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Lentiviral Vectors Interfering with Virus-Induced CD4 Down-Modulation Potently Block Human Immunodeficiency Virus Type 1 Replication in Primary Lymphocytes

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CD4 down-modulation is essential for the production of human immunodeficiency virus (HIV) infectious particles. Disease progression correlates with enhanced viral induced CD4 down-modulation, and a subset of long-term nonprogressors carry viruses defective in this function. Despite multiple pieces of evidence highlighting the importance of this function in viral pathogenesis in vivo, to date, HIV-induced CD4 down-modulation has not been used as a target for intervention. We describe here HIV-based vectors that deliver truncated CD4 molecules resistant to down-modulation by the viral products Nef and Vpu. Infection of cells previously transduced with these vectors proceeded normally, and viral particles were released in normal amounts. However, the infectivity of the released virions was reduced 1,000-fold. Lentiviral vectors expressing truncated CD4 molecules were efficient at blocking HIV-1 infectivity and replication in several cell lines and in CD4-positive primary lymphocytes. The findings presented here provide proof-of-principle that approaches targeting the virus-induced CD4 down-modulation may constitute the basis for novel anti-HIV therapies.

CD4 plays a dual role during human immunodeficiency virus (HIV) infection. CD4 is required for entry of HIV into most permissive cells (14). However, during late stages of infection, the viral receptor exerts inhibitory effects on the infectivity of the released particles (25). To overcome these effects, HIV has evolved mechanisms that ensure the removal of the CD4 receptor from the surface of infected cells (reviewed in reference 24). Three viral proteins participate in this process: Nef, Vpu, and Env. The effects of Nef (early product) and Vpu/Env (late products) are quantitative and qualitatively distinct. Nef enhances CD4 internalization from the cell surface and targets the receptor for degradation into lysosomes, whereas Env interferes with transport of CD4 to the cell surface, and Vpu targets CD4 for degradation in proteasomes (16, 33, 34). Unlike Nef, Env and Vpu exert their effect only on newly synthesized receptor molecules. Nef acts as a connector, by bridging together the cytoplasmic domain of CD4 with the heterotetrameric clathrin adaptor protein complexes AP-1, AP-2, and AP-3 (6, 13, 18, 28, 32, 38; reviewed in references 11 and 14). Nef also binds directly to the cytoplasmic domain of the CD4 receptor (19, 35, 36). This domain is sufficient to confer Nef-induced down-modulation to heterologous proteins (1, 3). By binding to the cytoplasmic domain of CD4 and to components of the cellular trafficking machinery, Nef targets the viral receptor to specific trafficking pathways. The interaction between Nef and AP-2, which is involved in endocytosis from the plasma membrane, appears weak. However, direct interactions of Nef with AP-1 and AP-3 suggest that in addition to enhancing endocytosis, Nef may also exert its effects by targeting CD4 to the endosomal/lysosomal system and preventing the recycling of CD4 to the cell surface (21). Similar to Nef, Vpu also acts as a connector between CD4 and the cellular degradation machinery. Vpu interacts with the cytoplasmic domain of CD4 and h-βTrCP, a key connector in the ubiquitin-mediated cellular proteolytic machinery (29).

Studies with simian immunodeficiency virus (SIV) Nef have highlighted the importance of this protein in viral pathogenesis. SIVs carrying deletions in *nef* replicate poorly in rhesus macaques and usually do not cause AIDS (22). However, it has been difficult to determine which of the in vitro functions of Nef contribute to the pathogenesis of HIV or SIV in vivo (reviewed in reference 41). Other findings have highlighted the importance of CD4 down-modulation in virus pathogenesis in vivo. Mutations in SIV Nef that disrupt the ability to down-modulate CD4 strongly reduce viremia in infected monkeys and revert quickly to restore the CD4 down-modulation function (20). Insights into the role of CD4 down-modulation in HIV pathogenesis have also come from analysis of long-term nonprogressors (LTNPs). One study has shown that *nef* alleles isolated from LTNPs are less efficient in CD4 down-modulation than those isolated from progressors or asymptomatic carriers (40). Another report has shown that *nef* alleles isolated during "early" asymptomatic stages of infection poorly down-modulate CD4. In comparison, "late" *nef* alleles isolated after progression to AIDS show enhanced ability to down-modulate the HIV receptor (8). Recent findings have directly correlated the activity of Nef to down-modulate CD4 with enhanced viral replication in CD4-positive cells, including primary lymphocytes (4, 17, 27). Supporting these observations, overexpression of CD4 in HIV producer cells interferes with Env function and leads to drastic reductions in viral infectivity (25).

The above findings suggest that CD4 down-modulation may play an important role in AIDS pathogenesis and imply that

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interfering with this function might delay progression to disease and result in clinical benefits. To date, the HIV-induced down-modulation of CD4 has not been targeted for intervention, and no specific inhibitors of this viral function have been characterized. We describe here a strategy to interfere with this viral function. Our results indicate that lentivirus-mediated delivery of CD4 molecules resistant to HIV-induced down-modulation potently reduces viral infectivity and replication in CD4-positive cells, including primary lymphocytes. These findings demonstrate proof-of-concept that specific inhibition of this function may constitute the basis for novel approaches to treat HIV infection.

MATERIALS AND METHODS

Cells. 293T, MAGIC5, and MAGIC5B cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). MAGIC-5 and MAGIC-5B cells (kindly provided by M. Matsuda, Osaka University) are CD4-positive CCR5-positive derivatives of HeLa cells (39). These cells contain an integrated copy of the β-galactosidase gene under the control of the HIV-1 long terminal repeat (LTR) promoter. MAGIC5B cells express CD4 receptor levels 12-fold higher than MAGIC5 cells (4). Transformed T-cell lines (SupT1, C8166, and Jurkat Low-CD4) were maintained in RPMI 1640 medium supplemented with 10% FCS. Ficoll-purified peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors and cultured in RPMI 1640 medium supplemented with 10% human AB serum. PBMCs were activated with 5-μg/ml phytohemagglutinin (PHA) (Sigma) for 2 days prior to transduction with lentiviral vectors or infection with HIV-1. After PHA stimulation, cells were maintained in RPMI 1640 medium supplemented with 10% human AB serum containing 50 U of interleukin-2 (IL-2)/ml. All culture media used here were supplemented with 100 U of penicillin/ml, 100 μg of streptomycin/ml, 1 mM sodium pyruvate, and 2 mM glutamine.

Construction of lentiviral vectors expressing modified CD4 molecules. To engineer lentiviral constructs expressing CD4 variants, we utilized a PCR-based approach to amplify either full-length CD4 (CD4WT), or CD4 lacking its cytoplasmic domain (CD4Δcyt). The latter construct expresses a truncated CD4 protein in which the transmembrane domain of wild-type CD4 (ending with Val at position 395) is followed by a short 6-amino-acid cytoplasmic domain (sequence GSWPAS). Similarly, we also engineered a CD4 chimera in which the extracellular and transmembrane domains of CD4 were fused to the HIV-1 MA protein (CD4-MA). The CD4 variants were PCR amplified and cloned into the lentiviral transfer vectors PPT-PGK-GFP, pHR′-CMV-GFP-WPRE, and pWPI. PPT-PGK-GFP is a lentiviral vector with a chimeric Rous sarcoma virus (RSV)-HIV 5′-LTR and a deletion in the 3′-LTR that renders the vector self-inactivating (SIN) (15). Expression of the transgene is driven by the human phospoglycerate kinase (PGK) promoter. pWPI is also a SIN lentiviral vector in which the gene of interest is transcribed from an internal human EF1-alpha promoter. The transcribed unit also contains an internal ribosome entry site (IRES) element from encephalomyocarditis virus that allows internal initiation of translation of green fluorescent protein (GFP). In pHR′-CMV-CD4-WPRE, the transgene is expressed from the internal human cytomegalovirus (CMV) immediate-early promoter (30). Unlike PPT-PGK-CD4 and pWPI, pHR′-CMV-CD4-WPRE can be mobilized upon infection with replication-competent HIV. All lentiviral vectors used here contain a posttranscriptional regulatory element from the woodchuck hepatitis virus (WPRE) that enhances expression of transgenes (44).

Vector particle production. For preparation of HIV-based lentiviral vector particles coated with the vesicular stomatitis virus glycoprotein (VSV G) we used a previously described three-plasmid cotransfection procedure (30). 293T cells were cotransfected with 10 μg of CD4 lentiviral vector (PPT, pWPI, or pHR′ based), together with 10 μg of a packaging construct (pCMVΔR8.91) (45), and 2.5 μg of a plasmid encoding VSV G (pMD.G). Forty-eight hours later, vector particles were collected from culture supernatants, filtered through 0.45-μm-pore-size nitrocellulose membranes, aliquoted, and frozen to −80°C until use. All transductions described here were performed with unpurified vector-containing supernatants from 293T cells.

Transduction with lentiviral vectors and viral replication assays. 293T, MAGIC-5B, SUPT-1, C8166, and PBMCs were transduced following a previously described spinoculation procedure (12). Briefly, 0.5×10^6 cells were incubated with viral or vector supernatants (200 to 500 ng of p24 antigen) in 24-well plates in the presence of 4-μg/ml Polybrene and 10 mM HEPES (pH 7.4). Mixtures of cells and vectors were centrifuged at room temperature for 90 min (2,500 rpm) in a tabletop centrifuge (Sorvall RT6000B). After centrifugation, cells were washed with propagation medium and then incubated in the same medium for 2 to 3 days at 37°C. Transduced cells were infected with HIV-1 (1 μg of p24 protein) following the above protocol. Viral replication assays in PBMCs were performed by incubating cells with supernatant-containing viruses in the absence of Polybrene. Routinely, PBMCs were activated for 48 h with 5-μg/ml PHA, transduced with lentiviral vectors, and sorted for GFP expression 2 to 3 days later. Three to 4 days after sorting, 10^6 transduced PBMCs were infected with 2,000 50% tissue culture infective doses (without additional PHA treatment) in 150 μl of propagation media. After 2 h at 37°C, cells were washed twice and distributed into 96-well plates (2×10^5/well). Cells were maintained in the presence of 50 U of IL-2/ml. Replication assays in Jurkat Low-CD4 cells were performed by incubating 10^6 cells with HIV-1 R9 (50 ng of p24).

HIV-1 virus production. Infectious HIV-1 (R9) was produced by transient transfection of 293T cells with HIV-1 proviral clones. VSV G-pseudotyped R9 HIV-1 was generated by cotransfecting proviral DNA (10 to 20 μg) with 2.5 μg of pMD.G plasmid DNA. The CCR5-tropic HIV-1 BaL virus was harvested and purified from the supernatant of infected monocyte-derived macrophages.

Single-round infectivity assays. Infectivity assays were performed with MAGIC5 cells using equal amounts of input virus (measured as nanograms of p24 protein) in the presence of 20-μg/ml DEAE dextran. Infection was allowed for 48 h. Routinely, after 8 h of infection, 1 μM zidovudine (AZT) was added to block second rounds of virus replication. Infected cells were scored after staining for β-galactosidase with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

Protein analysis. Incorporation of cellular and viral proteins in HIV particles was determined by Western blot with the following antibodies (NIH AIDS Research Reference Reagent Program). For HIV-1 Env, a gp120-specific sheep antiserum was used. For CD4, the T4-4 rabbit antiserum was used. gp41 was probed with the 2F5 human monoclonal antibody (MAb), and p24 was analyzed with the 183-H12-5C murine MAb. Loading controls for cell lysates were performed by probing with an α-tubulin MAb (Sigma). Virion-associated proteins were pelleted through a 20% sucrose cushion at 26,000 rpm in a SW28 rotor (Beckman) for 1.5 h at 4°C. Equal amounts of p24 protein were separated onto a sodium dodecyl sulfate–10%-polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and then probed with specific antibodies. Antibody binding was detected by the Amersham ECL enhanced chemiluminescence Western blotting kit, using horseradish peroxidase-conjugated secondary antibodies (Amersham Bioscience, Inc.).

Flow cytometry and cell sorting. Surface staining of CD4 was performed with either the OKT4 MAb, followed by addition of goat anti-mouse Cy5-conjugated antibody, or with CD4-V4 MAb (phycoerythrin [PE] conjugated; Becton Dickinson). The epitopes recognized by these MAbs do not overlap with the gp120-binding domain (37). Intracellular staining of HIV-1 Gag was performed with a p24-specific MAb (fluorescein isothiocyanate [FITC]-conjugated KC57 clone; Coulter), using a cell permeabilization kit following the recommendations of the manufacturer (Caltag Lab). PBMCs transduced with lentiviral vectors were sorted for enrichment of double-positive (GFP-positive/CD4-positive) cells. PBMCs were resuspended at 20×10^6 cells/ml in PBS with 2% FCS and stained with a CD4 MAb (CD4-PE-Cy5 conjugated; DAKO) at room temperature for 15 min. Then the cells were washed twice and resuspended in RPMI supplemented with 5% human AB serum at 10 to 25×10^6 cells/ml. Cells were immediately sorted with a FACSVantage SE system (Becton Dickinson), collected in RPMI supplemented with 20% human AB serum, centrifuged, and resuspended in propagation media supplemented with 50 U of IL-2/ml.

RESULTS

Lentiviral vectors expressing CD4 molecules resistant to modulation by HIV-1 Nef and Vpu. Since Nef and Vpu require the cytoplasmic domain of CD4 to connect the viral receptor with cellular degradation pathways, we hypothesized that truncated CD4 molecules lacking the cytoplasmic domain would be poorly down-modulated by HIV-1 and subsequently inhibit the infectivity of the newly synthesized particles. We designed lentiviral vectors delivering full-length CD4 (wild type), CD4 lacking its cytoplasmic domain (CD4Δcyt), or the extracellular and transmembrane domains of CD4 fused to the full-length HIV-1 matrix protein (CD4-MA). Production of these engi-

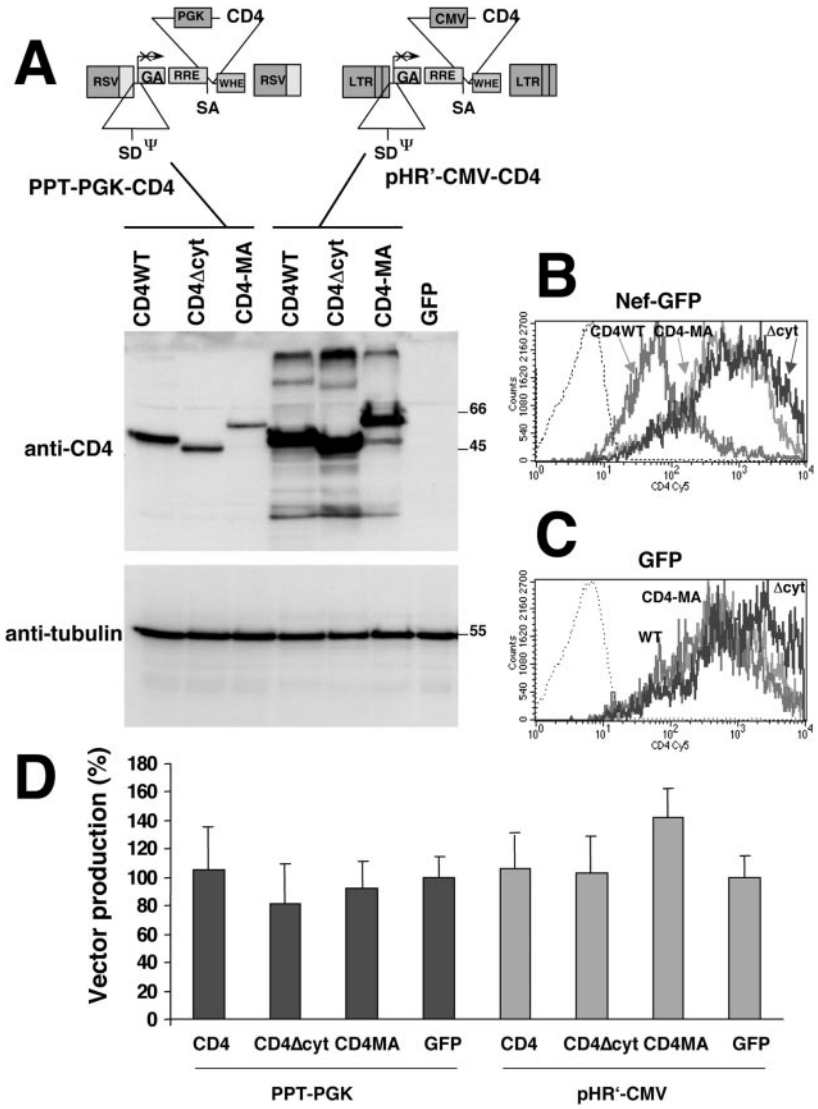

FIG. 1. Lentiviral vectors expressing CD4 molecules resistant to the down-modulatory effect mediated by HIV-1 Nef and Vpu. (A) Schematic drawing of the lentiviral vectors PPT-PGK-CD4 and pHR′-CMV-CD4. These vectors were used to express GFP alone (GFP), full-length CD4 (CD4WT), CD4 lacking its cytoplasmic domain (CD4Δcyt), or the extracellular and transmembrane domain of CD4 fused to HIV-1 matrix (CD4-MA). Lysates of 293T cells transduced with these vectors were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis-Western blot with CD4-specific antibodies. In panels B and C, 293T cells were transduced with pHR′-CMV vectors expressing either wild-type CD4, CD4-MA, or CD4Δcyt and transfected with a plasmid expressing Nef (NA7) fused to GFP (B) or GFP alone (C). Histograms represent CD4 levels in GFP-positive cells. Results for an IgG isotypic control are shown as dotted lines. (D) Vector production in 293T cells was analyzed 48 h after transfection by estimating the levels of HIV-1 p24 antigen in culture supernatants.

neered proteins was confirmed in 293T cells transduced with these vectors (Fig. 1A). CD4-MA fusion protein was expressed at lower levels than full-length or truncated CD4. This was more evident in cells transduced with PPT-PGK vectors. The reason for this is unknown. To test the ability of the various CD4 proteins to be down-modulated by HIV-1 Nef, 293T cells transduced with lentiviral vectors were then transfected with plasmids expressing either the HIV-1 NA7 primary *nef* allele fused to GFP (Nef-GFP) or GFP alone (Fig. 1B and 1C, respectively). Expression of Nef-GFP decreased 15-fold the surface levels of full-length CD4, but did not alter expression of CD4Δcyt or CD4-MA, consistent with the requirement of the CD4 cytoplasmic domain for efficient Nef-induced down-modulation. Elevated levels of CD4 have been previously shown to interfere with HIV production in an HIV-1 Env-independent manner (5, 12). To test whether expression of CD4 affects vector production, we estimated the amount of viral p24 protein in cultures of 293T producer cells. 293T cells were cotransfected with packaging and VSV G plasmids, together with either control transfer vectors (GFP) or vectors expressing the CD4 variants. As shown in Fig. 1D, no significant reduction in p24 released was observed with any of the vectors (PPT-PGK- or pHR′-CMV-based), suggesting that expression of the various CD4 transgenes does not interfere with vector production in 293T cells.

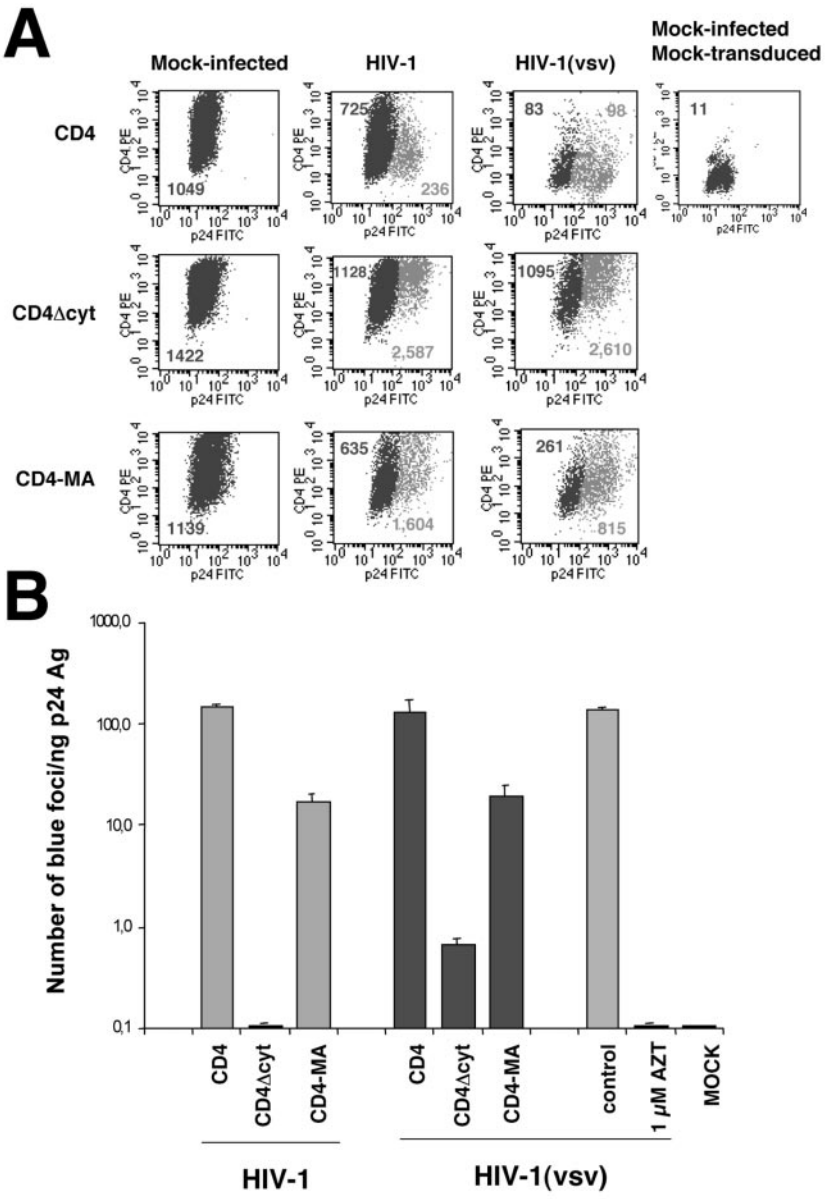

FIG. 2. Suppression of HIV-1 infectivity by lentiviral vectors expressing CD4 variants. (A) Twenty-four hours after transduction with pHR′-CMV lentiviral vectors expressing CD4 variants, 293T cells were either mock infected (left columns) or infected with HIV-1 (R9) (middle columns) or HIV-1 R9 pseudotyped with VSV G (right columns). After 48 h of infection, CD4 surface expression and intracellular accumulation of HIV p24 protein were estimated by flow cytometry. Mock-infected cells were used to set up staining levels for p24-negative cells. p24-positive HIV-infected cells are shown in gray dots. Inlet numbers indicate mean values of CD4 expression in p24-negative, and p24-positive infected cells (upper and lower inlets, respectively). (B) Infectivity of the released particles was estimated in MAGIC5 cells infected with equal amounts of p24 input virus. Control infection was performed with virus produced in 293T cells infected with HIV-1 pseudotyped with VSV G. The effect of addition of 1 μM AZT before infection of MAGIC5 cells with HIV-$1_{(vsv)}$ is also shown.

Suppression of HIV-1 infectivity by lentiviral vectors expressing truncated CD4 proteins. Interference with HIV-1 infectivity was first analyzed in CD4-negative 293T cells. These cells express the CXCR4 coreceptor, and upon transduction with CD4 lentiviral vectors, they become permissive to infection with HIV-1. This strategy ensured that release of HIV-1 particles would occur only from the lentiviral vector-transduced cells. We were interested in evaluating the inhibitory effect of the vectors under optimal conditions of HIV infection, in which overexpression of Nef, Vpu, and Env could overcome the block imposed by CD4. To achieve this goal, we infected cells with HIV-1 R9 (1,000 ng of p24) following a spinoculation procedure (31). We also evaluated whether the above lentiviral vectors could inhibit the infectivity of VSV G-pseudotyped HIV-1 [HIV-$1_{(vsv)}$], which infects cells to a higher extent than HIV-1 particles using the natural viral envelope (Env) for entry. Figure 2A shows that more than 90% of the cells became transduced and expressed CD4, as estimated by surface staining with anti-CD4 antibodies. Levels of surface CD4 were significantly lower in the p24-negative cells present in HIV-

treated cultures than in mock-infected cultures (Fig. 2A, compare the values 725 versus 1,049 in CD4-transduced cells). This is likely due to the fact that Nef is incorporated into viral particles. The amount of Nef protein encapsidated into virions is enough to down-modulate surface CD4 even in the presence of reverse transcriptase or integrase inhibitors (43; and data not shown). This fact is more notable in infections with HIV-$1_{(vsv)}$, which resulted in p24-negative cells with very low levels of CD4. Furthermore, cells expressing higher levels of CD4 are more susceptible to infection with HIV-1. Upon infection, these cells move out from the p24-negative region, further contributing to the impression that CD4 down-modulation occurs in p24-negative cells. Upon infection with HIV-1, full-length CD4 was efficiently down-modulated in p24-positive cells, presumably by the combined action of Nef, Vpu, and Env proteins (Fig. 2A, top-middle plot). In contrast, the levels of receptor remained elevated in HIV-1-infected cells transduced with CD4Δcyt and CD4-MA, with surface levels 11- and 7-fold higher, respectively, than those observed in infected cells transduced with wild-type CD4.

As expected, pseudotyping HIV-1 with VSV G led to enhanced infection (4- to 5-fold higher, estimated as percentage of p24-positive cells) and higher levels of p24 expression in infected cells (52% higher). Consequently, the level of down-modulation of full-length CD4 occurred to higher extents. Under these conditions, surface levels of CD4-MA were partially reduced, as compared to cells infected with HIV-1. However, CD4Δcyt molecules still remained completely resistant to down-modulation by HIV-$1_{(vsv)}$ (Fig. 2A, right-center panel). These results demonstrate the feasibility of a lentiviral vector system to achieve elevated levels of expression of CD4. Our findings also demonstrate that expression of Env in HIV-1-infected cells is not sufficient to down-modulate receptor molecules insensitive to the action of Nef and Vpu.

293T cells tranduced with CD4 vectors and then infected with HIV-1 did not show significant differences in the release of viral particles, estimated by p24 enzyme-linked immunosorbent assay (ELISA), as compared to mock-tranduced cells (data not shown). The infectivity of HIV-1 particles produced from 293T-transduced cells was analyzed in single-round infectivity assays in MAGIC5 cells. MAGIC5 cells were challenged with equal amounts of p24, and the number of blue foci was estimated (Fig. 2B). Lentiviral-based expression of CD4Δcyt reduced the infectivity of HIV-1 by more than 1,000-fold. The extent of inhibition was comparable to that observed by addition to target cells of 1 μM AZT, an inhibitor of the HIV reverse transcriptase (Fig. 2B). These results prove the ability of these lentiviral vectors to efficiently block HIV-1 infectivity in the presence of a full set of CD4 down-modulator genes (*nef*, *vpu*, and *env*). Particles synthesized in cells transduced with CD4-MA expressed smaller amounts of the fusion protein on the surface of infected cells, as compared to CD4Δcyt (mean value: 1,604 versus 2,587, respectively), but still reduced HIV-1 infectivity by 88%. Interestingly, the viral progeny from cells transduced with CD4Δcyt and then infected with VSV G-pseudotyped HIV-1 showed severely reduced infectivity (100-fold), suggesting that higher levels of viral products were not sufficient to overcome the inhibitory effects of truncated CD4. Expression of full-length CD4 by itself did not affect HIV-1 infectivity, suggesting that the inhibitory effects mediated by CD4Δcyt and CD4-MA were likely due to the expression of the transgenes and not to the lentiviral vectors, which might interfere with viral replication at different stages (7, 23).

We next wanted to determine the effect of expression of the truncated receptor in CD4-positive cells. For this purpose, we tested several cell lines permissive to HIV-1 infection: SupT1, C8166, and MAGIC5B. These cells were selected because they express high levels of the viral receptor (Fig. 3A, bottom plots). Transduction efficiencies with GFP control vectors were higher than 90% in all cell lines, as estimated by flow cytometry (data not shown). Infection of mock-transduced cells with HIV-1 led to efficient down-modulation of CD4. Receptor down-modulation was also efficiently achieved in cultures transduced with full-length CD4. In these cultures, a population of Low-CD4/p24-positive infected cells that had undergone receptor down-modulation was observed. In contrast, infection of cells previously transduced with CD4Δcyt failed to down-modulate the receptor, which maintained surface levels ~3- to 4-fold higher than those observed in mock-infected cells. Of note, p24-positive cells with low levels of the receptor were no longer observed in cultures of SupT1 and C8166 cells transduced with CD4Δcyt. The infectivity of viral particles released from transduced cells was determined as in Fig. 2A. Overexpression of full-length CD4 led to a small reduction in infectivity in the three cell types, as compared to cells transduced with control GFP vectors. In contrast, cells transduced with truncated CD4 produced viral particles with largely diminished infectivity (Fig. 3B). Mobilization of pHR′-CMV vectors by HIV-1 did not significantly contribute to the inhibitory effect observed in the above experimental settings, since similar results were observed in SupT1 cells transduced with the self-inactivating PPT-PGK lentiviral vector expressing CD4Δcyt (Fig. 3B).

Inhibition of HIV-1 replication in primary lymphocytes. The experiments described above with transformed T cells were not feasible with primary cells, since treatment of PBMCs with lentiviral vectors resulted in transduction efficiencies of only 20 to 40%, as estimated by the number of GFP-positive cells by flow cytometry (Fig. 4). To overcome this problem, we utilized CD4 lentiviral vectors derived from pWPI. In addition to the transgene, cells transduced with these vectors also express GFP under the control of an IRES element. Transduced cells can thus be readily identified by gating GFP-positive cells. Primary cells were transduced with either GFP alone, full-length CD4, or with the most potent inhibitor of HIV infectivity, CD4Δcyt. Three days after transduction with bicistronic vectors, PBMCs were infected with HIV-1 (R9) and analyzed by flow cytometry for surface CD4 and GFP fluorescence (Fig. 4). Infection with HIV-1 resulted in the appearance of a population of GFP-positive cells that had undergone CD4 down-modulation (Fig. 4C; 2.14% of the culture). In contrast, transduction of PBMCs to similar levels with a CD4Δcyt lentiviral vector resulted in a 13-fold reduction in the percentage of Low-CD4/GFP-positive cells (Fig. 4F; 0.16%). These results indicate that CD4Δcyt is resistant to down-modulation in primary T cells infected with wild-type HIV-1. To rule out the possibility that truncated CD4 may interfere with expression of HIV proteins, aliquots of the cultures were stained intracellularly with antibodies specific for HIV-1 p24. A total of 1.92% of the cells transduced with full-length CD4 and infected with HIV stained positive

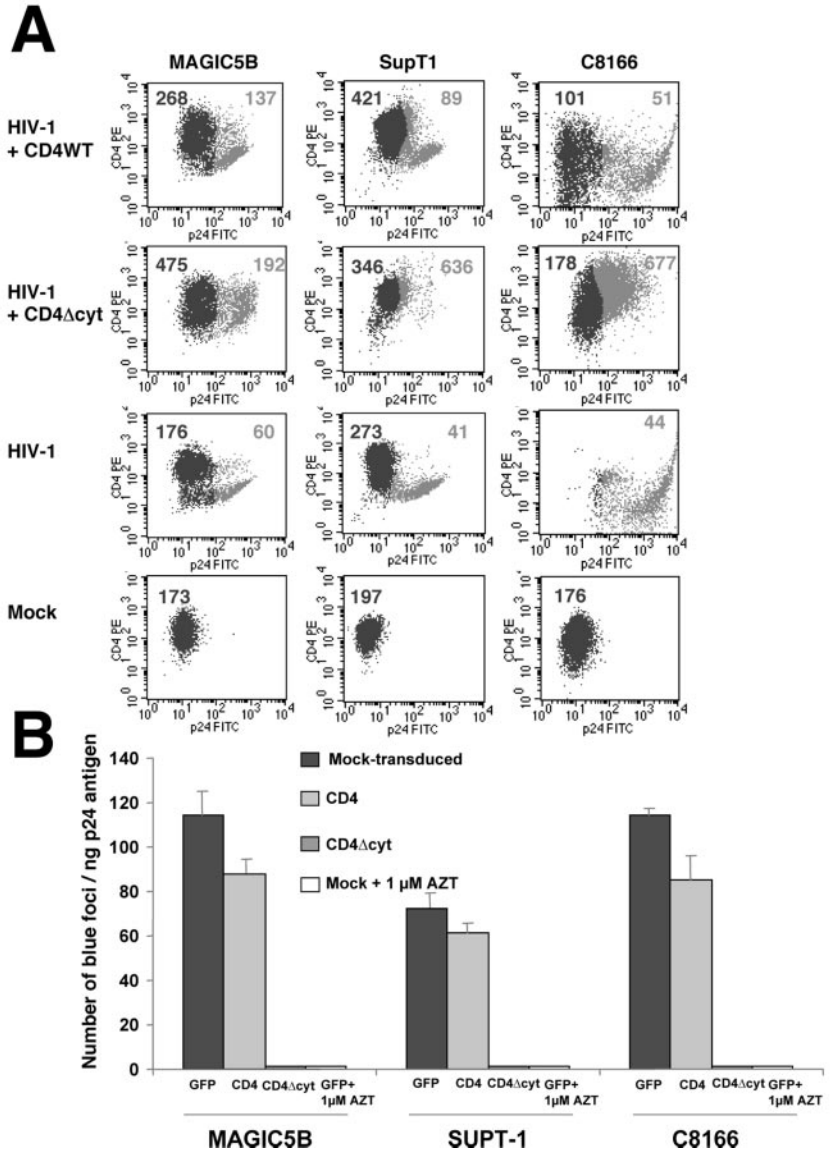

FIG. 3. Truncated receptors interfere with HIV-1 infectivity in CD4-positive transformed cells. (A) CD4-positive MAGIC5B and C8166 cells were mock treated or transduced with lentiviral vectors (pHR′-CMV) expressing either full-length CD4 or truncated CD4. CD4-positive SupT1 cells were transduced with similar PPT-PGK vectors. Twenty-four hours after transduction, cells were infected with HIV-1 (R9). The extent of CD4 down-modulation in HIV-infected cells (p24 positive) was analyzed 48 h after infection, as shown in Fig. 2. The left and right numbers indicate the mean CD4 values in uninfected and infected (gray dots) cells, respectively. Note that due to very high levels of p24 expression, proper compensation could not be performed in infected C8166 cells. (B) Infectivity of the released particles estimated in MAGIC5 cells, as shown in Fig. 2A.

for p24 antigen. This value is comparable to the fraction of cells that underwent CD4 down-modulation in the same culture (2.14%). Cultures of cells transduced with truncated CD4 showed slightly lower numbers of p24-positive cells (1.21%; Fig. 4H). However, this difference cannot account for the 13-fold reduction observed in CD4 down-modulation, as compared to cells transduced with full-length receptor. These results suggest that expression of CD4Δcyt does not interfere with the steps of the viral life cycle that lead to expression of the late Gag viral protein. The reduction in the number of p24-positive cells is likely due to a block in second rounds of infection, since the output virus produced under conditions in which CD4 down-modulation is blocked is unable to infect new target cells (12). In summary, these results prove that expression of truncated CD4 in primary lymphocytes is resistant to down-modulation by HIV-1.

We then performed viral replication assays with transduced primary lymphocytes. Cultures of PBMCs were incubated with bicistronic vectors and enriched in transduced cells by sorting for GFP expression. This procedure resulted in cultures containing ~90% GFP-positive cells (Fig. 5A). Enriched cultures were challenged with the CCR5-tropic HIV-1 BaL strain. The extent of viral replication, estimated as the amount of p24 antigen in cultures, is shown in Fig. 5B. Transduction with

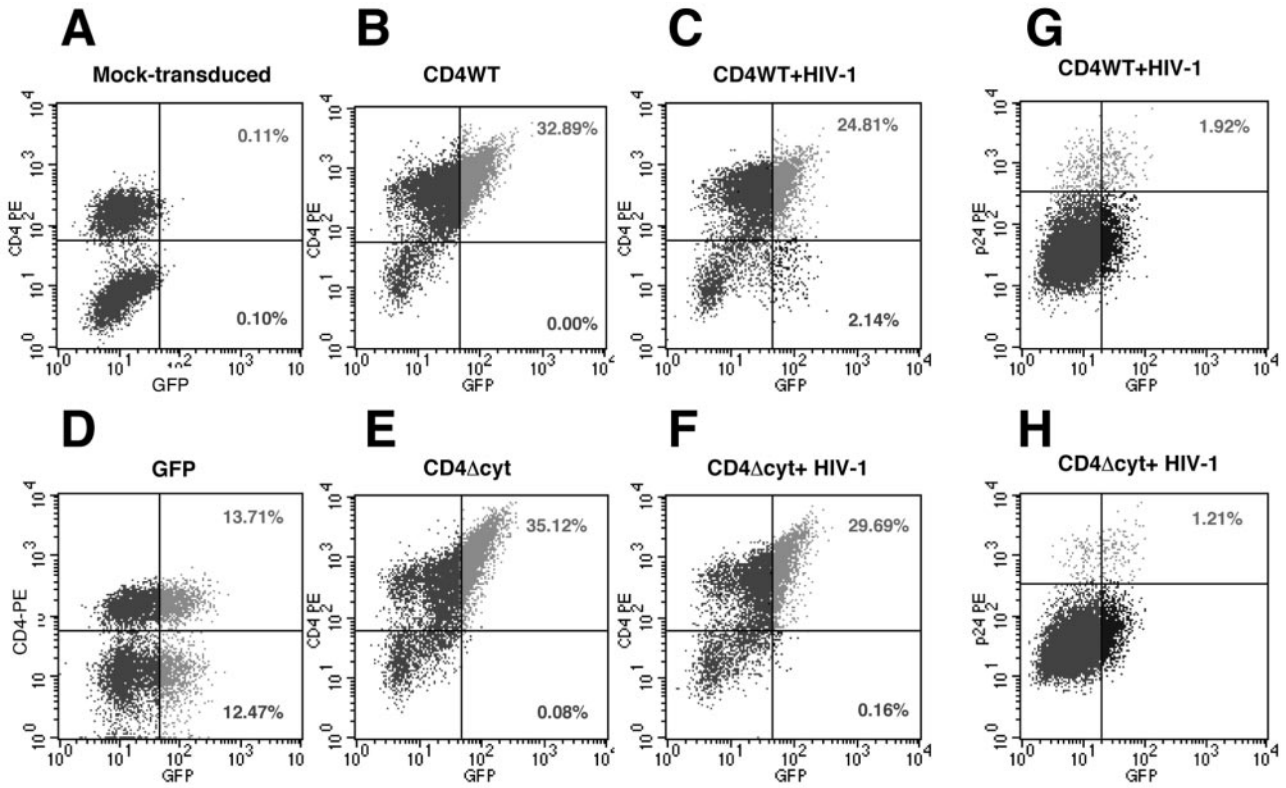

FIG. 4. Transduction of primary T cells with CD4/GFP bicistronic vectors. PBMCs were transduced with lentiviral vectors (pWPI) expressing either GFP alone (D), GFP and full-length CD4 (B, C, and G), or GFP and CD4Δcyt (E, F, and H). Transduced PBMCs were either mock treated or infected for 48 h with HIV-1 (R9) and then stained for surface CD4 (A through F) or intracellular p24 (G and H). Inlet numbers (A through F) represent the percentage of transduced (GFP positive) cells with high or low levels of surface CD4 (upper and lower numbers, respectively). Numbers in panels G and H represent the percentage of GFP-positive/p24-positive cells.

full-length CD4 resulted in faster replication profiles than those observed in cells transduced with control vectors (GFP alone). This may be due to the enhancement in HIV entry promoted by the elevated levels of receptor. Conversely, cells transduced with CD4 molecules resistant to the action of Nef and Vpu showed severely reduced HIV-1 replication. The residual level of HIV-1 replication observed in these cultures may be due to viral production from the fraction of untransduced cells still present in the enriched cultures (5 to 10%). Viral replication assays were also performed in Jurkat Low-CD4 cells infected with the CXCR4-tropic R9 strain (Fig. 5D). Enrichment of these cultures was not necessary, since treatment with lentiviral vectors resulted in transduction efficiencies higher than 95%, estimated as a percentage of GFP-positive cells (Fig. 5C). As shown in primary cells, transduction of Jurkat cells with full-length CD4 resulted in faster viral replication. This enhancement was more pronounced than the one observed in primary cells, probably due to the much lower levels of CD4 receptor present in Jurkat Low-CD4 cells (data not shown). Expression of truncated CD4 resulted in complete abrogation of HIV-1 replication. Of note, we failed to detect revertant viruses after more than 60 days in two independent experiments in which Jurkat Low-CD4 cells were transduced with CD4Δcyt vectors and exposed to HIV-1. The above findings demonstrate the ability of lentiviral vectors to interfere with CD4 down-modulation and HIV-1 replication in primary cells and highlight the potency of the observed inhibitory effect.

Mechanism of inhibition of HIV-1 infectivity. To address the mechanism of inhibition of HIV-1 replication mediated by CD4 lentiviral vectors, transduced 293T cells were infected with HIV-1 pseudotyped with VSV G. The extent of inhibition mediated by CD4Δcyt estimated in single-round infectivity assays was greater than 99%. Viral particles were purified through sucrose cushions and analyzed by Western blot with antibodies specific for CD4, gp120, gp41, and p24. In parallel, total cell lysates of the producer cells were analyzed (Fig. 6). Expression of CD4 molecules lacking the cytoplasmic domain (CD4Δcyt and CD4-MA) resulted in accumulation of a 160-kDa protein band immuno-recognized with anti-gp120 antibodies. This band was also recognized by gp41-specific antibodies (not shown) and represents the gp160 envelope precursor. Concomitantly, a reduction in the steady-state levels of gp41 was evident in cells transduced with CD4Δcyt. The mature gp120 Env subunit was not detected in cell lysates, probably due to the poor sensitivity of the polyclonal serum and the fact that only a small fraction of the Env precursor (5 to 10%) is transported to the cell surface and cleaved into the mature forms (42). Virions produced in cells expressing truncated CD4 molecules incorporated the receptor in their membranes, whereas the levels of gp120 incorporation were reduced. CD4 incorporation was proportional to gp120

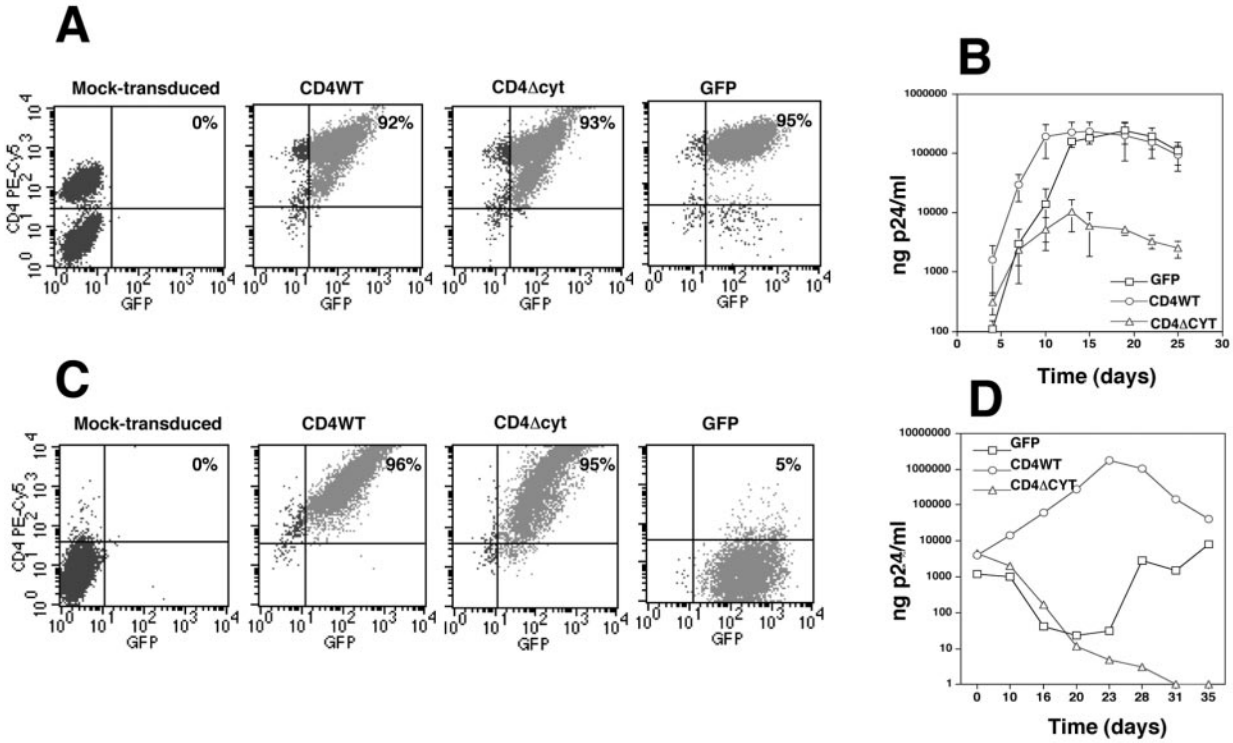

FIG. 5. Inhibition of HIV-1 replication in primary and transformed T cells. (A) PBMCs were transduced with pWPI lentiviral vectors expressing either GFP alone, or together with CD4, or CD4Δcyt. Cultures were enriched with transduced cells by sorting GFP-positive/CD4-positive cells in a flow cytometry apparatus. Mock-transduced cells are shown for comparison. Numbers indicate the percentage of CD4-positive/GFP-positive cells. (B) Three days after sorting, cells were infected with the CCR5-tropic HIV-1 BaL strain. Viral replication was estimated by measuring accumulation of p24 protein in cultures. (C) Jurkat T cells were transduced with pWPI vectors and analyzed by flow cytometry with CD4-specific antibody (without further enrichment). Jurkat cells were infected with the CXCR4-tropic R9 HIV-1 strain, and the extent of viral replication was estimated as described above.

exclusion, which was more severe in virions produced in the presence of CD4Δcyt, the most potent inhibitor of HIV-1 infectivity. Reductions in gp41 incorporation were also observed in membranes from virions produced in the presence of CD4Δcyt and CD4-MA. These results are in agreement with previous reports and suggest that the observed inhibitory effects are likely due to interference with incorporation of Env into viral membranes (25). Furthermore, CD4 molecules incorporated into viral membranes could also interfere with Env function by saturating its CD4 binding sites.

DISCUSSION

We have evaluated virus-induced receptor down-modulation as a possible target to inhibit HIV-1 replication. To block this viral function, we engineered mutant CD4 molecules lacking their cytoplasmic domain. Lentiviral vector-mediated expression of truncated CD4 led to potent inhibition of HIV-1 infectivity in several transformed cell lines: Jurkat T, C8166, SupT1, MAGIC5B, and 293T. The inhibitory effect was also revealed in primary CD4 lymphocytes, which exhibited a 100-fold reduction in replication of the CCR5 BaL strain following transduction with CD4Δcyt. Interestingly, the HIV-1 envelope by itself was not sufficient to promote down-modulation of the presumably Env-sensitive CD4Δcyt, suggesting that efficient down-modulation of newly synthesized receptor molecules requires the combined action of both Env and Vpu proteins. Previous findings have shown that Env can independently down-modulate full-length CD4 in the absence of Nef and Vpu (9). It seems possible that in situations with elevated levels of receptor (e.g., those achieved with truncated CD4) the need for a complete set of HIV genes is revealed, and Env alone cannot exert its effect. Expression of CD4Δcyt in transformed and primary T cells resulted in severe reductions in the number of infected cells in which CD4 down-modulation had efficiently occurred. We cannot exclude the possibility that overexpression of truncated receptor also interferes with down-modulation of endogenous full-length CD4. CD4Δcyt, which maintains the HIV-1 envelope binding domain, might compete with full-length CD4 for binding to gp160, thus interfering with down-modulation of the endogenous receptor. However, our experiments did not directly address this point, and additional studies will be required to test this hypothesis.

Our studies indicate that expression of truncated CD4 results in the production of defective virions with severely reduced incorporation of gp120 and gp41 proteins. The mechanism of exclusion is not completely understood, but may be due to a reduction in the availability of processed gp120 envelope protein on the surface of infected cells. Supporting this explanation, the gp160 precursor accumulated in cells expressing truncated CD4, whereas levels of the mature gp41 protein

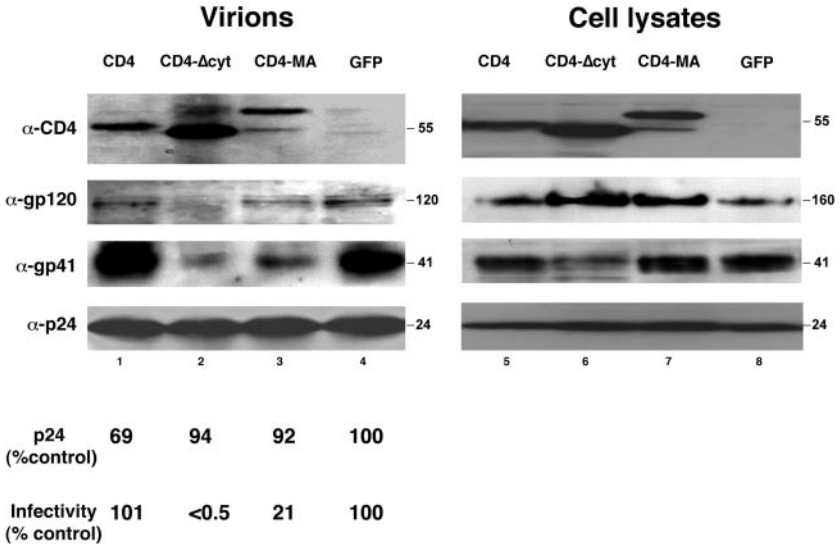

FIG. 6. Lentiviral vectors expressing truncated CD4 interfere with Env incorporation into viral membranes. 293T cells previously transduced with lentiviral vectors (pHR′-CMV) expressing either GFP or CD4 variants were infected with HIV-1 pseudotyped with VSV G. Forty-eight hours postinfection, culture supernatants were collected and used to purify virions through sucrose cushions. Cell lysates from producer cells (right panels) and purified virions (left) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis-Western blot with antibodies specific to CD4, gp120, gp41, and p24. Molecular masses in kilodaltons are shown. The numbers at the bottom left indicate the amount of HIV-1 particles released from infected cells (estimated by p24 ELISA) and their infectivity (estimated in MAGIC5 cells). Values are given as percentages of those observed in virions from cells transduced with the pHR′-CMV-GFP control vector.

were reduced. Furthermore, the CD4 receptor, which is efficiently excluded from viral membranes under normal conditions (12), becomes incorporated when receptor down-modulation is inhibited. CD4 molecules incorporated into viral membranes associate with gp120, and it is likely that they interfere with the binding of envelope to receptor molecules on the surface of target cells (26). Recent studies have shown that the relative density of envelope spikes in HIV-1 virions is surprisingly low. Compared to influenza virus virions, which contain 200 to 300 spikes per particle, HIV-1 virions incorporate an average of 7 to 14 spikes per particle (10). It is therefore not surprising that approaches aimed at either reducing incorporation of Env or functionally inactivating it may lead to potent inhibition of HIV infectivity. We can also speculate that antiviral therapies targeting HIV entry (e.g., inhibitors of CCR5) could result in synergistic effects with drugs targeting CD4 down-modulation.

The findings presented here provide proof-of-concept that targeting the virus-induced receptor down-modulation may constitute the basis for novel approaches to block HIV replication and encourage the identification of small-molecule inhibitors of this function. However, as demonstrated with all known antiretrovirals, the emergence of resistant strains may pose important problems. Inhibition of CD4 down-modulation might result in the appearance of viral strains with enhanced receptor down-modulation activity in the Env, Nef, or Vpu products. Interestingly, Nef variants with enhanced CD4 down-modulation have been characterized in vivo. As patients progress to AIDS, *nef* alleles with enhanced activity emerge during the course of infection (4, 8). Alternatively, revertant viruses with either higher density of envelope spikes or enhanced envelope fusogenic activity could be anticipated. In this regard, compensatory changes in gp41 have been characterized in *nef*-deleted SIVs after passage in rhesus macaques. Interestingly, the changes in gp41 observed in these viruses increase the fusogenic activity of the SIV envelope and may be compensating for the poor viral entry of *nef*-deleted SIVs (2). In the absence of small-molecule specific inhibitors, the availability of lentiviral vectors to block CD4 down-modulation will provide essential tools to evaluate in vitro the therapeutic value of targeting this function.

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