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Dissociation of the CD4 Downregulation and Viral Infectivity Enhancement Functions of Human Immunodeficiency Virus Type 1 Nef

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Recent evidence indicates that the *nef* gene of human immunodeficiency virus type 1 augments rather than inhibits viral replication in both cell culture and in vivo models. In addition, *nef* alters various normal cellular processes, including the display of CD4 on the cell surface. However, it remains unknown whether the enhancement of infectivity and the downregulation of CD4 represent linked or independent biologic properties of this single protein. In the present studies, mutational analyses were performed to define structure-function relationships within the Nef protein that mediate these effects. To assess the functional consequences of these mutations, sensitive and reliable assays were developed to quantitate the viral infectivity enhancement and CD4 downregulation functions of Nef. The results indicate that membrane-targeting sequences at the N terminus of Nef are important for both functions of Nef, while certain other conserved regions are dispensable for both functions. A conserved proline-X-X repeat segment in the central core of the protein, which is reminiscent of an SH3-binding domain, is critical for the enhancement of infectivity function but is dispensable for CD4 downregulation. However, the downregulation of CD4 by Nef appears to involve a two-step process requiring the initial dissociation of p56^{lck} from CD4 to permit engagement of the endocytic apparatus by CD4. Together, these findings demonstrate that the infectivity enhancement and CD4 downregulation activities of human immunodeficiency virus type 1 Nef can be dissociated. Thus, these processes may be independent of one another in the viral replication cycle.

The replicative life cycle of human immunodeficiency virus type 1 (HIV-1) is regulated by the expression of several auxiliary genes contained within its genome, a characteristic which is shared by other lentiviruses (reviewed in references 12 and 20). Among these genes is the *nef* open reading frame, which is located at the 3′ end of the virus, partially overlapping the U3 region of the 3′ long terminal repeat (LTR). This gene, which is expressed abundantly via multiply spliced transcripts during early viral transcription (26, 42, 46), encodes a 27- to 29-kDa cytoplasmic protein that is targeted to the plasma membrane and to other cellular membranes by an *N*-myristyl group added posttranslationally at glycine 2 (16, 21, 24, 54). The Nef protein thus far remains an enigmatic element in the HIV-1 life cycle.

Early investigation of Nef suggested that it exerted negative regulatory effects on viral replication through inhibitory effects on transcription of the HIV-1 LTR, perhaps contributing to the development or maintenance of viral latency (1, 8, 31, 37). Subsequent studies described either neutral or enhancing effects of Nef on viral transcription and infection of activated T lymphocytes (22, 27). Compelling evidence for a crucial positive role for the *nef* gene in primate lentiviral replication and in disease pathogenesis was obtained in experiments employing wild-type and mutant forms of a related virus, simian immunodeficiency virus from macaque (SIV_{mac239}) (25). In these in vivo studies with the rhesus monkey model system of AIDS, molecularly cloned viruses containing a *nef* deletion exhibited profoundly impaired replication competence and pathogenicity. Additionally, viruses containing a point mutation within the *nef* gene exhibited a strong tendency toward reversion to wild-type sequence as early as 2 weeks after inoculation. Recent cell culture studies have demonstrated clearly that *nef* increases HIV-1 replication in primary human peripheral blood lymphocytes (35, 50). This enhancement of replication appears to reflect an increase in the intrinsic infectivity of virus particles, as measured in single-cycle infection systems (9, 35). Furthermore, the Nef-mediated enhancement of infectivity of HIV-1 occurs through a mechanism that requires Nef expression during the production of viral particles but is independent of either gp160 or viral entry into targets (36).

Infection by HIV-1 results in a significant decrease in the cell surface expression of the CD4 molecule, a type I integral membrane protein that serves as a key component of the cellular receptor for HIV-1 (13, 30). Part of this effect is related to interactions of gp160 with CD4 in the endoplasmic reticulum (receptor interference), which can involve the intracellular degradation of CD4 in conjunction with the viral Vpu protein (10, 11, 23, 52, 53). Interestingly, both HIV-1 (7, 17, 18, 21, 50) and SIV Nef (7, 15) proteins have been shown to downregulate the surface display of CD4 even in the absence of other viral gene products. This effect is a characteristic of numerous HIV-1 Nef alleles (3, 34) and is exerted on murine as well as human CD4 (3, 17). Both the prevention of superinfection (7) and the modulation of cellular activation (8, 32) have been suggested as potential biologic functions of CD4 downregulation by Nef. Nonetheless, the full significance of these processes in the viral life cycle remains undetermined.

The molecular mechanism underlying Nef-mediated CD4

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modulation is largely unknown. This process in T cells appears to involve the accelerated elimination of surface CD4 via an endocytic pathway leading to lysosomal degradation (2, 41, 45). The internalization of CD4 by Nef depends upon the CD4 cytoplasmic tail (2, 3, 17) and specifically upon a pair of leucine residues within the 20 membrane-proximal amino acids that may constitute a "dileucine degradation motif" shared by other lymphocyte surface molecules that are subject to a similar intracellular fate (2). Recent evidence suggests that there is some degree of overlap between this motif and the element that binds $p56^{lck}$ (4, 44). An active area of investigation is the specific means of exploitation of the cellular internalization machinery by viral proteins such as Nef to promote downregulation of surface molecules involved in the immune response.

The cumulative evidence that Nef acts both to increase the intrinsic infectivity of HIV and to downregulate the surface expression of CD4 raises the question of whether Nef has a single or multiple mechanisms of action. To address this critical question, the present studies were undertaken to define structure-function relationships within the Nef protein relating to the enhancement of infectivity and to the downregulation of CD4. Analyses of a panel of site-directed mutants of Nef identified protein regions that are either contributory or noncontributory to each of these functions. The results indicate a large degree of overlap in the functional architecture with regard to these two functions but also reveal certain amino acids within Nef that differentially affect the infectivity and CD4 downregulation phenotypes. Thus, these findings suggest that Nef exerts multiple, partially independent effects on cells infected with HIV-1. The present studies further revealed that the downregulation of CD4 by Nef may be a multistep process that is contingent upon the dissociation of $p56^{lck}$ from CD4.

MATERIALS AND METHODS

Generation of Nef expression vectors and proviral vectors. The *nef* gene was amplified by PCR with the HIV-1 proviral clone HXB nef+ as a template. This clone contains a chimeric HXB2-IIIB *nef* gene in which the premature stop codon of the HXB2 allele was repaired by recombination (35). The primer sequences used for amplification are 5′-GCGCACGCGTATCGATAGACAG GGCTTGGAAAGG-3′, which anneals to the region just upstream of the *nef* start codon and includes a *Cla*I site for cloning, and 5′-TCCCTTGTAGGATC CACGCGTTCAGCAGTTCTTGAAGTA-3′, a reverse primer which overlaps the 3′ end of the *nef* gene and contains *Mlu*I and *Bam*HI restriction sites just downstream of the *nef* stop codon. The amplified *nef* gene was introduced into the unique *Cla*I and *Bam*HI restriction sites of a cytomegalovirus-based expression vector (pCMV4Neo, provided by M. Feinberg) (19), which had been modified slightly by eliminating the unique *Xho*I, *Bgl*II, and *Kpn*I sites by blunt-end ligation of the Klenow-filled restriction sites. The *nef* mutants were generated by PCR amplification of portions of this construct with primers that encoded the specific mutations described in this report. These plasmids (pNef2 and related constructs) were used for studies of CD4 downregulation.

Nef-expressing proviral clones were generated by subcloning the wild-type and mutant *nef* genes into a *nef*-minus provirus analogous to the ΔN-term clone described previously (35). In this construct, the N-terminal portion of the *nef* gene of HXB2 from the start codon to the *Bgl*II site 256 bp downstream was deleted and replaced by an *Mlu*I restriction site, into which the *nef* variants were introduced. In these viral constructs, the restored *nef* cDNA, which contains a stop codon, is followed immediately by the native LTR containing a duplication of the 3′ region of the *nef* gene (see Fig. 2A).

Generation of virus stocks and cell lysates. 293T cells (3×10^6 to 5×10^6; human embryonal kidney cells expressing the simian virus 40 large T antigen [provided by D. Baltimore]) (38) were transfected with 50 μg of HIV proviral DNA by a standard calcium phosphate transfection method (29). After the cells were cultured overnight in the presence of the DNA precipitate, the medium was replaced with fresh Dulbecco's modified Eagle's medium (DMEM; Mediatech, Herndon, Va.) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, Gaithersburg, Md.), 2 mM L-glutamine, and antibiotics. The cultures were maintained for 48 h, after which time the cell supernatant was collected and clarified of cell debris by centrifugation at $400 \times g$ for 5 to 10 min. The Gag p24 concentration was determined by a commercially available antigen capture enzyme-linked immunosorbent assay (ELISA; Coulter Immunology, Hialeah, Fla.). Virus stocks were uniformly diluted and stored in aliquots at −70°C. All virus stocks underwent one freeze-thaw cycle prior to infection studies.

To obtain cellular lysates for expression studies, the transfected 293T cell monolayers were harvested in TEN (40 mM Tris-Cl [pH 7.5], 1 mM EDTA [pH 8.0], 150 mM NaCl), pelleted as above, and lysed in 100 μl of TEN containing 1% Triton X-100. The lysates were clarified in a microcentrifuge at $15{,}000 \times g$ for 10 min. The soluble portion of the lysate was retrieved and stored at −70°C.

Infectivity assay. A modification of the multinuclear activation of a galactosidase indicator (MAGI) assay (28) was developed with the β-galactosidase (β-gal) enzyme assay system kit (Promega, Madison, Wis.). In this modified assay, the readout of viral infectivity is the relative β-gal activity measured in bulk lysates of all infected cells rather than quantification of the number of individual cells infected; this modification proved to be reliable, reproducible, and rapid. HeLa-CD4-LTR/β-gal cells (MAGI cells; NIH AIDS Research and Reference Program, catalog no. 1470) were maintained in complete Iscove's medium (Mediatech) supplemented with 10% FBS. One day prior to infection, 2.5×10^5 MAGI cells were plated in 2 ml in a six-well plate (Corning, Corning, N.Y.). On the day of infection, the medium was removed and replaced with 2 ml of diluted supernatant containing the indicated amount of HIV Gag p24. After a 15- to 18-h infection period, the viral supernatants were removed and replaced with complete Iscove's medium containing 10% FBS and 2 μg of soluble CD4 (a generous gift from Genentech) per ml, added to inhibit viral spread and syncytium formation. At 65 h after initial infection, the confluent cell layers was washed twice in calcium- and magnesium-free phosphate-buffered saline (PBS). The cell layer was harvested in 1 ml of TEN, and the cells were pelleted by brief centrifugation. The TEN supernatant was removed by aspiration, and the cells were resuspended in 100 μl of 1× reporter lysis buffer supplied in the β-gal enzyme assay system kit. The cell suspension was subjected to vigorous vortexing for more than 10 min to ensure complete nuclear lysis. The cell lysates were centrifuged at $15{,}000 \times g$ for 7 min at 4°C. A 50-μl volume of the soluble fraction was placed in each well of a flat-bottom 96-well plate (Falcon, Lincoln Park, N.J.), to which 75 μl of 2× assay buffer (120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, 1.33 mg of *o*-nitrophenyl-β-D-galactopyranoside [ONPG]) was added. The optical densities at 405 nm were monitored every 20 s for 90 min with the SOFTMax 881 software program for running a Vmax Kinetic Microplate Reader (Molecular Devices Corp., Menlo Park, Calif.). The rate of ONPG conversion during the linear phase of the reaction was used as the indicator of β-gal induction by HIV Tat produced during viral infection.

Assay of CD4 downregulation. Transient transfections were performed with the *nef* expression constructs described above and a human CD4 expression plasmid (pCD4Neo) prepared as follows. A 1.7-kb *Eco*RI-*Bam*HI fragment containing the coding sequence for CD4 (33) (from plasmid pF4.4; provided by D. Littman) was subcloned into pBluescript to yield pBSCD4 and then transferred into the *Hin*dIII-*Bam*HI sites of pCMV4Neo. The CD4-415 expression plasmid was prepared by PCR and subcloning into pCMV4Neo and encodes a CD4 protein that terminates after amino acid 415 (2). The $p56^{lck}$ expression plasmid was prepared by subcloning the murine *lck* cDNA from *lck*/pUC19 (provided by R. Perlmutter) into the *Hin*dIII site of pCMV4Neo. The kinase domain mutant of $p56^{lck}$ (provided by D. Littman) was also subcloned into pCMV4Neo. The human interleukin-2 (IL-2) receptor α expression plasmid pCMV4-IL-2Rα was described previously (5).

COS7 cells (obtained from the American Type Culture Collection) were passaged in Iscove's medium (Cellgro; Mediatech) supplemented with 10% FBS, glutamine, and antibiotics. Approximately 18 h prior to transfection, 3×10^5 cells were plated into each well of a six-well plate (Falcon) containing 2.0 ml of DMEM supplemented with FBS (10%) and antibiotics (complete DMEM). A total of 1.0 μg of plasmid DNA was first added to 100 μl of Opti-Mem low-serum medium (GIBCO BRL) and then combined with a second 100-μl aliquot of Opti-Mem containing 7 μg of the Lipofectamine reagent (GIBCO BRL) for a 30-min incubation at room temperature. The DNA-Lipofectamine mixture was then diluted fivefold with Opti-Mem and added to the cells, which had been washed once with 2.0 ml of Opti-Mem medium. Following a 5-h incubation at 37°C, 1.0 ml of DMEM containing 20% FBS was added and the cultures were incubated for 16 h. The medium was then aspirated and replaced with 2 ml of complete DMEM, and the cells were cultured further for 30 h.

For cell surface marker analysis, cells prepared as described above were washed once with PBS. The cells were then incubated in 2 ml of TEN for 10 min, washed once in staining buffer (SB; PBS with 10% FBS), and then recovered from the dish by being scraped into 4 ml of SB. A 0.8-ml portion of the cell suspension was set aside for immunoblot analysis (see below), and the remaining cells were washed again in SB prior to immunostaining. Cells were stained in 40 μl of SB plus 10 μl of either unlabelled or fluorescein isothiocyanate-conjugated anti-CD4 or anti-IL-2 receptor (CD25) monoclonal antibody (Becton Dickinson) for 60 min on ice in the dark. Samples stained with unlabelled primary antibody were washed twice in ice-cold SB and then stained with fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Becton Dickinson) as described above for the primary antibody. Cells stained by either method were washed twice with ice-cold SB and resuspended in SB containing propidium iodide (1 μg/ml) for fluorescence analysis.

Immunostained samples were analyzed with a FACScan (Becton Dickinson). Gating on live cells was performed by using forward scatter and propidium iodide exclusion, and the fluorescein signal was detected as an indicator of surface

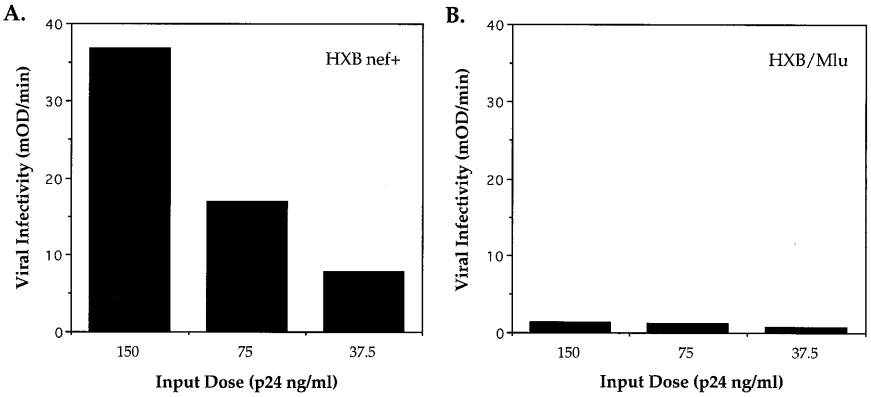

FIG. 1. Dose-response comparison of infectivity functions of HIV-1 clones. Infections were performed by using the MAGI system (28), and induction of β-gal was measured by a liquid β-gal kinetic assay method. Enzyme activity as represented by the rate of ONPG conversion is shown for representative infections at the indicated doses of virus. (A) Parental HXB nef+ clone. (B) *nef*-deleted derivative HXB/Mlu. OD, optical density.

expression of the indicated marker. A total of 10^4 cells events were measured for each sample.

Immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% polyacrylamide gels followed by electrophoretic transfer onto nitrocellulose membranes were conducted by standard methods. Immunoblot analyses were performed with either an anti-CD4 rabbit antiserum (1:1,000) raised against recombinant soluble CD4 (NIH AIDS Repository, catalog no. 806) or an anti-HIV-1BH10 Nef antiserum (1:2,000) (NIH AIDS Repository, catalog no. 2121). Immunoblots were developed by the enhanced chemiluminescence system (Amersham).

RESULTS

Viral infectivity enhancement by Nef: a quantitative assay. Previously, this laboratory demonstrated that expression of wild-type Nef augments HIV-1 infectivity (35). This work employed an established assay of HIV-1 infectivity in which production of the viral Tat protein from the successfully integrated viral genome promotes expression of β-gal via an HIV LTR-driven reporter construct (28). In the original assay system, expression of Tat-induced β-gal was measured in situ by visual detection of blue color derived from conversion of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). To facilitate the present quantitative analyses, this system was modified by measuring β-gal activity in the liquid phase with a kinetic, colorimetric determination of substrate (ONPG) conversion. For each such experiment, the indicator MAGI cells were exposed to virus and cultured for 65 h to allow induction of the reporter construct; this was followed by detergent lysis of the sample and assay of its enzymatic activity in an ELISA format with a microplate reader. In the range employed for these studies, the induction of β-gal activity in cell cultures exhibited a linear and reproducible relationship with the amount of input virus as determined in dose-response experiments with virus stocks prepared from the previously described (35) functional HXB nef+ molecular clone of HIV-1 (Fig. 1A). Furthermore, results from this detection method generally correlated well with those obtained by the visual detection method (data not shown). This convenient approach thus provided a reliable and standardized method for quantitating viral infectivity.

Viral infectivity enhancement by Nef: complementation in *cis*. A method of *cis* complementation was developed to evaluate various site-directed mutants of the *nef* gene. This method utilized a mutant of the HXB2 provirus (referred to as HXB/Mlu) in which approximately 250 nucleotides spanning the 5′ end of the *nef* open reading frame (including the translation initiation codon) were deleted, leaving a unique *Mlu*I restriction site just upstream of the 3′ LTR. As has been observed previously with *nef*-deleted viruses in the visual MAGI cell assay system, virus stocks derived from the *nef*-deleted HXB/Mlu construct exhibited strikingly diminished infectivity in the modified colorimetric assay (Fig. 1B). As expected, no Nef protein was detected by immunoblotting of lysates prepared from cells used to produce this virus (see Fig. 3C).

For complementation studies, wild-type or mutant *nef* cDNAs that included an initiator ATG were transferred from the vectors in which they were prepared into the *Mlu*I restriction site of the HXB/Mlu plasmid (Fig. 2A). The newly derived proviral clones thus contained a novel *nef* cDNA insert upstream of the 3′ LTR in place of the 5′ region of the endogenous *nef* gene. Virus stocks prepared from an HXB/Mlu proviral construct reconstituted with a wild-type *nef* gene in this position (hereafter referred to as Mlu/nef_{wt}) expressed abundant levels of Nef protein as detected by immunoblot analysis (see Fig. 3C). Importantly, such virus stocks also exhibited substantially restored function in the infectivity assay system (Fig. 2B). β-Gal expression in cultures infected with Mlu/nef_{wt} viruses was typically at least 10-fold higher than was β-gal expression in cultures infected with HXB/Mlu viruses, confirming in the *cis* configuration the contribution of Nef to enhanced viral infectivity.

The *cis* complementation assay was developed to provide a reliable, quantitative means of comparing the infectivity function of various *nef* variants. Complementation in *trans* has previously been described (36), but this method exhibits a wider range of variability due in part to variations in cotransfection efficiencies and to cellular toxicity from overexpression of Nef. Therefore, in the present studies, comparative analyses of *nef* variants emphasized the *cis* complementation strategy.

It should be noted that in the *cis* method the infectivity function from *nef*-complemented viruses was somewhat less than that of the parental HXB nef+ viruses. This diminution may have resulted from genetic perturbations ensuing from duplication of part of the U3 region of the 3′ LTR in the *nef* cDNA insert. For example, some small degree of viral spread may occur in cultures with native viruses prior to the addition of soluble CD4, and this type of spread may be impaired in cultures with *cis*-complemented viruses if the genetic construction results in aberrant secondary rounds of replication. Nevertheless, these studies were designed specifically to permit comparison within a panel of viruses complemented in *cis* with various *nef* cDNAs, rather than comparison with parental strains. The dynamic range and reproducibility obtained with

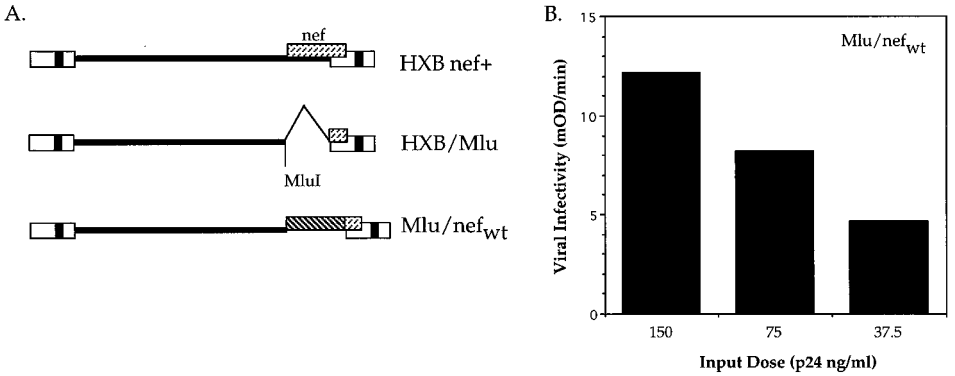

FIG. 2. *cis* complementation strategy for assessing infectivity enhancement. (A) Schematic of proviral constructs, including the parental HXB nef+ clone, the *nef*-deleted HXB/Mlu construct, and the reconstituted virus Mlu/nef$_{wt}$ in which a *nef* cDNA was inserted into the *Mlu*I restriction site of HXB/Mlu. (B) Infectivity of the Mlu/nef$_{wt}$ virus in the single-cycle infectivity assay described in the legend to Fig. 1. OD, optical density.

the *cis* complementation strategy in conjunction with the liquid β-gal assay permitted a reliable, quantitative comparison of the infectivity phenotypes of these variants. Notably, similar phenotypes were also apparent in *trans* rescue studies (see below).

Viral infectivity enhancement by Nef: mutational analyses. Several regions of the *nef* gene were targeted for site-directed mutagenesis in part on the basis of their conservation among viral isolates, as described by Shugars et al. (47) (Fig. 3A). These mutant cDNAs were inserted into the *nef*(−) HXB/Mlu backbone for evaluation in the infectivity complementation assay (Fig. 3B). For example, disruption of membrane targeting of Nef either by elimination of the myristylation site (Mlu/

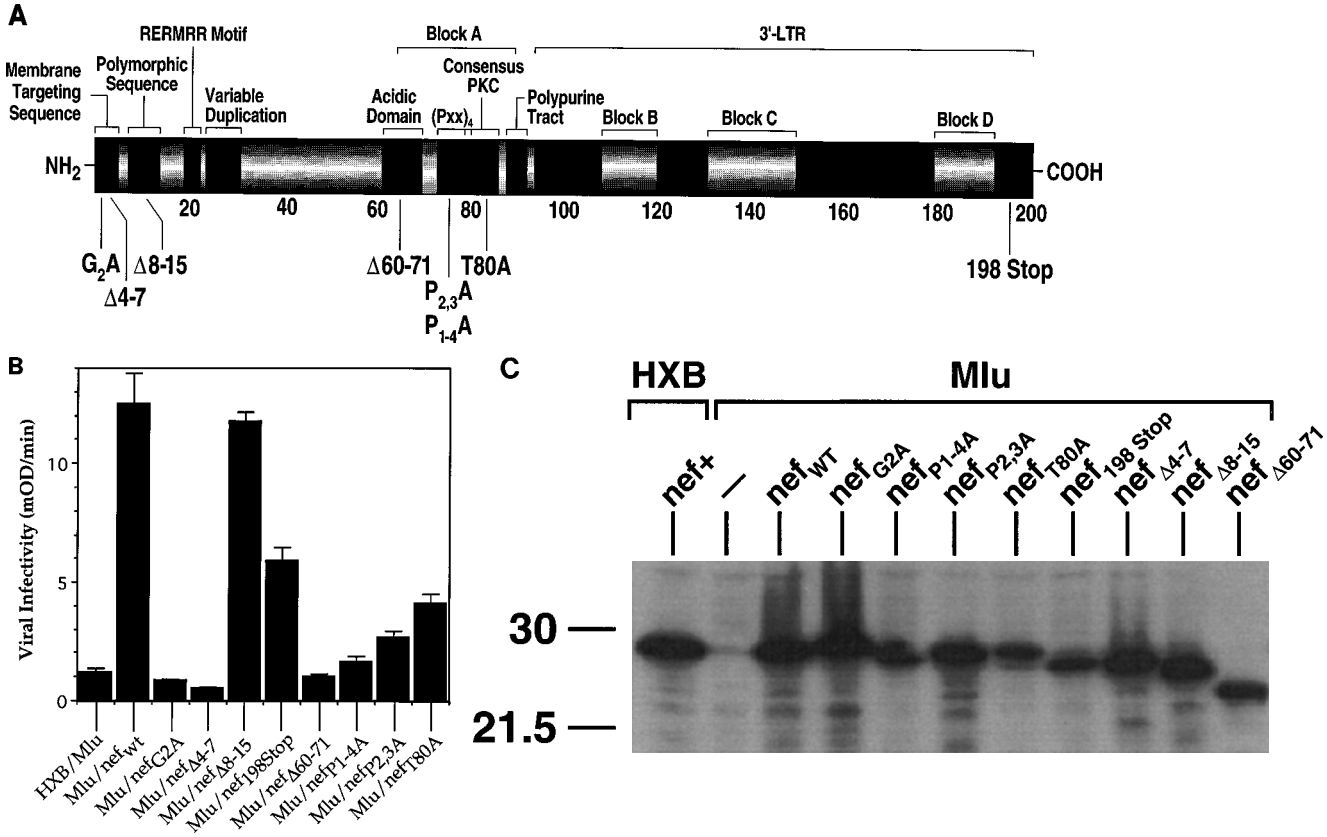

FIG. 3. Mutational analysis of viral infectivity enhancement by Nef. (A) Schematic of conserved regions of the Nef protein as described previously (47). Deletion and substitution mutants used in the present studies are indicated below the schematic: G2A, containing alanine in place of glycine 2; Δ4–7, deleted of residues 4 to 7; Δ8–15, deleted of residues 8 to 15; Δ60–71, deleted of residues 60 to 71; P2,3A, containing alanines in place of proline 72 and proline 75; P1–4A, containing alanines in place of proline 69, proline 72, proline 75, and proline 78; T80A, containing alanine in place of threonine 80; and 198Stop, deleted of residues 199 to 206. (B) Assessment of infectivity functions of viral constructs containing the indicated *nef* mutant inserted into the *nef*-deleted HXB/Mlu clone. Viral infectivity was measured as in the experiment in Fig. 1. These infections were performed at a viral input dose of 150 ng/ml. Each bar represents the mean and standard error of the mean (n = 9) from a representative experiment. OD, optical density. (C) Preservation of protein expression of the Nef mutants functionally assessed in panel B. Whole-cell lysates from 293T cells transfected with the indicated proviral constructs during virus production were analyzed by immunoblot analysis with an anti-Nef antiserum.

nef_{G2A}) or by deletion of adjacent amino acids that may either interact with polar phospholipid head groups or affect myristylation (Mlu/$nef_{\Delta 4-7}$) dramatically impaired enhancement of infectivity by these Nef variants; these viruses were largely indistinguishable from the *nef*(−) parental virus HXB/Mlu. Interestingly, deletion of a nearby region of the gene encoding a highly polymorphic protein segment near the N terminus (Mlu/$nef_{\Delta 8-15}$) had essentially no significant effect on viral infectivity. Importantly, both these functional and nonfunctional Nef proteins were detectable by immunoblot analysis with an anti-Nef antiserum to probe lysates of virus-producing cells (Fig. 3C), demonstrating that the observed functional defects were not merely the result of protein instability. Although some differences in protein levels did result from certain mutations, there was no evident correlation between protein level and degree of functionality. We cannot exclude, however, that protein stability may influence the phenotype of a given mutant.

C-terminal truncations were also prepared to define the minimal protein backbone required for this function. Of six such truncations prepared, one (Mlu/$nef_{198Stop}$) exhibited substantial preservation of function (Fig. 3B). However, this truncation was also the only one that was detectable in the immunoblotting studies (Fig. 3C), implying that all other truncated proteins were too unstable to evaluate functionally (data not shown).

Several mutations corresponding to the conserved central core of the Nef protein resulted in significant impairment of the infectivity function. For example, deletion of a region encoding a conserved glutamic acid-rich segment of the protein (Mlu/$nef_{\Delta 60-71}$) virtually abrogated the enhancement of infectivity (Fig. 3B). In addition, three point mutations resulting in nearby amino acid substitutions diminished enhancement activity. Specifically, disruption of a conserved proline-X-X repeat segment by substituting alanine for either the internal two prolines (Mlu/$nef_{P2,3A}$) or all four prolines (Mlu/nef_{P1-4A}) significantly impaired this activity. However, multiple experiments performed with independent virus preparations of these mutants revealed a very small but persistent level of function above that obtained with HXB/Mlu or the membrane-targeting mutants Mlu/nef_{G2A} and Mlu/$nef_{\Delta 4-7}$. Moreover, the Mlu/$nef_{P2,3A}$ mutant consistently displayed marginally greater activity than did the Mlu/nef_{P1-4A} mutant. Finally, elimination of a potential protein kinase C phosphorylation site contiguous with the proline repeat stretch (Mlu/nef_{T80A}) partially reduced the viral infectivity enhancement function of Nef. In summary, since Nef proteins from all of these mutants were readily detectable by immunoblotting (Fig. 3C), these analyses indicate that conserved regions both near the N terminus and within the central core of the protein substantially influence the viral infectivity enhancement function of Nef.

Although the *trans* rescue method has proven to be less quantitative in our hands, the phenotypes described above were also evident in experiments performed by this approach. For such experiments, virus stocks were prepared by cotransfecting 293T cells with a *nef*-deleted virus (R7-Xho) (35) and an expression vector encoding various Nef mutants. These viral stocks were then used to infect MAGI cells, and cells positive for β-gal expression were scored by visual inspection as in the original assay system (28). In such an experiment in which complementation by wild-type Nef is defined as 100%, the *nef*-deleted virus exhibited 30% residual infectivity function. The mutant $nef_{\Delta 4-7}$ exhibited approximately 25% activity, and the nef_{P1-4A} mutant exhibited approximately 40% function. Thus, although this method is less useful for quantitative comparisons, it confirmed that such mutants exhibited markedly impaired infectivity activity as seen in the *cis* complementation system.

Modulation of CD4 cell surface expression by Nef: a quantitative assay. To identify other functional domains within Nef, its CD4 downregulation activity was evaluated by a transient-transfection system. Since the modulation of surface expression of CD4 by Nef has been observed in many primate and murine cell types, a rapid assay method was developed by using the readily transfectable COS-7 mammalian cell expression system. The use of a cationic lipid transfection vehicle (Lipofectamine) permitted the reproducible, high-level expression of human CD4 on the surface of COS-7 cells as detected by anti-CD4 monoclonal antibodies in conjunction with either FACS (Fig. 4B) or radiolabelled second antibody (data not shown). In numerous immunofluorescence experiments, approximately 15 to 30% of input cells subjected to transfection with the CD4 expression plasmid displayed the CD4 molecule, while fewer than 1% of cells transfected with an empty vector fluoresced with the anti-CD4 reagents (Fig. 4A).

With this method of gene transfer, double transfection was also readily achieved. Cotransfection studies were performed with a mixture of plasmids encoding two independent cell surface molecules (human CD4 and IL-2 receptor α chain). Dual-color FACS following transfection with equimolar amounts of the two plasmids revealed that approximately 50% of cells expressing one marker concomitantly expressed the second marker. This proportion was increased to 85% by simply adjusting the ratio of input plasmids (data not shown). Thus, this method provided a potential cotransfection approach to assessing the effects of Nef on the surface expression of CD4.

As measured by both detection methods (fluorochrome-conjugated versus radiolabelled second antibody), cotransfection of the Nef expression construct (pNef2) with the CD4 expression vector (pCD4Neo) resulted in a 50 to 70% reduction in CD4 display on the cell surface (Fig. 4A; data not shown). Recent work has demonstrated that this effect of Nef is attributable to enhanced endocytosis and degradation of CD4 rather than to alterations in transcription or translation of CD4 (2, 41). Consistent with this mechanism, immunoblot analyses with an anti-CD4 antiserum revealed no differences in the overall expression of CD4 protein in cells transfected with or without Nef (Fig. 4C). In view of the high levels of CD4 obtained in this overexpression system, we cannot exclude the possibility that Nef increased degradation of a relatively small pool of CD4 protein displayed at the cell surface. The essential observation, however, is that the Nef-mediated downregulation of CD4 in this system is not an artifact resulting from interference with CD4 protein expression. Furthermore, the specificity of the surface modulation activity was demonstrated by the lack of an effect by Nef on the surface display of two other integral membrane proteins, human IL-2 receptor α (Fig. 4B) and CD8 (data not shown). These results indicated that the cotransfection strategy offered a reliable, specific, and quantitative assay of the CD4 surface downregulation activity of Nef.

Modulation of CD4 cell surface expression by Nef: mutational analyses. To identify regions of the Nef protein that contribute to the downregulation phenomenon, the Nef mutants with site-directed mutations described above (Fig. 3A) were evaluated in the transient-transfection assay system. For these studies, all mutant *nef* cDNAs were cloned into the same vector by employing a cytomegalovirus promoter-enhancer to regulate expression of the insert as well as an simian virus 40 origin sequence to permit episomal amplification by the simian virus 40 T antigen constitutively expressed within COS-7 cells.

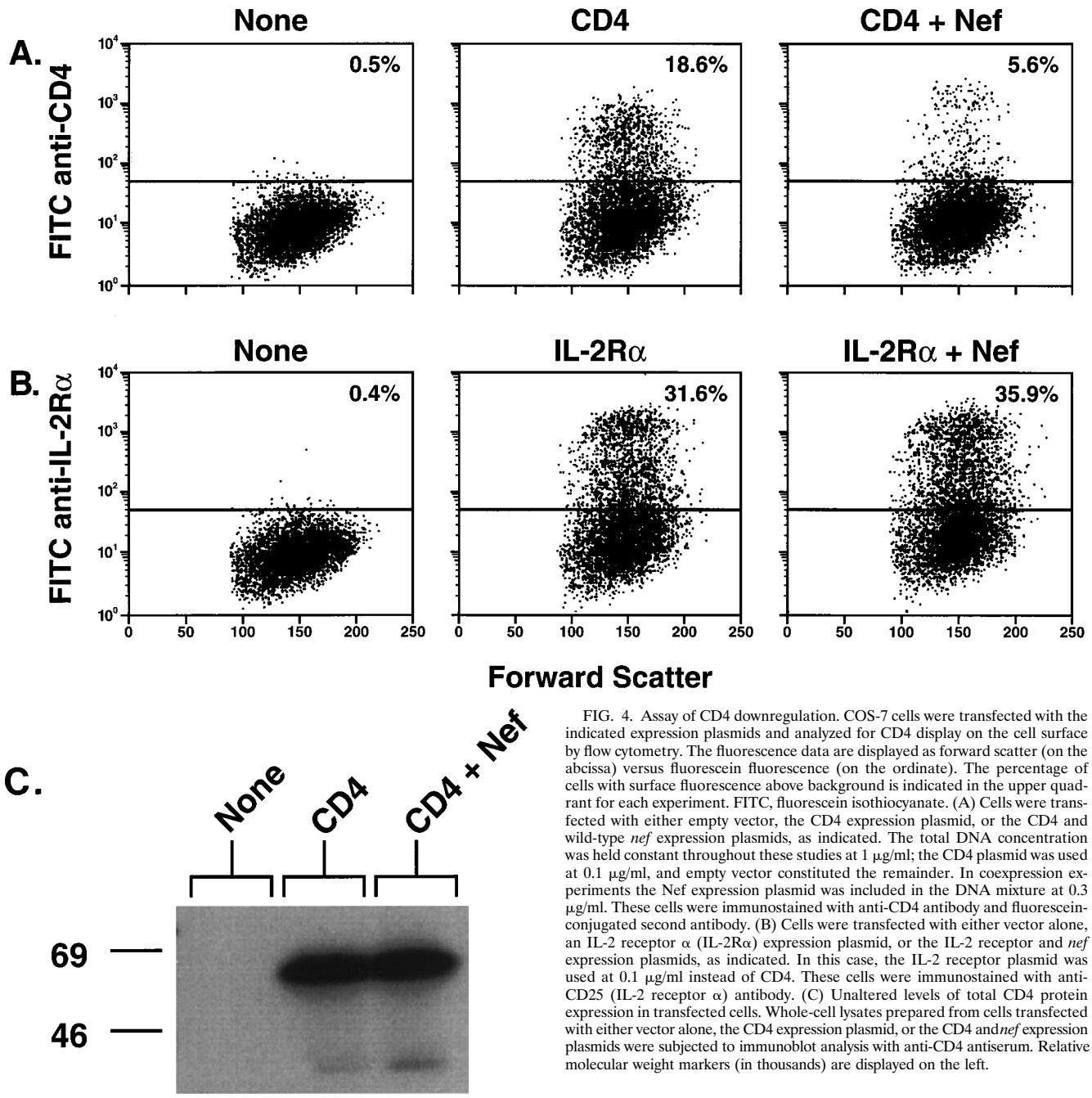

FIG. 4. Assay of CD4 downregulation. COS-7 cells were transfected with the indicated expression plasmids and analyzed for CD4 display on the cell surface by flow cytometry. The fluorescence data are displayed as forward scatter (on the abcissa) versus fluorescein fluorescence (on the ordinate). The percentage of cells with surface fluorescence above background is indicated in the upper quadrant for each experiment. FITC, fluorescein isothiocyanate. (A) Cells were transfected with either empty vector, the CD4 expression plasmid, or the CD4 and wild-type *nef* expression plasmids, as indicated. The total DNA concentration was held constant throughout these studies at 1 μg/ml; the CD4 plasmid was used at 0.1 μg/ml, and empty vector constituted the remainder. In coexpression experiments the Nef expression plasmid was included in the DNA mixture at 0.3 μg/ml. These cells were immunostained with anti-CD4 antibody and fluorescein-conjugated second antibody. (B) Cells were transfected with either vector alone, an IL-2 receptor α (IL-2Rα) expression plasmid, or the IL-2 receptor and *nef* expression plasmids, as indicated. In this case, the IL-2 receptor plasmid was used at 0.1 μg/ml instead of CD4. These cells were immunostained with anti-CD25 (IL-2 receptor α) antibody. (C) Unaltered levels of total CD4 protein expression in transfected cells. Whole-cell lysates prepared from cells transfected with either vector alone, the CD4 expression plasmid, or the CD4 and *nef* expression plasmids were subjected to immunoblot analysis with anti-CD4 antiserum. Relative molecular weight markers (in thousands) are displayed on the left.

As was observed in the infectivity assay, mutations designed to disrupt membrane targeting (nef_{G2A} and $nef_{\Delta 4-7}$) significantly reduced the CD4 downregulation activity of Nef (Fig. 5A). Likewise, the internal deletion of 12 amino acids encompassing the conserved glutamic acid-rich segment ($nef_{\Delta 60-71}$) largely abrogated the CD4 downregulation function, indicating that this gross change disrupted both the infectivity and downregulation functions of Nef. However, as in the infectivity assays, a deletion corresponding to a nonconserved segment near the N terminus of the protein ($nef_{\Delta 8-15}$) had little effect on the downregulation function. Additionally, a mutation resulting in a short C-terminal truncation of Nef ($nef_{198Stop}$) had no impact on the downregulation activity. As was observed in the viral infectivity studies, all of these mutant Nef proteins were readily detectable by immunoblot analyses of lysates of transfected cells (Fig. 5B). These findings thus indicate that certain Nef regions that are either dispensable or required for the infectivity enhancement function contribute similarly to the CD4 downregulation function.

A more complex relationship was observed with certain mutants with mutations in the central core of the protein. In contrast to the conserved structure-function relationships described above, the proline-X-X repeat segment exhibited differential contributions to the two Nef activities. Although both the partial ($nef_{P2,3A}$) and complete (nef_{P1-4A}) abolition of these prolines substantially impaired the infectivity enhance-

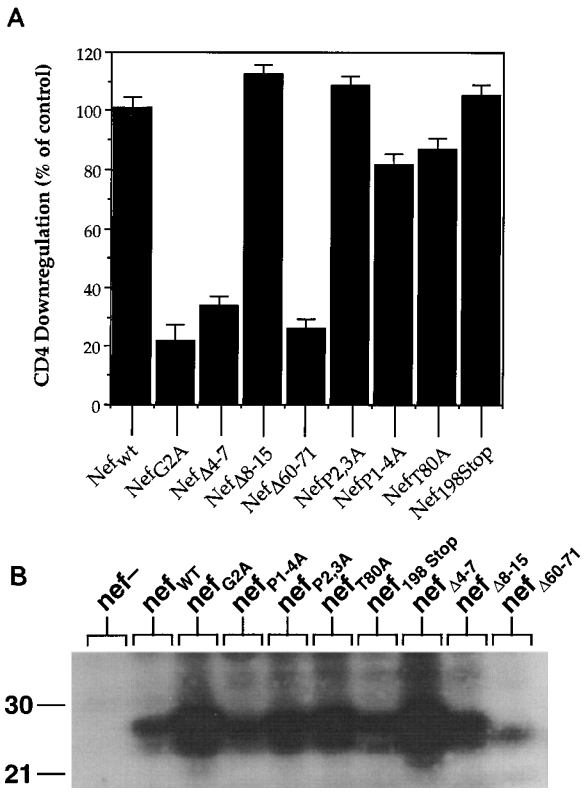

FIG. 5. Mutational analysis of CD4 cell surface expression by *nef*. CD4 downregulation assays were performed in COS-7 cells as described in the legend to Fig. 4. Cells were transfected with the CD4 expression plasmid and expression plasmid encoding wild-type or mutant Nef. The mutants used were as described in the legend to Fig. 3. Relative CD4 downregulation was calculated as the ratio of the percentage reduction in CD4 surface display in the presence of the indicated mutants divided by the reduction in surface CD4 display in the presence of the wild-type Nef in each experiment. Each bar represents the mean and standard error of the mean for determinations from multiple experiments (n = 4), each expressed relative to the wild-type function for each assay. (B) Preservation of protein expression for the mutant Nef proteins used in panel A. Whole-cell lysates from cells transfected with the indicated constructs were evaluated by immunoblotting with an anti-Nef antiserum.

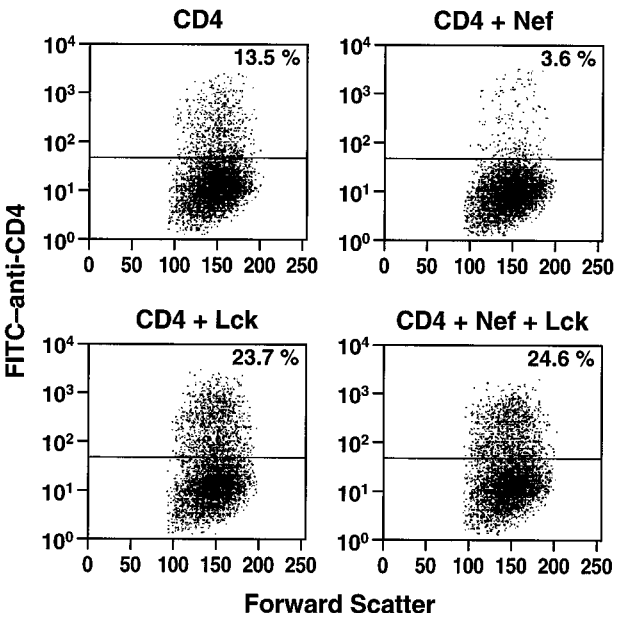

FIG. 6. Abrogation of Nef-mediated CD4 downregulation by p56^{lck}. CD4 downregulation assays were performed with COS-7 cells as described in the legend to Fig. 4. Cells were transfected with the indicated expression plasmids at the following concentrations (total DNA, 1 μg/ml): CD4, 0.1 μg/ml; Nef, 0.3 μg/ml; p56^{lck} (Lck), 0.6 μg/ml; vector, 0.1 to 0.9 μg/ml. FITC, fluorescein isothiocyanate.

ment function (Fig. 3B), neither of these substitutions significantly disrupted the CD4 downregulation activity (Fig. 5A); both mutants displayed well-preserved downregulation activity compared with wild-type Nef in multiple experiments. Interruption of the potential protein kinase C phosphorylation site (nef$_{T80A}$) had a modest effect on the CD4 downregulation activity. All of the mutant constructs abundantly expressed protein in both the infection (Fig. 3C) and transfection (Fig. 5B) systems, although it is possible that the phenotypes of certain mutants (e.g., nef$_{\Delta 60-71}$) are affected by protein instability. Regardless, the differential effects with these mutants suggested that the infectivity enhancement and CD4 downregulation activities are dependent upon distinguishable domains within Nef. Therefore, these processes do not appear to be completely interdependent, since CD4 downregulation is preserved even in the absence of enhanced infectivity.

Inhibition of the CD4 downregulation activity of Nef by overexpression of p56^{lck}. The downregulation of CD4 by Nef has been shown to be accompanied by the liberation of the T-cell-specific *src* kinase p56^{lck} from the cytoplasmic tail of CD4 (41), implying a possible direct or indirect role for this kinase in the downregulation process. However, the CD4 cytoplasmic motif specifying internalization in response to Nef is functional even in the absence of p56^{lck} binding to CD4 (2, 17). To explore further these processes, CD4 surface expression was studied in the transient-transfection system in the presence and absence of p56^{lck}. In multiple independent experiments, coexpression of p56^{lck} with CD4 resulted in augmentation of CD4 expression at the cell surface (Fig. 6), although the degree of enhancement varied somewhat. Strikingly, expression of p56^{lck} in these cells virtually abolished all CD4 downregulation activity by Nef in numerous experiments (Fig. 6); the level of CD4 expression in the presence of p56^{lck} was essentially unaltered by the presence of Nef. This effect was not due to alterations in Nef expression or stability in the presence of p56^{lck}, since no changes in Nef protein levels were observed by immunoblotting of extracts of cells transfected with expression plasmids for Nef with or without p56^{lck} (data not shown). Moreover, the abrogation of CD4 downregulation by Nef was specific for p56^{lck}, as it was not observed with other *src* family kinases (data not shown). Therefore, overexpression of p56^{lck} specifically inhibits the CD4 downregulating activity of Nef.

To determine whether this inhibitory effect depends upon

TABLE 1. Inhibition by p56^{lck} of CD4 downregulation activity[a]

CD4 used	CD4 expression (%) for transfection with[b]:			
	None	Nef	Nef + Lck	Nef + Lck(kin−)
CD4	100	38	86	108
CD4-415	100	49	41	

[a] Assays were performed as described in the legend Fig. 4. Cells were transfected with expression plasmids encoding either full-length CD4 (CD4) or a C-terminal truncation mutant of CD4 (CD4-415), and plasmids encoding wild-type Nef, wild-type p56^{lck} [Lck], and a kinase domain mutant of p56^{lck} [Lck-(kin$^-$)] as indicated.

[b] CD4 expression was defined as 100% for the single transfections with either the CD4 or CD4-415 expression plasmids. CD4 expression for the other conditions is presented as a percentage of the matched control.

the catalytic domain of $p56^{lck}$, a similar experiment was performed with a tyrosine kinase domain mutant of $p56^{lck}$. Like the wild-type $p56^{lck}$, the kinase-minus mutant virtually abrogated the effect of Nef on CD4 surface expression (Table 1). Therefore, this effect is exerted independently of the kinase activity of $p56^{lck}$.

An alternative hypothesis is that this effect is mediated by direct competition with the endocytic machinery for CD4, as suggested by the partial overlap of the $p56^{lck}$-binding site with the CD4 internalization motif (4, 44). To evaluate this possibility, a C-terminal truncation mutant of CD4 that lacks the ability to associate with $p56^{lck}$ but retains the downregulation motif was employed (2). This mutant (CD4–415) indeed retained downregulation responsiveness to Nef but was unaffected by coexpression of $p56^{lck}$ (Table 1). Thus, the inhibitory effect of $p56^{lck}$ on Nef-mediated downregulation of CD4 depends critically on the CD4 cytoplasmic domain necessary for binding $p56^{lck}$.

DISCUSSION

The *nef* gene of HIV-1 is expressed at high levels during the early phase of cellular infection prior to the expression of the *gag*, *pol*, and *env* genes. Both in vivo (25) and in vitro studies (35, 51) have demonstrated convincingly that *nef* markedly upregulates viral replication. The molecular basis of this augmenting effect involves enhancement of the intrinsic infectivity of the virus (36). It remains uncertain whether Nef modifies virus particles directly or instead exerts indirect effects that change the intracellular milieu to favor production of viruses with enhanced infectivity characteristics.

A variety of studies have demonstrated functional effects of HIV-1 *nef* on the biology of mammalian cells. The best studied of these effects is the downregulation of cell surface expression of the human CD4 molecule (7, 17, 18, 21, 50). Extraordinary although controversial effects of *nef* have also been observed with regard to cellular activation (8, 14, 32, 50). Therefore, it is tempting to speculate that the CD4 downregulation process may be part of a larger modulating activity that provides an optimal intracellular environment for HIV-1 genome replication and for formation of highly infectious viral particles. For example, release of the *src*-related kinase $p56^{lck}$ from the cytoplasmic tail of CD4 accompanies the CD4 internalization event (41), and liberation of this protein may influence the signal transduction or "activation" status within the cell.

The present studies were undertaken to explore the molecular mechanisms underlying these processes. Specifically, a panel of Nef mutants with site-directed mutations targeted to conserved sequences (47) was analyzed in assays of two recognized functions of Nef, enhancement of infectivity and downregulation of cell surface CD4. The results indicate that the highly conserved membrane-targeting sequences at the N terminus of the Nef protein contribute significantly to the infectivity function. Specifically, these findings confirm the importance of myristylation to this process, as has been suggested previously (55). Moreover, they reveal a role for the basic amino acids adjacent to the myristylation signal, a motif that may influence interactions with polar phospholipid head groups in membranes (48, 49) or may disrupt the N-myristylation process. These alterations at the N terminus of Nef exerted similar dramatic effects in the CD4 downregulation assay, confirming a role for membrane targeting in this process as well. In this regard, it has been proposed that the subcellular localization of Nef may regulate its effects on both viral replication and T-cell activation (6), an idea that is supported by the phenotypes reported here.

Analyses of certain mutations within the conserved central region of Nef uncovered a structure-function discordance regarding these two properties of Nef. Multiple mutations within this region of Nef interfered with its viral infectivity function, such as deletion of a segment encompassing a poly-glutamic-acid stretch. This deletion also interfered with CD4 downregulation function. The deleted segment is contiguous with a proline-X-X repeat region as well as a consensus protein kinase C target site. Importantly, point mutations disrupting the proline-rich segment largely abrogated the infectivity activity of Nef but had no discernible effect on its CD4-modulating activity. Although it remains impossible to exclude differential sensitivities between these assay methods to account for such differences, these observations strongly suggest that the protein domains required for these two activities are only partially overlapping. Moreover, they indicate that the CD4 downregulation event can occur without augmentation of viral infectivity. These findings are consistent with the report of Saksela et al. (43) that was published following completion of the present work.

It should be noted that in such mutational analyses it is difficult to distinguish between effects on domains surrounding a given mutation and distal effects on overall protein conformation. Therefore, we cannot state with certainty that the proline-X-X repeat region per se is involved in the infectivity function of Nef. The present results nonetheless provide a compelling demonstration that the infectivity and CD4 downregulation processes mediated by Nef are not completely linked to one another.

The specific basis for these differential structure-function relationships remains unknown, but several potential explanations can be offered. It is possible that the central proline repeat segment of Nef regulates interaction with a cellular protein required for enhancement of viral infectivity but not for induction of CD4 downregulation. Indeed, SH3 domains specifically bind proline-rich protein domains (40), and the recent report that certain SH3 domains interact with this region of Nef in vitro are intriguing in this regard (43). Although none of the present mutants exhibited *trans*-dominant negative phenotypes in the assay of CD4 downregulation by Nef (data not shown), it remains possible that Nef function requires formation of a heteromultimeric protein complex involving such cellular factors.

The present results strongly imply that CD4 internalization is not an indirect consequence of Nef-mediated events within the cell that influence viral infectivity, such as induction of an intracellular "activation" state. Similarly, enhanced infectivity must not be an inevitable consequence of CD4 downregulation. Thus, current models of Nef function must consider the possibility of more than one primary effect within the cell leading to these two outcomes. The apparent compound functionality displayed by Nef represents an interesting economical mechanism by which HIV-1 may exploit multiple aspects of the host cell to augment its own replication.

The specific role of $p56^{lck}$ in these functions has been controversial. That both the CD4 downregulation and viral infectivity enhancement properties of Nef can be manifested independently of $p56^{lck}$ is suggested by the present and prior work in which these functions were exhibited in cells lacking detectable $p56^{lck}$ (2, 4, 17, 35). However, it remains intriguing that the domains of CD4 mediating Nef-induced downregulation and $p56^{lck}$ binding are closely related to one another (2, 4, 44) and that Nef promotes the dissociation of $p56^{lck}$ from CD4 (41). It has also been reported that $p56^{lck}$ inhibits constitutive CD4 endocytosis by disabling its entry into coated pits (39). The present studies revealed that overexpression of $p56^{lck}$ also

abolishes Nef-mediated downregulation of CD4. This observation implies that the pathway of internalization normally employed by CD4 is similar to that which is promoted by HIV-1 Nef. However, the serine independence of Nef-mediated downregulation (18) and the possibly different fates of CD4 in Nef-dependent and Nef-independent internalization (45) represent distinctions between these two processes. The effect of $p56^{lck}$ appears to depend upon direct competition with a component of the internalization machinery for the CD4 cytoplasmic tail, rather than on its kinase activity. Although exaggerated effects of $p56^{lck}$ were observed in the present studies employing overexpression, a similar but less readily apparent interplay is likely to occur in native lymphocytes expressing these constituents at different relative levels. We therefore speculate that the downregulation of CD4 by Nef in HIV-infected cells is a two-step process requiring both the dissociation of $p56^{lck}$ from CD4 as well engagement of the cellular endocytic apparatus. Further investigation is needed to determine the biologic significance of each of these activities with regard to viral replication, cellular fate, and the development of disease.

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