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The Cytoplasmic Domain of CD4 Is Sufficient for Its Down-Regulation from the Cell Surface by Human Immunodeficiency Virus Type 1 Nef

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Human immunodeficiency virus type 1 Nef down-regulates surface expression of murine and human CD4 but not human CD8. We recently reported that the cytoplasmic domain of CD4 is required for its down-regulation by Nef. Using a chimeric molecule composed of the extracellular and transmembrane domains of human CD8 fused to the cytoplasmic domain of human CD4, we show here that the cytoplasmic domain of CD4 is sufficient for down-regulation by Nef. Since the cytoplasmic domain of CD4 is also the site of its association with p56^{lck}, we used a series of CD4 mutants to determine whether the regions of the cytoplasmic domain of CD4 required for down-regulation by Nef are the same as those required for p56^{lck} binding. Our results indicate that the portion of the cytoplasmic domain required for the down-regulation of CD4 by Nef overlaps with the binding site of p56^{lck}, but the cysteine residues which are essential for the association of CD4 with p56^{lck} are not required. This observation raised the possibility that Nef competes with p56^{lck} for binding to CD4. However, under conditions which are considerably milder than those permissive for coimmunoprecipitation of CD4 and p56^{lck}, we found no evidence for an association between Nef and CD4. While a decrease in total CD4 was observed in lysates of cells expressing Nef, the levels of p56^{lck} were not significantly affected. Pulse-chase experiments further revealed a decrease in the half-life of CD4 in Nef-expressing cells. These results show that the decrease in surface CD4 expression induced by Nef is mediated at least in part by a decrease in the half-life of CD4 protein. These results also indicate that a large portion of p56^{lck} is free of CD4 in T cells expressing Nef, which could have a significant effect on T-cell function.

CD4 is the major cell surface receptor for human immunodeficiency virus (HIV) (15, 32–34, 36) and is an important molecule for T-cell activation (30, 41). Macrophages and T cells infected with HIV in vitro have reduced surface CD4 expression (15, 19, 22, 26, 43, 49). The reduction in surface CD4 expression is due at least in part to the interaction between the HIV envelope protein gp160 and CD4 in the cytoplasm of the infected cell (13, 29, 48) together with the auxiliary effect of Vpu (58, 59). Work from our laboratory and others has demonstrated that the expression of HIV or simian immunodeficiency virus Nef in CD4$^+$ cells also suppresses the expression of both mouse and human CD4 at the cell surface (5, 7, 20, 21, 24, 35). The down-regulation of surface CD4 expression is a property of Nef both from laboratory strains and clinical isolates of HIV-1 (5, 7, 35).

The mechanism by which Nef suppresses CD4 expression still is not known. We recently reported that the cytoplasmic domain of human CD4 is required for its down-regulation by Nef (20). The cytoplasmic domain is highly conserved between mouse and human CD4, sharing 79% identical residues (41), and is the site of association with the Src family tyrosine kinase p56^{lck} (42, 52). Activation of p56^{lck} via engagement of CD4 at the cell surface is critical for antigen-driven stimulation of T cells through the CD3/T-cell receptor complex (12, 23, 50). The purpose of the present study was to determine whether the cytoplasmic domain of CD4 is sufficient for its down-regulation by Nef and whether the regions of the cytoplasmic domain required for Nef-mediated down-regulation are the same as those required for the association of CD4 with p56^{lck}. We have examined the effect of Nef on the surface expression of (i) a chimeric molecule composed of the extracellular and transmembrane domains of human CD8 and the cytoplasmic domain of human CD4 and (ii) a series of murine CD4 molecules containing truncations or mutations within the cytoplasmic tail which affect their association with p56^{lck} (51). Our results show that the cytoplasmic domain of CD4 is both necessary and sufficient for down-regulation by Nef and that the region of the cytoplasmic domain required for down-regulation overlaps the residues involved in the association of CD4 with p56^{lck}. Furthermore, the reduced levels of CD4 at the cell surface correlate with a reduction in steady-state levels of CD4 protein and a decrease in the half-life of CD4 in cells expressing Nef.

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MATERIALS AND METHODS

Construction of the human CD8/CD4 chimeric expression vector. The chimeric human CD8/CD4 construct SV-884 was generated by overlapping PCR, resulting in an in-frame hybrid coding sequence with a junction between amino acid 186 of human CD8 and amino acid 398 of human CD4. The chimeric protein is composed of the extracellular and transmembrane domains of human CD8 fused to the cytoplasmic domain of human CD4 (Fig. 1A). The CD8 and CD4 fragments were amplified separately, mixed, and reamplified with external primers containing a 5′ *Eco*RI site and a 3′ *Xba*I site. The full-length PCR fragment was then cleaved with *Eco*RI and

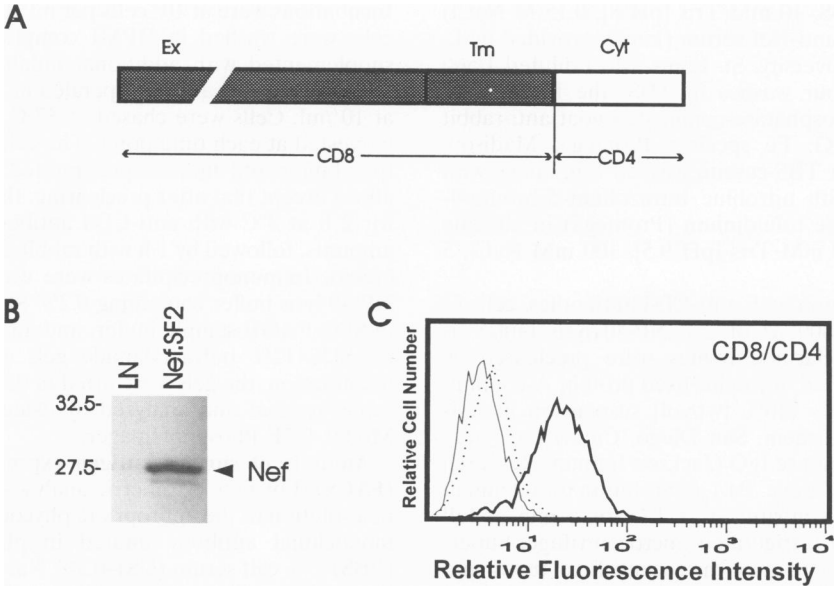

FIG. 1. The cytoplasmic domain of human CD4 is sufficient for down-regulation by Nef. (A) Diagram of the CD8/CD4 chimeric molecule. The extracellular (Ex) and transmembrane (Tm) domains of human CD8 were fused to the cytoplasmic (Cyt) domain of human CD4 as described in Materials and Methods. (B) Nef expression in NIH 3T3 cells expressing the chimeric CD8/CD4 molecule. One percent NP-40 lysates from NIH 3T3 cells expressing the chimeric CD8/CD4 molecules transduced with a control vector (LN) or the Nef expression vector LNefSN were analyzed by Western blotting using rabbit anti-Nef antiserum and alkaline phosphatase-conjugated anti-rabbit IgG (Fc specific). The migration of prestained molecular weight markers is indicated at the left in kilodaltons). The position of the Nef protein is indicated at the right. (C) Surface expression of the chimeric CD8/CD4 molecule is down-regulated by Nef. Cells expressing the chimeric CD8/CD4 molecule and transduced with LN or LNefSN were stained with an FITC-labeled mouse anti-human CD8 or an FITC-labeled control antibody and analyzed by flow cytometry. Data are presented as single-color histograms with FITC fluorescence (surface CD8 expression) along the *x* axis and relative cell number along the *y* axis. Thin solid line, cells transduced with the control vector LN and stained with control antibody; dark solid line, LN cells stained with FITC-labeled anti-human CD8; dotted line, cells transduced with LNefSN and stained with FITC-labeled anti-human CD8.

*Xba*I and cloned into the pSV-7d expression vector (33) to generate pSV-884.

Cell lines and culture conditions. The human T-cell lines HPB-ALL/LN and HPB-ALL/LNefSN and the human monocytic cell lines U937/LN and U937/LNefSN, transduced to express the neomycin phosphotransferase gene (Neo^r) only or HIV-1_{SF2} Nef and Neo^r, have been described previously (21). The AKR1-G1/LN and AKR1-G1/LNefSN murine T-cell lines (20) and HuT-78 human T cells expressing Neo^r or two different primary isolates of HIV-1 Nef (233 and 248) have also been described elsewhere (5). Murine L cells expressing wild-type murine CD4 (L3T4) (20) and L3T4 cDNA expression vectors (51) were generously provided by D. Littman (University of California, San Francisco). NIH 3T3 TK$^-$ cells expressing the murine CD4 mutants were generated by retrovirus-mediated gene transfer as described previously (20). Cells expressing murine CD4 on their surface were selected by fluorescence-activated cell sorting (FACS) as described below. Cells expressing the human CD8/CD4 chimeric molecule (see above and Fig. 1A) were generated by cotransfection of NIH 3T3 TK$^-$ cells with pSV884 and a thymidine kinase expression plasmid as described previously (37).

All human cell lines were cultured in RPMI 1640 medium with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah), penicillin and streptomycin (50 IU and 50 μg/ml, respectively), 2 mM L-glutamine, and 1 mM sodium pyruvate (RPMI complete medium). The human cell lines were maintained at 37°C in a humidified incubator with 5% CO_2. The murine L cells, NIH 3T3 TK$^-$ cells, and AKR1-G1 cells were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum and penicillin-streptomycin at 37°C in a humidified incubator with 10% CO_2.

Retrovirus-mediated transduction of HIV-1 *nef*. The control retroviral vectors LN and LXSH and the Nef expression vectors LNefSN and LNefSH were generated as previously described (20, 38, 39). Briefly, virus-containing supernatants were harvested from confluent plates of PA317 producer cells, filtered (0.45-μm-pore-size filter), and stored at −70°C. For transduction, the cells of interest were infected by overnight incubation with virus-containing supernatants and selected in hygromycin B (200 μg/ml) or G418 (1.5 mg/ml), using standard methodology as described previously (5, 20, 21).

Immunoprecipitation and Western immunoblot analyses. For determination of Nef expression, cells were lysed in 1% Nonidet P-40 (NP-40) buffer (1% NP-40, 20 mM Tris [pH 8], 0.15 M NaCl, 2 mM EDTA) containing 1 μg of leupeptin per ml, 1 μg of aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride for 20 min on ice. The lysates were clarified by centrifugation for 10 min at full speed (16,000 × *g*) in a refrigerated microcentrifuge at 4°C. The supernatant was transferred to a new tube, and 5× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer was added to give a final concentration of 10^6 lysed cells per 20-μl final volume. Protein concentration was determined by using a modified Bradford protein assay kit (Bio-Rad, Hercules, Calif.). Aliquots of lysates containing approximately 100 μg of total protein were separated on SDS–12% polyacrylamide minigels (Mini-Protean II; Bio-Rad) and transferred to nitrocellulose filters (Hybond-C; Amersham Life Science, Arlington Heights, Ill.), using a semi-dry electrotransfer apparatus (Bio-Rad). The filters were blocked with 1% casein in

Tris-buffered saline (TBS; 10 mM Tris [pH 8], 0.15 M NaCl) and probed with rabbit anti-Nef serum (kindly provided by L. Ratner, Washington University, St. Louis, Mo.) diluted 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.), diluted 1:3,000 in TBS-casein, washed four times with TBS, 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 $MgCl_2$).

For immunoprecipitation with anti-CD4 antibodies, cells (3×10^7) were lysed in 800 μl of 1% NP-40 lysis buffer as described above. The clarified lysates were precleared by addition of 60 μl of washed, formalin-fixed protein A-containing *Staphylococcus aureus* (10% [wt/vol] suspension in lysis buffer; Pansorbin; Calbiochem, San Diego, Calif.) precoated with 8 μg of rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) and rotation for 60 min at 4°C. The samples were centrifuged at 4°C for 5 min at full speed (16,000 × *g*) in a refrigerated microcentrifuge. Supernatants were transferred to new microcentrifuge tubes and incubated with 60 μl of *S. aureus* precoated with 4 μg of rabbit anti-mouse IgG plus 2 μg each of the mouse anti-human CD4 monoclonal antibodies L83 and L92.5 (21) for another 60 min at 4°C with rotation. For control immunoprecipitations, the mouse anti-human CD4 antibodies were substituted with an isotype-matched mouse IgG1 (MPC11; Sigma Immunochemicals). For precipitation with anti-Nef antibodies, cells were lysed as described above and precleared with 60 μl of *S. aureus* precoated with 10 μl of normal rabbit serum. Precleared lysates were incubated with 2 μl of rabbit anti-Nef antiserum for 60 min at 4°C on a rotator, 30 μl of *S. aureus* was added, and the lysates were rotated for another 60 min at 4°C. The precipitates were washed three times with 1% NP-40 lysis buffer and once with TBS (10 mM Tris [pH 7.2], 0.15 M NaCl) and resuspended in 40 μl of SDS-PAGE sample buffer. For experiments with digitonin, cells were lysed in buffer containing 1% digitonin (Sigma Chemical Co., St. Louis, Mo.) in place of NP-40 and immunoprecipitated as described above. All washes were also performed with digitonin in place of NP-40. The precipitates were separated on SDS–12% polyacrylamide gels and analyzed for the presence of Nef, $p56^{lck}$, and human CD4 by Western blotting. The Western blotting protocol was essentially as described above except that ^{125}I-protein A (Amersham catalog no. IM.144), 0.2 μCi per blot, was used in place of the secondary antibody, and development was by autoradiography. The rabbit anti-human CD4 antiserum (T4-4) has been described previously (17) and was donated to the AIDS Research and Reference Reagent Program (catalog no. 806) by R. Sweet (SmithKline Beecham Pharmaceuticals, King of Prussia, Pa.). The rabbit anti-$p56^{lck}$ antiserum was a generous gift from K. Amrein (F. Hoffmann LaRoche Ltd., Basel, Switzerland). All antisera were used at a 1:500 dilution for Western blotting. For experiments to determine CD4 and $p56^{lck}$ levels in lysates of human $CD4^+$ cells, Western blots were developed with ^{125}I-protein A, and the integrated optical densities of bands on autoradiographs were quantitated by using a Kodak Visage 110 BioImage system.

Metabolic labeling and immunoprecipitation. U937/LNefSN and U937/LN cells were starved for 20 min in methionine/cysteine-free RPMI 1640 (ICN, Costa Mesa, Calif.) supplemented with L-glutamine, sodium pyruvate, and antibiotics as described above and then labeled for 45 min in the same medium containing 10% dialyzed fetal bovine serum and 100 μCi of Tran-[^{35}S]-label (ICN) per 5×10^6 cells. Incubations were at 10^7 cells per ml at 37°C. After labeling, the cells were washed in RPMI complete medium (see above) supplemented with additional unlabeled amino acids (from 50× stock; ICN) and resuspended in RPMI complete medium at 10^7/ml. Cells were chased at 37°C, and 4×10^6 cells were harvested at each time point. The cells were lysed in 600 μl of lysis buffer and immunoprecipitated essentially as described above except that after preclearing, the lysates were incubated for 2 h at 4°C with anti-CD4 antibodies alone, in saturating amounts, followed by 1 h with rabbit anti-mouse IgG-coated *S. aureus*. Immunoprecipitates were washed three times in 1% NP-40 lysis buffer containing 0.2% SDS, solubilized by boiling in SDS-PAGE sample buffer, and analyzed by electrophoresis on SDS–12% polyacrylamide gels as described above. For quantitation, the gels were dried in the absence of any fluorography reagent and analyzed by using a Molecular Dynamics Model 425F PhosphorImager.

Analysis of surface antigen expression by flow cytometry (FACS). For flow cytometric analyses, cells were resuspended in a solution of the appropriate phycoerythrin (PE)-conjugated monoclonal antibody diluted in phosphate-buffered saline (PBS)–5% calf serum (CS)–0.1% 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/5×10^5 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, 100 μl/10^6 cells. For cell sorting, cells were stained essentially as described above except with filter-sterilized PE-labeled anti-CD4 and were sorted on a Becton Dickinson FACStar Plus instrument. Bulk populations of sorted cells were washed twice in complete medium and cultured as described above. NIH 3T3 cells expressing the CD8/CD4 chimeric protein were stained with fluorescein isothiocyanate (FITC)-labeled mouse anti-human CD8 (Becton Dickinson). The samples were analyzed on a Becton Dickinson FACScan instrument equipped with LYSYS II software. All fluorescence data were collected in the log mode.

RESULTS

The cytoplasmic domain of CD4 is sufficient for down-regulation by Nef. We reported previously that the cytoplasmic domain of CD4 is necessary for down-regulation by Nef (20). To further determine whether the CD4 cytoplasmic domain is sufficient for down-regulation by Nef, we took advantage of our previous observation that expression of human CD8 is not affected by Nef (20). We constructed a chimeric molecule composed of the extracellular and transmembrane domains of human CD8 and the cytoplasmic domain of human CD4 (Fig. 1A). NIH 3T3 cells expressing the chimeric CD8/CD4 molecule were transduced in parallel with the Nef expression vector LNefSN and the control vector LN. Expression of HIV-1_{SF2} Nef in CD8/CD4-expressing cells transduced with LNefSN is shown in Fig. 1B. No Nef or cross-reacting protein was detected in lysates of cells transduced with the control vector LN (Fig. 1B). Analysis of surface expression of the chimeric CD8/CD4 molecule by FACS shows that this molecule is efficiently down-regulated by Nef (Fig. 1C). Our previous results showed that neither human CD8 nor a chimeric molecule with the extracellular domain of human CD4 and the cytoplasmic and transmembrane domains of human CD8 was affected by Nef (20). Together with the data presented here,

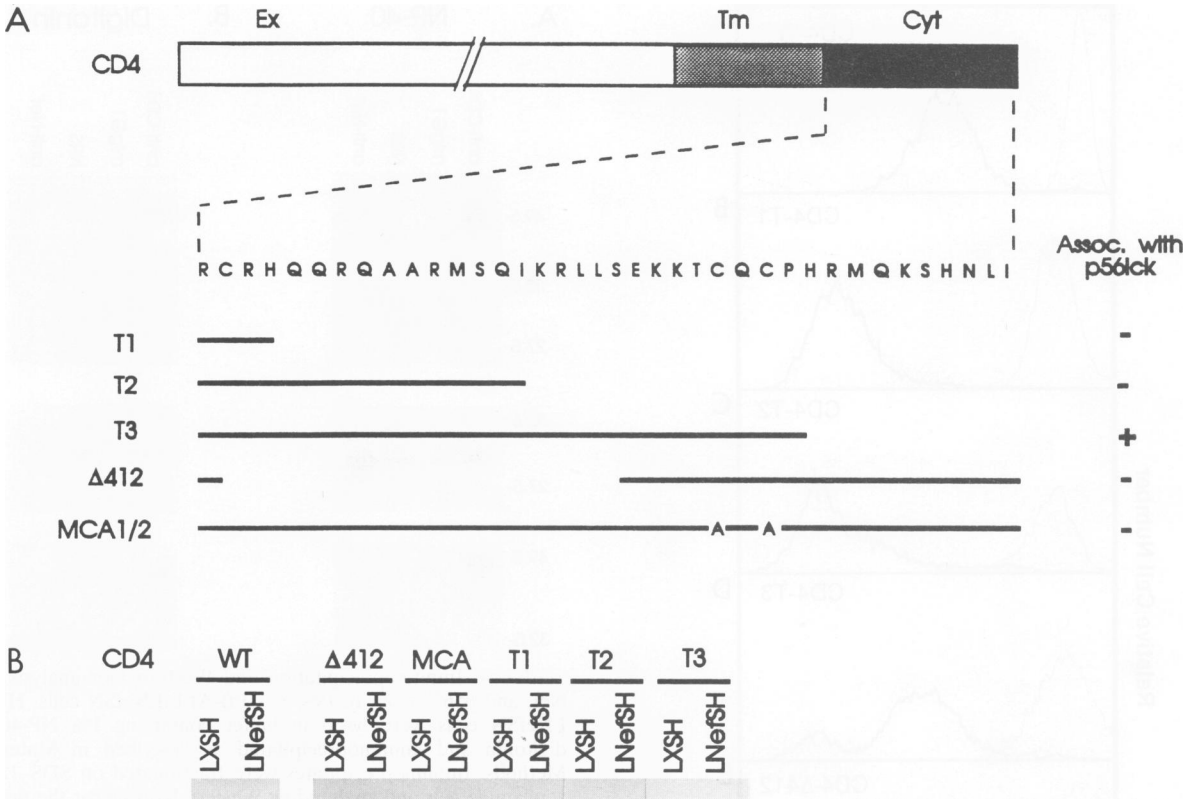

FIG. 2. Nef expression in cell lines expressing murine CD4 mutants. (A) Amino acid sequence of the cytoplasmic tail of wild-type murine CD4 and diagrams of the mutants used in this study. Ex, extracellular domain; Tm, transmembrane domain; Cyt, cytoplasmic tail. The ability of the CD4 mutants to coimmunoprecipitate $p56^{lck}$ is indicated. (Adapted from Turner et al. [51].) (B) Nef expression in transduced cells expressing wild-type murine CD4 or CD4 mutants. One percent NP-40 lysates from L cells expressing wild-type (WT) murine CD4 or NIH 3T3 cells expressing mutant CD4 proteins transduced with a control vector (LXSH) or the Nef expression vector LNefSH were loaded directly onto an SDS–12% polyacrylamide gel, transferred to nitrocellulose, and analyzed by Western blotting. The blots were probed with rabbit anti-Nef antiserum and developed with alkaline phosphatase-conjugated anti-rabbit IgG (Fc specific). Each lane contains approximately 100 μg of total protein. The Nef protein is indicated at the right.

these results demonstrate that the cytoplasmic domain of CD4 is both necessary and sufficient for down-regulation by Nef.

The $p56^{lck}$ binding domain of CD4 overlaps but is different from the region required for cell surface down-regulation by Nef. Since the cytoplasmic domain of CD4 is also the site of its association with $p56^{lck}$, we proceeded to determine whether the region of the CD4 cytoplasmic domain required for down-regulation by Nef is the same as that required for $p56^{lck}$ binding. The residues in the cytoplasmic domain of CD4 involved in the association with $p56^{lck}$ were mapped previously, using a series of mutated murine CD4 molecules (51). Since Nef is able to down-regulate both murine and human CD4 under similar conditions (5, 21), we used this well-characterized set of murine CD4 mutants to determine whether the requirements for binding to $p56^{lck}$ are the same as those required for down-regulation by Nef. Figure 2A shows a diagram of the murine CD4 mutants and their association with $p56^{lck}$ (adapted from Turner et al. [51]). Mutants T1, T2, and T3 are truncation mutants lacking the C-terminal 34, 23, and 10 amino acids of the cytoplasmic domain, respectively. In addition, we tested the mutant MCA1/2, in which the two cysteine residues critical for $p56^{lck}$ association are substituted with alanines, and Δ412, which has a deletion of 18 amino acids within the membrane-proximal portion of the cytoplasmic domain. Of these, only CD4 T3 was able to associate with $p56^{lck}$ (51). Murine L cells expressing wild-type murine CD4 or NIH 3T3 cells expressing the CD4 mutants were transduced with LNefSH or with a control vector (LXSH) in parallel. Transduced cells then were selected in medium containing hygromycin and analyzed for Nef expression by Western blotting. In all cases, we analyzed cell populations rather than individual clones. As shown in Fig. 2B, all of the cell lines transduced with LNefSH express HIV-1_{SF2} Nef. In contrast, no Nef was detected in cells transduced with the control vector LXSH.

To determine which mutant CD4 molecules were down-regulated by Nef, the Nef-expressing and control cells were analyzed for surface expression of CD4 by flow cytometry. The data in Fig. 3 are representative of at least two independent experiments for each CD4 mutant. As shown in Fig. 3A, wild-type murine CD4 was efficiently down-regulated by Nef in L cells, in agreement with our previous results (20). The significant degree of wt CD4 down-regulation occurred despite lower levels of Nef expression (Fig. 2B), indicating that Nef was not limiting in these cells. Of the CD4 truncation mutants T1, T2, and T3, only T3 was significantly down-regulated by Nef. T2 was not affected to a significant extent, and T1 was unaffected. The deletion mutant Δ412 also was not down-regulated by Nef (Fig. 3). Thus, for the deletion and truncation mutants of murine CD4, the sensitivity to down-regulation by

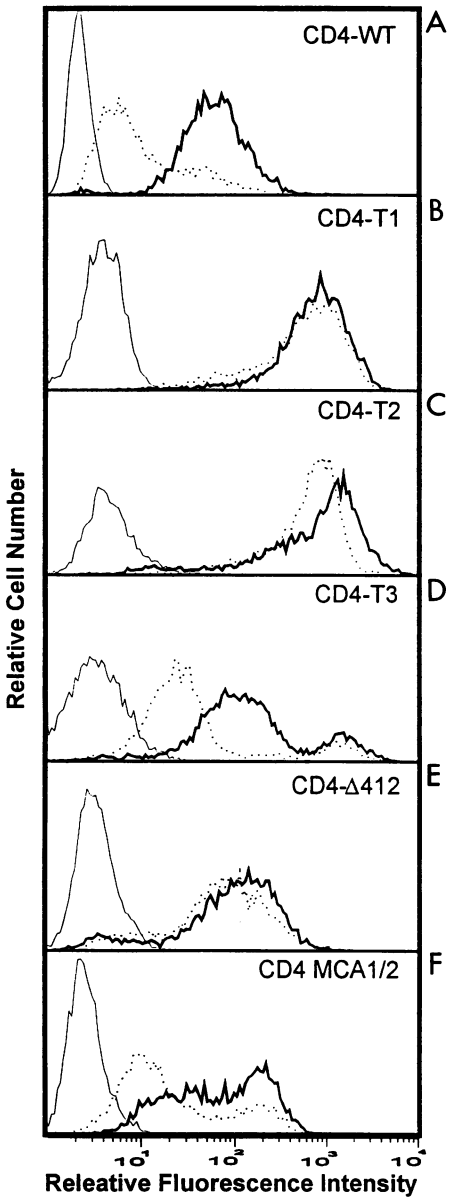

FIG. 3. Surface expression of wild-type or mutant CD4 in cells expressing Nef. L cells expressing murine CD4 (CD4-WT) or NIH 3T3 cells expressing the indicated CD4 mutants (analyzed for Nef expression in Fig. 2B) were stained with a PE-conjugated anti-mouse CD4 monoclonal antibody or isotype-matched control antibody and analyzed by flow cytometry. Bulk populations were analyzed for each of the CD4 variants. Data are presented as single-color histograms with PE fluorescence (CD4 expression) along the *x* axis and relative cell number along the *y* axis. Thin solid lines, cells transduced with the control vector LXSH and stained with the isotype-matched negative control antibody; dark solid lines, LXSH cells stained with anti-CD4; dotted lines, cells transduced with LNefSH and stained for CD4.

Nef correlates with the ability to associate with p56^{lck}. However, this correlation does not hold for the mutant MCA1/2. This mutant lacks by substitution the cysteine residues which are critical for the association of CD4 with p56^{lck} (51). It does, however, produce a full-length CD4 molecule. Despite its failure to associate with p56^{lck}, MCA1/2 is significantly down-regulated by Nef (Fig. 3F).

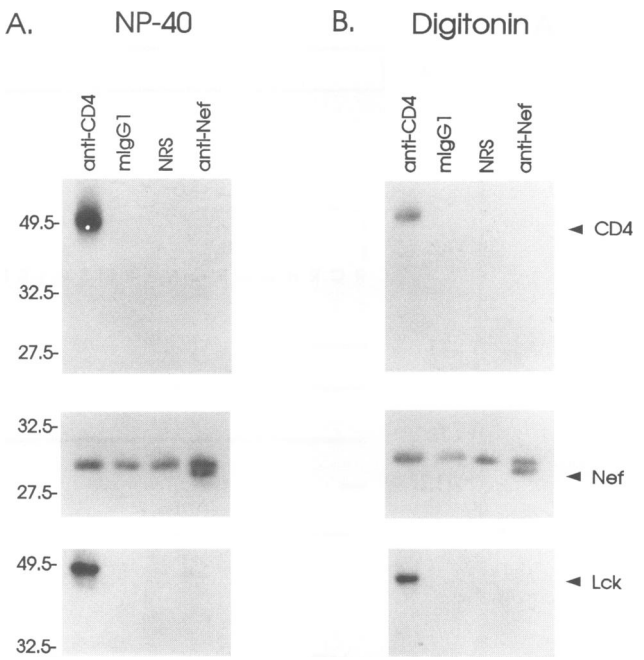

FIG. 4. Immunoprecipitation and Western blot analysis of CD4, Nef, and p56^{lck} from lysates of HPB-ALL/LNefSN cells. HPB-ALL/LNefSN cells were lysed in buffer containing 1% NP-40 or 1% digitonin and immunoprecipitated as described in Materials and Methods. Immunoprecipitates were fractionated on SDS–12% polyacrylamide gels and analyzed by Western blotting for the presence of human CD4, p56^{lck}, or HIV-1 Nef. The blots were probed with the appropriate rabbit antisera and ^{125}I-protein A and analyzed by autoradiography. Each lane contains material immunoprecipitated from the clarified lysates by the indicated antibodies: anti-CD4, monoclonal anti-human CD4; mIgG1, isotype-matched control antibody; NRS, normal rabbit serum; anti-Nef, rabbit anti-Nef antiserum. (A) NP-40 lysates; (B) digitonin lysates. Labels at right identify the antisera used for Western blot analysis and the positions of the CD4, Nef, and p56^{lck} proteins. Note that in the middle panel, Nef appears as the lower of two bands in the fourth lane of each set. The upper band (all lanes) is frequently observed as a nonspecific contaminant in Western blots of immunoprecipitates probed with this anti-Nef antiserum.

Thus, of the CD4 mutants described above, only T3 and MCA1/2 showed significant down-regulation by Nef. All other CD4 mutants were unaffected or minimally affected despite similar levels of Nef expression. These results indicate that the binding site for p56^{lck} overlaps the region of CD4 required for down-regulation by Nef but is different in that the cysteine residues critical for the CD4-p56^{lck} binding are not required for Nef-induced down-regulation.

Failure to coimmunoprecipitate Nef and CD4. The results described above and the similarities between p56^{lck} and Nef suggested that Nef might interact directly with CD4 in a manner similar to p56^{lck} and in so doing displace p56^{lck} from its association with CD4. Therefore, we investigated the possibility of an association between Nef and CD4 by coimmunoprecipitation under conditions significantly milder than those in which CD4-p56^{lck} coprecipitation has been observed (3, 42, 52). One percent NP-40 lysates of Nef-expressing HPB-ALL (human) T cells were immunoprecipitated with anti-CD4 or anti-Nef antibodies and analyzed for the presence of CD4, p56^{lck}, and Nef by Western blotting. Results of a representative experiment are shown in Fig. 4. Immunoprecipitation with anti-CD4 precipitated a protein recognized by the anti-CD4

TABLE 1. Total CD4 and p56^{lck} levels in human CD4$^+$ cells expressing Nef

Cell line	Surface CD4 (CD4 MCF [%])[a,b]	IOD (%)[b,c]		CD4/p56[d]
		Total CD4	Total p56^{lck}	
HPB-ALL/LN	1,348	21.9	4.0	1.0
HBB-ALL/LNefSN.SF2	106 (8)	3.6 (16)	1.7 (43)	0.4
HuT-78/LN	210	10.1	1.2	1.0
HuT-78/LNefSN.233	27 (13)	2.1 (21)	1.9 (160)	0.1
HuT-78/LNefSN.248	33 (16)	1.7 (17)	1.6 (133)	0.1
U937/LN	113	5.3	0	—
U937/LNefSN.SF2	8 (7)	0.3 (6)	0	—

[a] Determined by flow cytometry of cells stained with PE-conjugated anti-human CD4. Data were collected in the log mode. MCF, mean channel fluorescence.

[b] For Nef-expressing cells, percentages of control (LN) levels are indicated in parentheses.

[c] Determined by computerized image analysis of ^{125}I autoradiographs of Western blots probed with anti-CD4 or anti-p56^{lck}. IOD, integrated optical density.

[d] Determined by dividing the normalized values (as percentages) of CD4 by the normalized value (as a percentage) for p56^{lck}. Values of CD4 and p56^{lck} for LN cells were taken as 100%. —, not applicable.

anti-serum in Western blots (Fig. 4A, top). Likewise, the anti-Nef antiserum precipitated Nef (Fig. 4A, middle). However no Nef was detected in CD4 immunoprecipitates, nor did we detect CD4 in Nef immunoprecipitates. Under the same conditions, p56^{lck} was detected in anti-CD4 immunoprecipitates (Fig. 4A, bottom) but not in the anti-Nef precipitates.

Because the interaction between CD4 and Nef might be disrupted by NP-40, we performed parallel experiments in which the cells were lysed in 1% digitonin instead of NP-40. As expected, we were able to demonstrate an association between CD4 and p56^{lck} by coimmunoprecipitation in digitonin lysates of HPB-ALL/LNefSN cells (Fig. 4B, top and bottom). However, we were unable to detect any association of CD4 with Nef in the digitonin lysates of HPB-ALL/LNefSN cells with this technique (Fig. 4B, top and middle). Thus, we found no evidence for a physical association between Nef and CD4 by coimmunoprecipitation under conditions in which the association of CD4 and p56^{lck} is demonstrated. Furthermore, a significant amount of the residual CD4 in HPB-ALL cells expressing Nef is still associated with p56^{lck} (Fig. 4), indicating that the presence of Nef does not completely disrupt the association between CD4 and p56^{lck}. Similar results were obtained with AKR1-G1 murine T cells (not shown).

The ratio of CD4 to p56^{lck} is decreased in T cells expressing Nef. In T cells, up to 95% of p56^{lck} is tightly associated with CD4 expressed at the cell surface, although estimates vary significantly from cell line to cell line (25, 28). T cells expressing Nef have 5 to 20% of the normal levels of surface CD4 as determined by flow cytometry (5, 20, 21). Thus, it was of interest to determine whether total CD4 and total p56^{lck} levels were reduced in parallel with the decrease in surface CD4 expression. HPB-ALL and HuT-78 human CD4$^+$ T cells and the CD4$^+$ human monocyte/macrophage line U937 transduced with LNefSN or the control vector LN were analyzed for CD4 and p56^{lck} expression. Figure 5A to C show that surface CD4 expression was lower in HPB-ALL, U937, and HuT-78 cells expressing Nef than in cells transduced with the control vector LN. Total CD4 and total p56^{lck} levels in these cells were determined by Western blot analysis of whole lysates (Fig. 5D) and quantified by computerized image analysis of ^{125}I autoradiographs (Table 1). In all cell lines tested, there was a

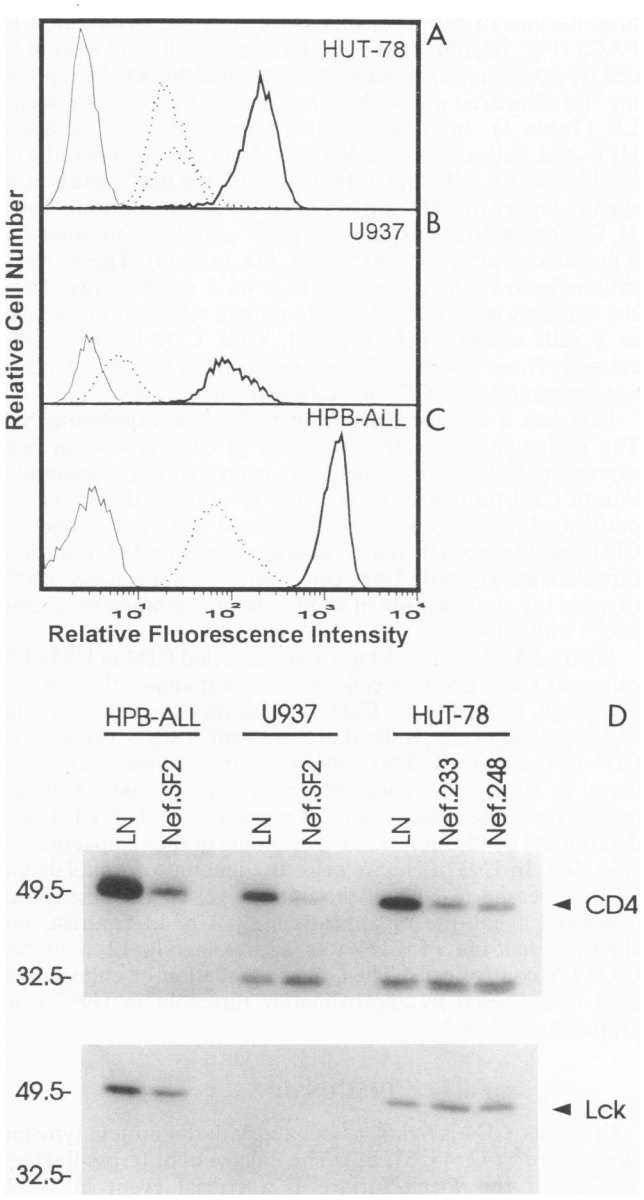

FIG. 5. Analysis of total CD4 and p56^{lck} levels in human CD4$^+$ cell lines expressing Nef. (A to C) Surface CD4 expression in human CD4$^+$ cell lines transduced with HIV-1 Nef. All cells were analyzed for surface CD4 expression by flow cytometry at the time at which the lysates were made. Data are presented as single-color histograms with PE fluorescence (CD4 expression) along the *x* axis and relative cell number along the *y* axis. Thin solid lines, cells transduced with the control vector LN and stained with the isotype-matched negative control antibody; dark solid lines, LN cells stained with anti-CD4; dotted lines, cells transduced with LNefSN and stained for CD4. The Nef isolate is from HIV-1$_{SF2}$ for HPB-ALL and U937 cells and from two primary isolates of HIV-1 (233 and 248, [5]) for HuT-78 cells. (D) Western blot analyses of CD4 and p56^{lck} levels in lysates of human CD4$^+$ cells. Cells were lysed in buffer containing 1% NP-40, clarified, and fractionated by SDS-PAGE on 12% gels. Each lane contains approximately 100 μg of total protein. The relative amounts of CD4 and p56^{lck} protein in the lysates were determined by Western blot analysis and ^{125}I autoradiography. Migration of prestained molecular weight markers is indicated at left in kilodaltons. Labels at right identify the antiserum used for Western blot analysis and the positions of the CD4 (top) and p56^{lck} (bottom) proteins.

decrease in total CD4 as determined by Western blotting proportional to the level of CD4 expression determined by FACS (Fig. 5D, top; Table 1). The decrease in total CD4 in all cell types was approximately three- to fivefold in cells expressing Nef compared with cells transduced with the control vector LN (Table 1). In contrast, the decrease in p56^{lck} levels in HPB-ALL cells expressing Nef was about 50% compared with the LN control cells, and in HuT-78 cells, the p56^{lck} levels were slightly higher in cells expressing Nef (Fig. 5D, bottom; Table 1). U937 cells do not express any p56^{lck} and therefore served as a negative control for p56^{lck} (Fig. 5D, bottom). These observations have been reproduced in several experiments. Thus, the tendency is for p56^{lck} levels to remain relatively unchanged in T cells expressing HIV-1 Nef, while CD4 levels are decreased. These results indicate a net increase in p56^{lck} which is not associated with CD4 in Nef-expressing T cells.

CD4 has a shorter half-life in U937 cells expressing Nef. The reduction in steady-state levels of CD4 protein in cells expressing Nef indicated that there is no significant accumulation of CD4 protein in these cells. To address this point, we performed pulse-chase experiments to determine whether CD4 degradation is increased in cells expressing Nef. For these experiments, we chose U937 cells, which do not express p56^{lck}, to avoid the complication of coimmunoprecipitation of labeled p56^{lck} with CD4.

Figure 6A shows the decay of radiolabeled CD4 in U937/LN cells and U937/LNefSN cells. By 4 h post-chase, there was a significant reduction in CD4 immunoprecipitated from the Nef-expressing cells, while there was only a slight decrease in CD4 in the control (LN) cells. In both cell lines, a gradual decay in surface CD4 was observed over the next 8 h. To quantitate these results, the amount of labeled CD4 was determined by PhosphorImager scanning of the radioactive gel (Fig. 6B). In U937/LNefSN cells, the amount of radiolabeled CD4 decreased to 50% of the initial level by 4 h post-chase, indicating a half-life of approximately 4 h. In contrast, the apparent half-life of CD4 was approximately 12 h in the U937/LN control cells. Thus, the degradation of endogenous CD4 is increased by approximately threefold in U937 cells expressing HIV-1 Nef.

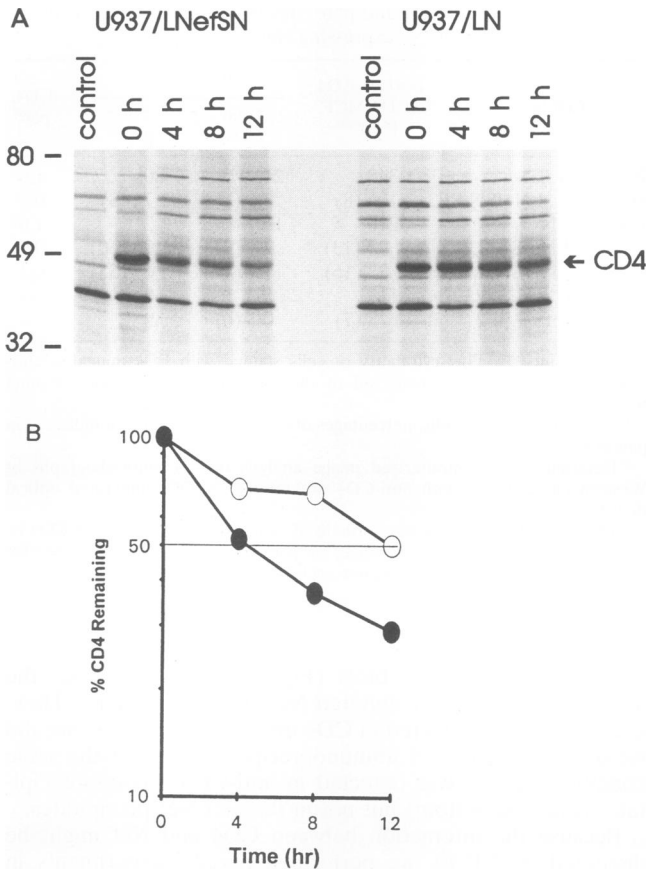

FIG. 6. Nef increases degradation of endogenous CD4. U937/LNefSN and U937/LN (control) cells were pulse-labeled for 45 min with [^{35}S]methionine/cysteine and chased for the indicated times. Cell lysates were immunoprecipitated with monoclonal antibodies to human CD4 as described in Materials and Methods, and the immunoprecipitates were analyzed by electrophoresis on SDS–12% polyacrylamide gels. (A) Autoradiograph of anti-CD4 immunoprecipitates from U937/LNefSN and U937/LN cells. The position of the band corresponding to CD4 is indicated by the arrow (right), and the migration of prestained molecular weight markers is indicated at left in kilodaltons. Control lanes contain immunoprecipitates made with a mouse IgG1 isotype-matched control antibody. Results shown are from one of two independent experiments. (B) Quantitation of CD4 levels in U937/LN cells (○) and U937/LNefSN cells (●). The amount of labeled CD4 remaining at each time point was determined by PhosphorImager analysis of the radioactive gels. The percentage of CD4 at each time point was calculated by comparison with the amount of CD4 at the initial time (0 h). Values are averages of two independent experiments.

DISCUSSION

In T cells, CD4 is tightly associated with the protein tyrosine kinase p56^{lck} (42, 45, 51, 52). The engagement (cross-linking) of CD4 at the T-cell surface is a crucial event in T-cell activation (12, 23, 50, 53, 54). Our results indicate that the cytoplasmic domain of CD4 is sufficient for down-regulation by Nef and that the domain required for down-regulation overlaps the site of association with p56^{lck} (45, 51). However, the two cysteine residues which are essential for association with p56^{lck} are not required for down-regulation by Nef. The overlapping of the regions of the CD4 cytoplasmic domain required for Nef-induced down-regulation and p56^{lck} binding and the similarities between p56^{lck} and Nef raised the possibility that Nef also associates with CD4. Therefore, we attempted to coimmunoprecipitate Nef with anti-CD4, and vice versa, from HPB-ALL/LNefSN cells under conditions significantly milder than those reported for the coimmunoprecipitation of CD4 and p56^{lck} (3, 42, 52). Under the conditions tested, we found no evidence for a physical association between CD4 and Nef. However, the small amount of CD4 remaining in these cells was still associated with p56^{lck}.

The mechanism by which Nef suppresses CD4 expression is not known. The fact that down-regulation of surface CD4 expression by Nef occurs without a significant effect on steady-state mRNA levels (21) and requires the cytoplasmic domain of CD4 (20) suggests that the effect of Nef on CD4 is both posttranscriptional and posttranslational. CD4 is down-regulated from the surface of mouse and human T cells following exposure to phorbol esters such as phorbol myristate acetate (PMA) (1, 4, 8, 18, 27, 31, 46, 56). PMA activates protein kinase C, which in turn phosphorylates three serine residues in the cytoplasmic domain of CD4. This phosphorylation leads to the internalization and subsequent degradation of CD4 (8, 18, 27, 46). Mutant CD4 molecules in which the serine residues have been substituted with alanines are no longer sensitive to down-regulation by PMA (18, 46) but are down-regulated by Nef (21). This observation rules out an obligatory role for protein kinase C phosphorylation of CD4 in the down-regulation by Nef.

Dissociation of p56^{lck} from CD4 accompanies or is required for the down-regulation of CD4 by PMA (28, 47). In contrast, Nef is able to suppress the expression of CD4 in B-cell, monocytic, and fibroblast cell lines which do not express p56^{lck} (20, 21) as well as in T-cell lines. Thus, the ability of Nef to down-regulate CD4 is neither dependent on nor inhibited by the presence of p56^{lck}. Instead, it is likely that the residual CD4 expressed on the surface of Nef-expressing T cells has escaped the action of Nef and is able to associate with p56^{lck} (Fig. 4).

In a previous study, we failed to observe a significant decrease in total CD4 protein in HPB-ALL/LNefSN cells by immunoprecipitation of metabolically labeled CD4 (21). Our current results, obtained by using a more sensitive and reproducible Western blot analysis, indicate that there is a decrease in total CD4 protein levels in cells expressing Nef. By direct Western blot analysis of total lysates, we have observed a reduction in total CD4 protein levels in all human CD4$^+$ cells expressing Nef that we have tested. This reduction was observed in T-cell, B-cell, and monocyte/macrophage cell lines, and with one laboratory isolate (SF2) and two different primary isolates (233 and 248 [5]) of HIV-1 Nef (Fig. 5), and suggest a lack of intracellular accumulation of CD4 in cells expressing Nef. Results of pulse-chase experiments using improved methods for immunoprecipitation revealed an increase in CD4 degradation in U937/LNefSN cells compared with the U937/LN control cells. These results are consistent with either an increase in endocytosis of fully mature CD4 molecules from the cell surface and subsequent degradation of the internalized CD4 or with degradation of CD4 molecules before they reach the cell surface. Experiments to distinguish between these two possibilities are currently in progress.

Expression of HIV envelope protein (gp160) in CD4$^+$ cells inhibits expression of CD4 at the cell surface (13, 29, 48). CD4-gp160 complexes form in the endoplasmic reticulum and Golgi complex and are subsequently degraded in lysosomes (13, 57–59). Degradation of CD4 is further enhanced in the presence of Vpu (58, 59) and also occurs in vitro in the presence of Vpu alone (11, 55). Thus Env, Vpu, and Nef provide HIV with three different mechanisms by which it can suppress the expression of its cell surface receptor, CD4. The *nef* gene is expressed early after infection, while *env* and *vpu* are expressed later, as mature virions are forming (14). It has been postulated that suppression of CD4 expression is beneficial to the virus by preventing superinfection (7, 21) and by promoting shedding of the virus from infected cells (7). Infected T cells with reduced levels of surface CD4 also would be expected to be less susceptible to envelope-dependent fusion with other infected cells (7, 21, 48) or to the possible toxic effects of soluble gp120 (6).

A reduction in CD4 expression caused by Nef might also affect the activation and function of T cells by altering the levels or distribution of p56^{lck}. It is estimated that up to 95% of p56^{lck} is associated with CD4 in T cells and T-cell lines (25, 28). Our current results indicate that cells with Nef have about 5 to 20% of the normal levels of surface CD4 and also have less total CD4 than do control cells without Nef. In contrast, there is no concomitant decrease in the amount of total p56^{lck} in T cells expressing Nef. These observations suggest that a significant proportion of p56^{lck} in Nef-expressing T cells is no longer associated with CD4 but could associate with another cell surface receptor. Engagement of CD2 on the surface of T cells has been shown to induce T-cell activation (2, 9, 10, 40), probably involving p56^{lck} (16, 44). Observations by Haughn et al. (25) suggest that cells lacking CD4 and grown in the continuous presence of interleukin-2 (IL-2) (e.g., T-cell clones) receive compensatory p56^{lck} signaling through the IL-2 receptor and thus maintain normal responsiveness to anti-T-cell receptor stimulation. This situation could have important consequences for host-virus interactions at the cellular level by altering T-cell responses to antigenic stimulation. In the absence of normal CD4 signaling, an association of p56^{lck} with other signal-transducing receptor molecules, such as CD2 or the IL-2 receptor, might maintain the ability of T cells to respond to external stimuli. In this way, the HIV-infected cell, with lower levels of CD4, could still be activated and thereby replicate virus. HIV has three different mechanisms to suppress CD4 expression. As indicated above, only Nef is expressed early after infection. The significance of this receptor down-regulation mechanism for in vivo virus spread and pathogenesis remains to be elucidated.

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