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

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Nef is a HIV-1 accessory protein critical for the replication of the virus and the development of AIDS. The major pathological activity of Nef is the down-regulation of CD4, the primary receptor of HIV-1 infection. The mechanism underlying Nef-mediated CD4 endocytosis and degradation remains incompletely understood. Since protein ubiquitination is the predominant sorting signal in receptor endocytosis, we investigated whether Nef is ubiquitinated. The in vivo ubiquitination assay showed that both HIV-1 and SIV Nef proteins expressed in Jurkat T cells and 293T cells were multiple ubiquitinated by ubiquitin-His. The lysine-free HIV-1 Nef mutant (Δ10K) generated by replacing all 10 lysines with arginines was not ubiquitinated and the major ubiquitin-His attachment sites in HIV-1 Nef were determined to be lysine 144 (di-ubiquitinated) and lysine 204 (mono-ubiquitinated). Lysine-free HIV-1 Nef was completely inactive in Nef-mediated CD4 down-regulation, so was the Nef mutant with a single arginine substitution at K144 but not at K204. A mutant HIV-1 provirion NL4–3 with a single arginine substitution in Nef at K144 was also inactive in Nef-mediated CD4 down-regulation. Lysine-free Nef mutant reintroduced with lysine 144 (ΔK10 + K144) was shown active in CD4 down-regulation. These data suggest that ubiquitination of Nef, particularly diubiquitination of the lysine 144, is necessary for Nef-mediated CD4 down-regulation. ***The Journal of Immunology,*** **2008, 180: 7878–7886.**

Human immunodeficiency virus Nef is a 27-kDa accessory protein critical for viral replication, high virus load, and the development of AIDS (1–7). The major pathological activity of HIV-1 Nef is the down-modulation of cell surface CD4 (8), the primary receptor for HIV infection. Nef-mediated CD4 down-regulation augments viral production and infectivity (9–16). The increased infectivity by CD4 down-regulation could be explained by preventing the disadvantageous superinfection of host cells (10, 17). CD4 down-regulation promotes HIV progeny release by escaping CD4-mediated "envelope interference," a mechanism that inhibits the incorporation of envelope into virions (9, 12–16). 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).

The mechanism of Nef-mediated CD4 down-regulation has been extensively investigated (8, 18–31). Nef connects CD4 to the AP-2 adaptor protein complex that brings CD4 into the clathrin-coated pits for endocytosis (for reviews, see Refs. 32 and 33). Nef binding to CD4 is also responsible for the sorting of CD4 from the early endosome to late endosome/lysosome (34). However, there remains a considerable gap in our knowledge about Nef-mediated receptor down-regulation, especially the sorting signal for the internalization of a Nef receptor complex and its subsequent intracellular vesicular trafficking. The dileucine motif in Nef is unlikely to fully fulfill the sorting role because in many proteins it is the common binding site for AP-1, AP-2, and AP-3. Yeast, two- or three-hybrid studies show that the dileucine motif in HIV Nef interacts mainly with AP-1 and AP-3 and weakly with AP-2 (26, 27), whereas a GST-tagged HIV Nef binds to AP-1 but not to AP-2 (25–27, 35). AP complexes also exhibit some overlap in their cellular distribution and binding specificity (for review, see Ref. 36). Therefore, an additional specific sorting signal may be required for Nef-mediated receptor endocytosis and/or for Nef receptor complex trafficking.

Ubiquitination is a form of protein posttranslational modification which covalently attaches a 76-aa ubiquitin (Ub)[3] molecule to the ε-amine group on a lysine residue (or the N terminus) of a protein. This form of posttranslational modification has emerged as one of the most important general cellular regulatory mechanisms (for review, see Refs. 37–41). Like protein phosphorylation, protein ubiquitination is extremely complex and versatile. Ub can be conjugated to proteins as a monomer or dimer (mono-ubiquitination or di-ubiquitination) or as a polymer formed by ubiquitination of Ub itself (poly-ubiquitination) (39). Attachment of several mono-ubiquitin or di-ubiquitin to different lysine residues is referred to as multiple ubiquitination (multi-ubiquitination) (39–41). The poly-ubiquitin formed by ubiquitination of Ub lysine 48 (poly-Ub_{48}) targets a protein to proteasomes for degradation. Mono- and multi-ubiquitination are involved in a variety of other cellular functions, including receptor endocytosis, endosome sorting, and DNA repair (for recent reviews, see Refs. 40, 42, and 43). Receptors tagged with mono- or multi-ubiquitin chains may interact with proteins containing Ub-binding domains such as Eps15, epsin, Hrs, and Tsg10 (Ub-binding proteins) (44–46). The consecutive binding to various Ub-binding proteins localized at different membrane compartments may therefore sort a cargo protein from the plasma

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[3] Abbreviations used in this paper: Ub, ubiquitin; wt, wild type.

Table I. *Ubiquitination of Nef (NA7) mutants with arginine for lysine substitutions and their activities in CD4 down-regulation*[a]

Nef	K4	K7	K18	K39	K82	K92	K94	K144	K184	K204	Ubiquitination	CD4 Down-Regulation
wt	+	+	+	+	+	+	+	+	+	+	+++	+
Δ2K	−	−	+	+	+	+	+	+	+	+	+++	+
Δ5K	−	−	−	−	−	+	+	+	+	+	+++	+
Δ7K	−	−	−	−	−	+	+	−	−	+	++	−
Δ8K	−	−	−	−	−	+	−	−	−	+	++	−
Δ9K	−	−	−	−	−	+	−	−	−	−	+	−
Δ10K	−	−	−	−	−	−	−	−	−	−	−	−
K144/R	+	+	+	+	+	+	+	−	+	+	++	<10%
K184/R	+	+	+	+	+	+	+	+	−	+	+++	+
K204/R	+	+	+	+	+	+	+	+	+	−	++	+
K92/94R	+	+	+	+	+	−	−	+	+	+	++	+
K144/184/204R	+	+	+	+	+	+	+	−	−	−	+	−
Δ10K + K144	−	−	−	−	−	−	−	+	−	−	di-Ub	~50%
Δ10K + K204	−	−	−	−	−	−	−	−	−	+	Mono-Ub	<5%

[a] The levels of ubiquitination of these Nef mutants (high, +++; medium, ++; low, +) are based on the intensity and the numbers of ubiquitinated Nef bands determined in three repeats represented by Figs. 3 and 6. CD4 down-regulation activity (+ or −) was determined by cotransfection of GFP and Nef mutants into BYCD4 T cells as described in Figs. 4 and 6.

membrane or transGolgi network into the lumen of endosomal vesicles.

In this report, we determined that both HIV-1 and SIV Nef proteins are multiply ubiquitinated as determined by in vivo ubiquitination assay and that the substitution of lysine 144, a di-Ub attachment site in HIV-1 Nef, with arginine abrogates Nef-mediated CD4 down-regulation.

Materials and Methods

Cell lines and the transfection

Jurkat T cells and BYCD4 hybridoma cells (47) were cultured in RPMI 1640 medium supplemented with 10% FCS. 293T cells (a human kidney cell line) were cultured in DMEM supplemented with 10% FCS. For transient expression in Jurkat T cells and BYCD4 hybridoma cells, plasmid DNA was electroporated into the cells at 800 μF/250 V. For transient expression in 293T cells, DNA was transfected into the cells using the $Ca_3(PO_4)_2$ method. Briefly, 50 μg of DNA in 1.1 ml of double-distilled H_2O was added by155 μl of 2 M $CaCl_2$ while mixing by vortex. Then, 1250 μl of 2× HBS (8.0 g of NaCl, 0.37 g of KCl, 201 mg of $Na_2HPO_4{\cdot}7H_2O$, 1.0 g of glucose, and 5.0 g of HEPES/500 ml, pH 7.05) was added to the above solution dropwise with gentle mixing. Within 1–2 min after addition of 2× HBS, the mixture was added directly to the cell culture medium dropwise.

Antibodies

Anti-HIV-1 Nef rabbit serum was obtained from the National Institutes of Health AIDS Research and Reference Reagent Repository. Anti-CD4 mAb (Leu3a), PE-conjugated anti-CD4 mAb (Leu3a), and mAb against p21 were purchased from BD Biosciences, mAb against Ub (P4D1) from Santa Cruz Biotechnology, sheep anti-SIV Nef pAb from Exalpha Biologicals, ECL anti-rabbit and HRP-conjugated anti-mouse IgG $F(ab')_2$ Abs from Amersham Biosciences, and HRP-conjugated rabbit anti-sheep IgG Ab from Millipore.

Plasmids and chemicals

Plasmid Nef (pNA7)-GFP encoding the HIV-1 Nef-GFP fusion protein and the bicistronic expression plasmid containing the 239-Nef gene (a SIV Nef) and GFP cDNA were provided by Dr. J. Skowronski (48). Plasmid pNA7 encoding HIV-1 Nef (28) was used in this study to express wild-type (wt) Nef unless otherwise specified. Another HIV-1 Nef allele gene (NL4-3) was subcloned from the HIV-1 provirion NL4-3 obtained from the National Institutes of Health AIDS Research and Reference Reagent Repository. Nef mutants with arginine for lysine substitutions were generated by sequential substitutions using the MultiQuick Change Mutagenesis Kit (USB). The sequences of the eight primers used in these substitutions are available upon request. All lysine mutants, including Δ5K, Δ7K, Δ8K, Δ9K, and Δ10K (lysine free) contain the same substitutions in Δ5K at residues K_4, K_7, K_{18}, K_{39}, and K_{82}. The additional lysine residues were substituted in mutant Δ7K, Δ8K, Δ9K, and Δ10K (see detail in Table I). The Δ10K+K92, Δ10K+K94, Δ10K+K144, Δ10K+K184, and Δ10K+K204 Nef mutants were generated using Δ10K as the template. GFP-tagged Nef mutants were similarly constructed using wt Nef-GFP as the template, so was a HIV-1 provirion NL4-3 mutant with a single arginine substitution at Nef K144 using wt pNL4-3 as the template. Nef C142A mutant was generated by substitution of cysteine C142 with alanine. Human CD4 subcloned in pcDNA3 was described previously (49). All mutations generated in this study were confirmed by DNA sequencing. Ub-His plasmids were provided by the Pagano laboratory (50). MG132 was purchased from Calbiochem.

In vivo ubiquitination assay

HIV-1 Nef (or SIV Nef) and Ub-His plasmid DNAs were cotransfected into 293T cells by the $Ca_3(PO_4)_2$ method or into Jurkat T cells by electroporation. Sixteen hours posttransfection, the cells were treated with 20 μM MG132 for another 6 h before harvest. The cells were lysed in 1 ml of denature lysis buffer (6 M guanidinium chloride and 0.1 M sodium phosphate, pH 8.0; 10 mM imidazole) per 60 mm dish. The lysates were sonicated to shear DNA and then centrifuged to remove particulate material. Fifty microliters of lysates was mixed with an equal volume of 2× SDS sample buffer and the mixture was boiled. This is the whole cell lysate. The rest of the lysates were mixed with 100 μl of 75% slurry of Ni-NTA-agarose (Qiagen) and incubated with rotation at 4°C for 3 h. The beads were washed three times with denature lysis buffer, twice with Wash buffer I (lysis buffer diluted 1/4 in 25 mM Tris-HCl (pH 6.8) and 20 mM imidazole) and twice with Wash buffer II (25 mM Tris-HCl (pH 6.8) and 20 mM imidazole). The bound proteins were eluted by boiling the beads in 2× SDS sample buffer/100 mM EDTA and were analyzed by immunoblotting.

FACS analysis of Nef-mediated CD4 down-regulation

GFP-tagged Nef or Nef plus GFP plasmid DNAs in a ratio of 4:1 (w/w) were cotransfected into BYCD4 T cells (47) by electroporation. Sixteen hours after the transfection, cells were incubated on ice for 45 min with PE-conjugated anti-CD4 mAb (Leu3a) at a 1/100 dilution in PBS and then fixed in 2% paraformaldehyde. Cells were then subjected to two-dimensional FACS analysis on a FACScan (BD Biosciences). The percentage of CD4 down-regulation in the presence of the mutant forms of HIV-1 Nef is expressed as a value relative to that of the wt Nef (100%) based on the medium CD4 staining. Each value was the average of three independent experiments (mean ± SD).

Confocal microscopy

BYCD4 T cells were transfected with Nef-GFP DNA for 12–16 h as described above. The cells were stained with anti-CD4 (Leu3a) of 1/ 100 dilution for 30 min on ice followed by the Texas Red-conjugated anti-mouse IgG (1/1000) on ice for 30 min. The stained cells were seeded on coverslips precoated with 5 mg/ml polylysine in PBS. HeLa cells preseeded on coverslips the day before were transfected with 1 μg of Nef-GFP plasmid DNA using Lipofectamine 2000 (Invitrogen). Twelve to 16 h after transfection, cells on coverslips were surface stained with anti-CD4 (Leu3a) followed by the Texas Red-conjugated anti-mouse IgG. Cells were fixed with 2% of paraformaldehyde at room temperature for 15 min. Confocal microscopy was performed on a Zeiss LSM 510 laser scanning confocal microscope (Cancer Center, New York University Medical School).

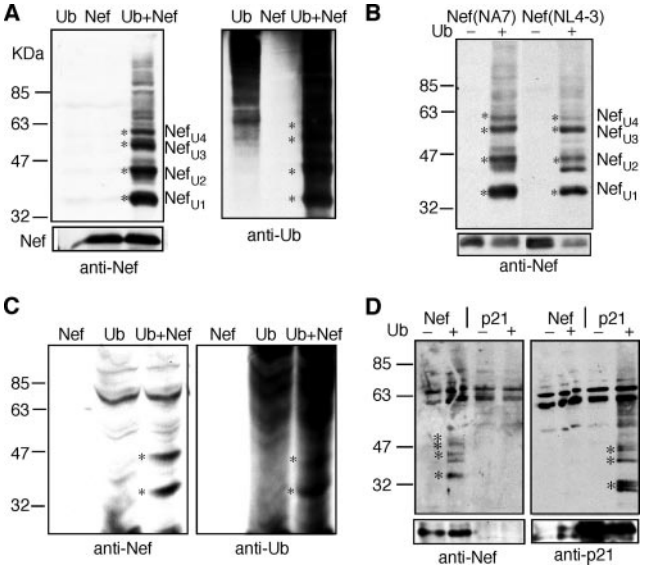

FIGURE 1. Ubiquitination of HIV-1 Nef expressed in 293T cells and Jurkat T cells. *A*, Ubiquitination of HIV-1 Nef (NA7). Plasmid DNAs of Ub-His, Nef, or Ub-His plus Nef were transfected into 293T cells. The Ub-His-conjugated Nef proteins (∗) were precipitated by Ni-NTA-agarose beads and analyzed by anti-Nef (*left*) and anti-Ub (*right*) immunoblotting (see *Materials and Methods* for details). The *bottom pan*el shows the levels of Nef expression in whole cell lysates of the transfected cells. *B*, Comparison of Nef (NA7) and Nef (NL4-3) ubiquitination. The equal amounts (50 μg) of Nef (NA7) or Nef (NL4-3) plasmid DNAs were cotransfected with Ub-His into 293T cells and determined for ubiquitination as described in *A*. *C*, Nef (NA7) and Ub-His were cotransfected into Jurkat T cells and assayed as in *A*. *D*, The pNL4-3 proviral plasmid and control p21 plasmid were cotransfected with Ub-His into 293T cells. The ubiquitination was determined by anti-Nef (*left*) or anti-p21 (*right*), respectively. The bands of ~30, 39, and 47 kDa of ubiquitinated p21 are marked with ∗.

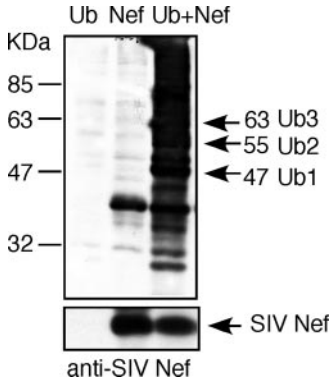

FIGURE 2. Ubiquitination of SIV_{239} Nef expressed in 293T cells. SIV_{239} Nef. Ub-His, SIV_{239} Nef, or Ub-His plus SIV $_{239}$ plasmids were transfected into 293T cells. The ubiquitination was determined by anti-SIV Nef immunoblotting.

Results

In vivo ubiquitination assay revealed that both HIV-1 and SIV Nef proteins are multiubiquitinated

Experiments attempting to demonstrate covalent Ub modification in vivo (meaning in cultured cells) using routine immunoblotting are challenging because of low steady-state levels of the ubiquitinated forms of the protein caused by proteasomal degradation and/or highly active deubiquitinating enzymes. This appeared to be the case with Nef proteins, because we were unable to detect ubiquitinated Nef proteins using standard anti-Nef immunoblotting. We then resorted to the in vivo ubiquitination assay (51) that is customarily used in the field. In this assay, a His-tagged Ub (Ub-His) is exogenously overexpressed to facilitate the detection of the ubiquitination of a protein of interest. We cotransfected DNAs encoding HIV-1 Nef and the Ub-His into 293T cells or Jurkat T cells. Proteins conjugated with Ub-His were precipitated using Ni-NTA beads and characterized using immunoblotting. In this procedure, highly denaturing conditions (8 M urea or 6 M guanidinium) were maintained to counter the activities of deubiquitinating enzymes (51).

Fig. 1 shows that an anti-Nef Ab specifically detected a number of Ub-His-conjugated proteins in cells cotransfected with Nef and Ub-His (Ub plus Nef) but not in cells transfected with Ub-His or Nef alone (Fig. 1*A*). In these experiments, Nef proteins were expressed at similar levels as determined by Western blotting with an anti-Nef Ab (Fig. 1*A, bottom panel*). Immunoblotting with an anti-Ub Ab confirmed that these proteins were conjugated with Ub-His. The major Ub-His- conjugated Nef proteins (indicated by the star symbols, Fig. 1) had apparent molecular masses of 35, 43, 51, and 59 kDa, respectively, which are likely to be the Nef molecules (27 kDa) linked with one, two, three, or four Ub-His (8 kDa) molecules, respectively. They are termed $NefU_1$, $NefU_2$, $NefU_3$, and $NefU_4$ in Fig. 1. The ubiquitination pattern of another Nef allele, NL4-3, was essentially the same as that of the Nef allele NA7 (Fig. 1*B*). To determine whether Nef was ubiquitinated in T cells, Nef and Ub-His were cotransfected into Jurkat T cells. The results indicated that Nef expressed in T cells was similarly ubiquitinated, except that the $NefUb_3$ and $NefUb_4$ were not evident (Fig. 1*C*). The difference is likely due to the low transfection efficiency commonly observed with suspended T cells. We also cotransfected the Ub-His and pNL4-3 into 293T cells (Fig. 1*D*). Anti-Nef blotting indicated that Nef encoded by pNL4-3 provirion was also ubiquitinated. To validate our ubiquitination assay, we included transfection of $p21^{Cip1}$; the ubiquitination of $p21^{Cip1}$-encoded protein was used as a positive control. Fig. 1*D* shows that anti-p21 immunoblotting detected several Ni-NTA-bound proteins in the lysates of $p21^{Cip1}$- and Ub-His-cotransfected cells but not in the lysates of the cells transfected with $p21^{Cip1}$ DNA alone. The pattern of $p21^{Cip1}$ubiqutination was similar to that reported by the others (the bands of ~30, 39, and 47 kDa as marked) (52, 53), indicating that our ubiquitination assay is reliable.

SIV Nef and HIV-1 Nef are well conserved in three regions (the N-terminal myristoylation site important for Nef membrane association, the proline-rich region (PXXP motif) important for interaction with SH3 domains, and the dileucine motif important for interactions with AP complexes) but differ in most of the remaining regions (54). To determine whether ubiquitination is a conserved posttranslational modification of Nef, we investigated SIV Nef ubiquitination using the same method. SIV_{239} Nef and Ub-His DNAs were cotransfected into 239T cells for the analysis. The results showed that, like HIV-1 Nef, SIV_{239} Nef was also ubiquitinated (Fig. 2). The major ubiquitinated SIV Nef bands (arrow-indicated) had the apparent molecular mass of 47, 55, and 63 kDa, which are likely to be the SIV Nef molecules (36 kDa) conjugated with one, two, or three Ub-His molecules. The results thus indicate that ubiquitination is a conserved posttranslational modification between HIV-1 Nef and SIV Nef.

The main Ub-His attachment sites in HIV-1 Nef (NA7) were determined to be K144 and K204

To determine which lysine residues in HIV-1 Nef are ubiquitinated, we made a series of arginine for lysine substitutions in Nef

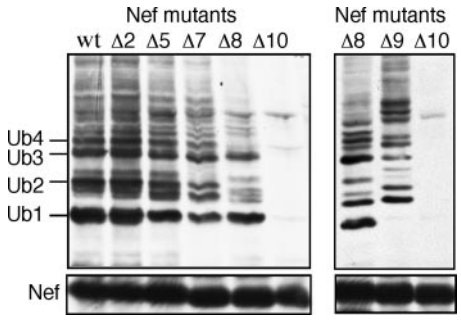

FIGURE 3. Ubiquitination of Nef arginine for lysine mutants (listed in Table I). The Nef mutants and Ub-His were cotransfected into 293T cells and determined for Nef ubiquitination as described in Fig. 1. *Left panel*, The ubiquitination of Nef mutants Δ2K, Δ4K, Δ7K, Δ8K, and Δ10K; *right panel*, Δ8K, Δ9K, and Δ10 K. *Top panel*, Ubiquitinated Nef proteins; *bottom panel*, the Nef expression in whole cell lysates.

(NA7) (Table I). Six of these mutants have more than one substitution and are termed Δ2K, Δ5K, Δ7K, Δ8K, Δ9K, and Δ10K (lysine free), having 2, 5, 7, 8, 9, or all 10 lysines replaced, respectively. These Nef mutants were subjected to the in vivo ubiquitination assay as described above. Fig. 3 shows two representative immunoblotting images demonstrating the ubiquitination of wt, Δ2, Δ5, Δ7, Δ8, and Δ10 (*left*) and Δ8, Δ9, and Δ10 (*right*), respectively (also summarized in Table I). The results (Fig. 3 and Table I) indicated that the ubiquitination of the Δ2K Nef mutant with two arginines for lysine substitutions at K4 and K7 and the Δ5K with five substitutions at K4, K7, K18, K39, and K82 was similar to that of wt Nef. With more substitutions in Δ7K, Δ8K, Δ9K, and Δ10K (lysine free), Nef ubiquitination was decreased in both the intensity and the number of the ubiquitinated bands. No ubiquitination was detected in the lysine-free Δ10K Nef mutant, indicating that Nef was ubiquitinated at the side chain of lysine residues exclusively. In contrast, there were both mono- and diubiquitinated Nef detected in Δ8K in which K144 and K204 are the only two lysine residues remaining (Table I), suggesting that K144 and K204 were either mono-ubiquitinated or di-ubiquitinated (see Fig. 6*A* also). We further examined the ubiquitination of Nef mutants with single substitution in the C-terminal region at K144, K184, or K204. The results showed that the ubiquitination was apparently affected by K144 and K204 substitutions but not significantly affected by K184 substitution (Table I). When all three C-terminal lysine residues (K144/184/204) were substituted, the mutant was ubiquitinated at a very low level (Table I). Taken together, we concluded that the main Ub-His attachment sites in HIV-1 Nef are K144 and K204. As shown below, further studies indicated that K144 is di-ubiquitinated, whereas K204 is mono-ubiquitinated (see Fig. 6*A*). Weak diubiquitination of K92 and K94 was also detected (Table I and see Fig. 6*A*). The Nef proteins tagged with three or four Ub-His molecules (Fig. 1) are likely to be formed by the Nef ubiquitination with one mono-Ub and one di-Ub or with two di-Ub molecules at different lysine residues.

Substitution of K144 with arginine abolished Nef-mediated CD4 down-regulation

To determine whether Nef ubiquitination is required for Nef-mediated CD4 down-regulation and, if so, which lysine residue(s) is critical, we examined the effects of the arginine for lysine substitutions on this Nef function (Table I and Fig. 4). We did this by cotransfection of Nef with GFP at a ratio of 4:1 into BYCD4 T cells, followed by FACS analysis of cell surface CD4 levels. We choose BYCD4 T cells (47) because, compared with other T cell lines tested, CD4 expression in our BYCD4 T cells is extremely stable and the cell line has a higher Nef DNA electroporation efficiency. As a result, data of Nef-mediated CD4 down-regulation in BYCD4 T cells are more reliable based on our previous studies (29). Fig. 4*A* shows that CD4 was down-regulated in cells expressing wt Nef or the Δ2K (data not shown) and Δ5K Nef mutants. In contrast, mutants Δ7K, Δ8K, Δ9K, and Δ10K were largely inactive in CD4 down-regulation. Because K144 is mutated to arginine in Δ7K, Δ8K, Δ9K, and Δ10K but is intact in Δ2K and Δ5K, the

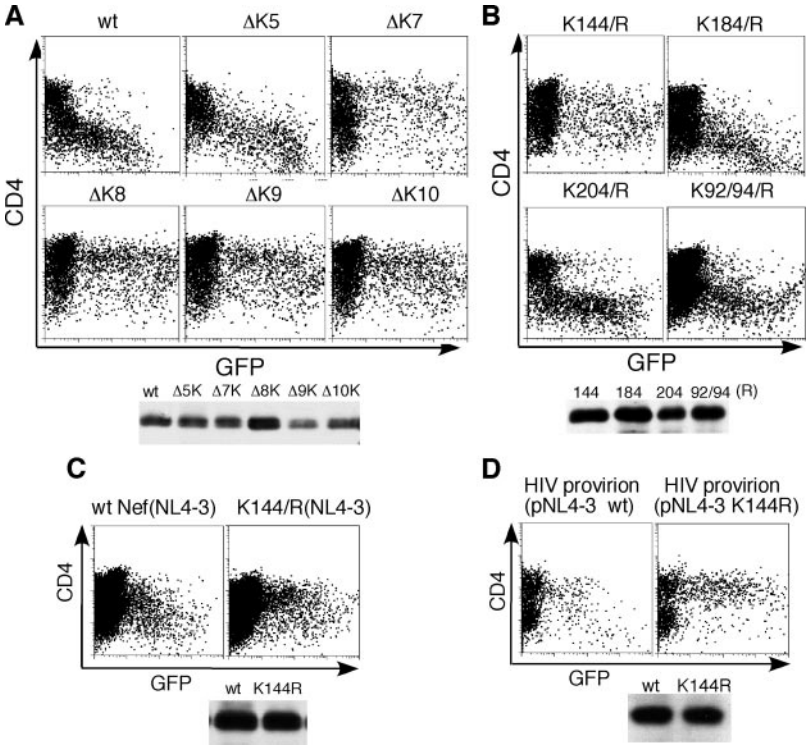

FIGURE 4. FACS analysis of CD4 down-regulation by Nef mutants with arginine for lysine substitutions. BYCD4 T cells were cotransfected with plasmids encoding Nef mutants and GFP at a ratio of 4:1 or 2:1 (w/w) for 16 h, surface stained with anti-CD4 mAb PE, and analyzed by two-dimensional FACS. *A*, CD4 down-regulation by Nef mutants containing multiple lysine to arginine mutations as indicated. *B*, CD4 down-regulation by Nef mutants containing single or double lysine to arginine mutations as indicated. *C*, CD4 down-regulation by Nef (NL4-3) K144 mutant subcloned in pcDNA3 plasmids. *D*, CD4 down-regulation by wt pNL4-3 or pNL4-3 provirion containing single Nef K144R mutation. *Bottom* of each *panel* shows the Nef expression determined by anti-Nef immunoblotting. Ten micrograms of wt Nef and 20–40 μg of Nef mutant DNAs were used in the transfection to adjust the Nef expression to similar levels.

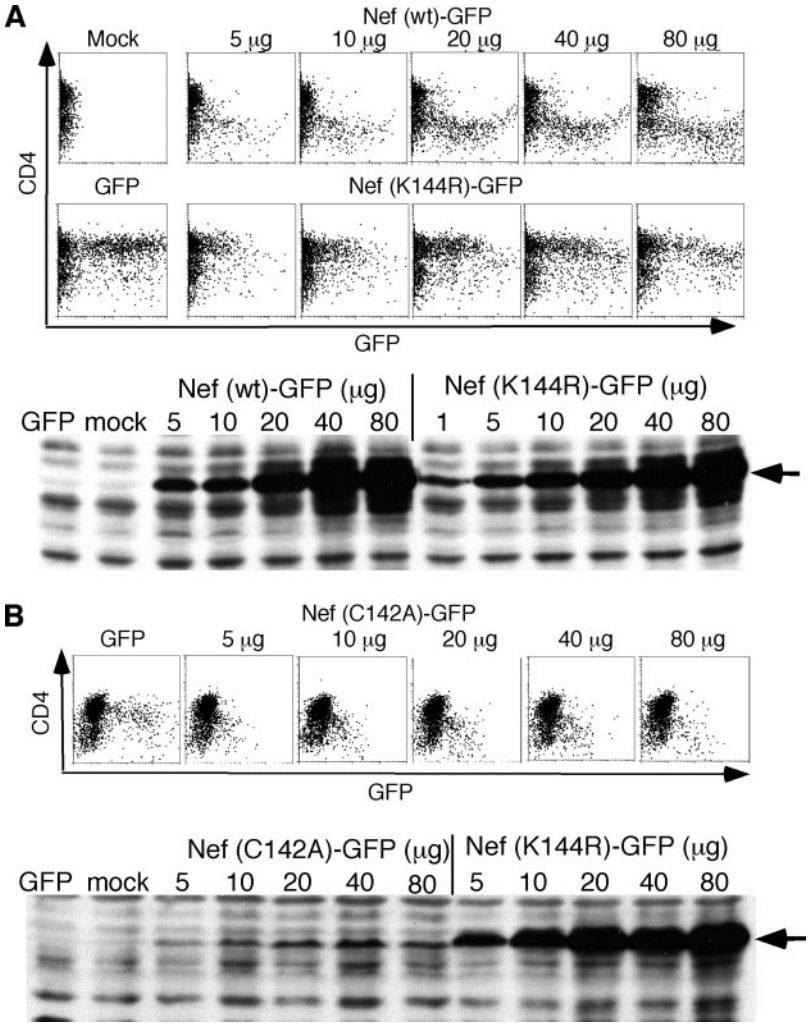

FIGURE 5. Analysis of CD4 down-regulation by GFP-tagged Nef K144R mutant. *A*, BYCD4 T cells were transfected with the indicated amounts of Nef (wt)-GFP or Nef (K144R)-GFP for 16 h. Cells were surface stained with anti-CD4-PE. *Top panel*, FACS analysis of the CD4 down-regulation; *bottom panel*, Nef-GFP expression determined by anti-Nef immunoblotting. *B*, BYCD4 T cells were transfected with Nef (C142A)-GFP mutant and determined for CD4 down-regulation and Nef expression as in *A*.

results indicate that K144 is required for Nef-mediated CD4 down-regulation (Table I). In this regard, it is worthy of note that K144 as well as the motif K_{144}LPV are conserved in all sequenced HIV-1 and SIVcpz Nef strains (55).

To confirm the critical role of K144 in Nef-mediated CD4 down-regulation, we analyzed CD4 down-regulation of three mutants with single substitution, K144R, K184R, and K204R in BYCD4 T cells. In addition, because K92 is weakly diubiquitinated (Fig. 3 and Table I), the effects of the double substitution of K92R/K94R on Nef-mediated CD4 down-regulation were also analyzed. Fig. 4*B* shows that the K144R substitution is the only mutation that abrogated Nef-mediated CD4 down-regulation (see also $<$10% of wt activity, Table I). To confirm the importance of K144 in Nef-mediated CD4 down-regulation and to determine whether it is not restricted to HIV-1 strain NA7, we analyzed CD4 down-regulation induced by the Nef K144R mutant of another HIV-1 strain (NL4-3) that has arginine at residue 184 as opposed to the K184 in NA7 Nef. BYCD4 cells were transfected with plasmid DNA of Nef NL4–3 subcloned into pcDNA3 vector (Fig. 4*C*) or HIV provirion DNA pNL4-3 (Fig. 4*D*). The FACS analysis showed that K144 to the R mutation in Nef NL4-3 also severely impaired the CD4 down-regulation. Importantly, HIV provirion NL4-3 with a single K144R Nef mutation is also inactive in CD4 down-regulation (Fig. 4*D*). In these experiments, Nef expression was at similar levels as determined by anti-Nef immunoblotting (Fig. 4, *bottom panels*).

In the above analysis, we transfected cells with Nef plus GFP plasmid DNAs and adjusted the Nef DNA amounts to make Nef expression at similar levels. For more quantitative analysis, we transfected cells with different doses of plasmids encoding Nef-GFP fusion proteins (Fig. 5*A*). The analysis by FACS (Fig. 5*A, top panel*) and by anti-Nef immunoblotting (Fig. 5*A, bottom panel*) both indicated that the expression levels of Nef (wt)-GFP and Nef (K144R)-GFP were DNA dose dependent at a range between 1 and 40 μg and were at about a 2:1 ratio in cells transfected with equal amounts of wt Nef-GFP or Nef (K144R)-GFP. Transfection with 5 μg of wt Nef-GFP DNA was sufficient for CD4 down-regulation, whereas transfection with up to 80 μg of Nef (K144R)-GFP did not result in significant CD4 down-regulation. The results excluded the possibility that the Nef K144R mutant failed to induce CD4 down-regulation due to a low level of expression.

To investigate whether a local structure change in the K144 located area abrogates the Nef-mediated CD4 down-regulation, we examined the effects of C142A mutation, which is in close proximity to K144, on Nef-mediated down-regulation. It was previously reported that residue C142 is critical for the correct folding of Nef and that a Nef mutant with C142 to A substitution is unstable (56). Fig. 5*B* shows that transfection with 5 μg of C142A Nef mutant was sufficient for CD4 down-regulation despite that the C142A Nef mutant was expressed at a much lower level compared with Nef (K144R; Fig. 5*B, bottom panel*). The results largely excluded the possibility that the K144R mutation abrogates Nef

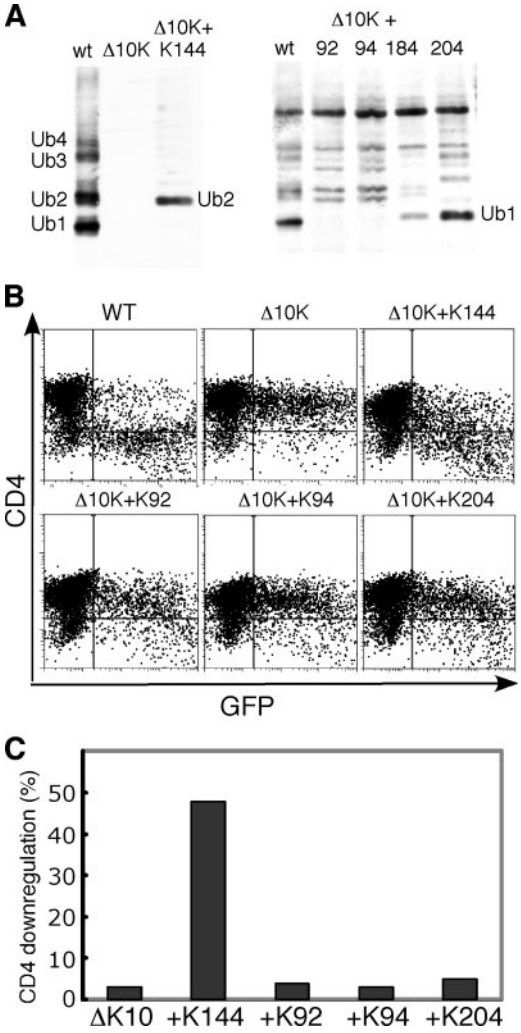

FIGURE 6. Nef ubiquitination and CD4 down-regulation by Δ10K Nef mutants that have regained a single lysine at different positions (K144 (Δ10K + K144), K92 (Δ10K + K92), K94 (Δ10K + K94), K184 (Δ10K + K184), or K204 (Δ10K + K204)). The Nef ubiquitination and CD4 down-regulation were performed as described in Figs. 1 and 4, respectively. *A, Left panel*, the ubiquitination of Nef ΔK10 + K144 mutant; *right panel*, the ubiquitination of Nef mutants Δ10K + K92, Δ10K + K94, Δ10K + K184, and Δ10K + K204. *B*, CD4 down-regulation by Nef mutants containing single lysine K144, K92, K94, or K204. *C*, Relative CD4 down-regulation (percent) in cells expressing high levels (GFP > 100) of the Nef lysine mutants as indicated. BYCD4 cells were cotransfected with GFP and Nef DNA (see *Materials and Methods* for the calculation).

activity in CD4 down-regulation by altering the Nef structure in the K144 area.

To provide further proof that residue K144 is critical for Nef-mediated CD4 down-regulation, we reintroduced K144 back into the lysine-free Nef mutant (Δ10K), resulting in Δ10K plus K144. Fig. 6 shows that the Δ10K plus K144 mutant was di-ubiquitinated (Fig. 6*A*) and that the Δ10K plus K144 Nef mutant was active in CD4 down-regulation (Fig. 6*B*). For comparison, we also reintroduced the K92, K94, K184, and K204 back into the lysine-free Nef mutant (Δ10K), respectively. The ubiquitination assay indicated that K204 and K184 were mono-ubiquitinated, whereas K92 and K94 were diubiquitinated. But K144 and K204 are the main Ub attachment sites in HIV-1 Nef based on the relative intensity of the ubiquitination (Fig. 6*A*). CD4 down-regulation analysis showed that the single K144 Nef mutant (Δ10K plus K144) was ~50% active in CD4 down-regulation in contrast to the inactive Δ10K

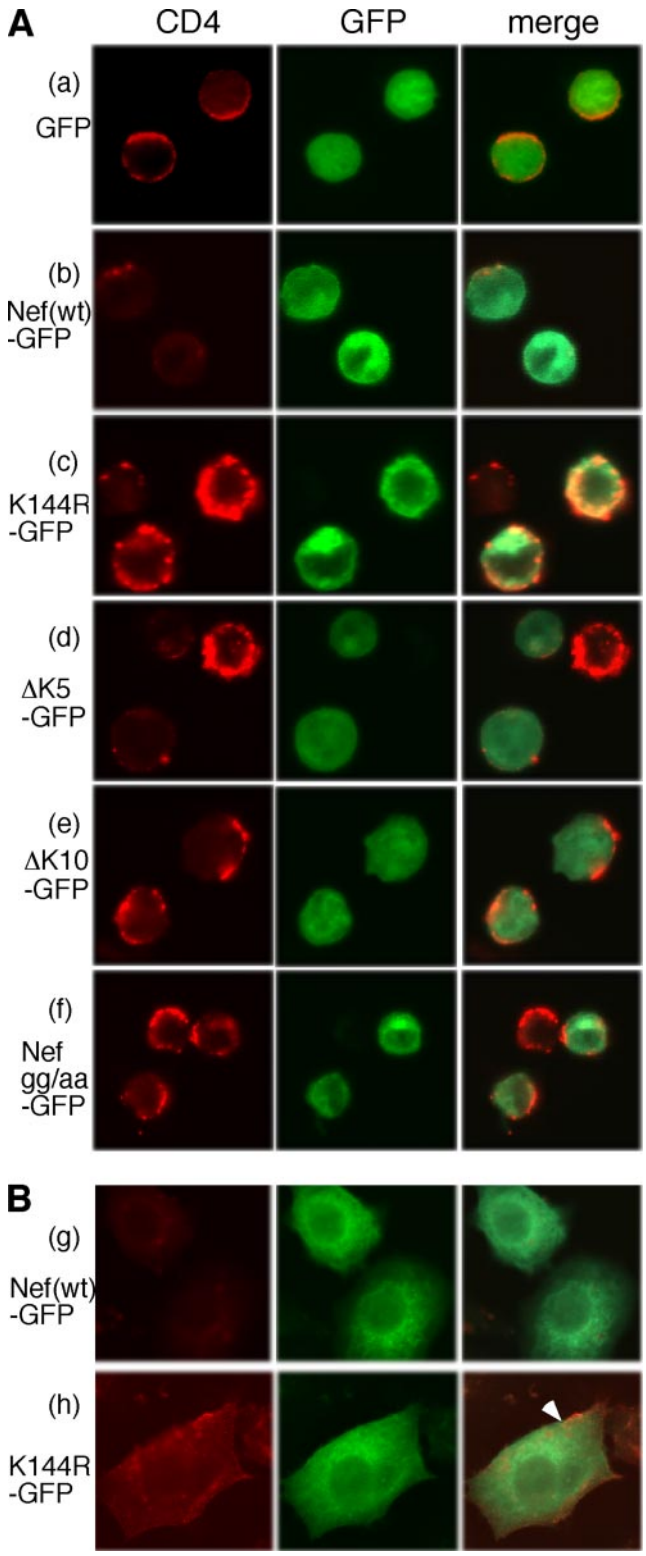

FIGURE 7. Confocal microscopy analysis of CD4 down-regulation in BYCD4 cells (*A*) and HeLa cells (*B*) transfected with Nef mutants. Cells were surface stained with anti-CD4 mAb (Leu3a) followed by the Texas Red-conjugated anti-mouse IgG. The results are representatives of three independent experiments. *A*, BYCD4 T cells were transfected with GFP (*a*), Nef (wt)-GFP (*b*), Nef (K144R)-GFP (*c*), Nef (ΔK5)-GFP (*d*), Nef (ΔK10)-GFP (*e*), and Nef (gg/aa)-GFP (*f*). *B*, HeLa cells were cotransfected with human CD4 and wt Nef-GFP (*g*) or with human CD4 and Nef (K144R)-GFP (*h*). The surface CD4 (red) is indicated by an arrowhead.

Nef, whereas the single K92 (Δ10K plus K92), single K94 (Δ10K plus K94), and single K204 (Δ10K plus K204) Nef mutants were slightly more active (< 5%) than Δ10K Nef (Fig. 6, *B* and *C*). The results indicate that K144 is the most critical lysine in Nef-mediated CD4 down-regulation, whereas other lysines, such as K92, K94, and K204, may contribute to CD4 down-regulation in the presence of K144.

Confocal microscopy confirmed that K144 was required for Nef-mediated CD4 down-regulation

To further examine the critical role of Nef K144 in Nef-mediated CD4 down-regulation, we used confocal microscopy. BYCD4 T cells were transfected with GFP-tagged Nef and surface stained with anti-CD4 (Leu3a). Fig. 7*A* shows that there was essentially no detectable cell surface CD4 (red) in BYCD4 cells expressing wt Nef-GFP (green; *b*) while a strong CD4 (red) staining in cells expressing GFP was observed (no Nef; *a*). Thus, the confocal microscopy correctly presented the Nef-mediated CD4 down-regulation phenotype. In contrast to cells expressing wt Nef-GFP, high surface CD4 expression was detected in cells expressing Nef (K144R)-GFP expressing Nef (K144R)-GFP (Fig. 7*Ac*), Nef (ΔK10)-GFP (Fig. 7*Ae*) or the control Nef (G_2G_3/AA)-GFP incapable of CD4 down-regulation (Fig. 7*Af*) whereas surface CD4 expression was low in cells expressing Nef (ΔK5)-GFP that retains the lysine K144 (Fig. 7*Ad*). We then examined the Nef-mediated CD4 down-regulation in HeLa cells cotransfected with human CD4 and Nef-GFP. Fig. 7*B* shows that CD4 was down-regulated from the surface in HeLa cells expressing wt Nef-GFP (*g*) but was not down-regulated in cells expressing Nef (K144R)-GFP (*h*). Thus, the confocal microscopy examination is in agreement with FACS data (Figs. 4–6), confirming that Nef K144 is required for the Nef-mediated CD4 down-regulation.

Discussion

We discovered that both HIV-1 Nef and SIV Nef are multiply ubiquitinated with Ub-His when Nef and Ub-His are exogenously expressed in BYCD4 T cells, Jurkat T cells, or 293T cells, suggesting that ubiquitination is a Nef posttranslational modification conserved among strains of HIV and SIV. Using 16 Nef mutants with arginine for lysine substitutions (not all are shown in this report), we determined the Ub attachment sites in HIV-1 Nef to be mainly at K144 (diubiquitination) and K204 (monoubiquitination). These Nef mutants were then examined for their activity in CD4 down-regulation by FACS and by confocal microscopy (Figs. 4–7 and Table I). Lysine-free Nef was completely inactive in Nef-mediated CD4 down-regulation and the K144R mutant, but not the K204R mutant, was greatly impaired in CD4 down-regulation. CD4 was down-regulated in cells transfected with 5 μg of wt Nef-GFP but was essentially not down-regulated in cells transfected with 20–80 μg of Nef (K144/R)-GFP. Introducing the Nef K144R mutation into HIV-1 provirion NL4-3 also abrogated its activity in CD4 down-regulation. Reintroducing K144 back into the lysine-free Nef mutant makes the resultant Nef mutant regain the function of Nef-mediated CD4 down-regulation. All of these data consistently show that K144 in Nef is both necessary and sufficient for Nef-mediated CD4 down-regulation.

A single conserved arginine for lysine substitution at residue 144 is unlikely to cause a global conformational change in Nef since mutants with multiple substitutions (Δ2K, Δ5K, and K92R/K94R) showed no impairment in Nef-mediated CD4 down-regulation. A structural alteration in this specific area is not likely to inactivate Nef since the C142A mutation, which is in proximity to K144 and known to affect the Nef structure (56), is active in CD4 down-regulation despite its low expression (Fig. 5*B*). Thus far, the regions essential for Nef to down-regulate CD4 include the N-terminal myristoylation site (residue glycine 2) necessary for membrane association of Nef, the CD4 interaction motif of Nef (WL57), and the AP-2 binding site of Nef (LL160). K144 is not in proximity to any of these three motifs, nor it is located in regions with other known Nef functions, such as the proline-rich region important for Nef to interact with the SH3 domains (54). Therefore, our data along with the fact that multiubiquitination serves as the signal for receptor endocytosis strongly suggest that the K144R loss of function results from the elimination of ubiquitination at that residue. However, we cannot completely exclude some other possibilities such as elimination of a yet unknown interaction that is exclusively lysine 144-dependent and is required for Nef-mediated CD4 down-regulation.

The current model of Nef-mediated CD4 down-regulation is that Nef does so by connecting CD4 to the AP-2 adaptor complex. Our finding suggests that this connection alone is not sufficient for Nef-mediated CD4 down-regulation. It is likely that Nef is ubiquitinated and the ubiquitination is required for sorting the CD4-Nef-AP-2 complex into the endocytic pathway. Mono- or multiple ubiquitination is known to serve as the signal for receptor endocytosis at the plasma membrane as well as for the intracellular trafficking of the endocytosed receptors to the early and late endosomes for degradation (39–41). Nef may play the role as a class of proteins called "ubiquitinated transport modifier" (39). The modifiers are themselves ubiquitinated but are not the ultimate targets of the Ub-dependent trafficking; instead, they regulate the trafficking of other proteins. One example is β-arrestin whose ubiquitination promotes the rapid internalization of the β2-adrenergic receptor (57, 58). Another example is the *Drosophila* integral membrane protein Commissureless (Comm) whose ubiquitination down-regulates Robo by diverting newly synthesized Robo in the form of a Robo-Comm complex from the secretory pathway to the lysosome (59, 60).

To overcome host defense responses, many viruses have developed a strategy in which a viral protein facilitates the ubiquitination of some host defense proteins, leading to their proteasomal degradation (for review, see Refs. 61 and 62). This strategy was first illustrated by the example of human papillomavirus E6 oncoprotein (63). The viral E6 protein induces the ubiquitination of the host p53 tumor suppressor, resulting in its degradation by 26S proteasome. Another example is the two transmembrane proteins MIR1 and MIR2 encoded by the human herpesvirus. Both proteins function as an E3 Ub ligase to ubiquitinate the MHC class I molecule, resulting in MHC class I's proteasomal degradation (64). There are three HIV-1 accessory proteins that apparently use the same strategy to facilitate the proteasomal degradation of their target proteins (for recent review, see Ref. 65). HIV-1 protein Vpu induces CD4 ubiquitination and proteasomal degradation by connecting CD4 to the Cullin-Ring Ub ligase (66–69). HIV-1 Vif induces the ubiquitination and proteasomal degradation of the cellular defense protein APOBEC3G by connecting it to a Ub ligase complex named Cul5-SCF (70, 71). HIV-1 Vpr induces the ubiquitination and proteasomal degradation of uracil DNA glycosylase and other cellular substrates (72, 73). Apparently HIV Nef uses a variation of the same theme that is through its own multiubiquitination to signal the CD4-Nef complex for endocytosis and to sort the endocytosed complex into the lysosomal degradation pathway.

The known ubiquitination-dependent sorting events involve the Ub binding to Ub receptors. For Nef, there is some evidence suggesting that Eps15, an AP-2-binding accessory protein that contains a Ub interaction motif, may be a Ub receptor that interacts

with Ub-Nef. Eps15 is localized at the clathrin containing endosomal membrane and has been implicated in sorting other ubiquitinated proteins into a multivesicular body, a subset of late endosomes (74–77). Our previous studies indicated that Nef-induced CD4 down-regulation was not significantly blocked by knockdown of AP-2 alone but was significantly blocked by a combination of the overexpression of a dominant negative mutant of Eps15 (DIII) and AP-2 RNAi (29). This suggests that Eps15 may be involved in the Nef-mediated CD4 down-regulation. The finding of Nef ubiquitination raised the possibility that Eps15 may interact with the Ub-Nef-CD4 complex in a Ub-dependent manner.

K144 and the sequence surrounding K144 ($FK_{144}LVP$) are conserved between all HIV-1 and SIVcpz sequences (55). The FKLVP motif we identified is located in a β-sheet secondary structure (β4) that is exposed to the surface (54, 78, 79). Functionally and structurally, this motif is different from the other known Nef functional regions, such as the N-terminal myristoylation site important for Nef membrane association, the proline-rich region important for interactions with the SH3 domains, and the dileucine motif important for interactions with AP complexes (54). Therefore, we propose that this motif in HIV-1 Nef is critical for its ubiquitination and intracellular trafficking.

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Disclosures

The authors have no financial conflict of interest.

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