JOURNAL OF VIROLOGY, Sept. 2005, p. 11422–11433 Vol. 79, No. 17
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.17.11422–11433.2005

Human Immunodeficiency Virus Nef Induces Rapid Internalization of the T-Cell Coreceptor CD8αβ

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Received 29 April 2005/Accepted 13 June 2005

Human immunodeficiency virus (HIV) Nef is a membrane-associated protein decreasing surface expression of CD4, CD28, and major histocompatibility complex class I on infected cells. We report that Nef strongly down-modulates surface expression of the β-chain of the CD8αβ receptor by accelerated endocytosis, while CD8 α-chain expression is less affected. By mutational analysis of the cytoplasmic tail of the CD8 β-chain, an FMK amino acid motif was shown to be critical for Nef-induced endocytosis. Although independent of CD4, endocytosis of the CD8 β-chain was abrogated by the same mutations in Nef that affect CD4 down-regulation, suggesting common molecular interactions. The ability to down-regulate the human CD8 β-chain was conserved in HIV-1, HIV-2, and simian immunodeficiency virus SIVmac239 Nef and required an intact AP-2 complex. The Nef-mediated internalization of receptors, such as CD4, major histocompatibility complex class I, CD28, and CD8αβ, may contribute to the subversion of the host immune system and progression towards AIDS.

The human immunodeficiency virus type 1 (HIV-1) Nef protein is a 27-kDa protein that is abundantly produced during the early phase of viral gene expression (28, 54). Nef is posttranslationally modified by phosphorylation and due to irreversible attachment of myristic acid to its N terminus, it is targeted to the cellular membrane. Nef has multiple distinct functions: it modulates cell surface molecules, such as CD4 (21), CD28 (63), major histocompatibility complex (MHC) class I (59), MHC class II and MHC class II-associated invariant chain (Ii, CD74) (62), interferes with signal transduction pathways (reviewed by Tolstrup et al. [65]), T-cell generation (61, 67), and enhances virion infectivity and viral replication (reviewed by Fackler and Baur [17]). The molecular mechanisms of most of these effects and their contribution to pathogenesis are only partially understood.

To modulate cell surface receptor expression, Nef utilizes several strategies, linked to distinct regions within the Nef protein. Like many other pathogenic viruses, HIV-1 down-regulates the cell surface expression of MHC class I and circumvents in this way the attack by cytotoxic T lymphocytes (59). Another profoundly investigated Nef-mediated effect is down-regulation of the CD4 receptor (21, 1), due to accelerated endocytosis via clathrin-coated pits followed by lysosomal degradation (51). In addition, CD4 down-regulation by HIV-1 and simian immunodeficiency virus (SIV) Nef proteins also involves intracellular retention mechanisms (55). As Nef has been shown to interact with the CD4 receptor as well as with the adaptor protein (AP) complex, either AP-1 (8, 16, 33), AP-2 (16, 22, 26), or AP-3 (33), it may act as a connector between components of the cellular endocytic machinery and the cytoplasmic tail of CD4 (13, 41, 42). A Nef dileucine sequence was found to be required for accelerated internalization of CD4 and CD28 from the cell surface to endosomes and lysosomes (8, 13, 25), rendering Nef the only nontransmembrane protein known to traffic via a dileucine-based motif (35).

The T-cell CD8 coreceptor exists as an αα homodimer, found on intestinal T cells, γδ T cells, thymic T-cell precursors, and NK cells, and an αβ heterodimer, most commonly expressed on thymocytes and on peripheral T cells (19, 31). The surface expression of the CD8 β-chain is dependent on expression of the CD8 α-chain, to which it becomes covalently linked in the endoplasmic reticulum (24). The cytoplasmic tail of CD8α comprises 30 amino acids and contains a motif of two vicinal cysteines for interaction with the Src kinase $p56^{lck}$ by means of a zinc chelate complex (68).

Although the tail of CD8β consists of only 19 residues and contains no known protein binding motifs, studies in mice indicated a role for CD8β and its cytoplasmic tail in thymic development and in activation of $CD8^+$ T cells (3, 4, 5, 32). Pathological conditions in which $CD8\alpha^+\beta^{low}$ and CD8αα T-cell receptor αβ T cells increase in the periphery include Wiskott-Aldrich syndrome, where peripheral blood $CD8^+$ T-cell receptor αβ T cells mostly express CD8αα homodimers (34), and HIV infection in which the appearance of a major CD8 subpopulation with reduced CD8β chains may occur (58).

Here, we report that HIV-1 as well as HIV-2 and SIVmac239 Nef down-regulate cell surface expression of the human CD8αβ receptor. The CD8 β-chain cytoplasmic tail contains an FMK amino acid sequence that allows Nef-mediated modulation. Based on our results we suggest Nef is using clathrin-mediated endocytosis, requiring AP-2, for accelerated down-

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regulation of CD8αβ and CD4. As a subset of $CD8^+$ T cells have been shown to be infected by HIV (39), down-regulation of CD8αβ might harm CD8 lymphocyte function and contribute in this way to HIV-mediated subversion of the immune system.

MATERIALS AND METHODS

Plasmid constructions. All retroviral *nef* constructs were made as previously described (61). The deletion mutant Del 3 was provided by J Zack (2), and SIVmac239 and HIV-2 Rod were obtained from J. Skowronski and R. Benarous, respectively. The CD8α (GenBank accession number NM_001768) gene, kindly provided by S. Bonatti, was subcloned in the LZRS vector, in which the internal ribosome entry site (IRES)-enhanced green fluorescent protein (EGFP) cassette was deleted by NotI digestion. The CD8β (GenBank accession number NM_004931) gene, also provided by S. Bonatti, was tagged with hemagglutinin (HA) for confocal imaging and was subcloned in the BamHI and EcoRI sites in LZRS-IRES-EGFP or BamHI and NotI sites in LZRS-IRES-ΔNGFR. In all experiments, control transductions were done with the parental LZRS vectors expressing only the marker gene.

The CD8α(EC-TM)–CD8β(IC) chimera was constructed using the primer pair CD8α S (BamHI): 5′-TATTGGATCCATGGCCTTACC-3′; CD8α EC+ TM AS (SalI): 5′-CTTACGTCGACGTGATAACCAGTGACAG-3′ and CD8β IC S (SalI): 5′-CTTACGTCGACACACCTGTGCTGC-3′; CD8β AS (NotI): 5′-ACTATAGCGGCCGCTTATTTGTAAAATTG-3′. The CD8α(EC-TM)–CD8α(IC) was constructed as a control, using the primers CD8α IC S (SalI): 5′-CTTACGTCGACACTTTACTGCAAC-3′; CD8α AS (NotI): 5′-TTTATAG CGGCCGCTTAGACGTATC-3′ for amplification of the CD8α cytoplasmic tail. For both constructs, the two PCR fragments were SalI digested and ligated. In the next step the CD8αβ and CD8αα chimers were amplified by PCR, BamHI-NotI digested, and ligated in the LZRS-IRES-ΔNGFR vector.

For confocal microscopy, LZRS-Nef.EGFP-N2 fusion protein constructs were designed. The EGFP-N2 gene was isolated from the pEGFP-N2 vector (Clontech, Palo Alto, CA) by BamHI-NotI digestion and cloned into the LZRS vector, in which the IRES-EGFP cassette was excised by NotI digestion. In a next step the *nef* genes NA-7 wild-type, PPAA, and LLAA were amplified by PCR, and BamHI-BamHI inserted 5′ of the EGFP sequence. The RNAi probes were BglII-HindIII cloned into the pSUPER vector (9) containing an extra EcoRI restriction site 3′ of the cloning site, further referred to as pSUPER(EcoRI).

To construct the 19-nucleotide hairpin short interfering RNA cassettes, two cDNA oligonucleotides were chemically synthesized (Invitrogen, Merelbeke, Belgium), annealed, and inserted immediately downstream of the H1 promoter: 5′-GATCCCC-19-TTCAAGAGA-19-TTTTTGGAAA-3′ and 5′-AGCTTTTCC AAAAA-19-TCTCTTGAA-19-GGG-3′. The target sequences for each of the genes were as follows: AP-2 μ2 subunit (GenBank accession number NM_004068), 5′-GTGGATGCCTTTCGGGTCA-3′ (47), clathrin heavy chain (GenBank accession number NM_004859), 5′-TATCTCGCTTGCTCAGCGT-3′, and dynamin-2 (GenBank accession number NM_004945), 5′-GACATGAT CCTGCAGTTCA-3′. The control short interfering RNA was a functional oligonucleotide with p53 as the target sequence and described elsewhere (9).

Lentiviral packaging plasmid p8.91 and vesicular stomatitis virus envelope plasmid (pMD.G) were kindly provided by D. Trono (Université de Genève, Geneva, Switzerland); transfer vector TRIPΔU3-CMV-EGFP was kindly provided by P. Charneau (Hôpital Necker, Paris, France). A PCR-amplified WPRE cassette (GenBank accession number J04514, nucleotides 1093 to 1685) (69) was inserted into plasmid TRIPΔU3-CMV-EGFP at the unique XhoI site downstream of the EGFP stop codon, resulting in TRIPΔU3-CMV-GFP-WPRE. Next, the H1 RNA interference (RNAi) cassettes were EcoRI-EcoRI transferred from the pSUPER(EcoRI) vector into the EcoRI-digested TRIPΔU3-CMV-EGFP-WPRE vector. Sequencing (ABI, Perkin Elmer, Foster City, CA) confirmed the integrity of all constructs.

Production of retroviral and lentiviral supernatants. The Phoenix-Amphotropic packaging cell line was transfected with the different retroviral constructs as previously described (61). For lentivirus production, 293T cells were seeded 24 h before transfection. Transfection of the three plasmids was done using a calcium phosphate transfection kit (Invitrogen) and viral supernatant was harvested 40 h later.

Cell culture and chemical products. All cells were cultured at 37°C in a humidified atmosphere containing 7.5% (vol/vol) CO_2 in air. Peripheral blood mononuclear cells were isolated by density separation (Lymphoprep, Nyegaard, Oslo, Norway) of buffy coats or whole blood. SupT1 (AIDS Research and Reference Reagent Program, National Institutes of Health, Bethesda, MD), Daudi, 293T, and other cells were cultured as described previously (61). Freshly isolated peripheral blood mononuclear cells were stimulated at day 0 with phytohemagglutinin (2 μg/ml) and interleukin-2 (50 IU/ml), and fresh interleukin-2 was added every 3 days of culture. 4-Hydroxytamoxifen was purchased from Sigma-Aldrich (Bornem, Belgium) and ikarugamycin was obtained from LKT Laboratories (St. Paul, MN).

Viral gene transfer. For retroviral transduction of cell lines and peripheral blood mononuclear cells, cells were mixed with viral supernatant which was incubated for 10 min with Dotap (Roche Diagnostics, Penzberg, Germany). To increase transduction efficiency, cells were spun (90 min, 2500 rpm, 32°C). Lentiviral transductions were carried out in the presence of Polybrene (4 μg/ml; Sigma-Aldrich). Transduction efficiency was evaluated by flow cytometry 48 to 72 h after transduction and varied between 20 and 50% with retroviral supernatant and between 50 and 100% with lentiviral supernatant. The degree of Nef-mediated down- or up-regulation of a receptor was evaluated by measurement of the surface level expression of the receptor at day 2 after transduction. The fraction of down- (or up-) regulation of a receptor was calculated by subtracting the mean fluorescence intensity (MFI) of cells with a fixed range of high marker gene expression (EGFP or ΔNGFR) from the MFI value of the cells lacking the marker gene and then dividing this result by the MFI of the cells lacking the marker gene (62).

Monoclonal antibodies, flow cytometry, and cell isolation methods. Mouse anti-human monoclonal antibodies used were CD28 (Leu-28; phycoerythrin; Becton Dickinson Immunocytometry Systems [BDIS], Mountain View, CA), CD4 (Leu-3a phycoerythrin or allophycocyanin; BDIS), CD8α (SK1 phycoerythrin or allophycocyanin; BDIS), CD8αβ (2ST8.5H7 phycoerythrin; Coulter, Miami, FL), CD74 (LN2; Immucor, Heppignies, Belgium), HLA-DR (L243 allophycocyanin; BDIS), HLA-A, B, and C (G46-2.6 phycoerythrin; BDIS), and nerve growth factor receptor (NGFR) low-affinity receptor (ME20.4 phycoerythrin or allophycocyanin; Chromaprobe, Maryland Heights, MO).

CD74 was detected through a phycoerythrin-conjugated goat anti-mouse $F(ab')_2$ antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The cells were analyzed on a FACSCalibur flow cytometer (BDIS), as previously described (66). Daudi cells transduced with CD8β-IRES-ΔNGFR or CD8α constructs were purified by positive selection using a phycoerythrin-conjugated monoclonal antibody assigned to NGFR and CD8α, respectively, followed by anti-phycoerythrin superparamagnetic microbeads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). SupT1 cells, transduced with (mutant) Nef-IRES-EGFP constructs, were sorted using a FACSVantage (BDIS).

Endocytosis assays. The fluorescence-activated cell sorting-based endocytosis assay (10), using a phycoerythrin-conjugated monoclonal antibody to CD8αβ, CD4, or CD28, was performed as described previously (50). For the calculation of internalization, a region was set on the EGFP-positive cells. The fraction of receptor internalized was calculated by subtracting the MFI of the background signal (the initial time zero acid wash) from all MFI values obtained and then dividing this result by the MFI of the total bound antibody (41).

Immunoblotting. Transduced SupT1 cells, sorted to homogeneity, were lysed in Laemmli sample buffer and equal amounts of proteins were run on 8 or 12% Novex Tris-glycine-polyacrylamide gels (Invitrogen) as previously described (61). Primary monoclonal antibodies used included mouse anti-Nef EH-1 (AIDS Research and Reference Reagent Program) (11), mouse anti-AP50 (Transduction Laboratories, Lexington, KY), mouse anti-clathrin heavy chain (Covance, Berkeley, CA), and rabbit anti-dynamin-2 (Affinity Bioreagents, Golden, CO).

Confocal microscopy. Transduced or transfected 293T cells were cultured on coverslips, fixed in methanol for 15 min at −20°C, and dried at room temperature. After a 10-min rehydration in phosphate-buffered saline, cells were blocked for 30 min at room temperature (0.4% fish skin gelatin [Sigma-Aldrich] in phosphate-buffered saline), followed by incubation for 60 min with a mouse anti-human CD8β primary antibody (5F2 [15], Serotec, Oxford, United Kingdom) or mouse anti-HA antibody (HA.11, Covance), both 1:100 diluted in blocking solution. After washing in phosphate-buffered saline, cells were stained with Alexa-Fluor 594-conjugated goat anti-mouse immunoglobulin G secondary antibody (Molecular Probes Inc, Eugene, OR) for 1 h (1:100 diluted in blocking solution). After a fourfold wash step, nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI)/methanol for 5 min, and after a final wash, coverslips were mounted onto glass slides using Vectashield (Vector Laboratories Inc, Burlingame, CA). Confocal images were collected with a blue diode Bio-Rad Radiance 2100 confocal laser scanning system (Bio-Rad) and were processed using the Confocal Assistant (CAS) program (Bio-Rad) and Adobe Photoshop (Adobe, San Jose, CA).

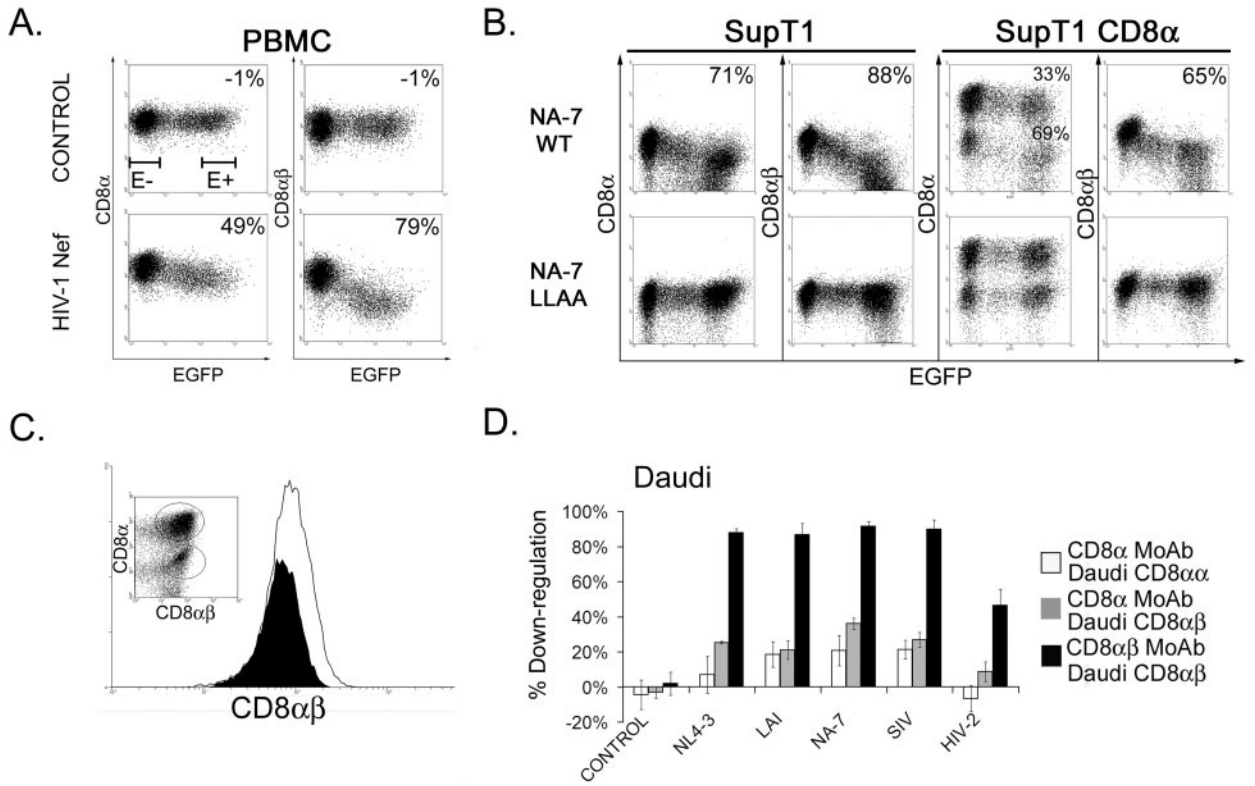

FIG. 1. Flow cytometric evaluation of Nef-mediated CD8 down-regulation in retrovirally transduced cells. (A) Bivariate dot plots (CD8α-allophycocyanin, CD8αβ-phycoerythrin versus EGFP) of flow cytometric measurement of Nef^- (control) and Nef^+ (NA-7 allele) transduced peripheral blood mononuclear cells, gated on $CD8^+$ cells, at day 3 after transduction. (B) Bivariate dot plots of flow cytometric measurement (CD8α-allophycocyanin, CD8αβ-phycoerythrin versus EGFP) of Nef NA-7 wild-type and NA-7 LLAA transduced SupT1 cells (left) and SupT1 cells overexpressing CD8α (right), at day 2 after transduction. (C) The solid and open histograms show the CD8αβ expression profile of SupT1 $CD8\alpha^+$ cells and SupT1 $CD8\alpha^+$ cells (CD8α-transduced population), respectively, gated as shown in the inset. (D) Daudi CD8αα cells and Daudi CD8αβ cells were transduced with control, HIV-1 (NL4-3, LAI and NA-7), SIV (mac239), and HIV-2 (Rod) Nef. Percent down-regulation is shown with white bars for CD8α in Daudi CD8αα, with gray bars for CD8α in Daudi CD8αβ, and with black bars for CD8αβ in Daudi CD8αβ. All percentages were calculated, as described in Materials and Methods, using the ranges E− and E+, as indicated in A.

RESULTS

Nef induces a decrease in CD8αβ surface expression in peripheral blood mononuclear cells and cell lines. A Nef-induced decrease in CD8αβ surface levels has been reported by our group in fetal thymic organ culture experiments, using human primary T-cell precursors (61, 67). In order to investigate whether this observation was conserved between primary cells, peripheral blood mononuclear cells were retrovirally transduced with bicistronic constructs expressing HIV-1 Nef and EGFP as a reporter protein. For flow cytometric analysis, cells were stained with a monoclonal antibody directed against the CD8αβ heterodimer or an anti-CD8α, the latter recognizing both αβ and αα dimers. Surface staining of CD8αβ revealed a dose-dependent decrease in the steady-state expression levels of this receptor with increasing Nef expression (alleles NA-7 [Fig. 1A], LAI, and NL4-3 [data not shown]), resulting in a 79% down-regulation in mean fluorescence intensity compared to nontransduced cells, whereas the decrease in CD8α staining was less pronounced.

We also verified this observation in cell lines. In the $CD8\alpha\beta^+$ T-cell line SupT1, expression of Nef evoked a similar decrease in CD8αβ surface levels (Fig. 1B, left panels). However, since Nef down-regulates several surface markers on T cells, decreased CD8αβ expression could be the consequence of Nef-mediated internalization of another surface molecule, such as CD4 or CD28, in close proximity to the CD8 receptor. Therefore, we generated Daudi B-cell lines stably expressing the CD8αα or CD8αβ receptor after retroviral gene transfer of only CD8α or both CD8 chains, further referred to as Daudi CD8αα and CD8αβ, respectively.

Figure 1D gives an overview of the ability of different wild-type HIV-1, HIV-2, and SIVmac239 Nef alleles to down-modulate CD8 in Daudi cells. Wild-type Nef-transduced cells are characterized by a strong decrease in CD8αβ surface expression levels, i.e., an 8- to 13-fold decrease in MFI for HIV-1 and SIVmac239. A lower functional activity was found with HIV-2 Rod Nef (a twofold decrease), an observation that was also made for other surface marker modulations (data not shown and (57)). These observations in the Daudi B-cell line demonstrate that down-regulation is independent of the presence of neighboring T-cell surface molecules that are known Nef targets. Nef-mediated down-modulation of the CD8 α-chain was three- to fourfold lower than that of the β-chain in Daudi CD8αβ cells (Fig. 1B). In the Daudi CD8αα cell line, NL4-3-

and HIV-2-mediated CD8α down-regulation did not differ significantly from control transduced cells, while LAI, NA-7, and SIVmac239 Nef caused CD8α down-regulation to a degree comparable to that observed in the Daudi CD8αβ cells (Fig. 1B).

An intriguing finding in Nef-transduced SupT1 cells was that, besides staining with CD8αβ monoclonal antibody, CD8α monoclonal antibody staining also showed a prominent decrease in surface staining (88% and 71% down-regulation, respectively) (Fig. 1B, left panels). Additional transduction of these cells with the CD8α chain resulted in a distinct population with a higher CD8α staining profile (Fig. 1C). However, only a slight increase in CD8αβ surface staining could be observed in these cells (Fig. 1C and 1B, right panels), suggesting that the amount of CD8β in these cells limits the formation of CD8αβ heterodimers, the excess of CD8α likely giving rise to CD8αα homodimer expression. Whereas this $CD8\alpha^{high}$ population showed a weakened down-regulatory response (33%), Nef-mediated down-regulation of the CD8αβ receptor remained strong (65%) (Fig. 1B, right panels). In conclusion, these data suggest that Nef strongly reduces CD8αβ surface receptor expression, while it reduces the CD8 α-chain surface expression only to a lower degree.

Concordance between CD8αβ down-regulation and Nef-mediated down-/up-regulation of other surface receptors. Retroviral transduction of SupT1 and Daudi cells with wild-type and mutant Nefs, aligned in Fig. 2A, was performed to assess their ability to down-regulate cell surface expression of CD4, MHC class I, CD28, CD8αβ (SupT1), and HLA-DR (Daudi) (Fig. 2B) or to up-regulate CD74 (Daudi) (Fig. 2C). As judged from Western blotting (Fig. 2D), all constructs were correctly expressed, their expression levels correlating with the mean fluorescence intensity of the sorted SupT1 cells (data not shown). Mutations within Nef that diminished its modulating capacity more than 50%, compared to the respective wild-type Nef, are underlined in Fig. 2B and 2C. The results indicate that the same mutations within Nef that abrogate down-regulation of CD4 also abolish down-regulation of CD8αβ.

Structurally, these can be divided into three groups: the N-terminal anchor region (G2A and Del3), the W57L58 sequence (WLAA), known as the binding site for the CD4 receptor (42), and the flexible loop harboring a dileucine (LLAA and LLGG) and E/D174D175 (EDAA and DDGA) sequence. With regard to Nef-mediated CD28 down-regulation, we found one more mutant (E4A) capable of disturbing the wild-type Nef effect. Of note, up-regulation of CD74 in Daudi cells can be prevented by the same mutations, with the exception of Del3, as those affecting down-regulation of CD4/CD8αβ. HLA-DR down-regulation was a rather weak effect, only abrogated by the G2A mutant, while the MHC class I down-regulating capacity of the panel of Nef mutants showed no resemblance to the other receptor modulations. In conclusion, these data suggest that down-regulation of CD4, CD28, and CD8αβ and the up-regulation of CD74 result from related functions of Nef.

Nef induces accelerated internalization of the CD8αβ receptor resulting in a down-regulated steady state surface expression. To explore the mechanism of down-regulation, a comparison of Nef-mediated CD4 and CD8αβ down-regulation was done in transduced SupT1 cells in function of time. Therefore, we used an inducible NA-7 Nef.ER fusion protein (61), activated at time zero of the experiment by adding 1 μM 4-hydroxytamoxifen to the cells. As shown in Fig. 3A, Nef-mediated down-regulation kinetics of both receptors were comparable: within 2 h, half of the steady-state level (reached after approximately 10 h) of down-regulation is achieved. These fast kinetics are due to accelerated internalization of cell surface receptors, as shown with a fluorescence-activated cell sorting-based assay using the inducible Nef.ER (Fig. 3B). Nef induced an acceleration of the endogenous internalization of CD4, CD8αβ, and CD28. The basal (Nef-negative) and Nef-mediated (Nef-positive) internalization kinetics for CD4 and CD8αβ were almost identical, while those measured for CD28 were both higher.

Mutational analysis of the CD8β cytoplasmic tail. In other known Nef-targeted surface receptors, essential residues in the cytoplasmic tail have been identified. To determine these residues in the cytoplasmic tail of the CD8 β-chain, series of Daudi cells, coexpressing CD8α and a CD8β deletion mutant, were transduced with wild-type Nef to evaluate the steady-state down-regulation levels (Fig. 4A and 4C) and internalization kinetics (Fig. 4B and 4D) of the CD8αβ receptors. In the absence of Nef, both wild-type and mutant CD8αβ showed low levels of down-regulation (Fig. 4C) and internalization kinetics (wild-type Nef-negative in Fig. 4B and data not shown). However, in the presence of Nef, truncation of the CD8 β-chain at amino acid 206 weakened down-regulation and internalization by Nef (Fig. 4A and 4B). Deletion of three more amino acids further decreased the steady state down-regulation ±2-fold, suggesting that amino acids 204 to 206 were important for this Nef effect. Further truncating the CD8β tail did not have any additional effect. Remarkably, deletion of the complete cytoplasmic β-tail could not entirely block CD8αβ down-regulation.

In a next step, we generated targeted amino acid substitutions in this particular cytoplasmic region and performed down-regulation and internalization experiments. As shown in Fig. 4C and 4D, the weakest down-regulation and internalization potential of Nef was observed with the mutant 204/5/6 Ala, followed by the mutants 204/5/6 Ser, 204/6 Ser, and 204 Ala, suggesting an important function for residue 204. Interestingly, for reasons that are unclear, the 206A mutant showed an even higher internalization rate than wild-type CD8β. However, this was not reflected by its Nef-induced down-regulation, which was lower than the control. In summary, our mutational analysis of the CD8β cytoplasmic tail revealed a C-terminal motif (FMK) implicated in Nef-mediated internalization, in which phenylalanine residue 204 seemed to be the most important. The residual down-regulation (approximately 20%) and internalization potential observed suggest some role for the CD8α cytoplasmic tail.

CD8α cytoplasmic tail is not required for Nef-mediated down-regulation of the CD8 β-chain. To investigate the role of the CD8α cytoplasmic tail in Nef-mediated down-regulation, we designed a CD8α(EC+TM)-CD8β(IC) chimera and stably expressed this molecule in Daudi cells. The CD8αβ chimeric receptor and the CD8αα chimeric control receptor showed comparable high expression levels, as judged by MFI (Fig. 5, control). In contrast to the construct with the CD8α cytoplasmic tail, which was only moderately affected by HIV-1 Nef or

A.

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LAI    MGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEAQEEEEVGFPV
NL4-3  MGGKWSKSSVIGWPAVRERMRRAEPAADGVGAVSRDLEKHGAITSSNTAANNAACAWLEAQEEEEVGFPV
NA-7   MGGKWSKRSAGGWSAVRKRMEQAEPAADGVGAVSRDLEKYGAITSSNTATNNADCAWLEAQEEEEVGFPV

          G2A                                                Del3          WLAA  E4A

PQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQGYFPDWQNYTPGPGVRYPLTFGW
PQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQGYFPDWQNYTPGPGVRYPLTFGW
PQVPLRPMTYKAAVDISHFLKEKGGLEGLIHSQRRQDILDLWIYHTQGFFPDWQNYTPGPGIRYPLTFGW

PPAA              D86A                    R106A

YKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDPEREVLEWRFDSRLAFHHVARELHPEYFKNC&
YKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDPEREVLEWRFDSRLAFHHVARELHPEYFKNC&
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                  LLGG        DDGA
                  LLAA        EDAA
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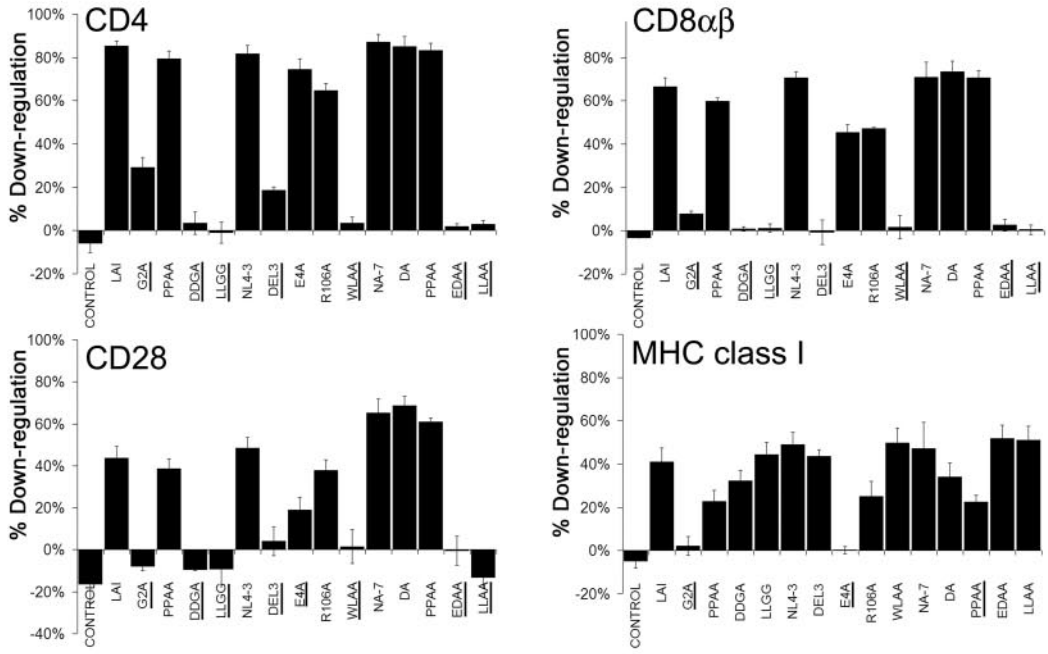
B.



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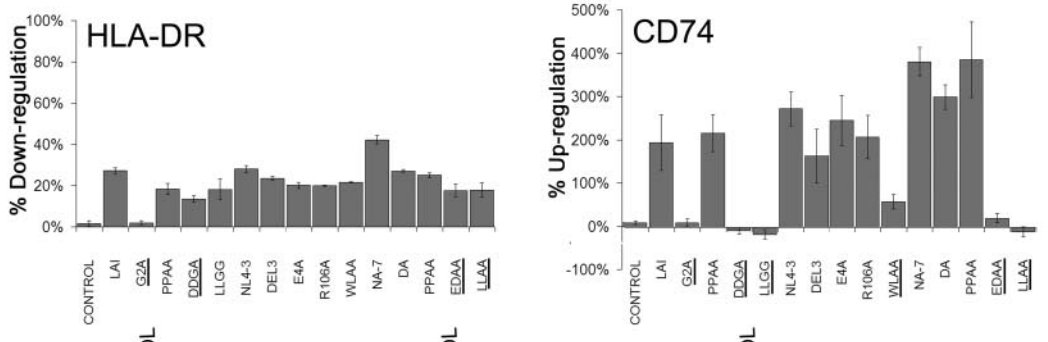

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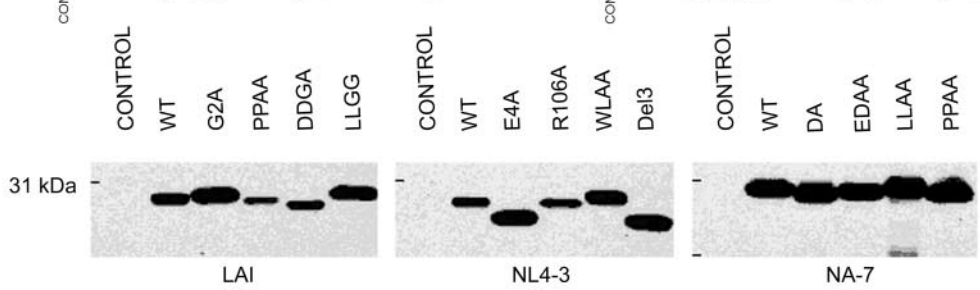

SIVmac239 Nef, down-regulation of the chimeric CD8αβ surface marker was very pronounced following Nef expression (Fig. 5). Indeed, by combining two CD8β cytoplasmic tails (in the chimera), instead of a CD8α and a CD8β cytoplasmic tail, the down-regulating capacity of Nef was elevated drastically, pointing towards a preferential, direct or indirect interaction between Nef and the short cytoplasmic CD8β tail. We conclude that, while the CD8α cytoplasmic tail is dispensable for Nef-mediated CD8αβ down-regulation, the CD8β cytoplasmic tail harbors a potent target sequence for down-regulation.

Colocalization of Nef and CD8 β-chain. To gain more insight into the intracellular localization of CD8αβ in Nef expressing cells, 293T cells expressing both CD8αβ and Nef.EGFP fusion protein were examined by confocal microscopy. Nef was localized in the cytoplasm and at the cellular membrane. In the absence of Nef, the CD8 β-chain was found at the cellular membrane and showed some cytoplasmic staining (Fig. 6, lower panel, Nef.EGFP-negative cells). In the presence of EGFP-tagged wild-type Nef and the PPAA Nef mutant, both being able to down-regulate HA-tagged CD8β (data not shown), the CD8 β-chain was localized in submembranous vacuoles (Fig. 6, upper and lower panels). In contrast, membranous CD8β expression was retained in cells coexpressing the Nef mutant LLAA, which fails to downregulate CD8β (Fig. 6, middle panel).

Repression of Nef-mediated CD8β endocytosis. Based on strategies, used to abrogate Nef-mediated CD4 endocytosis (37, 40, 55), repression of Nef-mediated CD8β endocytosis was tested. A decline in Nef-mediated CD8αβ internalization was observed after addition of ikarugamycin, a macrolide antibiotic postulated to be a general inhibitor of clathrin-coated pit-mediated endocytosis (40), to Nef-expressing Daudi CD8αβ cultures (Fig. 7A). To block distinct steps in endocytic trafficking, lentiviral RNA interference (RNAi) constructs were designed, expressing EGFP as well as a short hairpin RNA, to induce knockdown of three key molecules in clathrin-mediated endocytosis, i.e., AP-2 complexes by μ2 chain RNAi (AP-2 RNAi), clathrin heavy chain (Chc RNAi), and dynamin-2 (Dyn-2 RNAi).

Transduction of SupT1 cells with any of these RNAi constructs resulted in a strong depletion of the respective proteins (Fig. 7B), which had functional implications, as evidenced by a more than fivefold increase of the transferrin receptor surface expression (data not shown). Nef and control constructs were expressed from a bicistronic construct expressing a truncated human nerve growth factor receptor (ΔNGFR) as the reporter, allowing simultaneous flow cytometric evaluation of the expression levels of RNAi (EGFP), Nef (ΔNGFR), and a surface molecule (e.g., CD4) (Fig. 7C). Loss of AP-2 complexes in SupT1 cells markedly impaired HIV-1 or SIVmac239 Nef-mediated down-regulation of CD4 and CD8αβ compared to the control (p53 RNAi) (Fig. 7D). The knockdown of clathrin heavy chain had no effect on Nef-mediated receptor endocytosis. Remarkably, knocking down dynamin-2 hampered CD4 and CD8αβ down-regulation by HIV-1 Nef, but not by SIVmac239 Nef. This inhibition was less pronounced with the NA-7 allele (data not shown). In conclusion, these experiments demonstrate a common role for the AP-2 μ2 subunit in HIV-1 and SIVmac239 Nef-mediated receptor internalization and point to a similar mechanism used by Nef to down-modulate CD4 and CD8αβ surface expression.

DISCUSSION

In this study, we demonstrated that HIV-1, SIVmac239, and HIV-2 Nef functionally interact with the CD8 β-chain of the CD8αβ receptor, leading to an accelerated internalization of this receptor. Mutational analysis of several HIV-1 Nef alleles and mechanistic exploration indicated that Nef-mediated CD8αβ and CD4 endocytosis are closely related effects, suggesting that Nef utilizes similar mechanisms to internalize both receptors.

Based on our confocal images, showing colocalization of the CD8 β-chain with the HIV-1 Nef protein in perinuclear vacuoles, and our observation that the CD8 α-chain is dispensable for the down-regulation effect, we suggest that Nef interacts directly or indirectly with the CD8β cytoplasmic tail. Our attempts to demonstrate a molecular interaction between Nef and CD8 in mammalian cells all failed (data not shown), which might not come as a surprise, as the interaction between Nef and CD4, suggested to be weak and/or transient, has also only been demonstrated in vitro and in insect cells (27, 29, 52, 56).

Although the mechanism of Nef-mediated down-regulation of CD4, the receptor for HIV virions, has already been intensively studied for more than a decade, the ability of Nef to internalize the other T-cell coreceptor, i.e., CD8αβ, was never addressed in human cells. This can be explained by the fact that the most convincing evidence for the susceptibility of CD8 lymphocytes to HIV infection has been gathered only in the last years (7, 18, 30, 39, 44, 60). Moreover, most laboratories are using an antibody assigned to the CD8 α-chain for CD8 flow cytometric measurements instead of the 2ST8.5H7 clone, having an epitope on the CD8αβ heterodimer. The use of the CD8α monoclonal antibody clone also explains why Garcia et

FIG. 2. Flow cytometric mutational analysis of Nef-mediated down- or up-regulation of cell surface molecules in retrovirally transduced SupT1 and Daudi cells. SupT1 cells and Daudi cells were transduced with control virus or wild-type or mutant HIV-1 Nef (alleles NL4-3, LAI, and NA-7). Percent down-regulation was calculated, as described in the Materials and Methods, using the ranges indicated in Fig. 1A. (A) Alignment of the amino acid sequences of the HIV-1 Nef alleles LAI, NL4-3, and NA-7. Underlined amino acids represent the positions that are changed within the indicated mutant Nef protein, and shaded amino acids in NL4-3 and NA-7 are different from the LAI sequence. (B) Comparison of the down-regulating activity of control and Nef constructs in SupT1 cells by using monoclonal antibodies against CD4-allophycocyanin, CD28-phycoerythrin, CD8α-allophycocyanin, and CD8αβ-phycoerythrin. (C) Comparison of the modulating activity of control and Nef constructs in Daudi cells by using monoclonal antibodies CD74/goat anti-mouse-phycoerythrin and HLA-DR-allophycocyanin. The values in B and C are means and standard deviations calculated from the data generated from three independent experiments. Mutations that abrogate the modulating capacity of Nef for more than 50%, compared to the respective wild-type Nef, are underlined. (D) Western blot analysis of Nef expression in transduced SupT1 cells. Lysates of sorted SupT1 cells, transduced with virus as indicated, were stained for Nef. The marker indicates the position of 31 kDa.

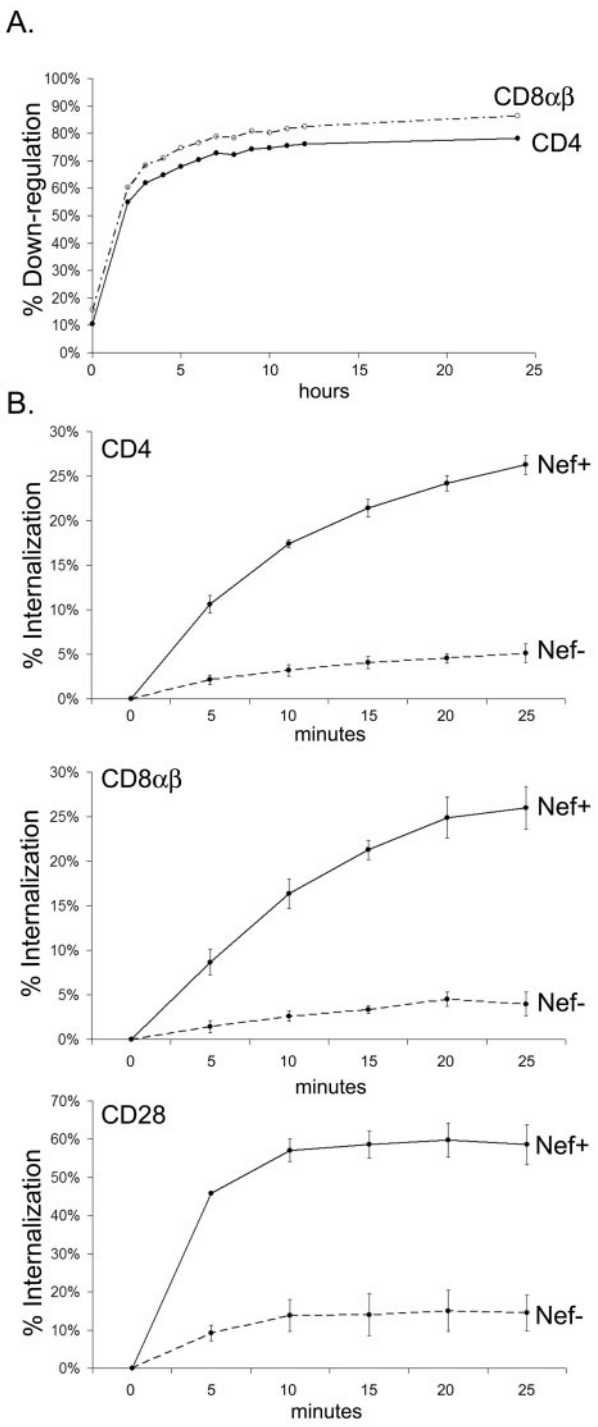

FIG. 3. Flow cytometric analysis of Nef-mediated receptor down-regulation and internalization in retrovirally transduced SupT1 cells. SupT1 cells were transduced with an inducible NA-7.ER construct. At time zero, 4-hydroxytamoxifen (1 μM) was added to the culture medium. (A) Percent down-regulation was calculated, as described in the Materials and Methods. CD4-allophycocyanin (solid line) and CD8β-phycoerythrin (dashed line) were measured as a function of time. (B) The figure shows the percentage of CD4, CD8αβ, and CD28 molecules internalized by HIV-1 NA-7.ER, calculated as described in Materials and Methods. In each graph, Nef-positive (EGFP expressing Nef, solid line) and Nef-negative (EGFP not expressing Nef, dashed line) cells are depicted. The EGFP ranges used for calculation are indicated in Fig. 1A.

al. claimed that Nef fails to affect the human CD8 receptor (20). However, our results in Daudi CD8αα cells indicate that there is some effect on the CD8 α-chain, suggesting a minor interaction of Nef with the CD8α cytoplasmic tail.

Previous reports have provided insight into the mechanisms involved in Nef-mediated CD4 down-regulation. Nef accelerates normal CD4 clathrin-dependent internalization by functioning as a connector between CD4 and the AP component of the clathrin complex. In a next step, the CD4 receptor is not recycled to the membrane, as occurs in normal conditions, but is misrouted by Nef to the lysosomes. In contrast to the dynamic trafficking properties of CD4, the CD8αα and CD8αβ receptors are slowly endocytosed, regardless of the presence of $p56^{lck}$ or phorbol esters (6, 43). In our internalization assays 0 to 4% of CD8αβ was found intracellularly in Daudi CD8αβ and SupT1 cells at equilibrium (25 min), increasing to 25 to 35% in the presence of Nef.

Efficient endocytosis of cell surface glycoproteins through clathrin-coated vesicles requires the presence of endocytosis signals (49). Whereas the cytoplasmic tails of CD4, CD28, CD74, and MHC class I all contain a putative sorting signal (35, 59, 63), this is not the case for the CD8α and β-chain cytoplasmic tails. However, as Nef can bring its own dileucine sorting signal, its recruitment, directly or indirectly, to the cytoplasmic tail may be sufficient to induce accelerated CD8αβ endocytosis, raising the question of whether a native CD8 endocytic signal would even be involved in this process. In this respect, mutational analysis of the CD8β tail showed a sequence (FMK), close to the CD8β carboxy terminus, to be essential for efficient Nef-mediated CD8β endocytosis.

Remarkably, we still observed a residual CD8αβ down-regulation of this mutant, as well as of the cytoplasmic tail-deleted CD8 β-chain mutant, probably reflecting the modulating capacity of Nef on the CD8 α-chain. The FMK sequence is to our knowledge not part of a known endocytic consensus sequence and, remarkably, is present in all membrane-associated splice variants of CD8β which, through alternative splicing, only start to differ immediately C-terminal of this sequence (48). Based on information from secondary-structure prediction methods (45), this FMK sequence would be part of a helix structure which comprises almost the complete CD8β cytoplasmic tail. Similarly, residues in the cytoplasmic tail of CD4, necessary for Nef-mediated CD4 down-regulation, are located within the helical part of the CD4 cytoplasmic tail. Based on mutational studies, a correlation was demonstrated between the presence of an α-helix in CD4 and its susceptibility to down-regulation by Nef (53), which might also be the case for Nef-mediated CD8αβ down-regulation.

Our results demonstrate that at least three sites within Nef are required for CD8αβ down-regulation, the myristoylation signal and N-terminal anchor regions, the C-terminal flexible loop, and amino acid positions 57 to 58. Consistent with all reported Nef functions, the myristoylation signal was found to be essential for CD8αβ down-modulation. The flexible loop contains a dileucine-based internalization motif, which is flanked by acidic clusters ($^{154}EEX_8LLX_8DD^{175}$), and is involved in enhanced internalization of the Nef-CD4 complex (23). This dileucine sequence, located at amino acid positions 164 to 165 of the Nef molecule, is involved in the association of HIV-1 Nef with the AP complex (8). Consequently, mutations

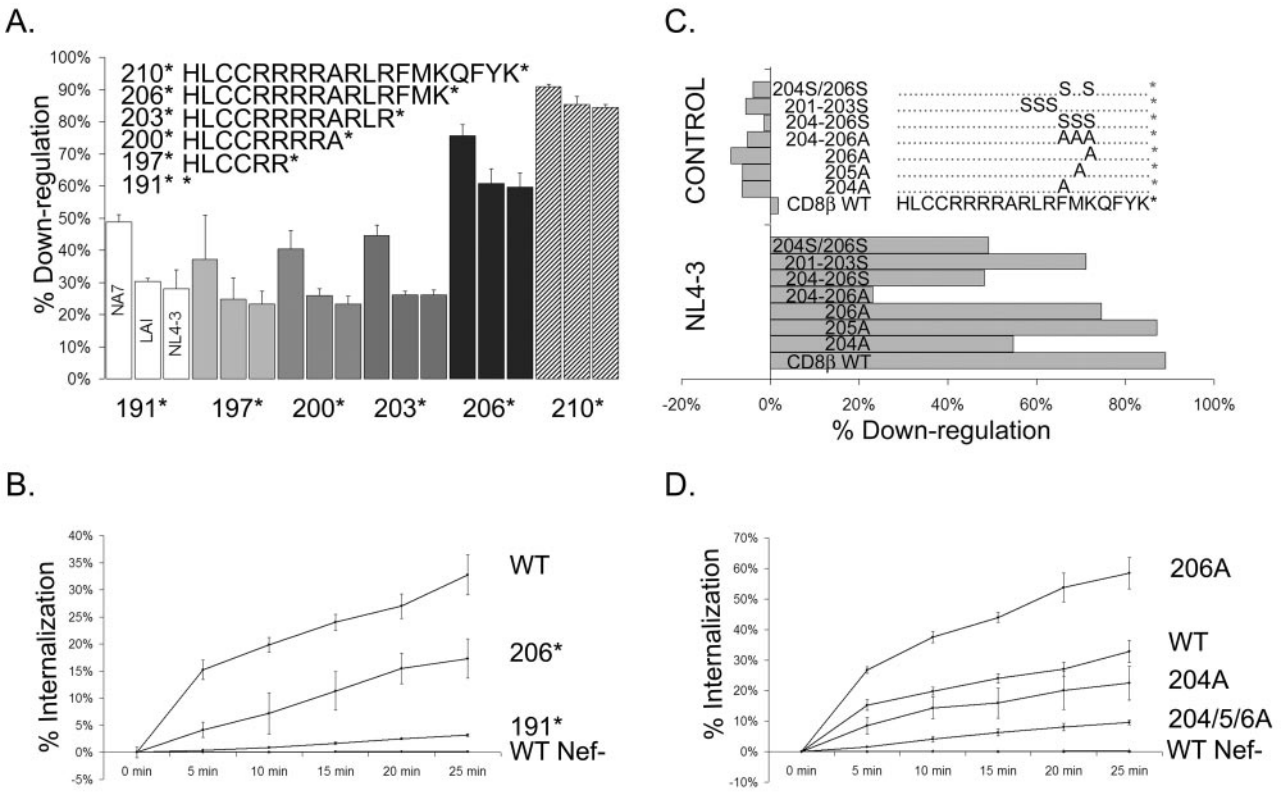

FIG. 4. Mutations in the CD8 β-chain and their effect on endocytosis and down-regulation. Daudi cells were cotransduced with CD8α, wild-type or mutant CD8β, and control or wild-type Nef. (A and C) Alignment of amino acid sequences of wild-type (210*) and mutant CD8 β-chain cytoplasmic tails. An asterisk indicates a stop codon. (A) The bar chart shows the percent down-regulation of (mutant) CD8αβ by HIV-1 Nef alleles NA-7, LAI, and NL4-3. In both B and D the percentage of (mutant) CD8αβ molecules internalized by wild-type HIV-1 NA-7 is shown, including in both the same data for a Nef-negative construct as a control (wild-type Nef−). (C) The bar chart represents the percentage of (mutant) CD8αβ down-regulation after transduction with either control virus or wild-type Nef NL4-3. Each bar represents a (mutant) CD8 β-chain, as indicated by the changed amino acid sequence compared with CD8αβ. Percent down-regulation and internalization were calculated as described in Materials and Methods, using the ranges indicated in Fig. 1A. In A, B, and D, mean values are shown and standard deviations are calculated from the data generated from three independent experiments. In B the results for NL4-3 are representative of the results with HIV-1 alleles NA-7 and LAI.

in the flexible loop at these dileucine sequences or at acidic positions 174 to 175 drastically changed the modulating capacities of Nef, preserving only MHC class I down-regulation (Fig. 2B) (57).

Mutation of amino acid positions 57 to 58, denoted the CD4 interaction site, abolished the capacity of Nef to modulate CD8αβ as well as CD4 surface expression. Furthermore, we found this sequence to be required for CD74 upregulation and, in agreement with Swigut and coworkers, for CD28 internalization (63). How Nef can use the same acceptor site (or overlapping sites) to form a complex with both CD4 and CD28, as well as the CD8 β-chain and CD74, even when the target sequences within these receptors are different, remains an open question. Even more intriguing is our observation that Nef apparently can use the same mechanism to down-regulate and to up-regulate (CD74) receptor surface expression levels.

A number of studies demonstrate that most if not all Nef functions are genetically separable (57). However, based on the results of our structure-function analysis, we could not genetically separate the ability of HIV-1 Nef to down-regulate CD8αβ and CD4. Mutation of the complete acidic cluster at amino acid positions 62 to 66 separated the effect of HIV-1 Nef

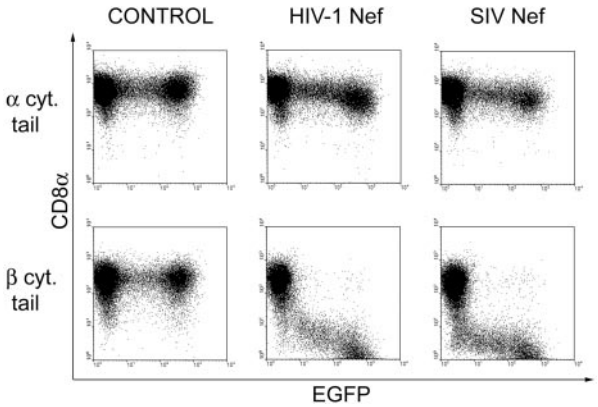

FIG. 5. Chimeric constructs. Daudi cells were retrovirally transduced with the CD8α(EC-TM)-CD8α(IC) chimera (cyt tail) or a CD8α(EC-TM)-CD8β(IC) chimera (cyt tail), using bicistronic constructs with ΔNGFR as the reporter. Bivariate dot plots are gated on ΔNGFR-positive cells, at day 2 after transduction of these cells with control virus, HIV-1 Nef (NA-7 allele), and SIV Nef (mac239), using bicistronic constructs with EGFP as the reporter. CD8α-phycoerythrin versus EGFP expression is shown.

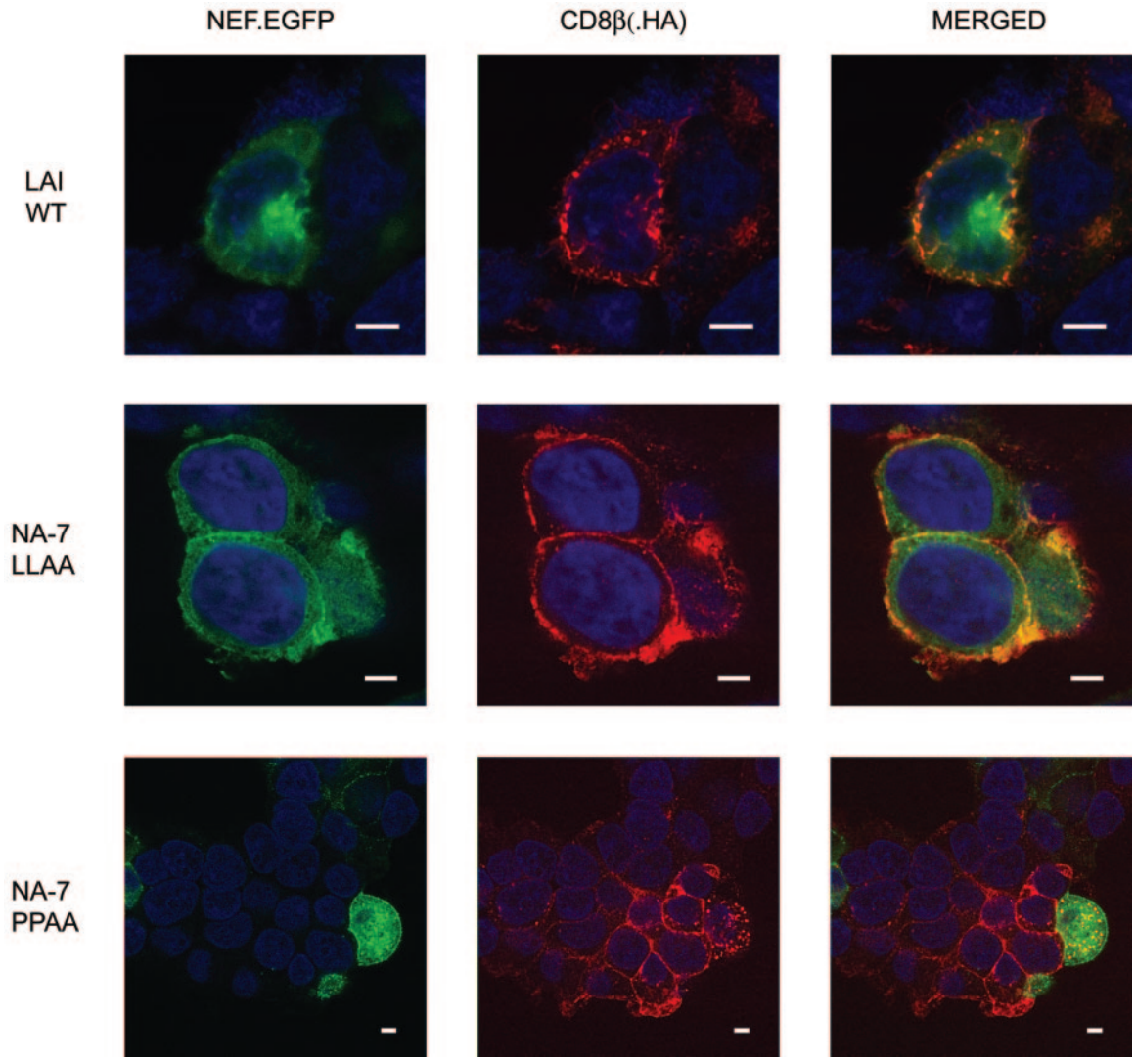

FIG. 6. Confocal images of 293T cells. Nef.EGFP was detected by direct fluorescence (green, left panels) and CD8β.HA or CD8β by monoclonal antibodies as indicated in Materials and Methods (red, middle panels). Nuclei were visualized by DAPI staining (blue). Right panels show the merged images from Nef.EGFP and CD8β. Areas of colocalization of Nef.EGFP/CD8β are shown in yellow. As indicated, the upper panels show cells expressing wild-type LAI, the middle panels show the LLAA mutant, and alower panels show the PPAA mutant. Scale bars represent 5 μm.

on CD8αβ/CD4 and CD28 expression, while amino acids 41 to 49 (removed in Del3) were required for CD8αβ/CD4/CD28 down-regulation but were dispensable for down-regulation of MHC class II and up-regulation of the Ii chain. Knockdown of the trafficking molecules AP-2, clathrin heavy chain, or dynamin-2 showed no profound difference in reversal of the HIV-1 Nef effect on CD4 and CD8αβ, corroborating our results with the mutant Nef panel.

Surprisingly, Nef-mediated endocytosis could still occur after knockdown of clathrin heavy chain but not AP-2, suggesting that trace amounts of clathrin heavy chain are sufficient for clathrin-coated endocytosis, while AP-2 is present at rate-limiting amounts. Although the LAI allele of HIV-1 Nef was affected more strongly by dynamin-2 RNAi than the NA-7 allele (data not shown), we did not observe any effect on the SIVmac239 Nef-mediated down-regulatory potency, pointing to a difference between HIV-1 and SIVmac239 Nef in the need for endocytic cargo molecules. However, we did not observe this difference after knockdown of AP-2, suggesting a central role for AP-2. This result is in contrast with Rose et al., who demonstrated a stronger inhibition of SIV than of HIV-1 Nef-mediated CD4 down-regulation (55). This dissimilarity in potency can be due to the different experimental set-ups (several electroporation steps of short interfering RNA) and cells (HeLa) used by Rose and coworkers.

Various mechanisms for HIV entry into CD8 lymphocytes have been proposed, with entry through a conventional CD4-dependent pathway as the most plausible one. This infection route can occur during intrathymic CD8 lymphocyte development, at the $CD4^+$ $CD8^+$ double positive stage (14), or upon activation of the mature CD8 lymphocyte, which leads to the coexpression of the CD4 receptor on the cell surface (18, 36).

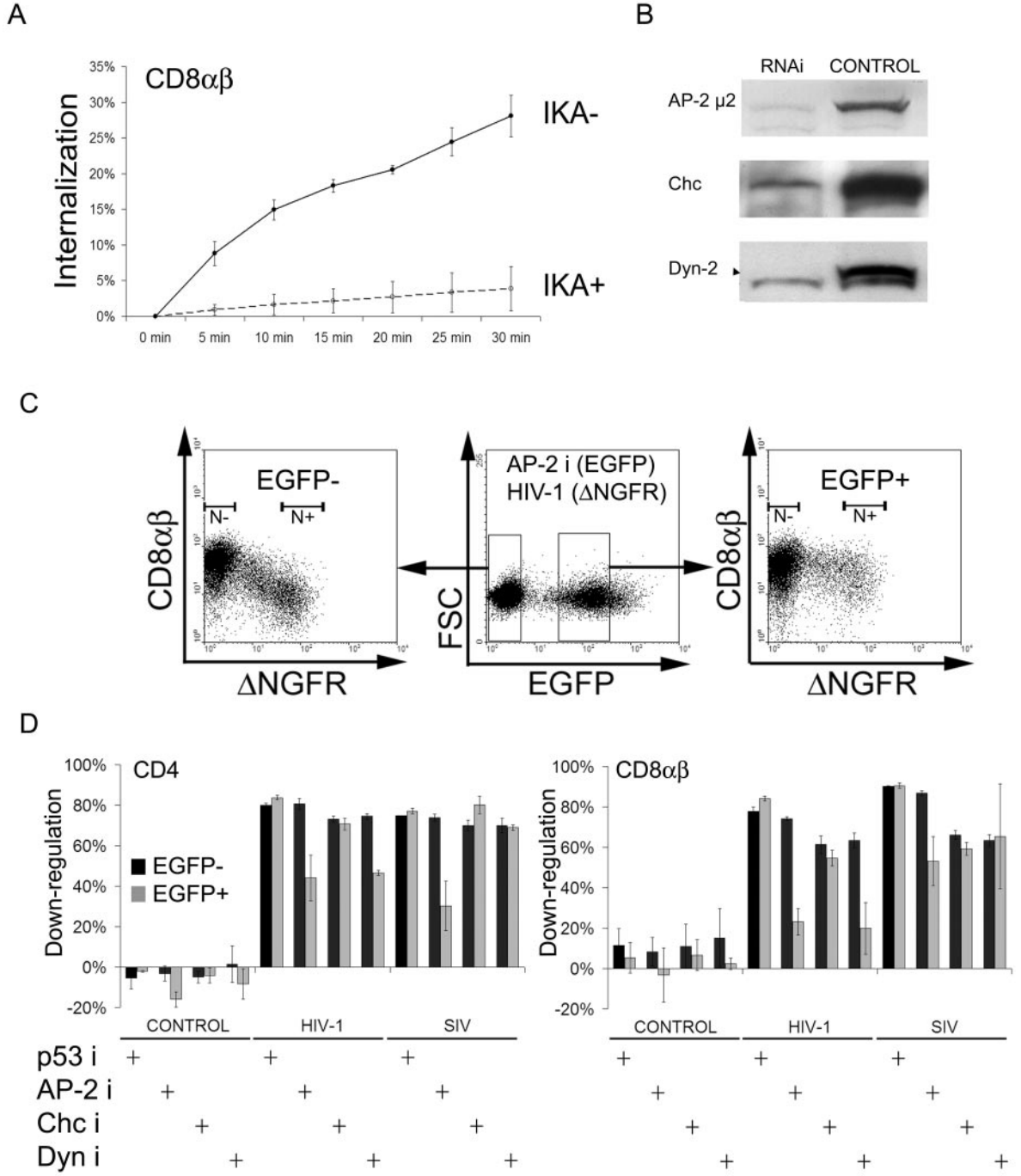

FIG. 7. Blocking Nef-mediated internalization and down-regulation by ikarugamycin and RNA interference. (A) The figures show the percentage of CD8αβ molecules internalized in Daudi CD8αβ cells by HIV-1 NA-7.ER. Cells were incubated for 2 h with (IKA+) or without (IKA−) ikarugamycin (2 μM) prior to the internalization experiment. At time zero, 4-hydroxytamoxifen (1 μM) was added to NA-7.ER-transduced Daudi cells. (B) Western blot, performed as indicated in Materials and Methods, shows protein expression levels of AP-2 μ2 subunit, clathrin heavy chain (Chc), and dynamin 2 (Dyn-2, arrowhead) in control and RNAi-transduced SupT1 cells, with equal amounts of protein loaded. (C) Bivariate dot plots of flow cytometric measurement of SupT1 cells transduced with AP-2i and HIV-1 Nef (LAI). CD8αβ (phycoerythrin) versus ΔNGFR (allophycocyanin) expression is shown, gated on EGFP-negative and EGFP-positive cells. (D) The bar charts represent the effect of AP-2, clathrin heavy chain (Chc), and dynamin 2 RNAi on HIV-1 Nef (LAI) and SIV (mac239) Nef-induced CD4 (left panel) and CD8αβ (right panel) down-regulation in transduced SupT1 cells, gated on EGFP-negative and EGFP-positive cells. Percent down-regulation was calculated, as described in Materials and Methods, using the ranges N− and N+, as indicated in C. Mean values and standard deviations are shown, calculated from data generated from three independent experiments.

Recently, several groups provided evidence that, while HIV-infected CD8 precursors from the thymus rarely reach the periphery, the majority of circulating infected CD8 lymphocytes acquired HIV through expression of CD4 during activation (7, 12).

HIV-infected $CD8^+$ T-cell precursors have been suggested to be depleted intrathymically. However, as previously reported, CD8αβ/CD4 down-regulation alone cannot explain Nef-mediated impaired T-cell development, and other functions of Nef and/or HIV are needed for its induction of thymic depletion (61). Although $CD8\alpha\beta^+CD4^{low}$ T lymphocytes account for a minor fraction of peripheral blood CD8 lymphocytes (<5%), HIV patients were found to harbor high levels of infection in this CD8 subset, approaching those found in CD4 lymphocytes, whereas $CD8\alpha\beta^+CD4^-$ T lymphocytes showed very low or even undetectable viral DNA loads (12). Possibly, the ability of HIV to infect this activated $CD8^+$ population may contribute to the decline in CD8 lymphocyte function which is at present predominantly ascribed to the lack of CD4 lymphocyte help and viral escape (reviewed by McMichael and Rowland-Jones [46]).

Remarkably, although the two chains of the CD8αβ receptor are covalently linked, suggesting a joined internalization of CD8α and CD8β, Nef modulates the surface expression of CD8α to a much lower extent than CD8β in peripheral blood mononuclear cells. A possible explanation may be the simultaneous expression of CD8αα and CD8αβ on the cell surface, with Nef predominantly affecting the CD8αβ surface expression. Whereas CD8αα is not expressed on resting naïve T cells in the periphery (38), coexpression of CD8αα and CD8αβ on activated conventional T cells and in T-cell leukemias has been reported (64). Hence, in the presence of Nef, activated lymphocytes might get skewed towards a predominant CD8αα receptor surface expression.

In conclusion, this work describes the down-modulation of the CD8αβ surface expression by Nef. Analysis of the mechanism by which Nef down-regulates CD8αβ revealed an accelerated AP-2 complex-mediated internalization of the receptor, closely related with the mechanism of Nef-mediated CD4 down-modulation. Further functional experiments are warranted to elucidate whether Nef impairs CD8 lymphocyte function, thus affecting the immune control of both HIV and opportunistic pathogens.

ACKNOWLEDGMENTS

We thank N. De Cabooter, E. Demecheleer, and S. De Keyser for technical assistance, Y. Taes for help with immunoblot imaging, C. Bonini for ΔNGFR, O. Schwartz, Y. Collette, D. Olive, W. Greene, R. Benarous, J. Zack, and the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, for the gift of the wild-type and mutant *nef* constructs, P. Charneau for providing the pTRIP vector, D. Trono for the p8.91 and pMD.G plasmids, S. Bonatti for the CD8α and CD8β constructs, and H. Göttlinger for the dynamin-2 RNAi sequence. We also acknowledge J. Hoxie for providing the hybridoma cell line EH-1.

V.S. is a Research Assistant, E.C. and C.S. are Postdoctoral Fellows, and B.V. is a Senior Clinical Investigator of the Fund for Scientific Research-Flanders (Belgium) (FWO –Vlaanderen). This work was supported by grants from the FWO –Vlaanderen.

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