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Cutting Edge: SIV Nef Protein Utilizes Both Leucine- and Tyrosine-Based Protein Sorting Pathways for Down-Regulation of CD4[1]

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The Nef protein is unique to primate lentiviruses and is closely linked to accelerated pathogenesis in both human and monkey hosts. Nef acts to down-regulate CD4 and MHC class I, two receptors important for immune function. A recent report demonstrated the presence of two tyrosine motifs in SIV Nef that contribute to its ability to down-regulate CD4 and to associate with clathrin adaptors. These tyrosine motifs are not present in HIV-1 Nef, which instead utilizes a leucine-based motif for its down-regulation of CD4. We now report that SIV Nef also contains a conserved leucine-based motif that contributes to CD4 down-regulation, functions to stimulate internalization, and contributes to the association of SIV Nef with clathrin adaptors AP-1 and AP-2. These results demonstrate that SIV Nef differs from HIV-1 Nef by its ability to use two parallel pathways of the protein-sorting machinery based on either tyrosine or leucine motifs. ***The Journal of Immunology,*** **1999, 163: 2977–2981.**

Because of the genetic and biological similarities between HIV-1 and SIV, SIV infection in macaques has emerged as a valuable animal model for HIV-1 infection in humans. Infection of macaques with live attenuated forms of SIV provides an important approach to assessing the safety and efficacy of a human HIV-1 vaccine (1, 2). Given the ability of both viruses to rapidly evolve, the identification of differences in the structure and function of viral gene products may not only determine the adequacy of the animal model, but also provide insights into functional motifs that are important for pathogenesis.

The Nef proteins of HIV-1 and SIV are 27- to 32-kDa myristoylated proteins that are found in both the membranes and cytosol of infected host cells (reviewed in Ref. 3). While not strictly required for viral infection and replication, attenuation of Nef expression in either HIV-1 or SIV is associated with markedly delayed progression of disease (1, 4, 5). The absence of Nef also decreases the intrinsic infectivity of SIV and HIV-1 in cultured cells, a characteristic that likely underlies the diminished viral loads observed with Nef-deleted viruses in vivo (6–9). Nef also mediates the down-regulation of two key immune modulators, CD4 (10–14) and MHC class I (15–17) in host cells. However, the relationship of these functions of Nef to its role in viral pathogenesis remains poorly understood.

Several observations show that distinct subregions of Nef are required for down-regulation of CD4 and MHC class I (18). First, CD4 down-regulation requires the presence of a dileucine sequence in Nef, which acts as an internalization signal, (19–21) and two discontinuous regions, including the sequence $W^{57}L^{58}$, that may form a binding site for the CD4 receptor tail (22, 23). In addition, a diacidic motif at positions 155,156 in HIV-1 Nef was recently identified as a lysosomal sorting signal involved in CD4 down-regulation (24). In contrast, MHC class I down-regulation is not affected by mutation of the dileucine sequence, but instead requires a proline-rich domain (not required for CD4 down-regulation), an upstream cluster of acidic amino acids, and an N-terminal α helical domain (16, 18, 25). Despite these genetically separable activities of Nef, its presence similarly leads to diminished CD4 and MHC class I expression involving increased endocytosis, blocked progression through the secretory pathway at the *trans* Golgi membrane, and enhanced sorting to the lysosome for degradation (11, 13, 17, 25).

Both HIV-1 and SIV Nef have recently been shown to form a complex with the clathrin adaptors AP-1 and AP-2 (19–21), which recruit clathrin to the Golgi membrane and the plasma membrane, respectively (26, 27). Both adaptors bind specific peptide motifs, including tyrosine-based sequences (Y-X-X-ϕ, where X may be any amino acid, and ϕ is an amino acid with a hydrophobic side chain) and leucine-based sequences (L-X, where X may be L, I, M, V, or F) on the cytoplasmic portion of transmembrane proteins. Thus, these adaptors link transmembrane receptors with tyrosine- or leucine-based sorting signals to the clathrin-based vesicles involved in protein trafficking and compartmentalization. However, the two sorting signals are not entirely interchangeable. Tyrosine- and leucine-based motifs can be independently saturated (28), and

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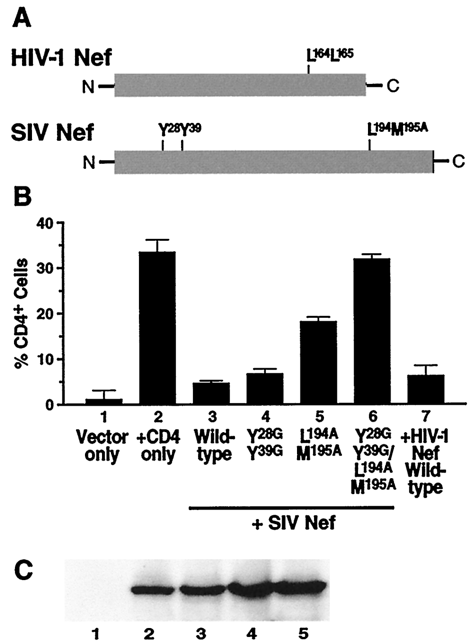

FIGURE 1. *A*, Structural comparison of HIV-1 Nef and SIV Nef, including the relative positions of two tyrosine-based internalization motifs at positions 28 and 39 in SIV Nef and a conserved leucine motif, at position 164,165 in HIV-1 Nef and 194,195 in SIV Nef. *B*, Relative expression of human CD4 on the surface of transfected 293 cells in the presence of wild-type and mutant forms of SIV Nef (*lanes 3*, *4*, *5*, and *6*), HIV Nef (*lane 7*), or in the absence of Nef (*lane 2*). CD4 down-regulation was measured by FACS analysis, as previously described (2). Values are reported as the mean of three samples, with error bars representing the standard deviaton. *C*, Expression of SIV Nef wild-type (*lane 2*), SIV Nef $Y^{28G}Y^{39G}$ (*lane 3*), SIV Nef $L^{194A}M^{195A}$ (*lane 4*), or SIV Nef $Y^{28G}Y^{39G}/L^{194A}M^{195A}$ (*lane 5*) in transiently transfected human 293 cells. The SIV Nef proteins were detected by immunoblotting using an anti-SIV Nef rabbit antiserum (a gift from Dr. Paul Luciw). *Lane 1* depicts the results obtained by immunoblotting lysate from mock-transfected cells.

the two motifs mediate different sorting reactions at the *trans* Golgi (29). The tyrosine- and leucine-based motifs thus appear to represent parallel but distinct sorting mechanisms within the cell.

As noted, different adaptor motifs are apparently employed by SIV Nef and HIV-1 Nef, raising the issue of whether these two viral proteins have evolved to use distinct and not entirely redundant protein sorting pathways. SIV utilizes two tyrosine-based motifs located near the N terminus (30) (Fig. 1*A*). In contrast, HIV-1 Nef lacks these tyrosine-based motifs and instead utilizes a highly conserved leucine-based motif located near the C terminus (Fig. 1*A*). Mutation of this motif leads to a loss of CD4 down-regulation and clathrin adaptor binding (19–21). We now describe the presence of a previously unrecognized leucine-based sorting motif in SIV Nef that is highly conserved in SIV Nef alleles. This leucine-based motif functions in a largely redundant manner with the previously reported tyrosine-based motifs to mediate CD4 down-regulation and assembly with clathrin adaptors. The tyrosine- and leucine-based motifs in SIV Nef contribute additively to the rate of internalization of a CD8-SIV Nef chimera, and mutation of both motifs is required to significantly diminish the ability SIV Nef to down-regulate CD4. These findings highlight the presence of a shared leucine-based trafficking motif in both SIV and HIV-1 Nef and the unique acquisition of additional tyrosine-based motifs in SIV Nef.

Materials and Methods

Abs and immunodetection

Rabbit serum to SIV Nef was provided by Dr. P. A. Luciw (University of California Davis, Davis, CA). OKT8 mAb specific for the human CD8 Ag was obtained from Dr. A. Weiss (University of California, San Francisco, CA). The following Abs were used for FACS analysis of transiently expressed cell-surface receptors: anti-CD4-Tricolor, anti-CD8-Tricolor (Caltag, South San Francisco, CA), anti-CD8-PE, and anti-CD25-FITC (Becton Dickinson, Mountain View, CA). Control cells used for determining background staining in the FACS analyses were transfected with empty expression vector only. Abs specific for AP-1 (clone 100/3, anti-adaptin γ; Sigma, St. Louis, MO) or AP-2 (anti-adaptin α; Transduction Laboratories, Lexington, KY) were used for immunoblotting, with a HRP-conjugated goat-anti-mouse secondary Ab (Zymax; Zymed, South San Francisco, CA). Immunoprecipitations were performed as described previously (19).

DNA constructions

The plasmids pSIV Nef and pSIV Nef $Y^{28G}Y^{39G}$, which express wild-type SIV Nef and a mutant of SIV Nef in which the tyrosine residues at positions 28 and 39 are replaced with glycines, were prepared by subcloning the SIV_{MAC239} nef sequences from CMX-SIV_{MAC239}Nef and CMX-SIV_{MAC239} Nef $Y^{28G}Y^{39G}$ (30) into the vector pcDNA3.1 (Invitrogen, San Diego, CA). To generate SIV_{MAC239} Nef mutants, in which the leucine and methionine at positions 194 and 195 are replaced with alanines, the plasmids pSIV Nef $L^{194A}M^{195A}$ and pSIV Nef $Y^{28G}Y^{39G}/L^{194A}M^{195A}$ were generated from the plasmids pSIV Nef and pSIV Nef ^{Y28GY39G}, respectively, by site-directed mutagenesis (Bio-Rad, Richmond, CA).

Plasmids expressing CD8-SIV Nef chimeras were prepared by amplifying wild-type and mutant SIV_{MAC}239 *nef* genes by PCR and inserting them into the pCN vector (31) as previously described (19). The resulting plasmids, pCD8-SIV Nef, pCD8-SIV Nef $Y^{28G}Y^{39G}$, pCD8-SIV Nef $L^{194A}M^{195A}$, and pCD8-SIV Nef $Y^{28G}Y^{39G}/L^{194A}M^{195A}$, all express chimeras in which the extracellular and transmembrane domains are derived from CD8, and the cytoplasmic domain consists of the entire sequence of SIV_{MAC239} Nef.

Results and Discussion

Comparison of SIV and HIV-1 Nef sequences revealed a highly conserved leucine in SIV Nef at position 194 corresponding to L164 in HIV-1 Nef (NL4-3 strain) (32) (Fig. 1*A*). An adjacent methionine at position 195 formed a consensus sequence for a leucine-based sorting signal. To assess the potential functional contribution of this conserved leucine-based motif to the SIV Nef-mediated down-regulation of CD4, the site was mutated by substitution of the leucine and methionine with alanines ($L^{194A}M^{195A}$). Additionally, glycine substitutions were introduced for Y^{28} and Y^{39} ($Y^{28G}Y^{39G}$) as previously described (30), and a composite mutant ($Y^{28G}Y^{39G}/L^{194A}M^{195A}$) was also prepared. Surprisingly, both the wild-type and $Y^{28G}Y^{39G}$ forms of SIV Nef reduced the cell surface expression of CD4 to approximately the same extent (Fig. 1*B*). Introduction of the $L^{194A}M^{195A}$ mutations resulted in a partial loss of CD4 down-regulation; however, the composite mutant (SIV Nef $Y^{28G}Y^{39G}/L^{194A}M^{195A}$) exhibited essentially no CD4 down-regulation. The partial loss of CD4 down-regulation by SIV Nef $L^{194A}M^{195A}$ and SIV Nef $Y^{28G}Y^{39G}/L^{194A}M^{195A}$ mutants could not be explained by poor expression, as all of the SIV Nef analogues were expressed at nearly the same steady state level (Fig. 1*C*). We conclude that the leucine-based motif in SIV Nef plays an important role in CD4 down-regulation, and that the N-terminal tyrosine-based motifs also play a role best revealed in the context of the composite mutation.

To further characterize the functional properties of both the tyrosine- and leucine-based motifs in SIV Nef, we constructed chimeric proteins with a truncated form of human CD8 comprising the extracellular and transmembrane domains fused to wild-type SIV Nef, or mutant SIV Nef sequences as a cytoplasmic domain (19, 31, 33). Steady state surface expression of the CD8-SIV Nef wild-type chimera was ~5-fold lower than that of CD8 (Fig. 2*A*), and the CD8-SIV Nef $Y^{28G}Y^{39G}$ and CD8-SIV Nef $L^{194A}M^{195A}$

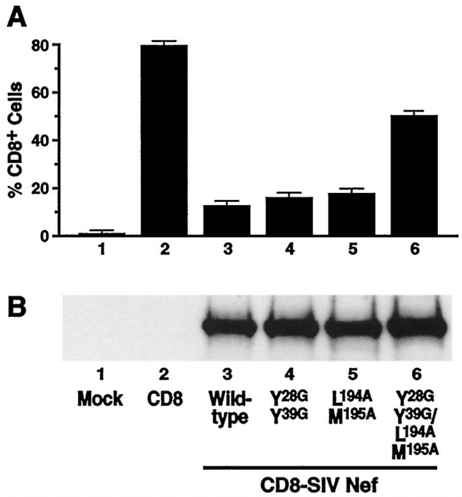

FIGURE 2. Steady state cell-surface expression of CD8 and CD8-SIV chimeric proteins in transfected human 293 cells. *A*, Cell-surface expression was quantitated by FACS; positive cells were scored as brighter than 95% of mock-transfected cells. Error bars indicate the SD from the mean value of three samples. *B*, Relative expression of CD8-SIV Nef chimeras detected by immunoblotting with anti-SIV Nef Ab, as described in *Materials and Methods*.

chimeras were similarly low. In contrast, the composite mutant CD8-SIV Nef $Y^{28G}Y^{39G}/L^{194A}M^{195A}$ was expressed at much higher levels at the cell surface. While all of the chimeras were comparably expressed (Fig. 2*B*), the composite mutant (CD8-SIV Nef $Y^{28G}Y^{39G}/L^{194A}M^{195A}$) was consistently expressed at lower levels on the cell surface than wild-type CD8. These findings suggest that a single mutation interrupting either the tyrosine- or leucine-based motifs in SIV Nef does not release the chimeric molecule from down-regulation. However, mutation of both motifs significantly impairs down-regulation, arguing that both play a role in intracellular protein sorting.

Expression of the CD8-Nef chimera does not demonstrate the partially impaired down-regulation activity found for SIV Nef $L^{194A}M^{195A}$ (Fig. 1*B*). Experiments using SIV Nef sequences in *cis* and in *trans* detect different aspects of its association with the intracellular protein sorting machinery. In the *trans* assay of CD4 down-regulation, at least three different properties of SIV Nef are required: 1) membrane localization; 2) coupling to CD4, possibly through a linker protein; and 3) association with clathrin adaptors and possibly other components of the protein sorting machinery. In the *cis* assay, the Nef sequences are not required for membrane localization, nor is assembly with the CD4 cytoplasmic tail needed. Thus, while the chimeric proteins allow a more direct measurement of the effects of SIV Nef sequences on internalization rates, their overall trafficking in the cell is less complex than that found with the soluble form of Nef. Accordingly, the *trans*- and *cis*- down-regulation assays are not fully equivalent, although both can detect the functional interplay of SIV Nef with the protein sorting machinery.

Endocytosis of both CD4 and MHC class I molecules is enhanced in the presence of Nef. To determine the relative contributions of the tyrosine- and leucine-based motifs in SIV Nef to the stimulation of endocytosis, we measured the kinetics of internalization of each CD8-SIV Nef chimera (Fig. 3). Internalization was reduced to approximately half the rate of CD8-SIV Nef wild-type with mutation of either the tyrosine- or leucine-based motif. Mutation of both motifs (CD8-SIV Nef $Y^{28G}Y^{39G}/L^{194A}M^{195A}$) further reduced the rate of internalization. These results demonstrate

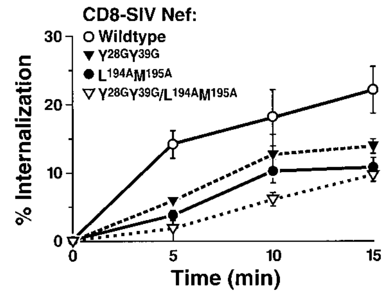

FIGURE 3. Both tyrosine motifs and leucine motifs contribute to the rate of CD8-SIV Nef internalization. Internalization rate of CD8-SIV Nef chimera in the absence of the N-terminal tyrosine motifs (CD8-SIV Nef $Y^{28G}Y^{39G}$), the C-terminal leucine-based motif (CD8-SIV Nef $L^{194A}M^{195A}$), or in the absence of both motifs (CD8-SIV Nef $Y^{28G}Y^{39G}/L^{194A}M^{195A}$). Error bars indicate the SD from the mean value of three samples.

that either motif can function independently as an internalization signal and that they both contribute additively to the overall rate of internalization.

We next investigated the effect of tyrosine- or leucine-based motifs on the ability of the CD8-SIV Nef chimeras to associate with the AP-1 and AP-2 adaptors in vivo. Mutation of either the tyrosine- or the leucine-based motif clearly diminished the association of the corresponding Nef analogues with both AP-1 and AP-2 (Fig. 4). The presence of the composite mutations (CD8-SIV Nef $Y^{28G}Y^{39G}/L^{194A}M^{195A}$) further reduced association with both adaptors. Thus, both motifs in SIV Nef contribute independently to an interaction with AP-1 and AP-2. The residual presence of AP-1 (13% of maximum signal) compared with AP-2 (0.2% of maximum signal, compare Figs. 4*A* and 4*B*, *lanes 7*) in complex with the composite mutant could reflect the contribution of Nef-associated proteins like NBP1 (34) in the immunoprecipitate that can themselves recruit clathrin adaptors through their own tyrosine- or leucine-based motifs.

While we detected relatively equivalent amounts of AP-1 associated with both HIV and SIV-1 Nef proteins, much less AP-2 was associated with HIV-1 Nef sequences (4% of SIV Nef signal) (Fig. 4*B*, *lanes 3* and *4*). These results could simply reflect fewer sorting

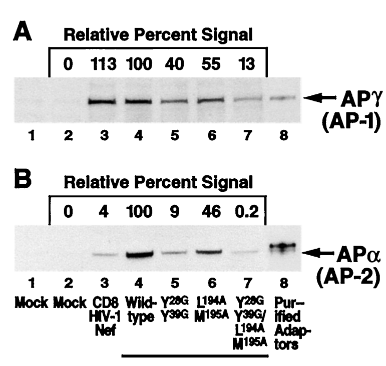

FIGURE 4. Coimmunoprecipitation of clathrin adaptors AP-1 (*A*) and AP-2 (*B*) with SIV Nef chimeras. Human 293 cells were mock transfected or transfected (*lane 2*) with CD8-HIV-1 Nef (*lane 3*) or CD8-SIV Nefs (*lanes 4*, *5*, *6*, and *7*). Cell lysates were immunoprecipitated with anti-CD8 (clone OKT-8, a gift from Dr. A. Weiss) (*lanes 2–8*) or with control IgG of the same isotype (*lane 1*). Purified bovine adaptors (a gift from Dr. F. Brodsky, University of California, San Francisco, CA) were electrophoresed and immunoblotted as a control (*lane 8*). Relative percent signal was determined by densitometry analysis and normalized to CD8-SIV Nef wild type in both panels.

motifs for AP-2 in HIV-1 Nef (a single leucine motif) than in SIV Nef (two tyrosine motifs and a leucine motif), though the same sites appear involved in the association of AP-1, which is comparable. Alternatively, SIV Nef may form a more stable complex with AP-2 under these immunoprecipitating conditions than HIV-1 Nef. Studies of AP-2 interactions with sorting motifs suggest that regulatory mechanisms may stabilize these interactions in vivo (27, 35, 36). Further studies on binding affinities of HIV-1 Nef and SIV Nef for each of the adaptors are required to explore this issue.

Together, our results indicate that SIV Nef has evolved to utilize two apparently parallel pathways for CD4 receptor internalization involving both tyrosine- and leucine-based motifs. In contrast, HIV-1 Nef contains a trafficking signal for only one of these pathways. The significance of the two independent, parallel pathways for internalization is not clear. Several cellular receptors have been described to contain multiple internalization signals using both the tyrosine- and leucine-based signals for internalization. Of note, once the receptor is internalized, these signals may participate in distinct sorting processes (37–41). Thus, it is possible that these motifs are not entirely redundant, but are utilized differently for sorting to specific end compartments within the cell.

Tyrosine-based motifs are highly conserved in pathogenic SIV strains, suggesting that genetic selection pressure is applied to the tyrosine-based motif in Nef in the context of SIV in macaques. However, the leucine-based motif is highly conserved both in SIV Nef and HIV-1 Nef. Therefore, leucine-based motifs may be fundamental components of Nef activity in both HIV-1 and SIV, while tyrosine motifs may contribute to a distinct part of SIV pathogenesis in macaques. Whether down-regulation of CD4 by Nef is clearly linked to accelerated HIV-1 or SIV pathogenesis remains to be determined. It has recently been observed that Nef is not absolutely required for pathogenesis (2), but its absence in SIV or HIV-1 significantly prolongs the onset of AIDS in adults (1, 4).

Infection of macaques with attenuated SIV is a leading model for testing the effectiveness and the safety of various HIV-1 vaccines. Therefore, it is important to establish the functional equivalence of the two viruses and the relationship of their viral products to pathogenesis. The maintenance of both tyrosine- and leucine-based sorting motifs in SIV Nef, but only a leucine-based motif in HIV-1 Nef, could be an indication of functional distinctions. These differences, if present, may emerge with further examination of the function of HIV-1 Nef within the context of SIV-HIV chimeric viruses (SHIVs).

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