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Reduced Cell Surface Expression of Processed Human Immunodeficiency Virus Type 1 Envelope Glycoprotein in the Presence of Nef

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***nef* genes from two laboratory grown human immunodeficiency virus type 1 (HIV-1) strains and from two proviruses that had not been propagated in vitro were introduced into CD4$^+$ lymphoblastoid CEM cells. The stable expression of all four Nef proteins was associated with an almost complete abrogation of CD4 cell surface localization. The consequences of the presence of Nef on gp160 cleavage, gp120 surface localization, and envelope-induced cytopathic effect were examined in CEM cells in which the HIV-1 *env* gene was expressed from a vaccinia virus vector. The presence of Nef did not modify the processing of gp160 into its subunits but resulted in a significant decrease of cell surface levels of gp120, associated with a dramatic reduction of the fusion-mediated cell death. Surface levels of mutant envelope glycoproteins unable to bind CD4 were not altered in Nef-expressing cells, suggesting that the phenomenon was CD4 dependent. The intracellular accumulation of fully processed envelope glycoproteins could significantly delay the cytopathic effect associated with envelope surface expression in HIV-infected cells and may be relevant to the selective advantage associated with Nef during the in vivo infectious process.**

In most isolates of human immunodeficiency virus type 1 (HIV-1), HIV-2, and simian immunodeficiency virus (SIV), a gene called *nef* (for negative factor) overlaps the 3′ long terminal repeat. It is expressed early in the viral cycle as a Rev-independent mRNA (29) and encodes a 27- to 30-kDa myristoylated protein, predominantly localized in the cytoplasm and partly associated with membranes (11, 17). In contrast with early observations, a positive role for *nef* in viral replication has been recently suggested. The SIV_{MAC} Nef protein was shown to be necessary for maintaining high virus loads and for inducing AIDS in infected rhesus monkeys (18), and several groups have reported that Nef accelerates HIV-1 replication in peripheral blood mononuclear cell (PBMC) cultures (8, 38). However, the molecular mechanism by which Nef improves the efficiency of viral replication remains unknown.

A clue might be provided by the observation that HIV-1 Nef down-modulates the cell surface levels of CD4, the receptor molecule recognized by the viral envelope glycoprotein. In the presence of Nef, CD4 is sequestered in an intracellular compartment (12, 13). In a productively infected cell, a large number of envelope glycoprotein precursors (gp160) are synthesized and processed into mature heterodimers (gp120/gp41) that reach the cell surface, where they are incorporated into virions. This process rapidly leads to cell death, primarily because the envelope glycoproteins can bind CD4 molecules on uninfected bystander cells and mediate syncytium formation (32). Binding to CD4 also occurs inside the infected cell, acts on the maturation of the envelope precursor (5, 10, 35), and can mediate fusion-independent cytopathic effect (21, 22). In this study, we have examined the consequences of Nef-mediated intracellular retention of CD4 on the transport and maturation of the envelope glycoprotein.

Four different HIV-1 *nef* genes were stably expressed in human CD4$^+$ T cells, resulting in the reduction of CD4 cell surface levels. The biosynthesis of the HIV-1 envelope glycoprotein molecules was studied in these cells, using vaccinia virus (VV) vectors. The envelope glycoprotein maturation was quantitatively and qualitatively unaltered in the presence of Nef. In contrast, we observed a reduced expression of the gp120/gp41 heterodimers at the cell surface that was dependent on the ability of the envelope to bind CD4. Envelope-induced cytopathic effect was also dramatically reduced in Nef-expressing cells. We discuss the possibility that in the presence of Nef, HIV-infected cells are less prone to death by fusion and can therefore maintain viral production for longer periods of time, because they accumulate mature envelope glycoprotein complexes in an intracellular compartment rather than at the cell surface.

MATERIALS AND METHODS

Blood samples and PCR. DNAs prepared from the PBMC of two HIV-1-infected children (2 to 6 months old) were obtained from A. Krivine (Hôpital St Vincent de Paul, Paris, France) and were used for amplification of *nef* alleles by polymerase chain reaction (PCR). The DNA plus-strand primer Nef5 (5′-CCAGGATCCAGTAGCTGAGGGGACA GATAG-3′) and DNA minus-strand primer Nef7 (5′-CCAG GATCCCCCTGGAAAGTCCCCAGCGGA-3′) were at positions 8280 to 8300 and 9055 to 9035, respectively, on the HIV_{LAI} sequence (33). Primers included *Bam*HI sites (underlined). Forty cycles of amplification were performed, using 1 μg of cellular DNA. Denaturation, annealing, and elongation conditions were 92°C for 30 s, 55°C for 1 min, and 72°C for 1 min 30 s, respectively.

Retroviral vectors. *nef* genes were inserted under the transcriptional control of the murine leukemia virus long terminal repeat in the LXSN retroviral vector (25), which carries a simian virus 40 early promoter-*neo* gene cassette. A

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*Bam*HI-*Sac*I 780-bp fragment containing the HIV-1_{LAI} *nef* gene (from plasmid pTG1147, a gift of B. Guy, Transgène, Strasbourg, France) was blunt-end ligated into dephosphorylated, *Hpa*I-cleaved pLXSN, yielding pLFLAI. Two recombinant vectors, pLFAO1 and pLFEO1, were obtained from PCR-amplified *nef* genes. PCR material was purified on an agarose gel, *Bam*HI digested, and ligated into dephosphorylated, *Bam*HI-cleaved pLXSN. The *nef* genes in plasmids pLFAO1 and pLFEO1 were sequenced by the dideoxy-chain termination method, using Nef5 and Nef7 PCR primers. Another pLXSN-derived vector (pCTR) which contained a 800-bp artifactual PCR product with no coding capacity was used as a control in all experiments. Nucleic acid sequences are available upon request.

Cells and retroviral infections. Amphotropic packaging ψCRIP cells (6) and human lymphoblastoid CEM cells were respectively grown in Dulbecco's modified Eagle medium and in RPMI medium supplemented with 10% fetal calf serum and 2 mM glutamine. ψCRIP cells were transfected with retroviral vectors by using calcium phosphate coprecipitations. Stable transfectants were isolated in the presence of 1 mg of G418 (GIBCO) per ml and examined for the production of infectious retroviral vectors as described previously (6). The amphotropic pLNefSN producer (12) was obtained from J. V. Garcia (St. Jude Children's Research Hospital, Memphis, Tenn.) and was used to transfer the HIV-1_{SF2} *nef* gene.

Exponentially growing CEM cells were suspended at a concentration of 2×10^5/ml and infected by a 72-h cocultivation with subconfluent producer cells. Infected cells were selected in the presence of G418 (1 mg/ml). Numerous G418-resistant cells started to grow after 4 to 6 days in selective medium, indicating that a significant fraction (5 to 10%) of cells had received the retroviral vector.

VV vector infections. Cells were infected for 1 h with recombinant VV at a multiplicity of infection of 50 PFU per cell, washed, and resuspended in fresh medium. Under these conditions, more than 95% of the cells were infected, as seen by fluorescence-activated cell sorting (FACS) analysis of VV antigen surface expression. Envelope-mediated syncytium formation was observed as soon as 4 h postinfection and could be easily distinguished from the VV-induced cell lysis that occurred between 24 and 48 h postinfection (26). Under our conditions, no cytopathic effect was observed 24 h after infection of CEM control or Nef^+ cells with wild-type VV. Envelope-mediated cell death was quantified 15 h postinfection by counting the surviving cells by trypan blue exclusion. Four different VV recombinants were used in this study. VV9.1 encodes the intact HIV-1_{LAI} gp160 precursor, which was shown to induce cell fusion in CEM cells (20); VV1139 encodes a HIV-1_{LAI} gp160 without cleavage sites, which is expressed at the cell surface, is not secreted, and does not mediate cell fusion (19); VV-env2-cs and VV-env5-cs are derived from VV1139 and encode HIV-1_{LAI} envelope glycoproteins deleted for residues 82 to 95 and residues 424 to 432, respectively. These two mutant envelope glycoproteins are correctly transported to the cell surface but have lost CD4 binding activity (4).

Immunoprecipitations. After starvation in methionine- and cysteine-free medium, cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine (100 μCi/ml) for 2 h and lysed in 0.5% Nonidet P-40–150 mM NaCl–20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES). Cell extracts were clarified by centrifugation. For Nef detection, lysates were incubated overnight with either a human anti-HIV serum or a rabbit serum against HIV-1_{LAI} Nef (a gift of E. Bahraoui, Hôpital Pitié-Salpetrière, Paris, France). Both antisera were used at a final dilution of 1:100. Immune complexes were precipitated with protein A-Sepharose (Pharmacia). After being washed in 20 mM Tris (pH 7.4)–0.1% deoxycholate–0.1% sodium dodecyl sulfate (SDS)–0.1% Triton X-100–150 mM NaCl, immunoprecipitates were subjected to electrophoresis on SDS–12% polyacrylamide gels followed by fluorography. Pulse-chase experiments of VV-infected cells were performed at 4 h postinfection. Cells were kept in methionine- and cysteine-free medium for 30 min and pulse-labeled for 30 min with [^{35}S]methionine and [^{35}S]cysteine (100 μCi/ml). One sample was lysed immediately, while others were chased by an excess of unlabeled amino acids for various lengths of time. Immunoprecipitations were performed with a human polyclonal anti-HIV serum.

Flow cytometry analysis. Cells were washed in PBS and incubated with monoclonal antibodies (MAbs) for 30 min at 4°C in PBA (1% bovine serum albumin–0.1% sodium azide in PBS). After staining, cells were fixed in PBA containing 1% paraformaldehyde and analyzed with a FACScan cytofluorometer (Becton Dickinson). The following MAbs were used: anti-CD4 (T4-fluorescein isothiocyanate [FITC]; Coulter), OKT4 (Ortho), Leu3a-phycoerythrin [PE]; Becton Dickinson), anti-CD8 (Leu2a-FITC; Becton Dickinson), and anti-gp120 (110.4 [23]), followed by PE-labeled goat anti-mouse antibody (Southern Biotechnology), anti-transferrin receptor (Becton Dickinson), anti-CD45RO (UCHL1; Dako), and anti-interleukin-2 receptor (Becton Dickinson). Nonspecific binding was measured with an anti-keyhole limpet hemocyanin immunoglobulin G1-PE MAb (Becton Dickinson).

For the analysis of VV-infected cells, dead and giant autofluorescent cells (representing up to 70% of VV9.1-infected CEM control cells) were excluded from the analysis, and viable cells were gated according to their volume and right-angle light scatter values (30).

RESULTS

Transfer and stable expression of different *nef* genes in CEM cells. Decreased amounts of cell surface CD4 were previously reported in cells expressing the *nef* gene of HIV-1_{LAI} (13) or HIV-1_{SF2} (12). We first examined whether Nef-induced CD4 down-regulation is a general property of the viral protein or a feature restricted to laboratory-grown strains. Two novel *nef* genes (AO1 and EO1) were isolated by PCR amplification on PBMC DNA obtained from two HIV-1-infected children. These *nef* genes were introduced in the pLXSN retroviral vector (see Material and Methods) and sequenced. The *nef* genes from HIV-1_{SF2} and HIV-1_{LAI} laboratory strains were inserted in the same vector. Sequence analysis indicated that AO1 and EO1 gene products share significant homologies with previously characterized Nef proteins (Fig. 1) (7, 8). Amino acid variation in pairwise comparison between SF2, LAI, AO1, and EO1 Nef proteins was approximately 12%. The four proteins possessed the N-terminal myristoylation site required for membrane association (17) and biological activity (14, 38). The AO1 and EO1 strains also bear sequences that were proposed to resemble the GTP binding site of G protein (KGGLEG at position 96; WRFD or WKFD at position 186 [13]). The SF2 *nef* gene contains a duplication of 12 nucleotides, corresponding to a four-amino-acid repeat (RAEP) at position 25, which is not found in LAI, AO1, and EO1 isolates.

Nef-encoding retroviral vectors and a control vector were

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            *       10         20         30         40         50         60         70
SF2  MGGKWSKRSM GGWSAIRERM RRAEPRAEPA ADGVGAVSRD LEKHGAITSS NTAATNADCA WLEAQEEEEV
LAI         S V V  PTV          ----        A                      A
AO1         C G     T           ----       P           V       TN            D D
EO1         SRV S  PTV          ----   V                        N  A

              80         90        100        110        120        130        140
SF2  GFPVRPQVPL RPMTYKAALD ISHFLKEKGG LEGLIWSQRR QEILDLWIYH TQGYFPDWQN YTPGPGIRYP
LAI      T              V  L               H      D                          V
AO1                        L               H  K   D     V         S          T
EO1                     RR  M  L               Y  K   D     V                    T F

              150        160        170        180        190        200        210
SF2  LTFGWCFKLV PVEPEKVEEA NEGENNSLLH PMSLHGMEDA EKEVLVWRFD SKLAFHHMAR ELHPEYYKDC Z
LAI                 D       K   T      V     D P  R   E      R     V         F N
AO1    Y      L                  C       Q     P  R     K    R                 N
EO1                           D N             P             N   R V    M
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FIG. 1. Alignment of SF2, LAI, AO1, and EO1 Nef amino acid sequences. The sequences are represented by the one-letter code; Z indicates a stop codon. Gaps are shown as dashes; the myristoylated glycine is indicated by an asterisk. Putative GTP binding sites are underlined.

transfected into ψCRIP amphotropic packaging cells (6). Populations of recombinant retrovirus producers with titers ranging from 10^4 to 10^5 G418-resistant CFU/ml were obtained. $CD4^+$ CEM cells were cocultivated with retroviral vector-producing cells, and infected cell populations were selected in the presence of G418. The transduced cells were labeled with [^{35}S]methionine and [^{35}S]cysteine, and cell extracts were immunoprecipitated with a human anti-HIV-1 serum (Fig. 2A, left). A specific immunoreactive product was detected in each CEM cell population obtained with *nef*-encoding retroviral vectors. As previously reported (27), the differences in apparent molecular size between the Nef-specific signals were attributed to the presence at position 58 of an alanine in the LAI and EO1 Nef isolates (27 kDa) versus an aspartic acid in AO1 and SF2 isolates (29 and 30 kDa, respectively). A strong specific signal was detected in CEM cells transduced with the AO1 and EO1 *nef* genes, using the human serum. These proteins were only weakly detected in the same extracts when a rabbit serum directed against the HIV-1_{LAI} Nef protein was used (Fig. 2A, right). In contrast, the LAI Nef reacted preferentially with the rabbit serum. The variation in antiserum affinities rendered difficult a direct quantitative comparison between Nef proteins encoded by different alleles.

In a previous study, we used a different retroviral vector (pMNF) to transfer and express the LAI *nef* gene in a variety of T-cell lines, including CEM cells (31). Using the rabbit anti-LAI Nef serum, we estimated that the levels of LAI Nef protein observed here in cells transduced with the pLFLAI vector were 5- to 10-fold higher than those previously reported.

Analysis of CD4 surface levels in Nef-expressing cells. We next examined whether the presence of CD4 at the cell surface was modified by the expression of Nef. Cells were stained with the anti-CD4 MAb or with the anti-CD8 MAb as a control and analyzed by flow cytometry (Fig. 2B). In cells infected with the control vector, surface levels of CD4 were equivalent to those of parental cells, indicating that the retrovirus-mediated gene transfer protocol did not lead to the selection of unrepresentative subclones. Cells expressing LAI, SF2, AO1, and EO1 Nef proteins and control cells displayed equivalent background staining with the anti-CD8 MAb. In contrast, the expression of all four Nef strains was associated with a significant reduction of cell surface CD4. This led to an almost complete abrogation of anti-CD4 MAb

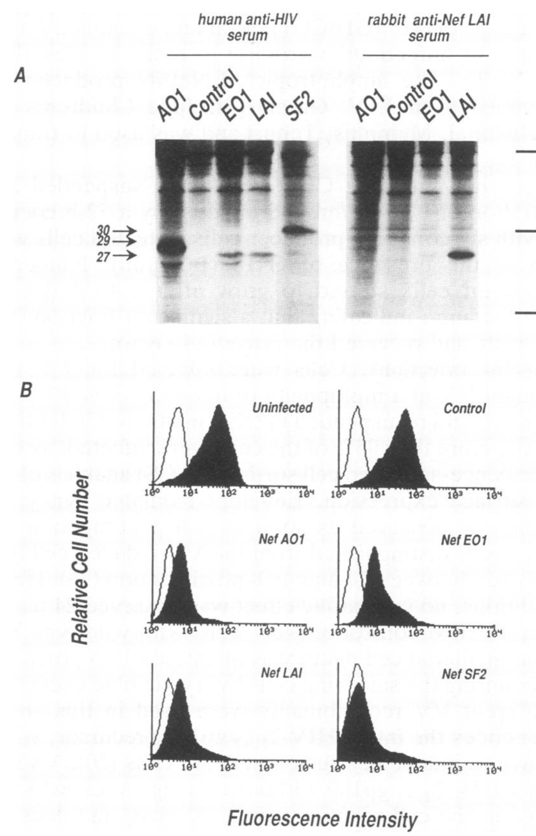

FIG. 2. Expression of Nef in CEM cells and CD4 down-regulation. (A) Immunoprecipitation analysis of Nef proteins. Populations of CEM cells infected with retroviral vectors encoding the AO1, EO1, LAI, and SF2 *nef* genes, respectively, were selected in the presence of G418 and labeled with [^{35}S]methionine and [^{35}S]cysteine for 2 h. Lysates were immunoprecipitated with a human anti-HIV-1 serum (left) or with a rabbit anti-Nef LAI serum (right) and analyzed on an SDS–12.5% polyacrylamide gel. Molecular weight markers are indicated in kilodaltons on the right. Arrows indicate Nef-specific bands. (B) Flow cytometry (FACS) analysis of surface membrane CD4 expression in CEM cells. CEM cells were stained with the control anti-CD8 MAb (Leu2a-FITC; white curves) or with the anti-CD4 MAb (T4-FITC; black curves) and analyzed with a FACScan. Similar results were obtained with other anti-CD4 MAbs (OKT4 and Leu3a).

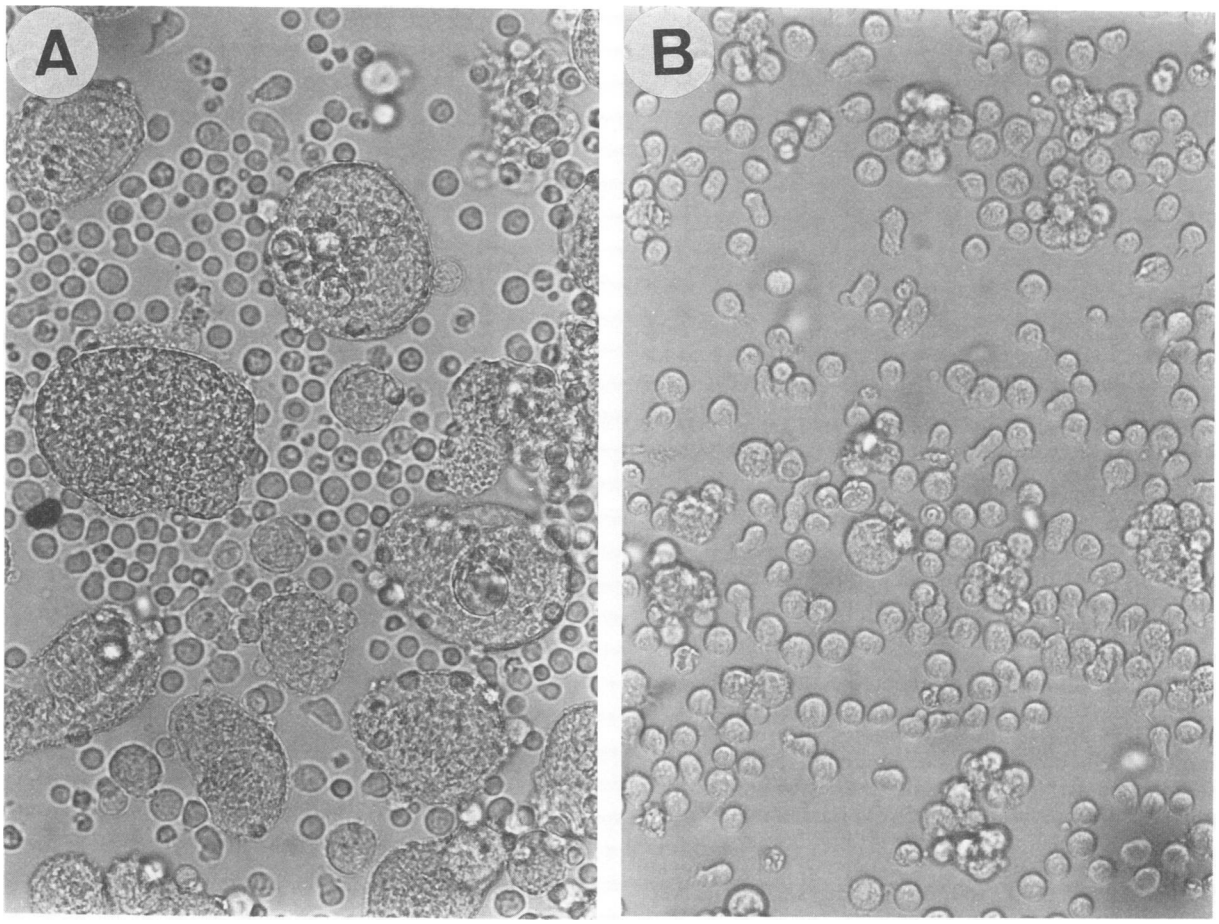

FIG. 3. Envelope-induced cytopathic effect in control and Nef$^+$ CEM cells. Control (A) and Nef AO1 (B) CEM cells were infected with VV9.1, a recombinant VV vector expressing HIV-1 gp160, at a multiplicity of infection of 50 PFU per cell. Cells were photographed 15 h postinfection. Large syncytia were observed in CEM control cells, 70% of which were killed at this time. The cytopathic effect was dramatically reduced in Nef AO1 CEM cells. Very few syncytia were observed, and cellular mortality was less than 15%. Similar results were obtained with CEM Nef SF2 cells.

binding in cells expressing AO1, LAI, and SF2 Nef proteins. Down-regulation of CD4 was less important in CEM Nef EO1 cells. Other surface molecules (CD29, CD45RO, interleukin-2 receptor alpha chain, and transferrin receptor) were unaffected by the presence of Nef (data not shown).

In each Nef-expressing population, only 3 to 10% of the cells had normal levels of CD4. When transduced CEM cells were maintained for several weeks in culture, an increased proportion of cells with normal CD4 surface expression appeared. Garcia and Miller previously reported a similar observation (12), suggesting that either the absence of Nef or the presence of normal surface levels of CD4 provided a growth advantage.

Since it was observed with molecules displaying significant amino acid variation, the capacity to down-regulate CD4 surface expression can be considered a characteristic property of Nef.

Processing and transport of the *env* gene products in CEM Nef$^+$ cells. VV recombinants have been widely used to express the HIV envelope glycoprotein in a variety of cells, and the synthesis and processing of the VV-encoded envelope glycoprotein closely mimic those of HIV-1-infected cells (9, 10, 20).

CEM cells expressing Nef and control cells were infected with the VV vector VV9.1, which encodes a wild-type HIV-1 envelope glycoprotein (20). The infected cells were observed at 15 h postinfection, before the onset of VV-induced cytopathic effect (Fig. 3). In control CEM cells, large syncytia were present, and 70% of the cells were killed, as determined by counting trypan blue-excluding cells. In contrast, only rare syncytia were observed in the presence of Nef, and cell mortality was less than 15%. At this stage, we interpreted this observation as the consequence of the low number of CD4 molecules at the surface of Nef-expressing cells.

To examine gp160 processing, CEM Nef$^+$ or control cells were infected with VV9.1. After 4 h, they were metabolically labeled for 30 min and analyzed after various times of chase (Fig. 4). In control cells, large amounts of gp160 were detected after pulse-labeling (time zero) and cleaved gp120 appeared 1 h 45 min later, while the amounts of unprocessed gp160 decreased (time 3.5 h). Identical kinetics were observed in CEM Nef$^+$ cells, indicating that Nef does not quantitatively or qualitatively alter the processing of gp160.

We next examined whether the presence of Nef would affect the surface localization of envelope proteins. CEM control and Nef$^+$ cells were infected with VV9.1, stained with the anti-gp120 MAb 15 h later, and analyzed by flow

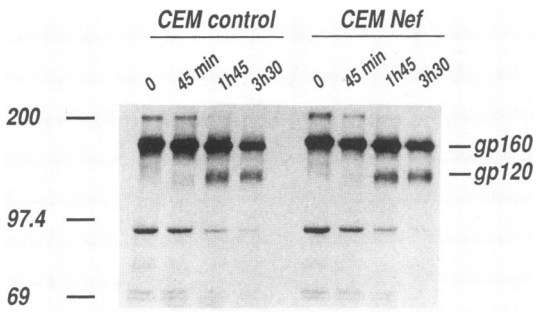

FIG. 4. HIV-1 gp160 processing in CEM control and Nef$^+$ cells. Control and Nef AO1 CEM cells were infected with VV9.1 for 4 h, labeled with [^{35}S]methionine and [^{35}S]cysteine for 30 min, and chased for the indicated periods of time. Cell lysates were immunoprecipitated with a human polyclonal anti-HIV-1 serum and analyzed by SDS-polyacrylamide gel electrophoresis (gp41 was not recognized by this serum). Molecular weight markers are indicated in kilodaltons on the left.

cytometry (Fig. 5). Control cells were found to express eight times more gp120 on their surface than did Nef$^+$ cells. Surface levels of CD29 were identical in the two cell populations.

The decreased amount of gp120 detected at the surface of Nef$^+$ cells could have resulted from the absence of CD4 molecules capable of binding soluble gp120 present in the culture media. It could also be due to an increased shedding of gp120 induced by Nef. To rule out these two possibilities, we repeated the experiment with a VV recombinant encoding a modified gp160 that is membrane bound and not secreted (VV1139) (19). Flow cytometry analysis again showed that less of the gp160 reached the surface in Nef$^+$ cells (Fig. 5).

We examined whether the intracellular retention of the envelope glycoprotein in the presence of Nef was dependent on its CD4 binding ability. Two VV recombinants (VV-env2-cs and VV-env5-cs), derived from VV1139 and encoding mutated envelope glycoproteins without CD4 binding activity (4), were used to infect CEM control and Nef$^+$ cells. In both cell populations, mortality measured after 15 h was less than 10% and no syncytia were observed. Surface levels of envelope glycoproteins produced by both VV-env2-cs and VV-env5-cs were unaltered in CEM Nef$^+$ cells (Fig. 5), suggesting that binding to CD4 is required for intracellular accumulation of the envelope.

Taken together, our data indicate that in the presence of Nef, gp160 is correctly processed to heterodimeric gp120/gp41 through the endoplasmic reticulum and Golgi compartments but is then impaired in its route to the cell surface. Alternatively, the assembled heterodimers could be immediately reinternalized in the presence of Nef. The intracellular accumulation of mature envelope glycoproteins certainly contributes to the reduction of the envelope-induced cytopathic effect that we observed in Nef-expressing cells.

DISCUSSION

Contradictory results concerning the capacity of Nef to down-regulate CD4 surface expression have been reported (3, 12, 13, 24). In a previous study, we observed that the constitutive expression of the LAI *nef* gene did not result in a significant decrease of CD4 cell surface amounts (31). In the present work, we have used a different retroviral vector for the expression of *nef* genes in lymphoblastoid CD4$^+$

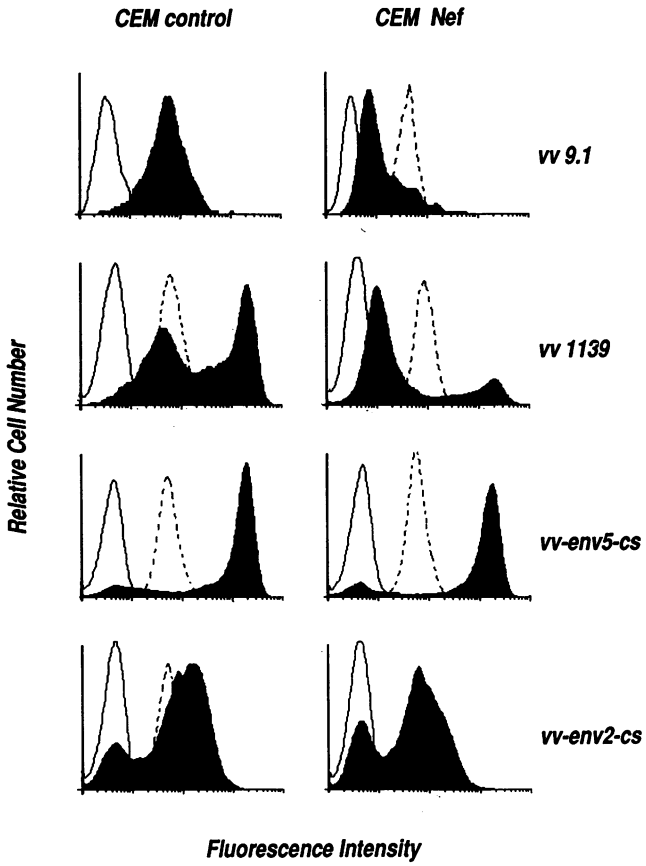

FIG. 5. HIV-1 envelope surface localization in control and Nef$^+$ CEM cells. Control and Nef AO1 CEM cells were infected with VV vectors for 15 h and stained with the 110.4 anti-gp120 MAb followed by a PE-labeled goat anti-mouse MAb (black curves). Anti-keyhole limpet hemocyanin (white curves, plain lines) and anti-CD29 (white curves, dashed lines) MAbs were used as negative and positive controls, respectively. Cells were analyzed by flow cytometry. Four different VV vectors were used. VV9.1 encodes an intact HIV-1_{LAI} gp160 precursor; VV1139 encodes a noncleavable gp160 precursor; VV-env2-cs and VV-env5-cs encode mutated envelope glycoproteins which are expressed at the cell surface but are unable to bind CD4. The presence of two distinct envelope-positive cell populations following VV infection was previously reported (4). Comparable envelope stainings were obtained with MAb 41.1 (Genetic Systems), which recognizes an epitope in the extracellular portion of the gp41 transmembrane protein. Similar results were obtained with CEM Nef SF2 cells.

CEM cells, resulting in 5- to 10-fold-higher levels of the transduced proteins and leading to an obvious effect on CD4 localization. Although our data suggest that a threshold level of Nef might be necessary for its activity on CD4, the data presented here do not directly address this point.

Our first purpose was to document whether CD4 down-regulation could be induced by *nef* genes from viruses present in infected individuals and not propagated in vitro. For this purpose, we have isolated two *nef* genes, AO1 and EO1, by PCR amplification of PBMC DNA from seropositive infants. The amino acid sequence comparison of AO1, EO1, SF2, and LAI Nef proteins indicated an overall variation of 12%. Flow cytometry analysis showed that the presence of each of the four Nef proteins in CEM cells was associated with an almost complete disappearance of CD4

molecules at the cell surface. Other surface molecules (CD29, CD45RO, interleukin-2, and transferrin receptors) were unaffected. We conclude that the ability of Nef to down-regulate CD4 surface expression is not restricted to laboratory-grown strains of HIV-1 and may therefore influence the replication rate or the pathogenicity of a natural virus in its infected host.

Receptor down-regulation is a common feature of retroviral infections, and this phenomenon, called interference, protects the virus-producing cells from lethal multiple superinfections (34). It is mediated by the envelope glycoprotein, which interacts with the receptor and impairs its transport to the cell surface (5, 16). Our interest here was to understand the role, during the HIV replicative cycle, of an additional mechanism for receptor down-regulation that would act early in the infection, before synthesis of the envelope glycoprotein begins.

The intracellular binding of the gp160 precursor to CD4 influences the trafficking of the envelope glycoproteins (5, 10, 36, 37), and it has been shown that modified CD4 molecules retained in the endoplasmic reticulum can block envelope processing and transport (2). We have examined in our experiments the consequences of a preestablished Nef-mediated down-regulation of CD4 on the production of the envelope glycoprotein. When the HIV-1 *env* gene was expressed in CEM cells from a VV vector, the kinetics of gp160 synthesis and cleavage into its subunits was unaffected by the presence of Nef. In contrast, the cell surface levels of gp120 were reduced by 1 order of magnitude in Nef^+ cells compared with control cells. This intracellular accumulation was not observed with mutant envelopes lacking CD4 binding activity. We concluded that in the presence of Nef, the envelope glycoprotein accumulates in an intracellular compartment through a mechanism dependent on its binding to CD4. Since the mature form of the *env* gene product is sequestered, the accumulation could take place in a late Golgi compartment. Alternatively, envelope-CD4 complexes could transit at the cell surface and become quickly and irreversibly reinternalized. This issue will now be addressed by localizing the trapped CD4 and envelope molecules in Nef-expressing cells.

Our observation is consistent with that of Binninger et al., showing that SIV_{MAC} mutants with inactivated *nef* genes exhibit an enhanced expression of viral proteins on the surface of infected cells (1). A mechanism preventing the cell surface expression of envelope glycoproteins could provide an advantage for the virus replication in vivo. Indeed, the expression of fusogenic gp120/gp41 at the cell surface induces the formation of lethal syncytia with $CD4^+$ cells (32). This phenomenon can be inhibited by preventing the correct maturation of the gp160 precursor (15) or by retaining it inside the cell (2). Nef is expressed early in the viral cycle from a Rev-independent mRNA (29) and can therefore modify CD4 localization before the synthesis of the *env* gene products. This process would then act on the trafficking of envelope glycoproteins. In certain cell types as monocytes/macrophages, the role of Nef may be to facilitate the incorporation of mature envelope molecules into intracellularly budding virions (28). This would allow for a more efficient production and accumulation of viral particles in infected cells by eliminating the risk of early death by fusion and could significantly contribute to the maintenance of high viral loads observed in vivo with viruses expressing a functional *nef* gene (18).

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