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AP-2 Is the Crucial Clathrin Adaptor Protein for CD4 Downmodulation by HIV-1 Nef in Infected Primary CD4$^+$ T Cells

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HIV-1 Nef-mediated CD4 downmodulation involves various host factors. We investigated the importance of AP-1, AP-2, AP-3, V1H-ATPase, β-COP, and ACOT8 for CD4 downmodulation in HIV-1-infected short hairpin RNA (shRNA)-expressing CD4$^+$ T cells and characterized direct interaction with Nef by Förster resonance energy transfer (FRET). Binding of lentiviral Nefs to CD4 and AP-2 was conserved, and only AP-2 knockdown impaired Nef-mediated CD4 downmodulation from primary T cells. Altogether, among the factors tested, AP-2 is the most important player for Nef-mediated CD4 downmodulation.

CD4 downmodulation is one of the hallmarks of human immunodeficiency virus type 1 (HIV-1) infection, and the viral Nef protein crucially contributes to this phenotype (1–4). In recent years, various cellular factors have been suggested to be involved in Nef-mediated CD4 downmodulation (5–12), and there is still disagreement concerning the importance of some of them (13–15). While most previous studies have been conducted in the well-characterized HeLa cell model, providing a good rational for molecular and cell biological experiments, our goal was to clarify the roles of the different cellular proteins in cells relevant to HIV-1 infection, such as the CD4$^+$ T cell line SupT1 and primary CD4$^+$ T cells. Furthermore, we aimed to investigate binding of host cell factors with Nef by our established fluorescence-activated cell sorter (FACS)-Förster resonance energy transfer (FRET) assay, allowing detection of transient protein interactions in living cells (16, 17).

We utilized cyan fluorescent protein (CFP)-tagged variants of the adaptor protein subunits AP-1σ, AP-2μ, AP-2σ, and AP-3μ; the thioesterase ACOT8; β-COP; the ATPase V1H; and CD4 (all ligated into Clontech pECFP/EYFP vectors as described previously [16]). AP-2μ and AP-3μ were not detectable by Western blotting (WB), while all the other CFP fusions migrated with their expected sizes (Fig. 1A). Transfection was extremely low for AP-3μ, as assessed by FACS and confocal microscopy (Fig. 1B and data not shown), and we therefore decided to omit the construct from further experiments. In contrast, AP-2μ–CFP showed specific fluorescence within the cytoplasm and vesicular structures (Fig. 1B), indicating its functional expression. FACS-FRET (measured with a MACSQuant VYB; Miltenyi) revealed significant FRET signals for HIV-1 NA7 Nef-yellow fluorescent protein (YFP) with AP-2μ-, ACOT8-, β-COP-, and CD4-CFP fusions coexpressed in 293T cells (Fig. 1C). CD4-YFP coexpression with the CFP-tagged cellular factors resulted in significant FRET with AP-2μ, β-COP, and CD4, indicating homo-oligomerization of CD4 (Fig. 1C). Importantly, the absence of FRET for Nef with AP-1σ, AP-2σ, and V1H does not preclude interaction between Nef and these factors but could stem from various reasons, including non-natural localization of the fusions (compare Fig. 1B) or cleavage of the CFP tag (Fig. 1A). Interestingly, upon cotransfection of untagged CD4, FRET values for Nef with CD4 or ACOT8 decreased while FRET for Nef with AP-2μ or β-COP was enhanced (Fig. 1D), following nearly perfect nonlinear regression (Fig. 1E). Altogether, these results demonstrate that Nef directly interacts with CD4, ACOT8, AP-2μ, and β-COP in living cells and further suggest formation of a complex between Nef, CD4, and AP-2μ or β-COP.

The *nef* open reading frame (ORF) is conserved among highly divergent lentiviruses, and all these Nef proteins are able to downmodulate human CD4 from the surfaces of CD4$^+$ T cells (18). We therefore tested if the interaction of various lentiviral Nefs with AP-2μ, ACOT8, β-COP, and CD4 is conserved (Fig. 2). Utilizing previously generated YFP-tagged versions of the various Nef proteins (19), we conducted FACS-FRET in 293T cells and found, as expected, that all the lentiviral Nef proteins tested interacted with CD4. In contrast, interaction of Nef with β-COP was weak and nonconserved, and of note, only HIV-1 NA7 and NL4-3 Nef bound to ACOT8. In agreement with previous data (19), HIV-2- and simian immunodeficiency virus (SIV)-derived Nef proteins potently interacted with AP-2μ (Fig. 2), most likely due to the presence of tyrosine-based AP-2 binding motifs, which are absent in HIV-1 and SIVcpz Nefs (15). In contrast, HIV-1 Nef showed only weak interaction with AP-2μ. This can be explained by the preferential binding of HIV-1 Nef to the AP-2α subunit through a dileucine motif in the C-terminal loop (20, 21). Unfortunately, AP-2α (similarly to AP-3μ) was not expressed as a CFP fusion protein (data not shown), precluding assessment of this interaction by FACS-FRET. Altogether, only interaction with human CD4 and AP-2 is largely conserved among various divergent lentiviral Nef proteins.

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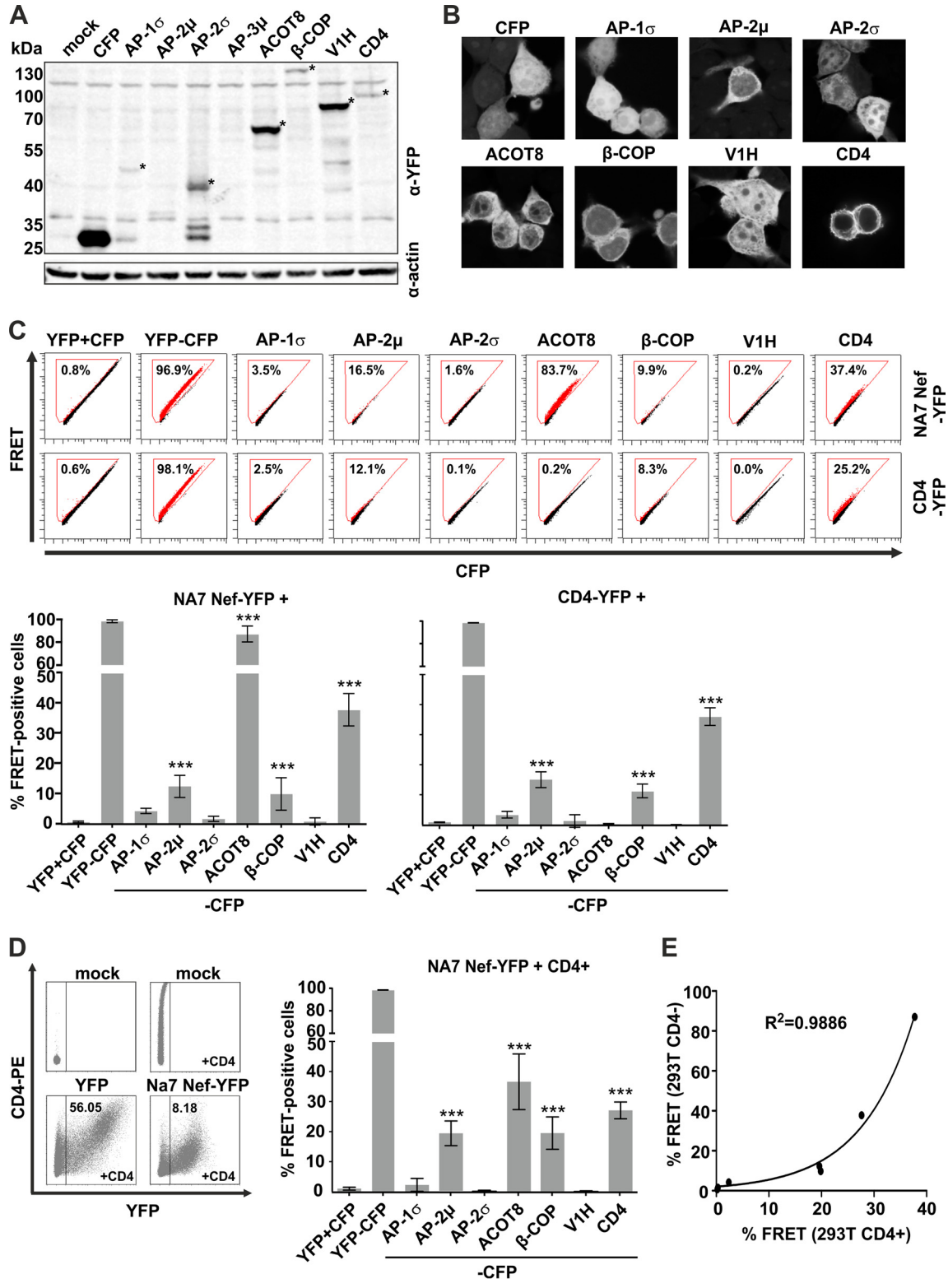

FIG 1 Interaction of Nef and CD4 with a panel of host cell factors. (A) 293T cells were $CaCl_2$ transfected to express different CFP-tagged host cell factors. Twenty-four hours later, lysates of the cells were analyzed by WB with an anti-GFP Ab (BioVision; number 3999; 1:1,000) and an anti-actin Ab (Abcam; ab3280; 1:1,000). The asterisks indicate the specific bands at the expected molecular masses. (B) Confocal microscopy (Zeiss LSM at ×63 magnification) with transfected and coverglass-embedded 293T cells to reveal the subcellular localization of the indicated fusion proteins. (C) 293T cells transfected to coexpress a panel of CFP-tagged host cell factors and YFP-tagged CD4 or NA7 Nef were harvested 24 h posttransfection and analyzed for FACS-FRET as described previously. (Top) Primary FACS plots of a representative measurement. The numbers indicate the percentages of $FRET^+$ cells (red) in the measurement. (Bottom) Mean values and standard deviations (SD) of the results of seven (NA7 Nef-YFP) and four (CD4 Nef-YFP) independent experiments. (D) Same setup as in panel C, but additionally, a CD4 expression plasmid was cotransfected. Mean values and SD were calculated from the results of three experiments with duplicate transfections. (C and D) Statistical significance versus the YFP-plus-CFP negative control was assessed by one-way analysis of variance (ANOVA) with multiple comparisons (Graph Pad Prism 5.0). ***, $P < 0.001$. YFP+CFP, cotransfection of YFP only and CFP only (FRET negative control); YFP-CFP, transfection of a YFP-CFP fusion protein (FRET positive control). (E) Regression analysis of the mean FRET values from panels C and D (Graph Pad Prism 5.0).

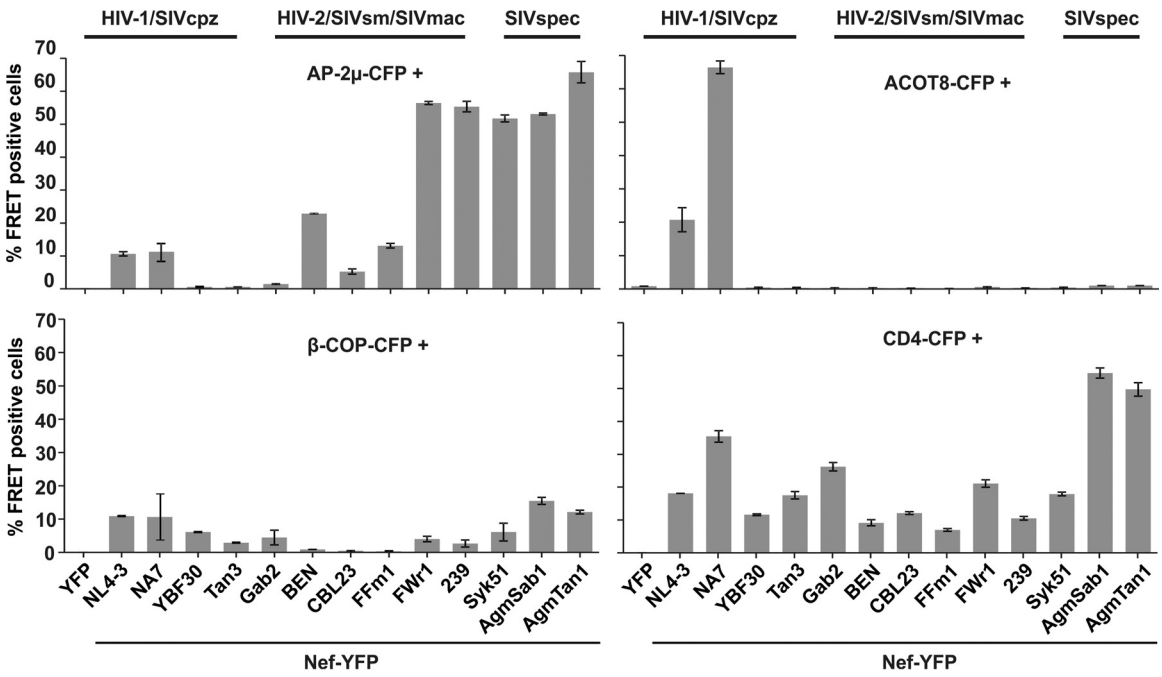

FIG 2 Interaction of AP-2μ, β-COP, ACOT8, and CD4 with highly divergent lentiviral Nef proteins. 293T cells transfected to coexpress the CFP-tagged host cell factors and the various lentiviral Nef-YFP fusion proteins were harvested 24 h posttransfection and analyzed for FACS-FRET as described previously. The graphs show mean values and standard deviations of the results of two independent experiments with duplicate transfections.

We next performed a comprehensive analysis of Nef mutants (20, 22–27) to identify domains necessary for interaction with host cell factors. Using site-directed mutagenesis to introduce amino acid changes in the NA7 Nef ORF, we generated YFP-tagged fusion constructs (16). WB for YFP showed that all the Nef mutants were expressed in 293T cells, albeit with different efficiencies (Fig. 3A). Importantly, this did not affect our FRET measurements, since the gating strategy allows analysis of only the YFP/CFP double-positive cell population (16). In addition, fluorescence microscopy indicated no major defects in subcellular localization (Fig. 3B). Specifically, all the mutants except those carrying the L110A change could be detected at the plasma membrane; a fraction of Nef was within the cytosol and in perinuclear accumulations, presumably the endoplasmic reticulum and the Golgi apparatus. As expected, the myristoylation-defective G2A mutant lost its membrane anchorage (25). To assess if YFP-tagged Nef was still functional, we electroporated SupT1 $CD4^+$ T cells with selected mutants using the Neon microporation device (Life Technology) and the following parameters: 1,325 V, 10 ms, and 3 pulses. Forty-eight hours later, cells were harvested and antibody (Ab) stained for CD4 and major histocompatibility complex class I (MHC-I) expression. FACS analysis revealed the capacities of the various YFP-tagged Nef mutants to downmodulate CD4 and MHC-I (Fig. 3C). Due to disrupted membrane localization, the G2A mutant failed to downmodulate CD4 and MHC-I. Mutations in the Nef core region, L110A and PD122AA, also impaired both Nef functions, whereas, in agreement with previous data, the PXXP motif (P72A/P75A) is largely responsible for MHC-I downmodulation (28) and the C-terminal dileucine motif mediates interaction with AP-2α and hence CD4 internalization (21). In sum, we conclude that the YFP-tagged Nef variants are functional and allow mapping of distinct binding domains by FACS-FRET. Interaction of Nef with AP-2μ and β-COP was clearly dependent on the membrane localization of Nef, since G2A mutation led to a complete loss of FRET (Fig. 3D). The PD122AA change in the Nef core domain also significantly impaired binding to AP-2μ, whereas mutations in other Nef regions did not influence interaction with either AP-2μ or β-COP. Of note, membrane localization of Nef does not appear to be crucial for interaction with the thioesterase ACOT8, since the G2A mutation impaired association between the two proteins but still yielded ~50% $FRET^+$ cells (Fig. 3D). In contrast, residues in the Nef core domain mediated binding between ACOT8 and Nef, and the same mutations impaired Nef interaction with CD4. The Nef Δ17-26 variant still bound CD4 but lost its ability to associate with ACOT8. Hence, we can conclude that similar, albeit not identical, Nef domains are involved in interaction with CD4 and ACOT8. Altogether, using FRET, we characterized for the first time the Nef domains necessary for binding to the diverse host cell factors in the physiological environment of living Nef-expressing cells.

Cellular factors acting downstream in the pathway of Nef-mediated CD4 internalization and degradation might be important without direct binding to Nef or CD4. Hence, we aimed to investigate the relevance of the various cellular factors for downmodulation of CD4 by Nef in HIV-1-infected T cells by short hairpin RNA (shRNA)-mediated gene knockdown. For this, we utilized pNL-SIN-CMV-BLR lentiviral shRNA delivery constructs (29) (kindly provided by Bryan R. Cullen, Howard Hughes Medical Institute) in which the BLR gene was replaced with green fluorescent protein (GFP), permitting detection of shRNA-expressing cells. ShRNA sequences against AP-1σ (ACCGAATTAAGAAAG TGGT), AP-2μ (GTGGATGCCTTT), AP-3μ (CGAGTCGAACA TTCTTAAA), ACOT8 (GGACGAGGATCTCTTCAGA), β-COP (AACTTCCTGGACTTCTGATGA), V1H (GTAGCGGTGTTG),

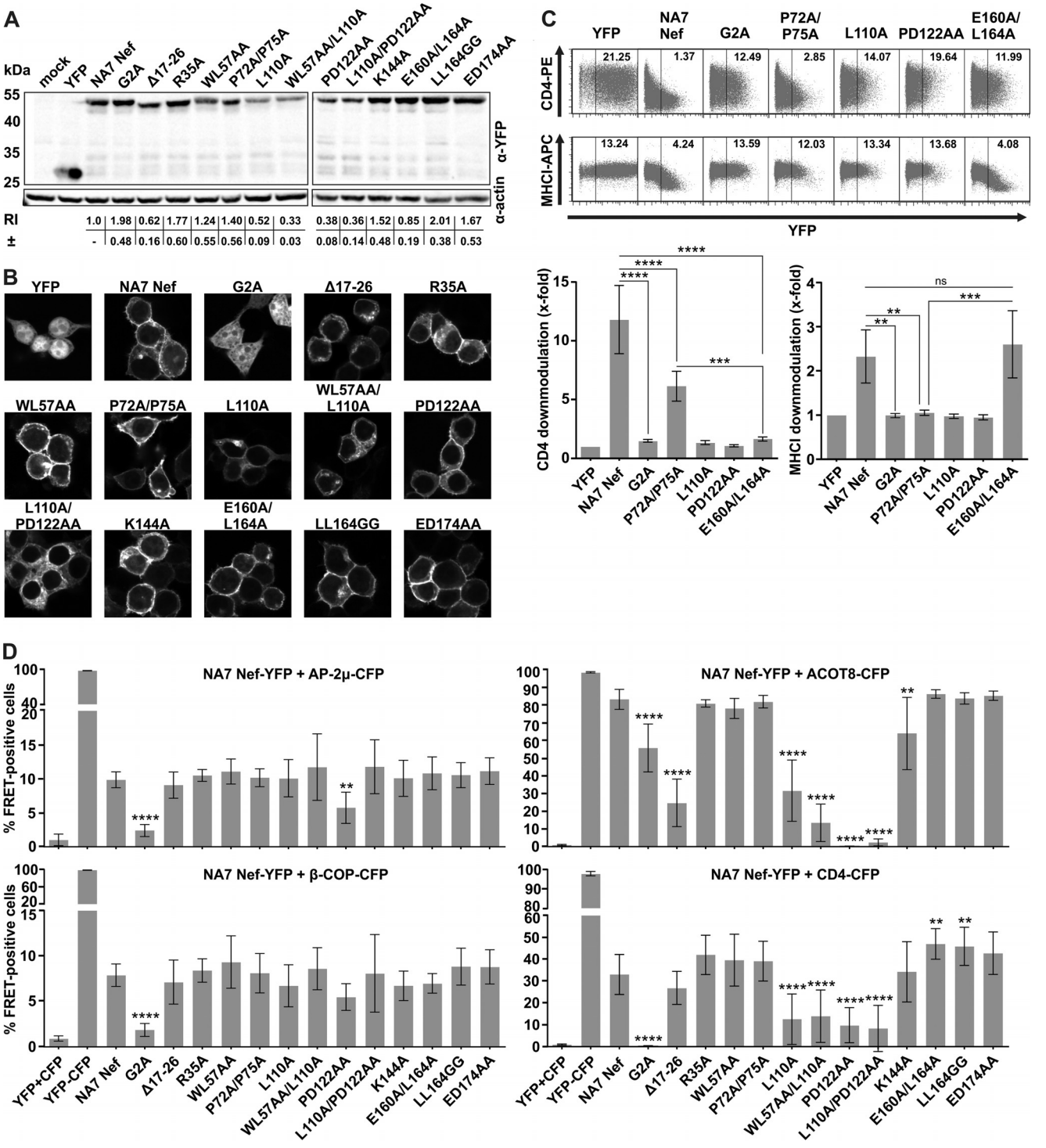

FIG 3 Mapping of Nef residues necessary for interaction with AP-2μ, β-COP, ACOT8, and CD4. (A) 293T cells were transfected to express a panel of YFP-tagged HIV-1 NA7 Nef mutants. Twenty-four hours later, cellular lysates were prepared and analyzed by WB for Nef expression, as described in the legend to Fig. 1A. As a loading control, we used an anti-actin Ab (Abcam; ab3280). The relative expression intensity (RI) of three independent blots was calculated by densitometric analyses (Licor Image Studio Lite Version 5.0) and is indicated together with the standard error (±) below the representative WB. (B) 293T cells seeded on coverslips and transfected to express the Nef mutants were analyzed by confocal microscopy to investigate the subcellular localization of the NA7 Nef mutant YFP fusion proteins. (C) SupT1 $CD4^+$ T cells (2×10^5) were electroporated with the Neon microporation device using 2 μg DNA. Forty-eight hours later, cells were harvested, and the cell surface was stained with antibodies against CD4 (Biolegend; 300508) and MHC-I (Biolegend; 316312) for FACS analysis. (Top) Primary FACS plots of a representative measurement. The numbers indicate the mean fluorescence intensity (MFI) in the YFP^+ and hence Nef- or YFP-only-expressing population of cells. The graph shows mean values and SD of *x*-fold Nef-mediated receptor modulation calculated by dividing the phycoerythrin (PE) or allophycocyanin (APC) MFI of the YFP-only control by the MFI of the cells expressing the respective Nef-YFP variant (the values were calculated from the results of four independent experiments). (D) 293T cells transfected to coexpress the CFP-tagged host cell factors and the different NA7 Nef-YFP mutants were harvested 24 h posttransfection and analyzed for FACS-FRET as described previously (16). The graphs show mean values and SD of the results of at least eight independent transfections. (C and D) Statistical significance was calculated using a one-way ANOVA with multiple comparisons (Graph Pad Prism 5.0). (D) NA7 wild type (WT) was used as a reference for the statistical assessment. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant. YFP+CFP, cotransfection of YFP only and CFP only (FRET negative control); YFP-CFP, transfection of a YFP-CFP fusion protein (FRET positive control).

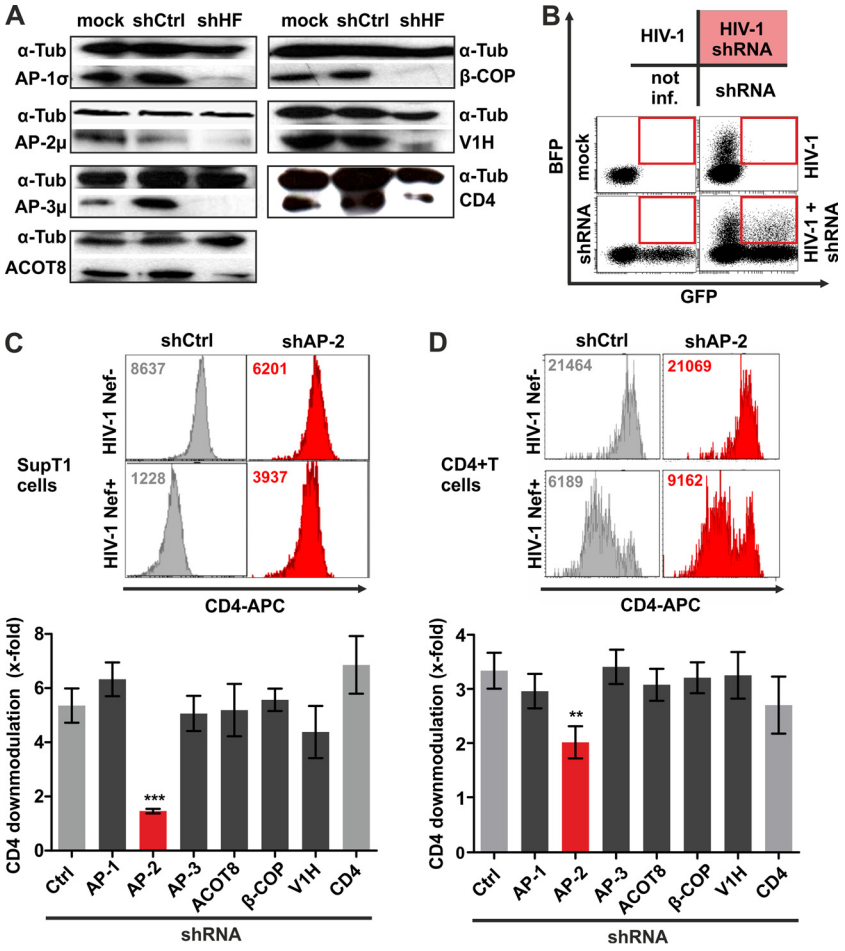

FIG 4 Importance of host cell factors for Nef-mediated CD4 downmodulation from T cells. (A) SupT1 cells were transduced with shRNA- and GFP-expressing lentiviral particles. Five days postransduction, the GFP^+ and hence shRNA-expressing population of cells was sorted with a FACS Aria II (BD Bioscience), and lysates of the cells were prepared for WB. As a control, we used mock-transduced cells and SupT1 cells transduced with lentiviruses expressing a scrambled shRNA cassette (shCtrl) and GFP. Knockdown efficiency was measured by WB with specific antibodies against the cellular factors at a concentration of 1:500: AP-1σ (US Biological; A2298-48B), AP-2μ (Santa Cruz; sc-99026), AP-3μ (Abcam; ab87092), ACOT8 (Novus; H00010005-B01P), β-COP (Abcam; ab2899), V1H (GeneTex; GTX110778), or CD4 (Sigma-Aldrich; HPA004252). The secondary antibodies used were anti-mouse and anti-rabbit horseradish peroxidase (HRP) (1:1,000; Sigma-Aldrich). α-Tub, α-tubulin; shHF, sh host factor. (B) Schematic presentation of the gating strategy used to specifically identify HIV-1-infected (inf.) (BFP^+)- and shRNA (GFP^+)-expressing cells and representative FACS plots. (C) $CD4^+$ SupT1 cells (1×10^6) were transduced with the shRNA-expressing lentiviral supernatants (200 ng p24) in 24-well plates for 6 h at 37°C. After incubation, the cells were washed with PBS and cultured for 5 days. Then, the cells were infected with 200 ng p24 of NL4-3 HIV-1 BFP (with an intact or disrupted Nef ORF [Nef^+ and Nef^-, respectively]) and incubated for 2 additional days before FACS analysis for CD4 cell surface expression. (Top) Primary FACS plots of a representative measurement. The numbers indicate the MFI of CD4-APC in the GFP/BFP^+ population of cells. Nef-mediated downmodulation of CD4 (*x*-fold) was calculated by dividing the CD4-APC MFI of the infection with HIV-1 (Nef^-) by the MFI of the cells infected with WT HIV-1 (Nef^+). Mean values and SD were calculated from the results of six independent experiments. (D) Same experimental setup as in panel C, but with $CD4^+$ primary T cells. The cells were isolated from PBMC by Rossette separation (StemCell Technologies) and prestimulated with 10 ng/ml interleukin 2 (IL-2) and 1 μg/ml phytohemagglutinin (PHA) for 2 days. For transduction with the lentiviral particles, we used 500 ng p24 and 1 μg p24 of HIV-1 to achieve sufficient rates of double-positive cells. Mean values and SD were calculated from the results of six independent experiments. (C and D) Statistical significance was assessed by one-way ANOVA with multiple comparisons (Graph Pad Prism 5.0). ShCtrl was used as a reference for the statistical assessment. **, $P < 0.01$; ***, $P < 0.001$.

or CD4 (TGATCGCGCTGACTCAAGA) were inserted into the H1 promoter within the 5′ long terminal repeat (LTR) in order to knock down endogenous host cell factor expression. Lentiviral particles were produced as described previously (29), and virus preparations normalized to p24 were used to infect SupT1 cells. We sorted the GFP^+ population, representing shRNA-expressing cells, using a FACS Aria II (BD Bioscience) and verified knockdown efficiency by WB (Fig. 4A). For simultaneous HIV-1 infection and analysis of CD4 downmodulation by Nef, we generated an HIV-1 NL4-3 variant coexpressing Nef and an improved blue fluorescent protein (BFP) version (30) (mTagBFP) via an internal ribosome entry site (IRES), as described previously (31), as well as a variant with a disrupted Nef ORF (Nef^-). In addition, we inactivated the Env and Vpu ORFs in these constructs to specifically monitor the effects on CD4 exerted through Nef (32). By this strategy, we were able to exclusively identify HIV-1-infected (BFP^+) and shRNA-expressing lentivirus-transduced (GFP^+) cells by FACS (Fig. 4B).

We infected $CD4^+$ SupT1 cells with the shRNA-expressing lentiviruses for 5 days, extensively washed them, and then infected

them with vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1 IRES-BFP. Two days later, we assessed CD4 downmodulation specifically in the HIV-1-infected and shRNA-expressing population of cells. Of note, only the knockdown of AP-2 strongly impaired the capability of Nef to downmodulate CD4 from infected SupT1 T cells (Fig. 4C). Importantly, we could reproduce this phenotype with the same experimental layout in primary $CD4^+$ T cells purified from human peripheral blood mononuclear cells (PBMC) (Fig. 4D).

In summary, we demonstrated for the first time that AP-2 is the crucial clathrin adaptor protein for Nef-mediated CD4 downmodulation in the context of primary HIV-1-infected $CD4^+$ T cells.

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