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Lysine 144, a Ubiquitin Attachment Site in HIV-1 Nef, Is Required for Nef-Mediated CD4 Down-Regulation

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Two Sorting Motifs, a Ubiquitination Motif and a Tyrosine Motif, Are Involved in HIV-1 and Simian Immunodeficiency Virus Nef-Mediated Receptor Endocytosis

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HIV-1 and SIV Nef proteins downregulate cell surface CD4 and MHC class I (MHC-I) molecules of infected cells, which are necessary for efficient viral replication and pathogenicity. We previously reported that K144 in HIV-1 Nef is di-ubiquitinated, and K144R substitution impairs Nef-mediated CD4 downregulation. In this report, we extend the role of ubiquitination at this lysine residue from Nef-mediated CD4 downregulation to Nef-mediated MHC-I downregulation and from HIV Nef to SIV Nef. All HIV-1 Nef mutants that contain K144R substitution are inactive in MHC-I downregulation. Tested MHC-I alleles include HLA-ABC endogenously expressed and HLA-A2 exogenously expressed in Jurkat T cells. CD4 downregulation by SIV Nef involves K176 that aligns with K144 in HIV-1 Nef, as well as an N-terminal tyrosine motif Y28Y39 not present in HIV-1 Nef. Dual mutation at K176 and Y28Y39 completely impaired SIV Nef-mediated CD4 and MHC-I downregulation, whereas a single mutation at K176 or Y28Y39 did not. The involvement of tyrosine motif in SIV Nef-mediated CD4 and MHC-I downregulation prompted us to investigate a putative tyrosine motif (Y202Y/F203) in HIV-1 Nef that is conserved among HIV-1 species. Single mutation at the tyrosine motif Y202F203 in HIV-1 Nef (NA7) greatly impaired Nef-mediated CD4 downregulation, which is similar to what we observed previously with the single mutation at lysine K144. Thus, our study demonstrated that Nef-mediated receptor endocytosis involves the ubiquitination motif and tyrosine motif. *The Journal of Immunology*, 2011, 186: 5807–5814.

Nef is a 27–35-kDa accessory protein unique to the primate lentiviruses HIV-1, HIV-2, and SIV. It plays an important role in HIV pathogenicity and disease progression to AIDS in infected individuals (1–6). Nef downregulates cell surface CD4 (7), the principal HIV receptor, and the MHC class I (MHC-I) Ags (8, 9). Nef-mediated CD4 downregulation augments viral production and infectivity. The increased viral infectivity by CD4 downregulation could be explained by preventing the disadvantageous superinfection of host cells (10, 11). CD4 downregulation also promotes HIV progeny release by escaping CD4-mediated “envelope interference,” a mechanism that inhibits the incorporation of envelope into virions (12–17). In the absence of Nef, fewer viral particles are released (13), and the released viral particles contain less envelope protein and more CD4 molecules and exhibit a lower infectivity (12). MHC-I presents HIV Ag to the immune system. Thus, downregulation of cell surface expression of MHC-I in infected cells may help virus

to escape recognition and lysis by virus-specific cytotoxic T cells (8, 9).

The mechanisms underlying Nef-mediated CD4 downregulation and Nef-mediated MHC-I downregulation are different (18, reviewed in Ref. 19). In CD4 downregulation, Nef motif W₅₇L₅₈ binds to the cytoplasmic tail of CD4, whereas the dileucine motif (ExxxL₁₆₄L₁₆₅) in the Nef C-terminal loop interacts with the clathrin adaptor protein complex AP-2, thus connecting CD4 to the clathrin-coated vesicles for endocytosis (reviewed in Refs. 19, 20). An N-terminal tyrosine motif, existing in SIV Nef but not HIV-1 Nef, was also involved in SIV Nef-mediated CD4 downregulation (21, 22). However, the role of this motif is not clear (22, 23). In MHC-I downregulation, Nef binds to MHC-I, preventing MHC-I from trafficking to the cell surface and promoting MHC-I endocytosis. Current models suggest that Nef retains MHC-I in the trans-Golgi network (TGN) by forming a complex with AP-1 in a mechanism not related to the Nef dileucine motif–AP-1 interaction. The MHC-I–Nef–AP-1 complex is not sorted to the plasma membrane, instead it follows an endosomal-degradation pathway (19, 24, 25).

Ubiquitination is a posttranslational modification, in which a protein is modified at its lysine ϵ -amino group with a covalent attachment of one (mono-), two (di-), or a chain (poly-) of 76-aa ubiquitin (Ub) molecules (reviewed in Refs. 26–30). Multi-ubiquitination (mono- and di-) is a signal in receptor endocytosis and lysosomal degradation (reviewed in Refs. 29, 31, 32). It provides the sorting signal for protein trafficking from the early endosomes to the late endosomes/lysosomes through special vesicular structures termed multivesicular bodies (reviewed in Refs. 33–36). Recently, we discovered that HIV-1 Nef is multiubiquitinated, and K144 is the major di-ubiquitinated lysine residue. Arg substitution at K144 impairs Nef-mediated CD4 downregulation, suggesting that K144 ubiquitination is the intracellular sorting signal for Nef-mediated CD4 downregulation (37).

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Abbreviations used in this article: EGFP, enhanced GFP; HB, homogenization buffer; JTAG, SV40 T Ag-transfected human leukemic Jurkat T; MHC-I, MHC class I; PNS, postnuclear supernatant; RT, room temperature; TGN, trans-Golgi network; Ub, ubiquitin; wt, wild-type.

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In this study, we determined that ubiquitination at the conserved Lys K144 in HIV-1 Nef or K176 in SIV Nef is also required for Nef-mediated MHC-I downregulation. We made a novel discovery that tyrosine-sorting motifs in HIV-1 and SIV Nef also function in Nef-mediated receptor endocytosis.

Materials and Methods

Plasmid construction

Plasmids encoding SIV Nef mutant K176/R (-K), Y₂₈Y₃₉/GG (-YY), K176/R plus Y₂₈Y₃₉/GG (-KYY), L₁₉₄M₁₉₅/AA (-LM), K176/R plus L₁₉₄M₁₉₅/AA (-KLM), and L₁₉₄M₁₉₅/AA plus Y₂₈Y₃₉/GG (-YYLM) were constructed by PCR mutagenesis using the template of SIV (wild-type; wt) mac239-IRES-GFP plasmid provided by Dr. J. Skowronski (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), following the protocol of the Multi-Quick Change Mutagenesis kit (USB). HIV-1 Nef (G₂G₃/AA)-GFP, Nef (L₁₆₄L₁₆₅/AA)-GFP, and (W₅₇L₅₈/AA)-GFP were constructed by PCR mutagenesis with the Nef (pNA7)-GFP template, as described previously (38). HIV-1 Nef (K144R)-GFP and Δ10K (lysine-free) with 10 Lys to Arg substitutions were described previously (37). HIV-1 Nef (NA7) Y₂₀₂F₂₀₃/GG (-YF) mutants were generated using PCR with wt Nef (NA7) or Nef (K144R) as the template. For bicistronic expression of Nef and GFP (Nef-IRES-GFP), HIV-1 Nef mutants were subcloned by PCR into the pCIneo/IRES-GFP vector (Promega) at the EcoRI site. All mutations generated in this study were confirmed by DNA sequencing.

Abs

Anti-HIV-1 Nef rabbit serum was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. PE-conjugated mAbs of anti-CD4 (Leu3a), anti-HLA-A2 (clone BB 7.2), and anti-HLA-ABC were purchased from Becton Dickinson (San Diego, CA); sheep anti-SIV Nef Ab was purchased from Exalpha; HRP-conjugated rabbit anti-sheep Ab was purchased from Upstate; anti-Ub (P4D1) mAb, anti-Rab7 Ab (H-50), and anti-Rab5 Ab (FL-215) were purchased from Santa Cruz Biotechnology; and ECL HRP-conjugated anti-rabbit and anti-mouse IgG Ab was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, U.K.).

Cell culture and development of Jurkat cell line that stably expresses MHC-I allele HLA-A2

SV40 T Ag-transfected human leukemic Jurkat T (JTAg) cells (39) and BY CD4 hybridoma cells (40) were maintained in RPMI 1640 supplemented with 10% FCS. HeLa cells were maintained in DMEM supplemented with 10% FCS. HeLa CD4 cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. JTAg-A2 cells were developed based on cell sorting. Briefly, JTAg cells were transfected with pcDNA3.1/Hygro plasmid encoding HLA-A2. Seven days after transfection, cells were surface stained with the PE-conjugated HLA-A2 mAb BB 7.2 and selected for HLA-A2 high cells by FACS. The selected cells were cultured and sorted three more times in a 1-mo period. The cells were maintained in RPMI 1640 medium containing no selective antibiotics and were sorted periodically.

DNA transfection and FACS analysis of Nef-mediated CD4 and MHC-I downregulation

JTAg cells (~0.5 × 10⁶ cells/well) or HeLa cells seeded overnight in 24-well plates (~80% confluent) were transfected with 1–5 μg Nef plasmid DNA using Lipofectamine 2000 (Invitrogen). BYCD4 cells were transfected by electroporation, as described (40). Sixteen to twenty-four hours after transfection, cells were surface stained on ice for 45 min with PE-conjugated mAbs at 1:100 dilution in PBS. The cells were fixed in 2% paraformaldehyde and subjected to FACS analysis on a FACScan (Becton Dickinson). The FACS data are plotted on a log scale. Percentages of downregulation were calculated based on the mean levels of HLA-A2 or CD4 staining of cells with a high level of transfection (GFP fluorescence >800).

Endosome separation

We used a well-established sucrose-gradient centrifugation to separate early endosomes from late endosomes (41). Briefly, Nef-transfected HeLa CD4 cells, grown in a 10-cm dish (~80% confluent), were scraped in 2 ml homogenization buffer (HB) (20 mM HEPES [pH 7.4], 0.25 M sucrose, 2 mM EDTA). The cells were pelleted by centrifugation at 800 × g for 5 min,

resuspended in 0.4 ml HB, and homogenized with 12 passages through a 30-gauge needle. The lysates were centrifuged at 800 × g for 5 min, and the supernatant was collected as postnuclear supernatant (PNS). In a Beckman 11 × 60-mm ultraclear centrifuge tube, 0.5 ml PNS (in 46% sucrose) was overlaid sequentially with 1.5 ml 35% sucrose, 1.2 ml 25% sucrose, and 1 ml HB containing 8% sucrose. The gradient was centrifuged for 60 min at 35,000 rpm using an SW60 rotor. After removing the top 1 ml HB, 10 fractions (0.25 ml) were collected from the top of the gradient in each tube. The fractions were boiled after the addition of 50 μl 5× SDS sample buffer and analyzed by anti-Nef immunoblotting.

Subcellular fragmentation of the cell lysates

Subcellular fragmentation of the cell lysates was performed, essentially as described previously, using differential centrifugation (42). Nef-transfected HeLa CD4 cells in one 10-cm dish (~80% confluent) were scraped in 2 ml PBS. The cells were pelleted by centrifugation at 800 × g for 5 min, resuspended in 1.0 ml hypertonic solution (42 mM KCl, 2 mM MgCl₂, and 10 mM HEPES [pH 7.4]), incubated for 15 min on ice, and passed through a 30-gauge needle 12 times. The homogenized cell lysates were centrifuged at 800 × g for 5 min, and the supernatant was collected as PNS. The PNS was transferred to a Beckman 11 × 34-mm ultracentrifuge tube and centrifuged for 30 min at 10,000 × g using an SW60 rotor. The 10,000 × g pellets were collected as heavy membrane. The supernatant was transferred to a fresh Beckman 11 × 34-mm polycarbonate tube and centrifuged for 120 min at 150,000 × g. The 150,000 × g pellets were collected as the light membrane fraction. The 150,000 × g supernatants were collected as the cytosolic fraction. All fractions were boiled in SDS sample buffer and analyzed by anti-Nef immunoblotting.

In vivo ubiquitination assay

The in vivo ubiquitination assay was performed, essentially as described previously (37). Briefly, plasmids encoding SIV Nef and Ub-His were cotransfected into 293T cells using Lipofectamine 2000. Sixteen hours posttransfection, the cells were treated with 20 μM MG132 for 6 h prior to harvest. The cells were lysed in 1 ml denatured lysis buffer (6 M guanidinium chloride, 0.1 M sodium phosphate [pH 8], 10 mM imidazole) per 60-mm dish. The lysates were sonicated to shear DNA and centrifuged to remove particulate material. One hundred microliters of 75% slurry of Ni-nitrilotriacetate-agarose (Qiagen) was added to the cleared lysates and rotated at 4°C for 3 h. After washing, the proteins bound to the Ni-beads were eluted by boiling the beads in 1× SDS sample buffer/100 mM EDTA and analyzed by immunoblotting.

Immunoblotting and densitometry

Lysates of Nef-transfected cells or cellular fractions were resolved on 11% SDS-PAGE. HIV-1 Nef immunoblotting was done with anti-HIV-1 Nef rabbit serum (1:10,000) at 4°C overnight, followed by HRP-conjugated anti-rabbit IgG (1:10,000) at room temperature (RT) for 1 h. SIV Nef immunoblotting was done with sheep anti-SIV Nef Ab (Exalpha) (1:2,000) at 4°C for 4 h or overnight, followed by HRP-conjugated rabbit anti-sheep IgG (Upstate) (1:10,000) at RT for 1 h. Ubiquitin immunoblotting was done with anti-Ub mAb (P4D1) (1:2,000) at 4°C overnight and HRP-conjugated anti-mouse IgG (1:10,000) at RT for 1 h. Films were developed using ECL at three levels of exposure, documented on Gel Doc 2000 (Bio-Rad), and quantitatively analyzed by densitometry using Quantity One software (Bio-Rad). The results are the means of three separate experiments.

Results

HIV-1 Nef mutant K144R was impaired in MHC-I downregulation

Following our previous observation (37), we first asked whether di-ubiquitination at K144 in HIV-1 Nef is also required for MHC-I downregulation. We transfected JTAg cells with plasmids encoding HIV-1 Nef (wt), Nef mutant K144R, Δ10K (where all 10 lysines were mutated), and Nef dileucine mutant (-LL) and investigated MHC-I downregulation using flow cytometry (Fig. 1). Fig. 1A shows the downregulation of the endogenous MHC-I (HLA-ABC) by wt Nef and Nef mutants expressed from the bicistronic IRES-GFP vector. The *top panel* shows that Nef-mediated MHC-I downregulation was less prominent than was

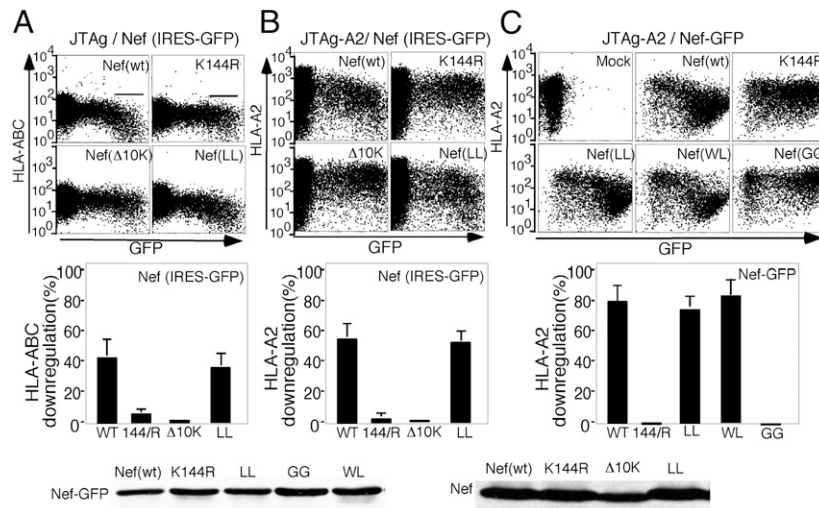


FIGURE 1. HIV-1 Nef K144R and Δ 10K mutants were impaired in Nef-mediated MHC-I downregulation. Cells were transfected with a bicistronic IRES-GFP plasmid encoding wt or mutant Nef proteins (A, B) or a GFP-fusion plasmid encoding wt or mutant Nef proteins (C). The top panels show flow cytometry results. The middle panels show the percentage of MHC-I downregulation relative to wt Nef-mediated downregulation in cells with high GFP levels (gated region on x-axis indicated by the horizontal bar, as seen in the top panel in A). The lowermost two panels show comparable Nef and Nef-GFP protein-expression levels in JTAG cells determined by anti-Nef immunoblotting. A, Downregulation of the endogenously expressed MHC-I allele ABC by HIV-1 Nef (wt), Nef (LL), Nef K144R, and Nef Δ 10K. Transfected JTAG cells were stained with anti-MHC-I (ABC) and analyzed by flow cytometry. B, Downregulation of the exogenously expressed MHC-I allele HLA-A2 in stably transfected JTAG cells by the same Nef mutants as in A. Cells were stained with anti-HLA-A2 and analyzed by flow cytometry. C, Downregulation of the exogenously expressed MHC-I allele HLA-A2 in stably transfected JTAG-A2 cells by Nef-GFP mutants Nef (wt)-GFP, Nef (K144R)-GFP, Nef (LL)-GFP defective for AP-2 interaction, Nef (WL)-GFP defective for CD4 interaction, and Nef (GG)-GFP defective for plasma membrane attachment.

Nef-mediated CD4 downregulation (data not shown) and was only seen clearly in cells with a high level of Nef expression (gated region). The middle panel shows the percentages of MHC-I downregulation in these cells. Nef (wt) and Nef (-LL) caused ~40% MHC-I downregulation, whereas Nef mutant K144R and Δ 10K resulted in no downregulation. Fig. 1B shows a similar pattern of downregulation of MHC-I allele HLA-A2 stably transfected in JTAG cells. Nef (wt) and Nef (-LL) caused ~50% HLA-A2 downregulation, whereas Nef mutant K144R and Δ 10K resulted in no downregulation. Fig. 1C shows a similar pattern of MHC-I HLA-A2 downregulation when Nef proteins were expressed as GFP-fusion proteins. We compared MHC-I downregulation by Nef (wt)-GFP, Nef (LL)-GFP, Nef (WL)-GFP (de-

fective for CD4 interaction), and Nef (GG)-GFP (defective for membrane attachment). Although Nef (wt)-GFP, Nef (WL)-GFP, and Nef (GG)-GFP resulted in marked MHC-I downregulation (~80%), Nef (K144R)-GFP had no effect. Our results for Nef (GG)-GFP, Nef (LL)-GFP, and Nef (WL)-GFP were in good agreement with the results reported by other investigators that N-terminal myristoylation is necessary for CD4 and MHC-I downregulation, whereas dileucine motif is required for CD4 downregulation but not MHC-I downregulation (18, 19). The expression levels of these Nef mutant proteins were comparable (Fig. 1C, bottom panel). The results indicated that Lys K144 also provides the critical sorting signal for Nef-mediated MHC-I downregulation (Figs. 2, 3).

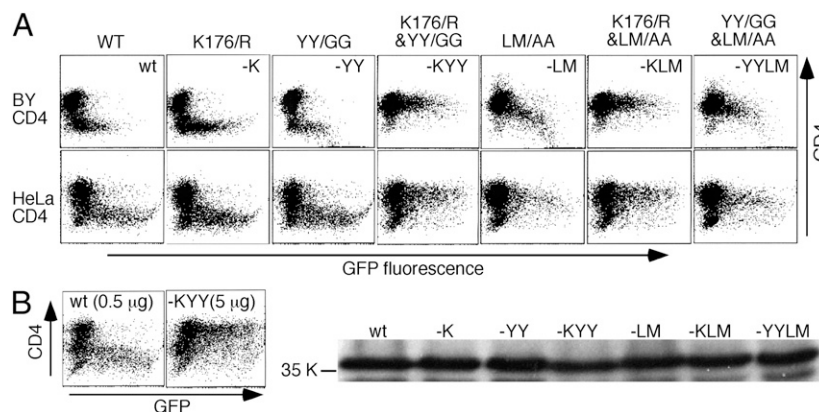


FIGURE 2. SIV Nef dual mutant (-KYY) with mutations at K176 and Y28Y39 was impaired in CD4 downregulation. A, CD4 downregulation by SIV Nef (wt); single mutants of K176/R (-K), Y28Y39/GG (-YY), and L184M185/AA (-LM); and dual mutants of K176/R plus Y28Y39/GG (-KYY), K176/R plus L184M185/AA (-KLM), and L184M185/AA plus Y28Y39/GG (-YYLM). Mutant and wt SIV Nef proteins were expressed from bicistronic IRES-GFP plasmids in transfected BY CD4 cells (upper panels) or HeLa CD4 cells (lower panels). The cells were surface stained with anti-CD4 mAb-PE for flow cytometry. B, CD4 downregulation in HeLa CD4 cells transfected with 0.5 μ g of SIV Nef (wt) (left) or with 10-fold more (5 μ g) plasmid encoding Nef mutant -KYY (right). Lower right panel, Comparable Nef protein-expression levels were noted in HeLa cells, as determined by anti-SIV Nef immunoblotting.

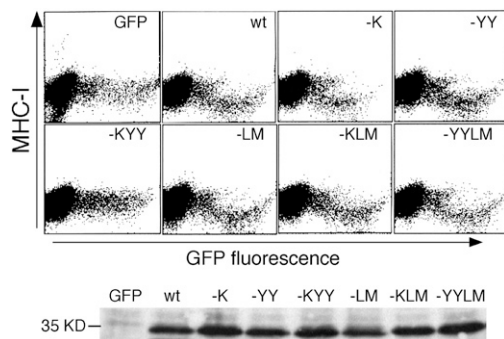


FIGURE 3. SIV Nef dual mutant (-KYY) was impaired in MHC-I downregulation. JTAG cells were transfected, as described in Fig. 2, and surface stained with anti-MHC (ABC) mAb for flow cytometry. *Bottom panel*, Comparable Nef protein-expression levels were noted in JTAG cells, as determined by anti-SIV Nef immunoblotting.

SIV Nef-mediated CD4 downregulation involves Lys K176 and the tyrosine motif (Y28Y39)

K176 in SIV Nef is homologous to K144 in HIV-1 Nef (43). To determine whether ubiquitination at this Lys residue is also required for SIV Nef-mediated CD4 downregulation, we substituted K176 with Arg in SIV Nef (-K). Considering that SIV Nef-mediated CD4 downregulation involves an additional N-terminal tyrosine motif (Y28Y39) that does not exist in HIV-1 Nef (21–23, 43), we mutated Y28Y39 alone (-YY) or with K176 (-KYY). The SIV Nef mutants in the bicistronic IRES-GFP vector were transfected into BY CD4 cells and HeLa CD4 cells and analyzed for CD4 downregulation by flow cytometry. Fig. 2A shows that mutations at K176 (-K) or at Y28Y39 (-YY) separately did not impair CD4 downregulation; however, mutation at both sites (-KYY) completely abrogated Nef-mediated CD4 downregulation in BY CD4 cells (*upper panels*) and HeLa CD4 cells (*lower panels*). Anti-SIV Nef immunoblotting showed that all of these Nef mutants were expressed at comparable levels (*bottom panel*). Fig. 2B shows that SIV Nef mutant -KYY did not downregulate CD4, even when 10-fold more of the mutant (-KYY) DNA was transfected. The results indicated that similar to K144 in HIV-1, K176 in SIV Nef is required for Nef-mediated CD4 downregulation, as is the N-terminal tyrosine motif Y28Y39. In agreement with a previous report (23), mutations at the SIV Nef leucine motif (L194M195) (-LM) greatly (~70–80%), but not completely, impaired CD4 downregulation (Fig. 2A, Table I). The double mutation at the leucine motif LM and at K176 (-KLM) resulted in complete abrogation of CD4 downregulation, whereas the double mutation at the tyrosine motif and the leucine motif LM (-YYLM) resulted in a significant reduction, but not complete elimination, of CD4 downregulation (Fig. 2A, Table I). The results suggested that

Table I. Comparison of the effects of SIV Nef mutations on Nef-mediated CD4 and MHC-I downregulation

Downregulation	wt	-K	-YY	-KYY	-LM	-KLM	-YYLM
CD4 (%)	+	+	+	–	~25	–	~10
MHC-I (%)	+	+	+	–	+	+	+

SIV Nef mutations: K176R (-K), Y28G,Y39G (-YY), L184M185/AA (-LM), and the combined mutation -KYY, -KLM, and -YYLM. Downregulation (%) is the ratio of the reduction in surface staining between cells transfected with Nef mutant and with wt Nef. CD4 downregulation is based on the results obtained from BY CD4 cells, and MHC-I (ABC) downregulation is based on the results from JTAG cells. +, complete downregulation (100%); –, no downregulation.

K176 and the tyrosine motif (Y28Y39) are involved in SIV Nef-mediated CD4 downregulation at different steps.

SIV Nef dual mutation at K176 and the tyrosine motif (Y28Y39) also completely abrogate Nef-mediated MHC-I downregulation

We also assessed the roles of K176, Y28Y39, and L184M185 in SIV Nef-mediated MHC-I downregulation. Fig. 3 shows that transfection with plasmids encoding wt SIV Nef mutant -K, -YY, -LM, -KLM, or -YYLM resulted in strong MHC-I (HLA-ABC) downregulation in JTAG cells; however, transfection with SIV Nef dual mutant -KYY did not result in downregulation of HLA-ABC. Similar results were obtained with regard to HLA-A2 downregulation using these mutants in JTAG-A2 and HeLa-A2 cells (data not shown). Table I summarizes the effects of mutations at K176, Y28Y39, and L184M185 on SIV Nef-mediated CD4 and MHC-I downregulation. A single mutation at K176 (-K) or the tyrosine motif (-YY) did not impair SIV Nef-mediated CD4 or MHC-I downregulation, but the dual mutation at both sites (-KYY) impaired CD4 and MHC-I downregulation, and mutation at the leucine motif (-LM) impaired CD4 downregulation but not MHC-I downregulation.

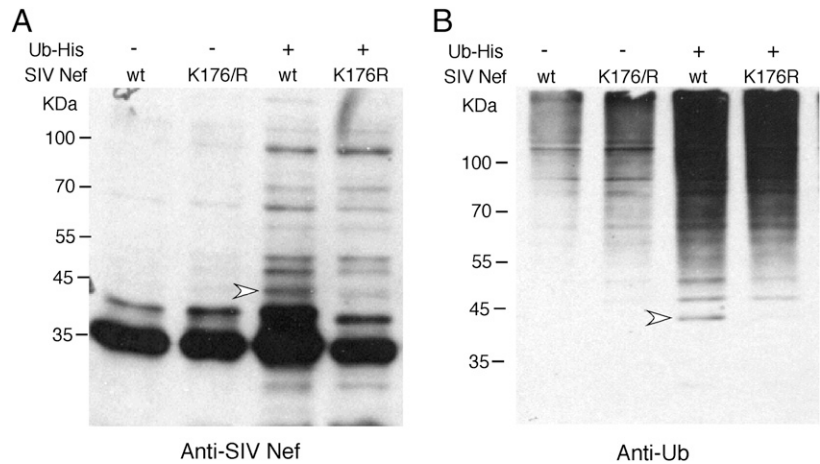
SIV Nef K176 is ubiquitinated

We previously showed that HIV-1 and SIV Nef were multi-ubiquitinated, and HIV-1 Nef K144 was di-ubiquitinated (37). To confirm that SIV Nef K176 is also a ubiquitination site, we performed an in vivo Ub assay, as described (37). Plasmid-encoding SIV Nef (wt) or mutant K176R (-K) was cotransfected into 293T cells with the Ub-His plasmid encoding His-tagged Ub. In vivo-ubiquitinated proteins that are covalently linked to Ub-His were isolated using Ni-beads and analyzed by immunoblotting with anti-Nef and anti-Ub Abs. Fig. 4A shows that with anti-Nef, several protein bands, ranging from ~44 to 85 kDa on SDS-PAGE, were detected in cells cotransfected with SIV Nef and Ub-His but not in cells transfected with Nef or Ub-His alone, indicating that SIV Nef protein was multiubiquitinated. Among them, a 44-kDa protein band (arrowhead) was detected in cells transfected with wt SIV Nef and Ub-His but not in cells transfected with Nef K176R and Ub-His, suggesting that the 44-kDa band is the SIV Nef protein ubiquitinated at K176. Anti-Ub immunoblotting confirmed that the 44-kDa SIV Nef band was ubiquitinated (Fig. 4B). Thus, the results indicated that SIV Nef K176 is also ubiquitinated, likely monoubiquitinated as judged by its molecular mass.

A novel tyrosine motif Y202Y/F203 in HIV-1 Nef is required for Nef-mediated CD4 downregulation

A putative C-terminal tyrosine motif (Y202Y/F203) is conserved among strains of HIV-1 Nef but is not found in SIV Nef (43). To investigate the possible role of this HIV-1 tyrosine motif in Nef function, we mutated the tyrosine motif in HIV-1 Nef (NA7) by substituting Y202F203 with two Gly residues. Plasmids encoding wt and Nef mutants were cotransfected with plasmid encoding cDNA of enhanced GFP (EGFP) into HeLa CD4 cells and analyzed for their role in CD4 downregulation. Fig. 5 shows that a single mutation at the tyrosine motif (HIV-1 Nef mutant -YF) resulted in a marked reduction in CD4 downregulation (~10% CD4 downregulation). Consistent with our previous report (37), a similar degree of impairment was seen with the mutation at K144 (K144R), whereas with the dual mutation at Y202F203 and K144 Nef mutant -KYF was completely impaired in CD4 downregulation. The four Nef proteins were expressed at comparable levels (Fig. 5, *bottom panel*). The results indicated that HIV-1 Nef tyrosine motif Y202Y/F203 is also required for Nef-mediated receptor endocytosis.

FIGURE 4. An in vivo Ub assay showed that SIV Nef lysine residue K176 is ubiquitinated. Plasmids encoding wt SIV Nef or SIV Nef mutant K176R were transfected into 293T cells with (+) or without (-) cotransfection of Ub-His. Cell lysates were incubated with Ni-beads to isolate Nef proteins ligated with Ub-His. *A*, Anti-SIV Nef immunoblotting of Nef proteins. *B*, Anti-Ub immunoblotting of Nef proteins. Arrowheads indicate a ~43-kDa ubiquitinated wt SIV Nef protein that is not detected in Nef mutant K176R.



HIV-1 Nef mutant proteins K144R and Δ10 K and SIV Nef mutant protein -KYY are missing from late endosomes and enriched in microsomes

It is known that mono (di)-ubiquitination is a sorting signal for intracellular vesicular trafficking of endocytosed receptor (29, 31, 32). To study whether Nef ubiquitination serves as a sorting signal for intracellular trafficking of the internalized Nef-CD4 and Nef-MHC-I complexes, we analyzed the distribution of Nef proteins on a discontinuous sucrose gradient that separates early endosomes from late endosomes (44). The lowest two rows in Fig. 6A show that the late endosomes (LE) were enriched in fractions 1 and 2 of the 25% sucrose gradient, identified by the late endosome marker Rab7, whereas the early endosomes (EE) were enriched in fractions 5 and 6 of the 35% sucrose gradient, identified by the early endosome marker Rab5 (45, 46). The upper six rows in Fig. 6A indicate that HIV-1 Nef (wt), Nef dileucine mutant (-LL), and non-CD4-associated Nef (-WL) proteins, all retaining the integrity of K144, were found in late and early endosomes. In contrast, K144R and Δ10K (both having the critical Lys 144 mutated) and the nonmembrane-associated Nef (-GG) mutant proteins were found in smaller amounts in the early endosomes and were not found in the late endosomes. The results suggested

that the late endosomal localization of HIV-1 Nef protein correlates with its ubiquitination at K144 but that it does not correlate with the Nef-AP-2 interaction, because the Nef mutant (-LL) defective for AP-2 interaction was not defective for localization in the late endosomes. The localization of Nef (-GG) mutant further supports the role of K144 ubiquitination in late endosome-bound vesicular trafficking, because the decrease in plasma membrane

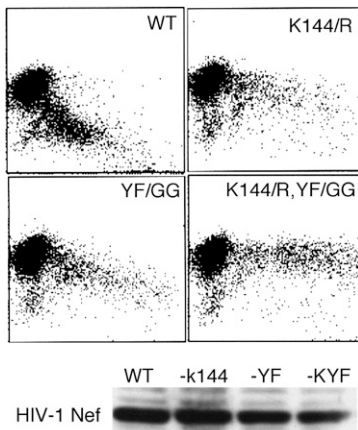


FIGURE 5. Effects of HIV-1 Nef mutation at tyrosine motif Y202F203 on Nef-mediated CD4 downregulation. Plasmids encoding HIV-1 Nef mutants with a single mutation at K144 (K144R) or at the tyrosine motif Y202F203 (YF/GG) and dual mutant at both motifs (K144/R, YF/GG) (-KYF) were cotransfected with EGFP into HeLa CD4 cells and analyzed for CD4 downregulation by flow cytometry, as described in Fig. 2. *Bottom panel*, Comparable Nef protein expression levels were noted in HeLa CD4 cells, as determined by anti-HIV-1 Nef immunoblotting.

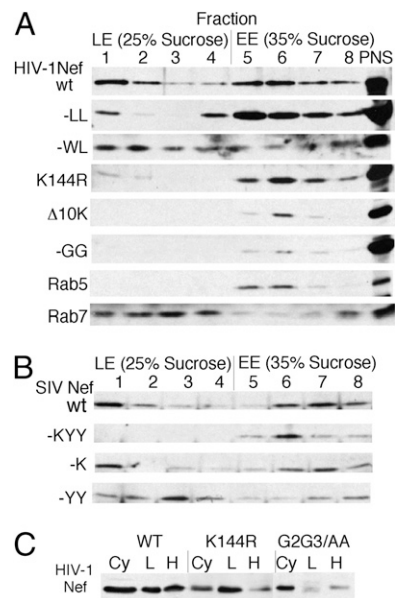


FIGURE 6. Effects of ubiquitination-deficient mutations on subcellular distribution of HIV-1 and SIV Nef proteins. *A*, Distribution of HIV-1 Nef wt and mutant proteins in early endosomes (EE), as indicated by EE marker Rab5, and late endosomes (LE), as indicated by LE marker Rab7. Nef (wt), Nef (LL/AA), Nef (WL/AA), K144R, Δ10K, and Nef (GG/AA) expressed in HeLa CD4 cells were separated on a discontinuous 25–35% sucrose gradient by centrifugation, as described in *Materials and Methods*. Fifteen percent of each collected sucrose fraction and 1.5% of the total PNS were loaded for SDS-PAGE. HIV-1 Nef was immunoblotted with anti-HIV-1 Nef (1:10,000). Rab5 was immunoblotted with anti-Rab5 (1:4,000), and Rab7 was immunoblotted with anti-Rab7 (1:2,000). *B*, Distribution of SIV Nef proteins wt, -KYY, -K, and -YY in EE and LE. The proteins were immunoblotted with anti-SIV Nef (1:10,000). *C*, Distribution of HIV-1 Nef proteins in different membrane fractions. Homogenized cell lysates were separated into cytosol (Cy), light membrane (L), and heavy membrane (H) fractions by differential centrifugation (*Materials and Methods*). Five percent of cytosol, 50% of light membrane, and 20% of heavy membrane fractions were loaded onto gels and immunoblotted with anti-HIV Nef for SDS-PAGE.

association of the mutant Nef protein should abolish endocytosis and the subsequent ubiquitination-dependent vesicular trafficking to the late endosomes. Fig. 6B compares the localization of SIV Nef (wt) and SIV Nef mutants (-K, -YY, and -KYY). SIV Nef (wt) and SIV Nef mutants -K and -YY were found in late and early endosomes, but mutant -KYY was only found in early endosomes. The results suggested that the late endosomal localization of SIV Nef protein requires the K176 and the tyrosine motif (Y28Y39).

The absence of the nonmembrane-associated HIV-1 Nef mutant (-GG) in the late endosomes and its marked reduction in the early endosomes (Fig. 6A) raised the concern that the defective localization phenotypes of HIV-1 Nef K144R, Nef Δ 10K, or -KYY could be due to the decrease in plasma membrane association rather than to the defect in vesicular sorting to the late endosomes. To rule out this alternative explanation, we analyzed the membrane association of these Nef mutants. Cell lysates from Nef-transfected HeLa cells were separated into cytosol, light membrane, and heavy membrane fractions by differential centrifugation, as previously described (42). The light membrane fraction contains microsomes mainly derived from the plasma membrane, endoplasmic reticulum, and Golgi membranes; the heavy membrane fraction contains mainly the larger organelles, including mitochondria and lysosomes (47). Fig. 6C shows that, although HIV-1 Nef (wt) protein was located in all three fractions, Nef proteins containing the K144R mutation were enriched in the light membrane fraction, whereas the nonmembrane-associated Nef mutant (-GG) protein was mainly found in the cytosolic fraction. Densitometrical scanning of the Western blots indicated that ~70% of HIV-1 wt Nef was in the cytosolic fraction, ~2% was in the light membrane fraction, ~7% was in the heavy membrane fraction, and ~20% was in the nuclei fraction (data not shown). Similarly, 70% of the HIV-1 Nef K144R mutant protein was in the cytosolic fraction; however, ~8% was in the light membrane fraction, and ~4% was in the heavy membrane fraction. The results are consistent with a vesicular-sorting defect but not with the alternative explanation of a plasma membrane-association defect for the HIV-1 Nef K144R mutant proteins. They suggested that, in the absence of critical sorting signal(s), Nef proteins are retained in the plasma and/or TGN membrane instead of being targeted to the late endosomes.

Discussion

We previously reported that HIV-1 Nef mutants that contain the K144R substitution are impaired in ubiquitination and Nef-mediated CD4 downregulation (37). In this study, we demonstrated that the ubiquitination signal is also required for Nef-mediated MHC-I downregulation (Figs. 1, 3, Table I). This is true for HIV-1 and SIV Nef-mediated CD4 and MHC-I downregulation (Figs. 2, 5). In addition, HIV-1 Nef K144R mutant protein and SIV Nef-KYY mutant protein were not found in late endosomes (Fig. 6). These results suggested that Nef ubiquitination is a common sorting signal in Nef-mediated receptor endocytosis from early to late endosomes.

A novel discovery made in this study is that the tyrosine-sorting motif also functions in Nef-mediated receptor endocytosis (Figs. 2, 3, 5). We first made this discovery in SIV Nef-mediated CD4 and MHC-I downregulation (Figs. 2, 3, Table I). We found that a single mutation at SIV K176 (homolog to K144 in HIV-1) or at tyrosine motif Y28Y39 did not impair SIV Nef-mediated CD4 and MHC-I downregulation, but the dual mutation completely impaired the downregulation of both. Following this, we discovered that a tyrosine motif Y202Y/F203 conserved among HIV-1 Nef strains is required for CD4 downregulation (Fig. 5). However, unlike SIV Nef, for which impairment of receptor downregulation

required dual mutation at both motifs, for HIV-1 Nef, single mutations at either Lys/ubiquitination motif or the tyrosine motif was sufficient to impair HIV-1 Nef-mediated receptor endocytosis, which suggests a difference in Nef-mediated receptor intracellular trafficking between HIV-1 and SIV.

The localization of the tyrosine-sorting motif (Y202Y/F203) in the flexible HIV-1 Nef C terminus is worth discussing. One needs to exercise caution when constructing a GFP-fusion protein at the HIV-1 Nef C terminus, because the GFP part of the fusion protein could obstruct access to the motif or stabilize the motif to enhance its activity. This may explain an observation made by daSilva et al. (48) that differed from ours. Their EGFP-fused HIV-1 Nef mutant (Nef [10K/10R]-EGFP) was normal in CD4 downregulation (48) in contrast to our finding that nonfused Nef K144R is essentially inactive (Fig. 5), and GFP-fused Nef K144R-GFP is completely inactive in CD4 downregulation (37). We constructed an EGFP-fused dual HIV-1 Nef mutant (K144R and Y202F203/GG-EGFP) in the same plasma encoding cDNA of EGFP (N1) vector as used by daSilva et al. (48), as well as in other vectors. The results (data not shown) demonstrated conclusively that EGFP-fused HIV-1 Nef dual mutant (-KYF-EGFP) is completely inactive in CD4 downregulation.

Our data also clarified the role of SIV Nef N-terminal tyrosine motif (Y28Y39) in Nef-mediated receptor endocytosis. Previously, using a yeast two-hybrid assay, the motif was shown to interact with the μ chain of the adaptor protein complex (21) and to be required for the colocalization of the SIV N-terminal portion (aa 1–45) with AP-2 (22), suggesting that the motif interacts with AP-2 for Nef-mediated CD4 downregulation. However, this model cannot explain our results that mutations at SIV Nef Y28Y39 alone did not affect CD4 downregulation, whereas mutations at the leucine motif L194M195 alone impaired CD4 downregulation by 70–80%, suggesting that Y28Y29 could not effectively compensate for the role of the leucine motif in AP-2 binding and CD4 downregulation (22, 23) (Fig. 3, Table I). Moreover, MHC-I downregulation is known to be independent of Nef-AP-2 interaction (18). Thus, our discovery that the tyrosine motif Y28Y39 performs a sorting role in SIV Nef-mediated MHC-I endocytosis (Fig. 4) provides this motif with a new function. By contrast, the N-terminal AP-2-binding leucine motif WL in SIV Nef is not required for MHC-I downregulation (Fig. 3). We concluded that the SIV Nef N-terminal tyrosine motif Y28Y39 mainly plays a role as a sorting motif for vesicular trafficking of Nef rather than in Nef's interaction with AP-2.

Subcellular fractionation showed that localization of HIV-1 Nef mutant proteins K144R, Δ 10K, and -KYY in endosomes was reduced, and their localization in the late endosomes was absent, whereas their localization in the plasma and TGN fractions was increased (Fig. 6). The results are consistent with the role of ubiquitination in receptor endocytosis from early endosomes to late endosomes/lysosomes (33–36). We also examined the subcellular localization of Nef (wt)-GFP, K144R-GFP, and Δ 10K-GFP in HeLa CD4 cells using confocal microscopy (data not shown). The results showed that Nef-GFP fusion proteins were mostly cytosolic and perinuclear, which is in agreement with the subcellular fractionation data that ~70% of Nef is cytosolic and ~15–20% is in the nuclear fraction (data not shown). A recent article confirmed this pattern of distribution, with Nef-GFP expressed in HeLa cells (49). However, as the result of strong fluorescent emission from the mutant Nef-GFP proteins in the cytosol, we could not precisely locate these fusion proteins in the early and late endosomes.

To evade the mammalian host immune-defense system, a number of viruses downregulate MHC-I by inducing MHC-I ubiq-

uitination. For example, two transmembrane proteins, MIR1 and MIR2 of human herpes virus, function as an E3 ubiquitin ligase to poly-ubiquitinate the MHC-I molecule, resulting in MHC-I's proteasome degradation (50). Other HIV-1 accessory proteins, Vpu (51–54), Vif (55, 56), and Vpr (57, 58), use ubiquitination to degrade host cellular proteins, thereby disarming host cell defense. Our studies indicated that Nef also uses ubiquitination to degrade MHC-I but with a slightly different mechanism. Nef binds to the MHC-I/AP-1 complex and, through its own ubiquitination, sorts the complexes for lysosome degradation.

In conclusion, our study revealed that Nef uses a ubiquitination motif and a tyrosine motif to sort endocytosed receptors. The ubiquitination motif is a Lys residue conserved between HIV-1 Nef and SIV Nef, whereas the tyrosine motif is not conserved in its location within Nef proteins (located at the C terminus of HIV-1 Nef and at the N terminus of SIV Nef). HIV-1 Nef-mediated receptor endocytosis may require both motifs, whereas SIV Nef-mediated receptor endocytosis requires only one of the two motifs.

Disclosures

The authors have no financial conflicts of interest.

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