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Nef from Primary Isolates of Human Immunodeficiency Virus Type 1 Suppresses Surface CD4 Expression in Human and Mouse T Cells

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The human immunodeficiency virus type 1 (HIV-1) *nef* gene was originally described as a negative regulator of transcription from the viral long terminal repeat promoter. This observation has been disputed, and the function of Nef remains unclear. In vivo experiments have indicated that an intact *nef* gene is required for disease progression in macaques infected with simian immunodeficiency virus, suggesting a role for Nef in the pathogenesis of AIDS. We and others have previously shown that expression of Nef in cells bearing surface CD4 results in a sustained decrease in surface CD4 expression. This was demonstrated for Nef from two laboratory strains of HIV-1, Bru and SF2. Because both of these isolates were passaged in vitro prior to molecular cloning and in vitro passage can result in mutations which might alter *nef* gene function, we have analyzed two primary isolates of Nef for their ability to suppress cell surface CD4 expression. The *nef* genes of HIV-1 isolates from two patients with fewer than 200 CD4$^+$ T cells per mm^3 of blood were introduced into human and mouse T-cell lines by retrovirus-mediated gene transfer. Expression of Nef from both isolates correlated with a decrease in surface expression of both human and mouse CD4. To determine whether the ability to suppress surface CD4 expression is a general function of Nef, we also tested an artificially generated consensus *nef* gene derived from analysis of 54 patient isolates of HIV-1. Expression of the consensus Nef protein also correlated with decreased cell surface CD4 expression in both mouse and human T-cell lines. These results suggest that the ability to suppress cell surface CD4 expression is an intrinsic feature of HIV-1 Nef.

The *nef* gene of the human immunodeficiency virus type 1 (HIV-1) codes for a 25- to 29-kDa protein which is expressed early after infection along with two other regulatory genes, *tat* and *rev* (24, 27, 36, 44). The functions of Tat and Rev are now fairly well understood, but the function of Nef remains unclear (42). Originally, Nef was reported to be a negative regulator of transcription from the viral long terminal repeat (LTR) (1, 5, 31, 32, 39), but this has been disputed (19, 25). Nef is not required for viral replication in vitro (11, 50), but evidence from studies with macaques infected with simian immunodeficiency virus (SIV) indicate that an intact *nef* gene is necessary for disease progression in vivo (23). Moreover, rhesus monkeys vaccinated with a *nef* deletion mutant of SIV were found to be protected from subsequent challenge with pathogenic SIV (8). These results suggest that Nef is an important factor in AIDS pathogenesis and may be useful in formulating vaccine strategies.

One of the hallmarks of HIV infection is the loss of CD4$^+$ T cells (30). In this regard, it is significant that Nef has been shown to suppress CD4 expression at the cell surface (14, 17). This has been demonstrated for Nef from two laboratory isolates of HIV-1, Bru and SF2. That Nef reduces CD4 expression at the cell surface is interesting since CD4 is both the receptor for HIV (7, 26, 33) and a costimulatory molecule for T-cell activation (21, 41).

There is significant polymorphism among the *nef* genes from laboratory isolates (43). It is possible that the ability to suppress CD4 expression is a characteristic of Nef which has resulted from serial passage in vitro (34, 47, 54). Therefore, it is important to determine whether the products of *nef* genes from primary clinical isolates of HIV-1 also have the ability to suppress cell surface CD4 expression. In the present study, we have addressed this question with two different clinical isolates of Nef. The *nef* genes were introduced into mouse and human CD4$^+$ T-cell lines by retrovirus-mediated gene transfer and tested for their ability to decrease CD4 expression at the cell surface. In addition, an artificially generated consensus *nef* gene was tested. We found that the products of all three of these *nef* genes were able to suppress surface CD4 expression to a degree comparable to the SF2 Nef. Our results demonstrate that the ability to suppress surface CD4 expression is an intrinsic feature of Nef.

MATERIALS AND METHODS

Isolation of *nef* sequences from clinical isolates of HIV and generation of retrovirus expression vectors. The clinical isolates of HIV-1 were obtained from patients 233 and 248. These patients are unrelated, HIV-1-seropositive males who were participants in the AIDS Clinical Trials Group at the University of North Carolina Memorial Hospitals (Chapel Hill, N.C.). At the time of sample collection, both patients had symptoms of AIDS and were taking 3′-azido-3′-deoxythymidine. CD4$^+$ T cells were 190 and 40 per mm^3 of blood for patients 233 and 248, respectively.

A detailed description of the isolation, amplification, and original cloning of the *nef* genes from these patients appears elsewhere in this issue (49). Briefly, uncultured peripheral

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blood mononuclear cells were isolated from heparinized blood following separation through lymphocyte separation medium (Organon Teknika-Cappel, Malvern, Pa.), and total DNA was purified by standard techniques. A 735-bp fragment containing the *nef* gene and flanking viral sequences (the 3′ ends of *env*, *tat*, and *rev* and the 5′ end of the 3′ LTR) was amplified from cellular DNA via two rounds of 30-cycle nested-primer polymerase chain reaction (PCR) amplification. The amplified fragment was then cloned into a sequencing vector, and the DNA sequence was determined by the dideoxy chain termination method (48).

For the present study, the *nef* genes from the isolates from patients 233 and 248 (submitted to GenBank as 233-23 and 248-3, respectively [49]) were reamplified to generate PCR products devoid of flanking viral sequences. The primers used for reamplification were Mut5′LXSN (5′ GCA-GTC-GAA-TTC-GCC-ACC-ATG-GGT-GGC-AAG-TGG-TCA-AAA-CGT-AGT-GTG-AGT-GGA 3′) and 3′LXSN (5′ ACG-TGC-GAA-TTC-TCA-GCA-GTC-TTT-GTA-GTA-CTC 3′). The Mut5′LXSN primer includes the ribosome-binding sequence (5′ GCC-ACC 3′) in front of the initiation codon for enhanced translation in mammalian cells (28) and encodes the first 12 amino acids of the consensus Nef protein sequence (M-G-G-K-W-S-K-R-S-V-S-G-). The 3′LXSN primer encodes the last six amino acids of the consensus Nef sequence (-E-Y-Y-K-D-C) followed by the termination codon. Both primers include restriction sites for *Eco*RI (underlined). Reamplification with these primers resulted in the following amino acid changes in the sequence of 233-23 Nef: Cys→Ser at position 8, Val→Ser at position 11, Phe→Tyr at position 203, and Asn→Asp at position 205. The *nef* genes were reamplified by PCR in reaction mixes containing a 1 μM concentration of each primer; approximately 200 ng of plasmid DNA; 200 μM each dATP, dCTP, dGTP, and dTTP; 10 mM Tris-HCl, pH 7.5; 1.5 mM $MgCl_2$; and 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn.). Amplification was carried out for 16 cycles under the following conditions: denaturation at 94°C for 30 s, annealing at ramped temperatures (see below) for 30 s, and elongation at 72°C for 30 s. Annealing temperatures were 37°C for the first four cycles, 42°C for the next four cycles, and 50°C for the final nine cycles. The *nef* products were gel purified and cloned directly with the pT7Blue T-vector system (Novagen, Madison, Wis.) for DNA sequence confirmation of both DNA strands. *Eco*RI fragments containing the *nef* sequences were then isolated and subcloned into the retrovirus expression vector pLXSN as described previously (35).

Generation of a retrovirus vector containing a consensus *nef* gene. A consensus HIV-1 Nef sequence was derived from an alignment of Nef amino acid sequences obtained from 54 noncultured patient isolates of HIV-1 (49). DNA encoding this consensus Nef protein was generated by overlapping-primer PCR amplification (10), using unique regions from four patient-derived *nef* genes to synthesize a full-length consensus *nef* gene. The primers described above were used in the subsequent amplification of the consensus *nef* sequence. After the nucleotide sequence had been confirmed, the consensus *nef* gene was cloned into the *Eco*RI site of pLXSN as described previously (14, 35) in both the forward and reverse orientations to generate vectors pLnefSN.cons and pLfenSN.cons, respectively.

Cell lines and culture conditions. HuT-78 cells were cultured in RPMI 1640 medium with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah), penicillin-streptomycin (50 IU and 50 μg/ml, respectively), 2 mM L-glutamine, and 1 mM sodium pyruvate. The human cell lines were maintained at 37°C in a humidified incubator with 5% CO_2. The murine PE501 (ecotropic) and PA317 (amphotropic) retrovirus-packaging cell lines, NIH 3T3 TK^- cells, and AKR1-G1 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and penicillin-streptomycin at 37°C in a humidified incubator with 10% CO_2 (13, 35).

Retrovirus-mediated gene transfer. The *nef* genes were transduced into cell lines by retrovirus-mediated gene transfer as described previously (14, 35). Briefly, 10 μg of plasmid DNA was transfected into PE501 ecotropic packaging cells (5×10^5 cells) by the calcium phosphate precipitation method or by electroporation (15). Supernatants from the transfected PE501 cells were used to infect PA317 amphotropic packaging cells. The PA317 cells were selected in G418 (1.5 mg/ml; Geneticin; GIBCO/BRL Life Technologies, Grand Island, N.Y.; 50% active), cloned, and expanded from individual colonies. The PA317 producer clones were then analyzed for Nef expression (see below) and virus titer on NIH 3T3 TK^- cells as described previously (35). HuT-78 and AKR1-G1 cells (5×10^5) were transduced by addition of virus-containing supernatant (0.25 to 2 ml) from the respective producer lines in the presence of 4 μg of Polybrene (Sigma Chemical Co., St. Louis, Mo.) per ml. Excess virus and Polybrene were removed 16 h later, and the cells were plated in medium containing 1.5 mg of G418 per ml. Populations of cells were examined for Nef and CD4 expression 10 to 14 days later as described below.

Immunoprecipitation and Western immunoblot analyses. For determination of Nef expression, 2×10^6 to 3×10^6 cells were lysed in 250 μl of RIPA buffer (50 mM Tris (pH 7.5), 0.15 M NaCl, 1% Nonidet P-40 [NP-40], 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing 10 mg of bovine serum albumin, 1 μg of leupeptin, and 1 μg of aprotinin per ml and 1 mM phenylmethylsulfonyl fluoride for 20 min on ice. The lysates were clarified by spinning for 5 min at full speed in a refrigerated microcentrifuge at 4°C. The supernatants were immunoprecipitated with 1 μl of rabbit anti-Nef antiserum (kindly provided by L. Ratner, Washington University, St. Louis, Mo.) for 60 min at 4°C on a rotator, after which 30 μl of protein A-containing *Staphylococcus aureus* bacteria (Pansorbin; Calbiochem, San Diego, Calif.) was added, and rotation was continued for another 60 min at 4°C. The precipitates were washed once with RIPA buffer, once with 3% NP-40 buffer (3% NP-40, 20 mM Tris [pH 8], 0.15 M NaCl, 2 mM EDTA), and once with TBS (10 mM Tris [pH 7.2], 0.15 M NaCl) and then resuspended in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (50 mM Tris [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.1% bromophenol blue). Alternatively, cells were lysed in 3% NP-40 buffer containing leupeptin, aprotinin, and phenylmethylsulfonyl fluoride for 20 min on ice. The lysates were clarified by spinning for 10 min at full speed in a refrigerated microcentrifuge at 4°C. The supernatant was transferred to a new tube with an appropriate amount of 5× SDS-PAGE sample buffer to give a final concentration of 10^6 cells per 20 μl. Immunoprecipitated proteins or lysates were separated on 12% polyacrylamide–SDS minigels (Mini-Protean II; Bio-Rad, Hercules, Calif.) and transferred to nitrocellulose filters (Hybond-C; Amersham Life Science, Arlington Heights, Ill.) with a semidry electrotransfer apparatus (Bio-Rad). The filters were blocked with 1% casein in TBS and probed with rabbit anti-Nef serum, 1:500 in TBS-casein. After four washes in

TBS, the filters were blotted with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG; Fc specific; Promega, Madison, Wis.), 1:3,000 in TBS-casein, washed, and developed with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium (Promega) in alkaline phosphatase buffer (100 mM Tris [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$).

Analysis of surface CD4 expression by flow cytometry. For flow cytometric analyses, 10^6 cells were resuspended in a solution of the appropriate phycoerythrin (PE)-conjugated monoclonal antibody diluted in phosphate-buffered saline (PBS) with 5% calf serum (CS) and 0.1% NaN_3 (PBS/CS/NaN_3) and stained for 20 min on ice. The cells were washed once in 2 ml of cold PBS/CS/NaN_3 and resuspended in 1 ml of PBS/CS/NaN_3. The PE-labeled antibody to human CD4 and an isotype-matched control antibody (both from Exalpha, Boston, Mass.) were used at a 1:10 dilution, 50 μl per 10^6 cells. The PE-labeled anti-mouse CD4 antibody (anti-L3T4; Becton Dickinson, San Jose, Calif.) and isotype-matched control antibody (Caltag, South San Francisco, Calif.) were used at 1:100 dilutions, 100 μl per 10^6 cells. The samples were analyzed with a Becton Dickinson FACScan instrument equipped with LYSYS II software. Data for PE fluorescence were collected in the log mode. Cells taking up propidium iodide (approximately 5 μg/ml final concentration) were excluded by electronic gating.

Protein sequence analysis. The predicted Nef protein sequences of HIV-1 laboratory isolates SF2 and Bru, clinical isolates 233 and 248, and the consensus *nef* gene were aligned with the PileUp program of the University of Wisconsin Genetics Computer Group sequence analysis software program (9).

RESULTS

Generation of retrovirus producer cell lines containing the *nef* genes from primary isolates of HIV-1. The *nef* genes of HIV-1 isolates from two patients with <200 $CD4^+$ T cells per mm^3 were used in the present studies (49). The *nef* genes were initially isolated by direct cloning of PCR fragments, as illustrated in Fig. 1. The *nef* genes from isolates 233 and 248 were then reamplified from the original PCR-generated fragments to remove flanking viral sequences, and the integrity of the original *nef* sequences was confirmed. The *nef* genes were subsequently subcloned into the retroviral expression vector pLXSN to generate the vectors pLnefSN.233 and pLnefSN.248 (Fig. 1). PA317 amphotropic retrovirus producer lines were established and analyzed for virus titer by using NIH 3T3 TK^- cells (35). Three PA317 clones of LnefSN.233 and three of LnefSN.248 with virus titers of 10^6 CFU/ml or greater were tested for the production of Nef protein by immunoprecipitation and Western blotting. Two of three LnefSN.233 clones and three of three LnefSN.248 clones screened for Nef expression produced significant amounts of Nef (not shown). Two independent PA317 producer line clones each of LnefSN.233 and LnefSN.248 were chosen for subsequent experiments.

Expression of Nef from primary clinical isolates of HIV-1 suppresses CD4 expression in HuT-78 human T cells. Supernatants from the LnefSN retrovirus producer lines were used to transduce the human T-cell line HuT-78. Following selection in G418, bulk cell populations were analyzed for Nef expression by immunoprecipitation and Western blotting. As shown in Fig. 2, HuT-78 cells transduced with two independently derived producer line clones each of LnefSN.233 and LnefSN.248 produced the corresponding Nef proteins in amounts comparable to those in cells transduced with the SF2 laboratory isolate of Nef (Fig. 2). Cells transduced in parallel with a control virus, LN (35), did not express a protein recognized by the anti-Nef serum. These results indicate that the retrovirus producer lines can efficiently transduce the *nef* gene into human T cells, leading to the expression of Nef protein. Interestingly, the Nef proteins encoded by the patient 233 and 248 isolates migrate slightly faster on SDS-PAGE than SF2 Nef. The slower migration of the SF2 Nef is probably due to four extra amino acids in this protein and to the presence of an aspartic acid residue at position 58 (40).

To determine whether the clinical isolates of Nef have an effect on surface CD4 levels, Nef-expressing HuT-78 cell populations were stained for surface CD4 expression and analyzed by flow cytometry. Cells expressing the SF2 Nef showed a significant decrease in surface CD4 levels compared with cells transduced with the control vector LN (Fig. 3A). HuT-78 cells transduced with virus from two different LnefSN.233 producer lines and expressing the 233 Nef protein showed a significant decrease in cell surface CD4 expression (Fig. 3B). Similarly, HuT-78 cells transduced with virus from two different LnefSN.248 producer lines and expressing the 248 Nef protein also showed a significant decrease in CD4 expression (Fig. 3C). In both cases, the reduction in cell surface CD4 expression was similar to that induced by SF2 Nef (Fig. 3A). Thus, the Nef proteins from two different clinical isolates of HIV have the same ability as SF2 Nef to suppress surface CD4 expression in human T cells. These results indicate that the ability to suppress CD4 expression is not an artifact developed during passage in vitro but is an intrinsic feature of HIV-1 Nef.

Suppression of CD4 expression in mouse T cells by clinical isolates of Nef. Recently we have shown that SF2 Nef suppresses the expression of mouse CD4 (13), indicating that the effect of Nef on CD4 is not species specific. As a further means of analyzing the functional capability of the clinical isolates of Nef, we transduced a mouse T-cell line, AKR1-G1, with LnefSN.233 and LnefSN.248. For comparison, these cells were also transduced in parallel with LnefSN.SF2 and a control vector which does not confer Nef expression. To facilitate our experiments, NP-40 lysates of transduced AKR1-G1 cells were used to screen directly for Nef expression. As shown in Fig. 4, Western blot analysis of lysates of AKR1-G1 cells reveals the presence of the SF2, 233, and 248 Nef proteins in the appropriate cell lines but not in cells transduced with the control vector. Omitting the immunoprecipitation step also eliminated the nonspecific contaminant band seen in Fig. 2. The relative levels of expression and mobilities of the different Nef proteins were the same as those observed in the HuT-78 cells, indicating similar processing of these proteins in both human and mouse cells.

The AKR1-G1 cells expressing Nef were stained with PE-conjugated antibodies to mouse CD4 and analyzed by flow cytometry. As shown in Fig. 5, expression of the 233 and 248 Nef proteins in the AKR1-G1 cells also correlates with a decrease in cell surface expression of mouse CD4. The decrease in CD4 expression induced by 233 Nef and 248 Nef in the mouse cells was similar to the decrease induced by the SF2 Nef. These results demonstrate that the clinical isolates of Nef are fully capable of suppressing cell surface CD4 expression in a species-independent manner, as is Nef from the SF2 laboratory strain of HIV-1.

Effect of a consensus *nef* gene on CD4 expression. By comparing 54 HIV-1 *nef* sequences from noncultured patient isolates, a consensus *nef* sequence was derived (49). In order

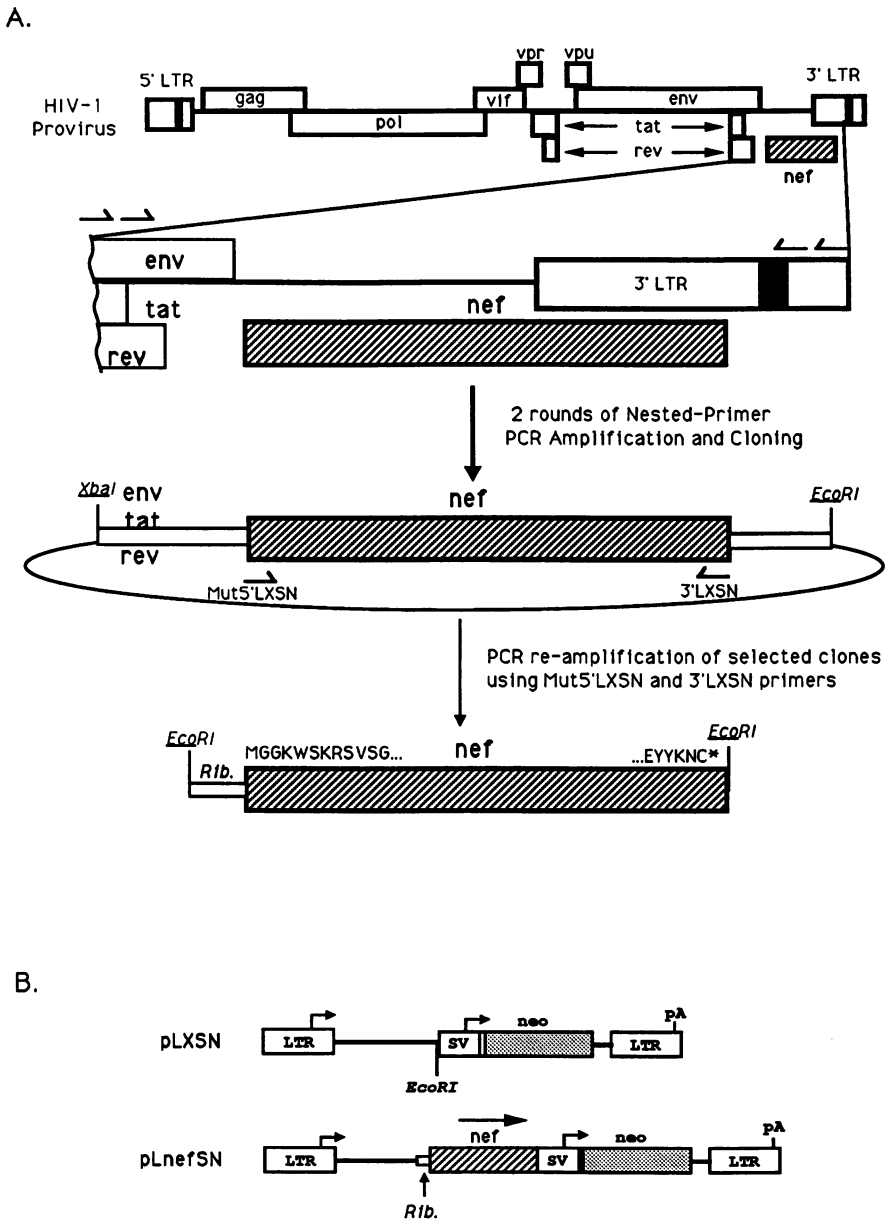

FIG. 1. Construction of LnefSN.233 and LnefSN.248 retrovirus vectors. (A) Genome organization of the HIV-1 provirus, PCR amplification, and cloning strategy. DNA fragments containing the *nef* gene and flanking sequences within *env*, *tat*, *rev*, and the 3' LTR were amplified by nested-primer PCR amplification of purified cellular DNAs (see Materials and Methods). The DNAs were prepared from uncultured peripheral blood mononuclear cells obtained from patients 233 and 248. After cloning and sequencing, the *nef* genes were reamplified with primers Mut5'LXSN and 3'LXSN to generate *nef*-containing PCR products that encode the "Kozak sequence" upstream of the initiator codon and lack flanking viral sequences. Also shown are the predicted Nef amino acid sequences encoded by the amplification primers (28). (B) Diagram of the retrovirus vectors LXSN and LnefSN. After subcloning the *nef* genes into the pT7Blue T-vector for sequence determination (not shown), the *nef* genes were cloned into the retroviral expression vector pLXSN at the *Eco*RI site. These vectors express the *nef* genes from the Moloney murine leukemia virus LTR and the *neo* gene from the simian virus 40 early promoter (SV). Rib, ribosome-binding site; pA, polyadenylation site.

to determine whether the suppression of CD4 is a common property of *nef* genes in general, we tested the ability of this consensus Nef to suppress cell surface CD4 expression. The consensus *nef* gene was cloned in both the sense and antisense orientations into the retrovirus vector pLXSN (LnefSN.cons and LfenSN.cons, respectively). These plasmids were used to establish retrovirus producer lines as described above. Pools of PE501 cells transfected with LnefSN.cons and selected in G418 were found to produce Nef, while cells transfected with LfenSN.cons did not (not shown). All clones of PA317 cells transduced with LnefSN.cons that were screened for production of the consensus Nef protein also were positive (not shown).

LNefSN.cons and LfenSN.cons then were used to transduce HuT-78 and AKR1-G1 cells as described above. Bulk populations of cells were analyzed for Nef and CD4 expres-

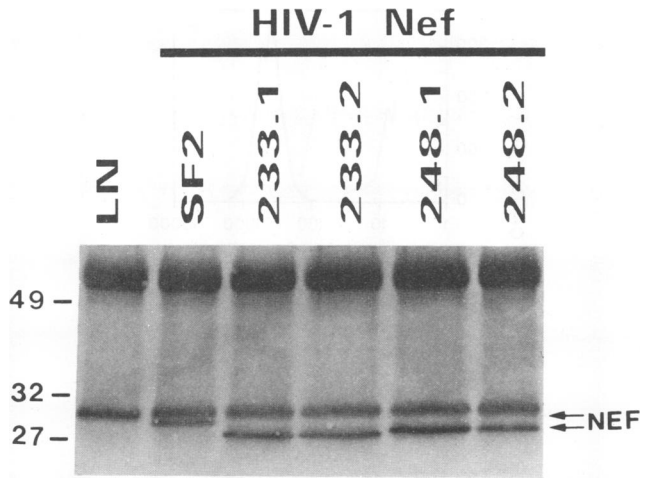

FIG. 2. Nef expression in HuT-78 cells transduced with LnefSN. Lysates from HuT-78 cells transduced with the control vector, LN, or the indicated LnefSN vectors were immunoprecipitated with rabbit anti-Nef antiserum and analyzed by Western blotting. The blots were probed with the same anti-Nef antiserum and developed with alkaline phosphatase-conjugated anti-rabbit IgG (Fc specific) Each lane contains precipitated material from 2×10^6 to 3×10^6 cells. The positions of prestained molecular size markers are indicated at the left (in kilodaltons). Arrows indicate the positions of the different Nef species. The dark band just above the 49-kDa marker is the heavy chain from the rabbit IgG used in the immunoprecipitation. An unidentified band of approximately 30 kDa appears as a non-specific contaminant in immunoprecipitates.

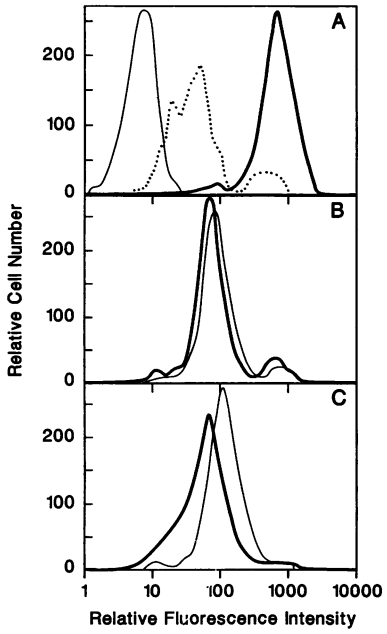

FIG. 3. Surface CD4 expression in transduced HuT-78 cells. Representative populations of cells were stained with a PE-conjugated anti-human CD4 monoclonal antibody or isotype-matched control antibody and analyzed by flow cytometry. Data are presented as single-color histograms with PE fluorescence (CD4 expression) along the x axis and relative cell number along the y axis. (A) HuT-78/LN (control cells), heavy solid line; HuT-78/LnefSN.SF2, dotted line; HuT-78/LN with control antibody, thin solid line. (B) HuT-78 cells transduced with two different LnefSN.233 producer clones. (C) HuT-78 cells transduced with two different LnefSN.248 producer clones. All samples were analyzed in the same experiment, and the data are shown in three separate panels for visual clarity.

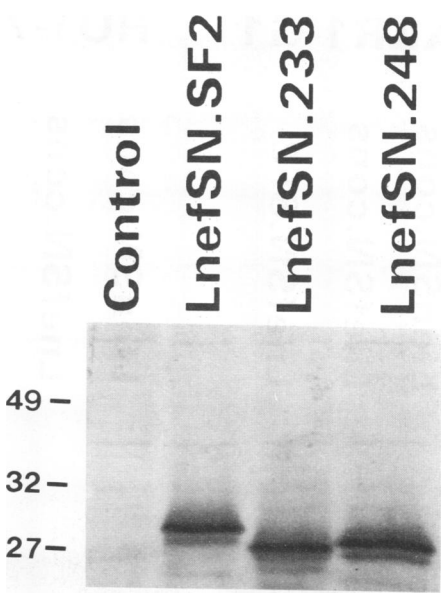

FIG. 4. Nef expression in transduced AKR1-G1 murine thymoma cells. The 3% NP-40 lysates from AKR1-G1 cells transduced with a control antisense Nef vector (LfenSN.cons) or the indicated Nef expression vectors were loaded directly onto a 12% polyacrylamide–SDS gel, transferred to nitrocellulose, and analyzed by Western blotting as described in the legend to Fig. 2. The positions of prestained molecular size markers are indicated at the left (in kilodaltons). Each lane contains lysate from 10^6 cells.

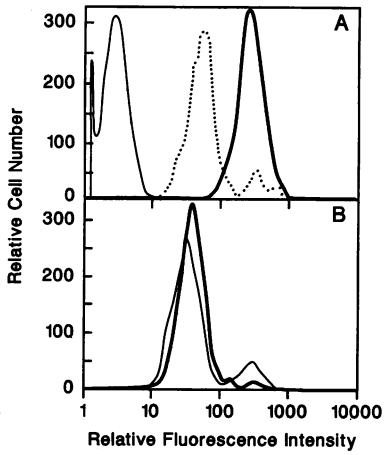

FIG. 5. Surface CD4 expression in transduced murine thymoma cells. Representative populations of Nef-expressing AKR1-G1 cells (Fig. 4) were stained with a PE-conjugated anti-mouse CD4 monoclonal antibody or isotype-matched control antibody and analyzed by flow cytometry. Cells transduced with a control antisense vector (LfenSN.cons) were analyzed in parallel. Data are presented as single-color histograms with PE fluorescence (CD4 expression) along the x axis and relative cell number along the y axis. (A) AKR1-G1/LfenSN.cons, heavy solid line; AKR1-G1/LnefSN.SF2, dotted line; AKR1-G1/LfenSN.cons with control antibody, thin solid line. (B) AKR1-G1/LnefSN.233, heavy solid line; AKR1-G1/LnefSN.248, thin solid line. All samples were analyzed in the same experiment, and the data are shown in two separate panels for visual clarity.

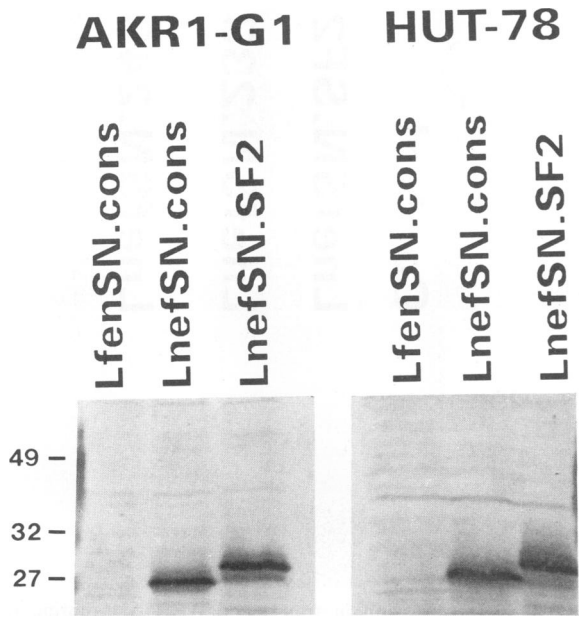

FIG. 6. Expression of a consensus Nef protein in human and mouse T-cell lines. HuT-78 and AKR1-G1 cells transduced with the consensus *nef* gene in the sense (LnefSN.cons) and antisense (LfenSN.cons) orientations were analyzed for Nef expression by Western blotting with anti-Nef antiserum as described in the legend to Fig. 4. Lysates from cells transduced with LnefSN.SF2 are included for comparison. The positions of prestained molecular size markers are indicated at the left (in kilodaltons). Each lane contains lysate from 10^6 cells.

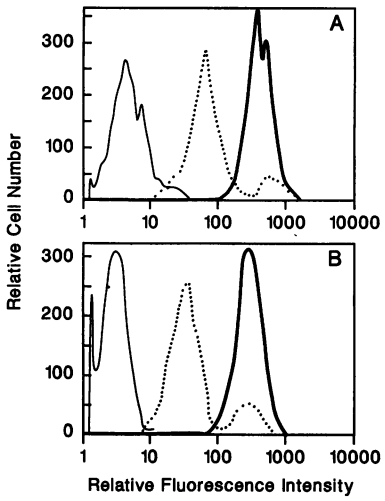

FIG. 7. Decreased surface CD4 expression in cells expressing a consensus Nef protein. Representative populations of cells transduced with the consensus Nef expression vector LnefSN.cons were stained for surface CD4 expression and analyzed by flow cytometry as described in the legends to Fig. 3 and 5. For controls, cells transduced with the antisense consensus Nef vector LfenSN.cons were analyzed in parallel. (A) HuT-78 human T cells. (B) AKR1-G1 mouse T cells. Heavy solid lines, cells transduced with LfenSN.cons; dotted lines, cells transduced with LnefSN.cons; thin solid lines, LfenSN.cons cells stained with control antibody.

sion following selection in medium containing G418. As shown in Fig. 6, both the HuT-78 and AKR1-G1 cells transduced with LnefSN.cons express levels of the consensus Nef protein similar to those in cells transduced with LnefSN.SF2. Interestingly, the consensus Nef has a faster mobility in SDS-PAGE than the SF2 Nef, similar to that of the 233 Nef and 248 Nef isolates. Like the 233 and 248 Nef proteins, the consensus Nef has four fewer amino acids than SF2 Nef and also has an alanine residue at position 54, which together could be responsible for the difference in mobility.

To test whether the expression of the consensus Nef protein also correlated with a decrease in surface CD4 expression, the transduced HuT-78 and AKR1-G1 cells were stained for human or mouse CD4 expression, respectively, and analyzed by flow cytometry. As shown in Fig. 7, the consensus Nef protein suppresses CD4 expression in both human (Fig. 7A) and mouse (Fig. 7B) T-cell populations, and the degree of suppression is similar to that induced by SF2 Nef. These results indicate that the ability of Nef to suppress cell surface CD4 expression is an intrinsic feature of most Nef isolates.

DISCUSSION

In the present study, we have shown that *nef* genes from two different clinical isolates of HIV-1 induce the suppression of surface CD4 expression in human and mouse T cells. This observation establishes the fact that primary isolates of HIV-1 have *nef* genes that encode a protein with a function thus far reported only for two laboratory strains, SF2 (14) and Bru (17), which were passaged in vitro prior to molecular cloning (47, 54). Moreover, we have shown that an artificially generated *nef* gene that expresses a consensus Nef protein can also suppress surface CD4 expression, suggesting that this is an intrinsic feature of most, if not all, *nef* alleles.

An alignment of the amino acid sequences of the SF2, Bru, 233, and 248 Nef isolates, as well as the consensus Nef, is shown in Fig. 8. A significant degree of sequence identity among all five of the HIV-1 Nef proteins is evident. Analysis of these sequences indicates that there are motifs of particular interest within this set of functional Nef proteins. All of these Nef proteins have a common myristylation signal (M-G-G-X-X-S-) at their N termini. This modification has been shown to be important for the association of Nef with cell membranes (12, 17, 22). It has been suggested that the K-G-G-L-E-G- motif (residues 97 to 103 of the SF2 Nef) has some homology with the nucleotide-binding domains of the Ras and Src proteins (17). Even though this motif is highly conserved among the functional Nef proteins, there is no conclusive evidence that Nef binds ATP or GTP or that Nef has kinase activity (2, 22, 37).

The threonine residue at position 15 of the Bru Nef has been shown to be a target for phosphorylation by protein kinase C (17, 18). However, it is clear from our studies that this residue is not critical for the effect of Nef on CD4 expression, since only two of the five isolates have a threonine at position 15 yet all the Nef proteins were able to suppress CD4 expression.

The SF2 Nef protein has a slightly higher apparent molecular weight by SDS-PAGE than the 233, 248, and consensus Nef proteins (Fig. 2, 4, and 6). This is due in part to the four extra amino acids found in SF2 Nef (Fig. 8). However, Obaru et al. (40) recently reported that isolates of Nef with an aspartic acid residue at position 54 (position 58 of SF2 Nef) migrate as 27-kDa proteins on SDS-PAGE, while Nef isolates with an alanine at the same position migrate as

```
          1                                                  50
SF2       MGGKWSKRSM GGWSAIRERM RRAEPRAEPA ADGVGAVSRD LEKHGAITSS
BRU       -------S-V V--PTV---- -....----- ------A--- ----------
233       ---------V S--PTV---- K....----- ------A--- ------L---
248       ---------V S--P-V---- -....----- -E-------- ----------
ConsNef   ---------V S--P-V---- -....----- -E-------- ----------

          51                                                 100
SF2       NTAATNADCA WLEAQEEEEV GFPVRPQVPL RPMTYKAALD ISHFLKEKGG
BRU       -------A-- ---------- ----T----- --------V- L---------
233       --V-N--A-- ---------- ---------- ------G--- L---------
248       ---HN--A-- ---------- ----K----- ------G-V- ----------
ConsNef   -------A-- ---------- ---------- --------V- L---------

          101                                                150
SF2       LEGLIWSQRR QEILDLWIYH TQGYFPPWQN YTPGPGIRYP LTFGWCFKLV
BRU       -----H---- -D-------- ---------- ------V--- ------Y---
233       -----Y--K- -D-----V-- ---------- ---------- ----------
248       -----H---- -D-----L-- ---------- ------V--- ----------
ConsNef   -----Y--K- -D-----V-- ---------- ---------- ----------

          151                                                200
SF2       PVEPEKVEEA NEGENNSLLH PMSLHGMEDA EKEVLVWRFD SKLAFHHMAR
BRU       ----D----- -K---T---- -V-----D-P -R---E---- -R-----V--
233       -----EI-K- ------C--- ---Q---D-P -R---Q-K-- -R--I-----
248       ------I--- -Q-------- -------D-P -----E---- -R-----V-K
ConsNef   ---------- ------C--- ---Q---D-P -----M-K-- -R--------

          201    211
SF2       ELHPEYYKDC
BRU       ------F-N-
233       --------N-
248       --------N-
ConsNef   --------N-
```

FIG. 8. Comparison of the amino acid sequences of five functional Nef proteins. The HIV-1 Nef amino acid sequences predicted from the *nef* genes of laboratory isolates SF2 and Bru, clinical isolates 233 and 248, and the consensus *nef* gene were aligned with the PileUp program (9). Only amino acids differing from those in the SF2 Nef are shown for the other proteins.

25-kDa proteins. Figure 8 shows that the 233, 248, and consensus Nef proteins all have an alanine at this position. The change in this amino acid residue from aspartic acid to alanine is predicted to have an effect on the conformation of the Nef protein (40). These observations could explain the differences in electrophoretic mobility between SF2 and the 233, 248, and consensus Nef proteins seen in our experiments. However, this potential conformational change did not affect the ability of these Nef proteins to suppress CD4 cell surface levels.

The ability of Nef to suppress CD4 expression at the cell surface is of particular interest for several reasons. The human CD4 molecule serves as the cell surface receptor for HIV (7, 25, 33). It is surprising that HIV should have two genes whose products are capable of suppressing the expression of its receptor (4, 6, 14, 17, 20). During HIV infection, the envelope protein precursor gp160 is processed very inefficiently within the host cell (55). The majority of gp160 is transported to the lysosome and degraded (55). Transient-expression studies have shown that CD4 processing and expression are also inhibited in these cells, probably as the result of direct binding between gp160 and CD4 (6, 56). We have shown that Nef also has the ability to block CD4 expression at the cell surface (13, 14; this study). The mechanism by which this occurs is unknown and is currently under investigation in our laboratories. In contrast to gp160, which does not bind mouse CD4 (29), Nef also blocks the expression of mouse CD4 (13). This result is important in view of the structural and functional similarities between mouse and human CD4 (21, 41). Moreover, the interaction of Nef with CD4 differs from that of gp160/gp120 in that the effect of Nef on CD4 appears to require the cytoplasmic domain of the CD4 molecule (13), while gp160 and gp120 bind to the extracellular domain of CD4 (29).

Infection with HIV is accompanied by a state of anergy within the $CD4^+$ T-cell population, resulting in the eventual depletion of $CD4^+$ T cells (45). The CD4 molecule on the surface of T cells is important for antigen-driven T-cell triggering via the T-cell receptor (21, 41). CD4 interacts with class II major histocompatibility complex molecules on antigen-presenting cells, serving as a coreceptor for antigen recognition (21, 41). Engagement of the CD4 molecule also activates the protein tyrosine kinase $p56^{lck}$, which has been shown to be important for T-cell activation (16, 21, 46, 51–53). Interference with the contribution of CD4 to proper T-cell signalling might have significant consequences for T-cell viability and function. Indeed, it has been demonstrated for both mouse and human T cells that removing CD4 from the cell surface by cross-linking with anti-CD4 antibodies, or with gp120 in the case of human CD4, renders the

cells unresponsive to T-cell receptor triggering and leads to cell death by apoptosis (3, 38). Thus, blocking CD4 expression at the cell surface could contribute to the depletion of $CD4^+$ T cells, which is the hallmark of AIDS.

Although Nef could have deleterious effects on cell function and is not required for viral replication in vitro (11, 50), there appears to be selective pressure to maintain a functional *nef* gene in vivo (23), suggesting that it offers some advantage to the virus. The work of Willey et al. suggests that the rate of processing of gp160 depends on the ratio of gp160, CD4, and Vpu inside the cell (55–57). The early expression of Nef during the virus life cycle may contribute to the processing of gp160 to gp120 and gp41 by interfering with the expression of CD4. Such an effect might be more evident at low levels of virus replication. Under such circumstances, the negative effect of Nef on CD4 surface levels could result in more efficient virus replication, which correlates with the results obtained in vivo with SIV (23).

We have shown that suppression of surface CD4 expression is a common function of the Nef protein of HIV-1. While the relevance of Nef to viral pathogenesis and disease progression has been clearly demonstrated (8, 23), the role of Nef-induced suppression of surface CD4 expression in the pathogenesis of AIDS is at present unclear. However, the fact that the ability to suppress surface CD4 expression is conserved in both primary and laboratory isolates of Nef suggests the probable importance of this function of Nef.

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