ml, and FK506 was added in subtoxic concentrations of 1–10 ng/ml for 60 min before stimulation. Cells were then washed and resuspended in complete RPMI containing 10% fetal calf serum (Hyclone Laboratories) and treated with pertussis toxin (100 ng/ml) or CD43 (clone OKT3, 0.25 μg/ml unless otherwise specified), anti-CD43 (MEM-59 at 3 μg/ml or L10 at 1 μg/ml), and anti-CD28 antibody (clone 9.3 at 1 μg/ml), and cross-linked with a goat anti-mouse IgG (2 μg/ml) in a final volume of 200 μl. Next, cells were incubated at 37°C for 8 h unless otherwise specified. Luciferase activity was determined following a previously described protocol (49).
Production of Virus Stocks and Virus Infection—Virus particles were produced by calcium phosphate transfection of 293T cells with virus-encoding vectors as previously described (50, 51). Pseudotyped HIV-1 particles were generated by cotransfection of 293T cells with pNL4-3-LUC-E-RΔ and an expression vector coding for the VSV-G full-length envelope protein. Virus stocks were normalized for virion content using an in-house sensitive double antibody sandwich enzyme-linked immunosorbent assay specific for the major core viral p24 protein (52).

Viral infection experiments were done using fixed amount of virus (5 ng of p24 protein) to inoculate 10^6 target cells (i.e. Jurkat, PBMCs, and purified CD4+ T lymphocytes). Cells infected with luciferase-encoding viruses were stimulated 48 h post-infection as described above and luciferase activity was monitored at 24 h post-stimulation. Cells infected with replication-competent viruses (i.e. NL4-3) were stimulated 8 h post-infection. Production of infectious viruses by NL4-3-infected CD4+ T lymphocytes at 3 days post-stimulation was assessed using the reporter LuSIV cell line. Briefly, cell-free culture supernatants were incubated with replication-competent viruses (i.e. BTATA-LUC, a molecular construct made of the luciferase reporter gene placed under the control of the minimal HIV-1 promoter region (Fig. 1B). This could be attributed to the deletion of the HIV-1 LTR negative regulatory elements in this construct. In both cases the L10 antibody showed much stronger co-stimulating activity as compared with MEM-59. Data from a time course experiment revealed that the optimal co-stimulating capacity of CD43 was maximal after 8 h of treatment (Fig. 1C). Interestingly, a significant induction of HIV-1 LTR-dependent luciferase activity was seen (30-fold increase over untreated cells) even with concentrations of OKT3 that were not sufficient to mediate activation by itself (i.e. from 0.05 to 0.2 ng/ml) (Fig. 1D). Moreover, the marked co-stimulating potential of the L10 antibody was not affected by a reduction in the concentration of OKT3 to as low as 0.05 ng/ml and was still observed at 0.025 ng/ml. In contrast, the co-stimulating potential of the MEM-59 antibody appears to be more dependent on the anti-CD3 concentration, suggesting that these two antibodies could act via different mechanisms.

Our next set of experiments was performed using pLTR-LUC, a vector that carries the reporter luciferase gene placed under the control of the complete HIV-1_LAI LTR region. As shown in Fig. 2A, the tested anti-CD43 antibodies (i.e. MEM-59 and L10) led to a significant increase in LTR activity when used in combination with a suboptimal dose of OKT3. Because the virus-encoded transactivating Tat protein is crucial for virus replication both in vivo and in vitro, we next wanted to assess the implication of Tat protein on the CD43-mediated co-stimulating capacity. To this end, Jurkat cells were co-transfected with pLTR-LUC along with a Tat expression vector (i.e. pCEP4-Tat). Data from Fig. 2B indicate that even when the LTR-driven expression is increased more than 100-fold by Tat, ligation of CD43 can still provide a co-stimulatory signal to a suboptimal TCR/CD3 triggering.

Previous findings have indicated that CD28 provides a co-stimulating potential with respect to HIV-1 replication and transcription (3). In agreement with such findings, an additive effect was noticed when a monoclonal anti-CD28 antibody (i.e. clone 9.3) was used along with L10, leading to a 100-fold increase in luciferase activity (Fig. 3). Because we have used a saturating concentration of anti-CD28 antibody to perform these studies (i.e. 1 ng/ml), this observation suggests that the CD43 co-stimulating potential is independent from CD28, and most likely uses a distinct signaling pathway.

**NFκB and NFAT Are Involved in CD43-mediated Cooperative Effect on HIV-1 Transcription**—Regulation of HIV-1 transcription that is seen with several stimuli, including CD3 and CD28 ligation, involves the NF-κB and NFAT transcription factors. CD43 ligation, the NF-κB complex via the two tandem conserved motifs located in the enhancer region (1). To test the involvement of the ubiquitous mammalian transcription factor NFκB in the co-stimulating activity of CD43, Jurkat cells were transfected with reporter constructs harboring either the full-
length LTR promoter (i.e. pLTR-LUC) or a LTR bearing mutated NF-κB-binding sites (i.e. pmxBLTR-LUC) in the presence of various combinations of anti-CD3 and anti-CD43 antibodies. As shown in Fig. 4A, the increase in HIV-1 LTR activity mediated by co-ligation of the TCR-CD3 complex and CD43 was significantly reduced but not totally inhibited in cells transfected with the NF-κB-mutated molecular construct. Previous findings have indicated that nuclear translocation and activation of NF-κB is mainly mediated by the degradation of the repressor IkBα, which sequesters the complex in the cytoplasm (54). To confirm the implication of NF-κB in the observed co-stimulating effect of CD43, we used a dominant negative version of IkBα mutated on serines 32 and 36, which is unable to be serine phosphorylated, and hence, degraded. When the pCMV-IκBα S32A/S36A vector was transfected along with the reporter plasmid pBTATA-LUC, the TCR/CD3- and CD43-dependent induction of virus transcription was severely reduced but again not completely abolished by this expression vector (Fig. 4B). Finally, we used a construct containing five consensus NF-κB binding sequences placed upstream from the luciferase gene along with a minimal promoter (i.e. pNFκB-LUC). As depicted in Fig. 4C, activation of NF-κB was indeed enhanced following co-engagement of the TCR/CD3 complex with CD43.

Although NF-κB is considered as a key regulator in HIV-1 expression, the NFAT family of transcription factors has also been shown to participate in virus gene expression (2, 55, 56). Given that mutations in the NF-κB-binding sites in the LTR region and the use of a trans-dominant repressor of IκBα do not completely abrogate the co-stimulatory activity of CD43 (Fig. 4,
A and B), NFAT could also play a role in the CD43-dependent signaling events. To test this hypothesis, we used the immunosuppressor FK506, which has been shown to block NFAT activation through the inhibition of calcineurin activity (57, 58). 1G5 cells were first pretreated with FK506 for 60 min and next stimulated with anti-CD3 and anti-CD43 antibodies. Data from Fig. 5A indicate that treatment with FK506 caused a 2-fold decrease of HIV-1 transcriptional activity that was the result of TCR/CD3 and CD43 co-engagement, therefore suggesting an implication of a calcineurin-dependent signal transducer such as NFAT. Similar results were obtained when using Jurkat cells transiently transfected with pLTRX-LUC where 1 and 10 ng/ml FK506 caused a 2- and 3-fold diminution of luciferase activity, respectively (Fig. 5B). Considering that the HIV-1 enhancer sequence present in the pLTRX-LUC vector bears only NF-κB- and NFAT-binding sites, these results strongly suggest the involvement of NFAT in HIV-1 LTR stimulation induced by TCR/CD3-CD43 co-ligation. To confirm this hypothesis, Jurkat E6.1 cells were transfected with pNFAT-LUC, a construct containing three NFAT-binding sites upstream from the minimal IL-2 promoter. Co-ligation of the TCR/CD3 complex and CD43 resulted in a 7-fold increase in luciferase activity, whereas only a 1.3-fold increase was observed following cross-linking of the TCR/CD3 complex alone (Fig. 5C). These results indicate that co-stimulation via CD43 acts not only via the NF-κB complex, but also via members of the NFAT family.

CD43-mediated Signal Transduction Cooperates with TCR/CD3 to Increase Nuclear Translocation of NF-κB and NFAT in Human T Lymphoid and Primary Cells—We were next interested in defining whether the CD43-mediated signaling pathway could either alone or in conjunction with TCR/CD3 stimulation modulate the level of HIV-1 enhancer-bound protein complexes. To this end, electrophoretic mobility shift assay experiments were conducted with a labeled probe containing the complete enhancer region of the HIV-1 LTR (−107/−77). Incubation of the HIV-1 enhancer probe with nuclear extracts from anti-CD3- or anti-CD43-treated Jurkat cells led to the formation of a single broad signal, which was much stronger upon co-ligation of TCR/CD3 and CD43 (Fig. 6A, compare lanes 2–6). It has already been shown that this signal can be the result of an overlapping of NF-κB and NFAT complexes (59). To discriminate the NFAT-related band from the NF-κB complex, supershift assays were performed using extracts from OKT3- and OKT3/L10-stimulated cells that were incubated with anti-NF-κB p50 and anti-NFAT1 antibodies (Fig. 6A, lanes 7–12). The upper part of the migrating complex was identified as the
NFAT complex, whereas the NF-κB complex was responsible for the lower part. The signal intensity of both complexes was increased following engagement of both CD43 and the TCR/CD3 complex (compare lanes 7–9 with 10–12).

Electrophoretic mobility shift assays were also performed with nuclear extracts from IL-2-starved human PBMCs. As shown in Fig. 6B, similar findings were made in such cells, except that the NFAT1-specific complex was very faint and could be seen only when the NF-κB complex was supershifted (lane 13). The specificity of the complexes was demonstrated by competition with a 100-fold excess of a specific or nonspecific oligonucleotide (lanes 7 and 8). Translocation of NFAT1 was also confirmed using an NFAT-specific labeled probe (data not shown). Altogether these results substantiate our observations indicating that co-ligation of CD43 and the TCR-CD3 complex induces more important NF-κB as well as NFAT-binding activities on the HIV-1 enhancer.

The Src Family Protein-tyrosine Kinase p56^lck^, the Adapter Molecules p36^lat^ and SLP-76, and Capacitative Entry of Calcium Are All Critical for HIV-1 LTR Activation by CD43-TCR/CD3 Co-ligation—Although CD43 demonstrates a potent co-stimulating effect on TCR/CD3-dependent induction of HIV-1 LTR-driven reporter gene activity, signal transduction events mediated through CD43 are not sufficient per se to up-regulate virus transcription. This may suggest that the TCR/CD3-oriented signaling pathway was involved in transducing the TCR/CD3-CD43 co-ligation signal. Previous work has reported that the TCR/CD3 signaling cascade was initiated by the Src family...
protein-tyrosine kinase \( p56^{\text{ck}} \), which phosphorylates immunoreceptor tyrosine-based activation motifs of the CD3\( \gamma \) chains (60). These phosphorylated motifs provide anchoring for the Syk family protein-tyrosine kinase Zap-70 that becomes activated (61) and was then responsible for phosphorylation of downstream effectors such as p36\( \text{LAT} \) (62) and SLP-76 (63). To assess the implication of these effectors in the co-stimulating activity of CD43, we used cell lines that are deficient for p56\( \text{ck} \) (JCAM1.6), p36\( \text{LAT} \) (JCAM2), or SLP-76 (J14-V29). When these cell lines were transfected with pEBTATA-LUC, no increase in luciferase activity was observed upon stimulation with anti-CD3 and anti-CD43 antibodies (Fig. 7, panels A–C). This unresponsiveness was not related to a lack of CD43 molecule, because flow cytometric analyses revealed that these cell lines express surface levels of CD43 similar to the parental Jurkat cell line (data not shown). Reconstitution experiments performed with JCAM1.6 cells transfected with a \( p56^{\text{ck}} \)-encoding vector indicate that expression of \( p56^{\text{ck}} \) partially restored induction of HIV-1 LTR activity following CD43 and TCR/CD3 co-engagement (Fig. 7A). The SLP-76-deficient cell line J14-V29 was also unresponsive to CD43 and TCR/CD3 stimulation, whereas the SLP-76-reconstituted J14-76-11 cells showed a partially restored response to this type of stimuli (Fig. 7C). Altogether these results demonstrate the importance of the most proximal TCR/CD3-mediated signaling events in the co-stimulating activity of CD43.

More distal events following TCR/CD3 stimulation include phospholipase C{\text{y}}1-dependent inositol triphosphate generation, an event leading to the release of calcium from intracellular stores. This initial burst of calcium is followed by an influx of extracellular calcium ions, also called capacitative calcium entry that is necessary for a sustained activation of calcium effectors and to replenish calcium stores (64). We analyzed the role of this signaling cascade in CD43 co-stimulation using Jurkat-derived cell lines demonstrating full (CJ), intermediate (CJ 5.13), or low (CJ 1.1) capacitative calcium entry, which were transiently transfected with pEBTATA-LUC and then stimulated with the indicated antibodies. CD43 antibodies. Cells were lysed to measure luciferase activity after an incubation period of 8 h. Results are presented as -fold induction in luciferase activity over untreated samples from the calculated mean ± S.D. of four different lysed cell samples in the same experimental setting. These results are representative of three different experiments.

To further assess the involvement of calcium-related events in this signal transduction pathway, we measured the extent of calcium mobilization following CD43 and/or TCR/CD3 ligation. Accurate measurements of intracellular calcium release can be achieved through the use of the Indo-1 dye by calculating the ratio of calcium-bound Indo-1 over calcium-free Indo-1. The addition of a suboptimal dose of anti-CD3 or anti-CD43 to Jurkat cells led to a slow but significant increase in intracellular calcium content. This increase was faster and much stronger when the anti-CD3 and the anti-CD43 antibody L10 were used in combination (Fig. 8). No such additive effect could be observed when the MEM-59 antibody was used in conjunction with anti-CD3.
permits the detection and quantification of single cycle HIV-1 infection because of the Tat-mediated expression of luciferase activity, which correlates with virus infectivity (37). Serial dilutions of the virus-containing culture supernatants were used to avoid saturation of the HIV-1-mediated signal. Results from Fig. 9C indicate that CD43 and TCR/CD3 co-ligation results in an enhancement of virus production, as estimated by luciferase activity, when compared with antibody-mediated engagement of CD43 or TCR/CD3 alone. These findings represent additional evidence of the biological significance of CD43 for the life cycle of HIV-1.

**DISCUSSION**

HIV-1 replication is controlled by many different external stimuli such as cytokines and antigens. Indeed, several agents known to induce T-lymphocyte activation have been found to stimulate HIV-1 transcription and replication. T-cell activation requires both antigen receptor-mediated biochemical events and signals provided by some specific co-stimulatory molecules (e.g. CD28). However, little is known about the implication of co-stimulating molecules other than CD28 with respect to activation of HIV-1 gene expression. In this report we show for the first time that one of these co-stimulating molecules, CD43, is a potent co-activator of the HIV-1 LTR, and can lower the threshold of signaling through the TCR/CD3 complex necessary to achieve activation of viral replication.

Our transient transfection experiments demonstrate that CD43 acts as a very potent co-stimulatory molecule that strongly potentiates TCR/CD3-induced HIV-1 LTR activation. These results are consistent with previous reports describing an enhancement of antigen-specific activation of T-cells by CD43 (14, 65) as well as a potentiation of proliferation and IL-2 secretion induced by CD3 triggering (13, 66). CD43 acts independently from CD28 because their co-stimulating effects are
additive, suggesting that the two receptors use different signal transduction pathways. Indeed, CD43 is a potent co-activator in murine intestinal intraepithelial lymphocytes, which are largely devoid of CD28 (66), and in T-lymphocytes from CD28-deficient mice (16). Hence, CD43 can be envisaged as a functionally important co-stimulating molecule in T-cells.

We did not observe any positive effect on HIV-1 transcriptional activity following engagement of CD43 alone, which was in line with the previous observations that reported the necessity of TCR/CD3 triggering for a CD43-mediated stimulation in T-cells. However, Santana and colleagues (22) reported an induction of IL-2 secretion in peripheral blood T-cells using MEM-59, whereas both CD69 and CD40L were induced following treatment with either L10 or MEM-59. The IL-2 promoter is predominantly activated by NFAT and activator protein-1, two transcription factors known to also activate the regulatory domain of HIV-1. Hence, signals that activate the IL-2 promoter should also activate HIV-1 LTR. This discrepancy could be related to the fact that in this study they used peripheral blood T-cells that were probably contaminated by other populations such as monocytes, which may have provided the necessary activating signal. Indeed, the CD43-induced proliferation of human T-cells was shown to be dependent on the presence of monocytes (12).

Our results indicate that upon CD43 cross-linking, very little anti-CD3 was necessary to potently activate HIV-1 transcription (Fig. 1D). At the concentrations used here, the anti-CD3 does not display any stimulating capacity by itself, suggesting that CD43 could influence the signaling threshold in T-cells. Moreover, when CD43 was multimerized using the L10 antibody, we did not observe a dose-dependent transcriptional increase in relation to anti-CD3. This observation suggests that when triggered by the L10 antibody, CD43 co-stimulates via its own transduction pathway rather than by enhancing the CD3-induced signaling. The situation was different when CD43 was engaged with the MEM-59 antibody because a dose dependence on anti-CD3 antibody was observed. These observations were supported by calcium mobilization studies. Indeed, when using the L10 antibody, there was a synergy between signal transduction pathways that were engaged following cross-linking of both CD43 and CD3 culminating in an augmented Ca\(^{2+}\) response (Fig. 8). On the contrary, no such enhancement in calcium mobilization was observed when CD43 was cross-linked by the MEM-59 antibody, indicating that CD43 engagement by MEM-59 and TCR/CD3 triggering share some common calcium-regulated effector(s). These findings are in agreement with a previous study (12) showing that the TCR/CD3 negative Jurkat-derived cell line J.TR3-T3.5 exhibits defective signaling upon CD43 cross-linking by MEM-59, suggesting that this specific anti-CD3 antibody acts via the TCR/CD3 transduction pathway. Moreover, a HPB-ALL-derived cell line severely defective in TCR/CD3 surface expression displays normal L10-induced CD43 signaling compared with the parental cell line (18). Thus, the engagement of cell surface CD43 by distinct antibodies that are specific for different epitopes initiates signal transduction events through different pathways. Considering that numerous ligands have been proposed for CD43, it is possible that different sets of genes will be modulated depending on the CD43 epitope that is being recognized by a given ligand.

Transient transfection experiments (Figs. 4 and 5) and DNA mobility shift assays (Fig. 6) indicate that the TCR/CD3- and CD43-induced activation of HIV-1 LTR is mediated primarily via NF-κB and to a smaller extent through NFAT. CD43 engagement alone could not induce any NF-κB or NFAT binding activity, but could cooperate with a suboptimal CD3 cross-linking to induce translocation of both transcription factors. Similar observations were made when the effect of CD43 cross-linking by L10 and MEM-59 on the distal NFAT site of the IL-2 promoter was tested (67). In that study, simultaneous cross-linking of CD43 by L10 and MEM-59 was sufficient to induce NFAT translocation, suggesting an additive effect of signals generated through each epitope. This observation confirms our hypothesis that CD43 engagement through different epitopes initiates signal transduction events through different pathways.

Our results raise the issue of the precise contribution of each of the CD43 and TCR/CD3 signaling pathways in HIV-1 LTR activation. The downstream effectors of the TCR/CD3 signaling cascade p56\(^{lat}\), SLP-76, and p36\(^{lat}\) were found to be crucial for TCR/CD3- and CD43-mediated induction of LTR gene expression (Fig. 7), thus suggesting that the activating signal was transduced mainly via the TCR-CD3 complex. However, these molecules could as well participate to the CD43-dependent signaling pathway. For example, ligation of CD43 has been reported to generate an interaction between CD43 and p56\(^{lat}\) (12, 21), leading to the tyrosine phosphorylation of Shc and the guanosine exchange factor Vav (20). SLP-76 has to be included in this pathway because of its known interaction with Vav. Also, CD43 is functionally coupled to the phospholipase C/phosphoinositides signaling pathway, most likely via the adaptor molecule p36\(^{lat}\), in a CD3-independent manner (18). A working model can then be proposed in which CD43 ligation would induce its association with p56\(^{lat}\) and a phosphorylation of this signal transducer, leading to the recruitment of SLP-76 and p36\(^{lat}\), possibly via ZAP-70, activation of phospholipase C\(_{y}\), and ultimately to a raise in intracellular calcium via the inositol triphosphates and the activation of the mitogen-activated protein kinase pathway via protein kinase C. This possible signaling pathway could be activated upon CD43 ligation by the L10 antibody, whereas ligation by the MEM-59 antibody could increase the TCR/CD3-mediated signaling pathway by a mechanism possibly involving a large complex including CD3 and CD43 (12). The protein-tyrosine kinase p59\(^{lat}\) could also play a role in the CD43-mediated activation because it was found to be associated with CD43 and is phosphorylated upon CD43 ligation (12, 21). This protein kinase could be involved in events leading to the noticed induction of NF-κB, because it has been shown that overexpression of p59\(^{lat}\) in T cell lines could stimulate HIV-1 LTR activity by NF-κB-like DNA-binding proteins (68). Further studies are needed to identify the various signal transducers participating to the CD43-mediated signaling cascade.

A supplementary role for CD43 in its co-stimulating activity could be in remodeling T-cell morphology. Recently, plasma membrane compartmentalization has been shown to take place following occupancy of the TCR/CD3 complex (reviewed in Refs. 69–71). TCR engagement promotes the integration of components of the TCR/CD3 signaling machinery, including ZAP-70, p36\(^{lat}\), and Vav, into lipid microdomains also called rafts, and the disruption of these microdomains attenuates TCR/CD3-dependent signal transduction events (71). Co-stimulatory molecules, with the exception of CD28, are also present in lipid rafts and it has been proposed that they exert their co-stimulatory effects by contributing to an enhanced association of TCR/CD3 with such raft domains (72). CD43 interacts with the actin-binding proteins moesin and ezrin via its cytoplasmic domain (73, 74). Interestingly, stimulation of T-lymphocytes with anti-CD43 antibodies increases this association and induces T-cell polarization as well as the redistribution of CD43 to the uropod (73). Moreover, CD43 seems to be excluded from the antigenic synapse formed between T-cells and dendritic cells (75–79). In
contrast, lipid microdomain clustering in T-cell induces a redistribution of receptors and adhesion molecules leading to a colocalization of CD43 and the TCR in a new microdomain (81). Also, in immature hematopoietic cells, CD43 cross-linking induces the formation of a long-lived cap and an increase in tyrosine phosphorylation through Syk and Lyn tyrosine kinases at the capping site. Interestingly, CD44, which demonstrates a co-stimulatory function very similar to that of CD43 (81), was shown to induce membrane reorganization including the recruitment of CD44 itself and the associated tyrosine kinase p56lck and p56fyn into lipid rafts (82). Because CD43 also interacts with these two tyrosine kinases (12, 21), it is tempting to speculate a similar scenario for CD43 stimulation. Experiments are now being conducted to shed light on this possibility.

We have shown here that CD43 functions as a potent co-stimulatory molecule for TCR/CD3-dependent induction of the HIV-1 LTR-driven transcription, which leads to an increased production of infectious viral particles. This co-stimulatory potential was observed both in the absence and presence of Tat, suggesting that CD43 could play a role in the early phase of the infection, to initiate viral transcription before Tat is produced, as well as in late stages, to enhance virion production. However, it should be noted that the involvement of CD43-mediated biochemical events in HIV-1 transcriptional activity once Tat is also present is most likely minimal. Indeed, the significant biochemical events in HIV-1 transcriptional activity once Tat is present is most likely minimal. Indeed, the significant

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