Journal of Virology

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J. Virol. **2000, 74(21):9836. DOI: 10.1128/JVI.74.21.9836-9844.2000.**

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JOURNAL OF VIROLOGY, Nov. 2000, p. 9836–9844 Vol. 74, No. 21
0022-538X/00/$04.00+0
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Disrupting Surfaces of Nef Required for Downregulation of CD4 and for Enhancement of Virion Infectivity Attenuates Simian Immunodeficiency Virus Replication In Vivo

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Received 31 May 2000/Accepted 4 August 2000

The multifunctional simian and human immunodeficiency virus (SIV and HIV) Nef proteins are important for virulence. We studied the importance of selected Nef functions using an SIV Nef with mutations in two regions that are required for CD4 downregulation. This Nef mutant is defective for downregulating CD4 and, in addition, for enhancing SIV infectivity and induction of SIV replication from infected quiescent peripheral blood mononuclear cells, but not for other known functions, including downregulation of class I major histocompatibility complex (MHC) cell surface expression. Replication of SIV containing this Nef variant in rhesus monkeys was attenuated early during infection. Subsequent increases in viral load coincided with selection of reversions and second-site compensatory changes in Nef. Our results indicate that the surfaces of Nef that mediate CD4 downregulation and the enhancement of virion infectivity are critical for SIV replication in vivo. Furthermore, these findings indicate that class I MHC downregulation by Nef is not sufficient for SIV virulence early in infection.

The Nef protein of simian and human immunodeficiency virus (SIV and HIV) is an important determinant of AIDS pathogenesis (12, 20, 23). Both HIV-1 and SIV Nef interact with cell signaling and protein sorting machinery and have several potentially important effects (for reviews, see references 9, 11, and 13), including (i) the downregulation of surface CD4 molecules (36, 45), (ii) the downregulation of surface class I major histocompatibility complex (MHC) molecules (8, 43), (iii) the induction of alterations in T-cell receptor signal transduction pathways (2, 3, 17, 19, 30, 42, 44), and (iv) the enhancement of viral replication in primary lymphocyte cultures infected prior to stimulation and the enhancement of virion infectivity in certain cell lines (2, 7, 30, 33, 39, 44). However, the importance of these functions for AIDS pathogenesis and of the surfaces of the Nef molecule that mediate them has only begun to be addressed (4, 5, 21, 28, 41).

The mechanism by which Nef induces CD4 endocytosis involves the recruitment of CD4 molecules to the endocytic machinery via the AP2 clathrin adapter complex at the plasma membrane (36, 45). This likely requires direct molecular contacts between an element in the N-terminal region of SIV Nef molecule and the AP2 complex, as well as an interaction between the C-terminal disordered loop in Nef with CD4 itself or other cellular factors (14, 29, 35). By decreasing CD4 cell surface expression, Nef can promote the release of progeny virions from the infected cells and facilitate Env incorporation into viral particles, thus enhancing the infectivity of progeny virions (27, 38). Consistent with this possibility is the observation that the positive effects of Nef on viral replication in vitro map to surfaces of Nef that are also involved in downregulation of CD4 expression (10, 29). However, additional evidence indicates that Nef also enhances viral replication via alterations of the activation state of the infected cells (2, 17, 39, 42, 44, 48).

The effects of Nef on CD4 expression, class I MHC expression, and the signal transduction machinery are genetically separable and map to different surfaces in HIV-1 and SIV Nef molecules (15, 19, 29, 36, 45, 47). Here we investigate the role of surfaces of the SIV Nef protein involved with CD4 downregulation and with the enhancement of SIV infectivity and replication in vitro for SIV replication in rhesus macaques. We constructed an SIVmac239 variant containing three amino acid substitutions in Nef which disrupted its ability to downregulate CD4 but had no detectable effect on downregulating CD3 or class I MHC, and in associating with the p62 serine/threonine kinase activity (28, 34, 40) or with the AP2 adapter/clathrin complex (19, 29). This mutant Nef did not stimulate SIV infectivity or replication in rhesus peripheral blood mononuclear cells (rPBMC). Six rhesus macaques inoculated with this SIVmac239 variant showed low plasma viral loads early in infection. Subsequent increases in viral loads coincided with the selection of amino acid changes that restored Nef function. Our results indicate that surfaces of the Nef protein that mediate molecular interactions important for CD4 downregulation are important for optimal SIV replication in vivo and that class I MHC downregulation by Nef is not sufficient for SIV virulence.

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MATERIALS AND METHODS

Construction of 239-Nef expression plasmids. Mutations were generated by oligonucleotide-directed mutagenesis of SIVmac239 *nef*(open) (239-*nef*), as previously described (19). Mutant 239-*nef* sequences amplified by PCR from proviral DNA were subcloned into the pCD3-β or pCG expression vector or into a modified pBR322 vector containing the full-length SIVmac239 proviral DNA using standard techniques (28, 44). The construction of the *nef*-defective SIVmac239 variant used in this study, 239(ΔNU), which has a 188-bp deletion in the unique region of *nef* together with a deletion of 325 bp in the long terminal repeat (LTR) U3 region, was previously described (16). The SIVmac239ΔUS (EDR) variant was constructed by deleting the same 325-bp fragment from the

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5′ LTR of the SIVmac239(EDR) provirus. All mutations and all constructs containing these mutations were verified by DNA sequencing.

Cell lines, transfections, and flow cytometry. Transfection of CD4-positive Jurkat T cells (provided by Dan R. Littman) and analysis of the effect of 239-Nef on CD4 expression and on CD3 signaling and expression were performed as previously described (19, 29, 47). At 18 to 24 h after transfection, cells were stimulated by overnight incubation with the anti-CD3 HIT3A monoclonal antibody (MAb) (PharMingen). At 30 to 36 h after transfection, cells were incubated for 1 h on ice with peridinin chlorophyll-α protein-conjugated anti-CD20 MAb (Leu-16; Becton Dickinson) and either a phycoerythrin-conjugated anti-CD4 MAb (Leu3A; Becton Dickinson) together with a fluorescein isothiocyanate-conjugated anti-HLA-A,B,C MAb (G46-2.6; PharMingen), anti-CD69 MAb (FN50; PharMingen), or anti-CD3 MAb (HIT3A; PharMingen). CD3, CD4, CD20, CD69, and class I MHC surface expression was analyzed using an Epics-Elite flow cytometer. For dose-response analysis, the levels of CD4, CD69, or class I MHC were represented by the peak channel number of red or green fluorescence on $CD20^+$ cells. The determination of relative stability of mutant 239-Nef proteins was performed as previously described (19).

Determination of viral replication and infectivity. Viral stocks were generated by transfection of proviral DNA into COS-7 or 293T cells as described (28), or following cocultivation of rPBMC with CEMx174 cells. The p27 antigen levels for these stocks were determined with a commercial HIV-1/HIV-2 antigen capture assay under conditions recommended by the manufacturer (Immunogenetics). For replication assays, aliquots of viral stocks containing 2 ng of p27 antigen were used to infect freshly isolated rPBMC. Cells were washed 16 to 18 h later to remove unadsorbed virus. At 6 days postinfection, rPBMC were stimulated for 2 days with phytohemagglutinin (4 μg/ml) (Sigma), and reverse transcriptase activity in these supernatants was determined as described (28). The infectivity of viral stocks in sMAGI cells was assayed as previously described (6, 28).

Infection of rhesus macaques and clinical assessment. Rhesus macaques were housed at the German Primate Center in Goettingen in accordance with the institutional guidelines. Rhesus macaques were infected intravenously with cell-free aliquots of viral stocks containing 10 ng of p27 antigen, prepared from COS-7 cells transfected with SIVmac239(EDR) or SIVmac239ΔUS(EDR) variants or control 239 *nef*(open) or SIVmac239ΔNU viruses. The animals were seronegative for SIV, type D-retroviruses, and simian T-cell lymphotropic virus type 1 at the time of infection. The amount of p27 antigen in the plasma was determined by antigen capture assay. Sera and cells were collected at regular intervals, and serologic, virologic, and immunologic analyses were performed as previously described (15, 28).

Viral DNA amplification and DNA sequence analysis. 239-*nef* sequences were amplified by PCR from DNA isolated from rPBMC or from rhesus organ biopsies with a nested PCR approach or from rPBMC-CEMx174 cocultures by a single round of amplification, as previously described (22, 28). All PCR fragments were purified and sequenced directly or after subcloning into the pCRII vector (Invitrogen), with the Prism sequencing kit (Perkin Elmer) and an automated Applied Biosystems 373 DNA sequencer. Nucleotide changes were quantitated as previously described (28).

RESULTS

Construction of a 239-Nef mutant impaired in CD4 downregulation. We have previously identified amino acid changes that disrupt the ability of 239-Nef to downregulate CD4 but not CD3 or class I MHC surface expression (19, 28, 47). For the purpose of animal experiments, we combined three such changes involving substitutions of glutamic acid for proline P73 (P73E), aspartic acid for alanine A74 (A74D), and arginine for aspartic acid D204 (D204R; referred to as the EDR mutation) on the same molecule [$239_{(EDR)}$-Nef]. We expected that combining these changes would disrupt the ability of Nef to downregulate CD4 expression even more severely than each mutation alone and delay selection of revertants, allowing us to better assess effects on SIV replication and pathogenesis.

As shown in Fig. 1A, dose-response experiments revealed that the P73E and D204R mutations severely disrupted the ability of 239-Nef to downregulate CD4; however, the A74D mutation had a much lesser effect (panel 1). Combining all three substitutions on the same molecule further impaired the residual activity. Importantly, the relative stabilities of mutant Nef proteins, including $239_{(EDR)}$-Nef, differed less than twofold from that of wild-type 239-Nef (panel 4), and in addition, fluorescence microscopy studies showed that $239_{(EDR)}$-Nef had cellular distribution indistinguishable from that of wild-type 239-Nef (data not shown) and associated with p62 phosphoprotein in in vitro kinase assays, similar to wild-type 239-Nef (data not shown). Furthermore, all mutant 239-Nef proteins tested, including $239_{(EDR)}$-Nef, retained wild-type ability to downregulate surface expression of class I MHC and of CD3 complexes (Fig. 1A, panels 2 and 3, and Fig. 1B). Thus, the EDR substitutions likely disrupt specific molecular interactions of 239-Nef required for CD4 downregulation without causing a global misfolding of the 239-Nef molecule.

$239_{(EDR)}$-Nef does not stimulate SIV replication and infectivity. Nef stimulates SIV replication induced from rPBMC infected prior to stimulation and infectivity of SIV virions to sMAGI cells. To assess the effect of mutations in 239-Nef on these functions, the P73E, A74D, and D204R mutations in Nef were introduced singly or in combination into the full-length SIVmac239 provirus. We then assayed their effect on SIV replication and on SIV virion infectivity (6, 28). rPBMC were infected at low multiplicity with mutant and control SIV and stimulated with phytohemagglutinin 6 days later, and the reverse transcriptase activity in the culture supernatants was determined at various times following stimulation. As shown in Fig. 2A, the *nef*-deleted (239ΔNU) virus and SIV containing the $239_{(EDR)}$-*nef* allele replicated less efficiently and with delayed kinetics compared to wild-type 239 (239wt). Similarly, the infectivity of the $239_{(EDR)}$-Nef variant in $CD4^+$ sMAGI indicator cells was comparable to that of *nef*-deleted virus and approximately fourfold lower than that of wild-type SIV (Fig. 2B). The P73E and D204R substitutions significantly reduced both SIV replication and infectivity, while the A74D mutation had little effect. The observation that these mutations also disrupt the ability of 239-Nef to downregulate CD4 is consistent with the links between CD4 downregulation and viral replication previously reported for HIV-1 and SIV Nef (27, 29, 38).

Attenuated replication of SIV containing $239_{(EDR)}$-*nef* in rhesus monkeys early in infection. Six rhesus macaques were infected with SIV containing $239_{(EDR)}$-*nef* using two different proviral constructs; three macaques (Mm8003, Mm8151, and Mm8155) were inoculated with $SIV239_{(EDR)}$, and three animals (Mm8493, Mm8494, and Mm8495) were infected with $SIV239\Delta US_{(EDR)}$. SIV $239_{(EDR)}$ contains nucleotide substitutions only in the *nef* open reading frame (ORF) at the 3′ end of the provirus, but not in the 5′ LTR. Since genomic transcripts initiate in the 5′ LTR downstream of the *nef* coding region present in U3, wild-type *nef* sequences should not be propagated during the viral replication cycle. The second construct, $SIV239\Delta US_{(EDR)}$, contains a 334-bp deletion in the 5′ LTR U3 region that spans the nonmutated *nef* sequence but does not affect important transcriptional elements or the genomic RNA sequence (36). This eliminated any possible interference by the *nef* sequence in the 5′ LTR.

In all animals, including controls, a peak of plasma antigenemia and viral RNA was observed at 2 weeks postinfection (wpi) (Fig. 3). Compared to 239wt infection, the average p27 plasma concentration was 70-fold lower in animals infected with *nef*-deleted SIV, and the viral RNA load was 100-fold lower (Fig. 3B and D). In the six animals infected with SIV containing the *nef* mutation, the levels of plasma antigenemia and the viral RNA loads were indistinguishable from those in animals infected with the *nef*-deleted virus at 2 wpi. These results show that, similar to large deletions in Nef, mutations P73E, A74D, and D204R consistently reduced SIVmac239 replication early in infection by almost 100-fold. Measurement of RNA loads showed that at later time points, SIV containing $239_{(EDR)}$-Nef replicated with an efficiency comparable to that of 239wt in four animals. The remaining two animals showed

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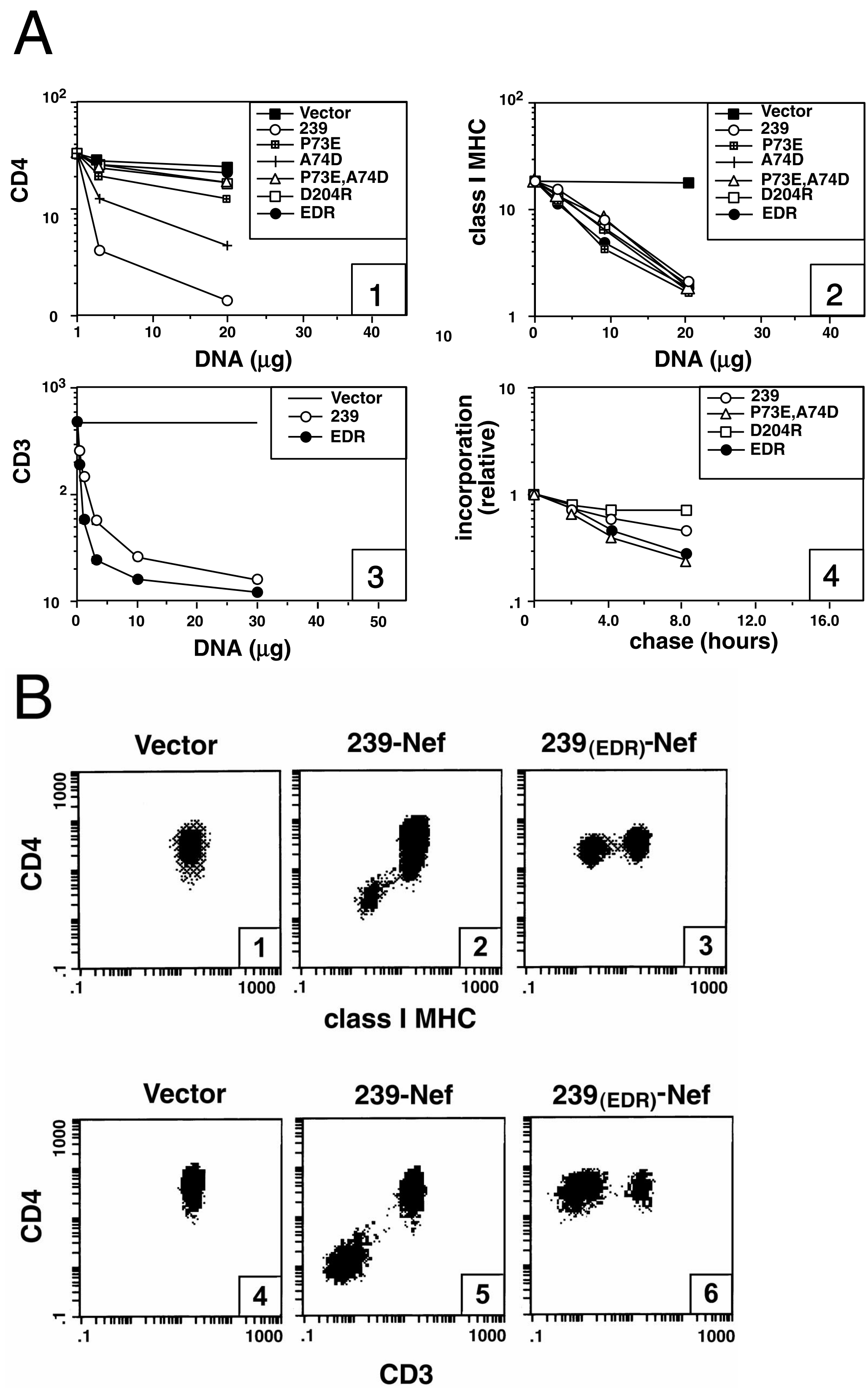

FIG. 1. EDR mutation disrupts the ability of 239-Nef to downregulate CD4 but not to downregulate CD3 or class I MHC. (A) Dose-response analysis of the effect of mutations in 239-Nef on the expression of CD4 (panel 1), class I MHC (panel 2), and CD3 (panel 3) on the surface of $CD20^+$ live cells is shown on the ordinate as peak channel number of CD4, class I MHC, and CD3 fluorescence, respectively. Panel 4 shows the relative stabilities of the indicated Nef proteins, represented as relative radiolabel incorporation over the indicated times. (B) Two-color flow cytometric analysis of CD4 and class I MHC or CD3 on the surface of cells transfected with 20 μg of control (panels 1 and 4), wild-type 239-Nef (panels 2 and 5), or $239_{(EDR)}$-Nef (panels 3 and 6) expression plasmids.

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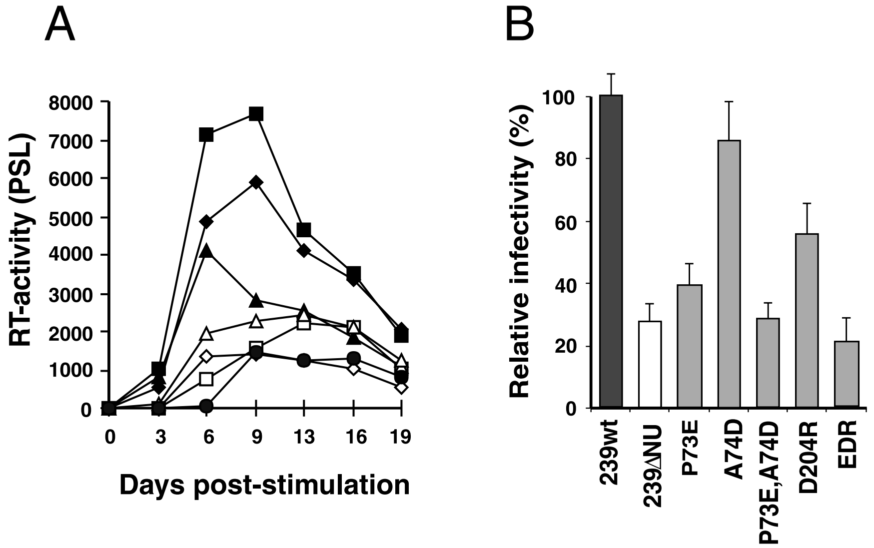

FIG. 2. P73E, A74D, and D204R mutations in Nef reduce replication and infectivity of SIVmac239. (A) Ability of SIVmac239 239wt (■) and of the variants with mutations in *nef*, including ΔNU (□), P73E (◇), A74D (◆), P73E,A74D (△), D204R (▲), and EDR (●), to replicate in rPBMC. Fresh unstimulated rPBMC were infected with virus stock containing 2 ng of p27 and stimulated with phytohemagglutinin at day 6, as described in the text. Reverse transcriptase (RT) activity of supernatants on the indicated days poststimulation was quantitated using a phosphorimager and is indicated on the ordinate as photo-stimulated light emission units (PSL). Similar results were obtained with PBMC from four different macaques. (B) Infectivity of SIVmac239 and of the indicated variants in the sMAGI indicator cell line. sMAGI cells were infected with aliquots of virus stocks containing 100 ng of p27. The values are percentages of 239wt activity and are the averages of 12 independent measurements of four different virus stocks.

RNA loads intermediate between those of animals infected with wild-type and *nef*-deleted SIV (Mm8003 and Mm8494).

SIV replication in the postacute phase of infection. After the acute phase, the course of infection differed between the six individual animals infected with SIV containing $239_{(EDR)}$-*nef*. Mm8003 remained healthy with stable CD4 counts throughout the observation period and was euthanized at 99 wpi. No plasma p27 antigen could be detected at any time (Fig. 3A), and the RNA copy numbers were about 100-fold reduced compared to wild-type SIVmac239 infection (Fig. 3C). At necropsy, this animal showed a moderate lymphoid hyperplasia, which was confirmed by histological examination. The second animal, Mm8151, also did not develop AIDS within the first year but showed clear signs of disease progression, including a declining number of $CD4^+$ T lymphocytes, lymphadenopathy, and weight loss. It died at 99 wpi. This animal showed an unusual second peak in the plasma p27 concentration at 16 and 20 wpi (Fig. 3A). Histopathologic analysis of this animal revealed a marked lymphoid involution and depletion which correlated with a systemic cytomegalovirus infection and a severe purulent bronchopneumonia induced by *Streptococcus pneumoniae*. The third infected macaque, Mm8155, showed characteristics similar to some rapid progressors of wild-type SIVmac239 infection. It generated a weak antibody response (data not shown), developed exceedingly high viral loads (Fig. 3C), and progressed to fatal disease within 21 wpi (24). Autopsy revealed a disseminated giant cell disease in the lungs, spleen, lymph nodes, and intestine with marked generalized depletion and fibrosis of the lymphatic tissue. In the lymph nodes, the lymphoid tissue was replaced by infiltrates of histiocytes and multinucleated giant cells of the macrophage-monocyte lineage.

The three animals infected with the SIV $239\Delta US_{(EDR)}$ variant also showed different courses of infection. Mm8493 showed a decline in the number of $CD4^+$ T cells after 16 wpi and developed lymphadenopathy and splenomegaly by 44 wpi. This animal died at 62 wpi because of an erosive, chronically active gastroenteritis induced by opportunistic organisms (*Giardia*, *Trichomonas*, *Trichuris*, and *Campylobacter* species). Histopathologic examination also revealed a moderate to severe follicular hyperplasia with progression to depletion of some follicles in lymph nodes and spleen. Furthermore, the animal developed lymphohistiocytic infiltrates with follicular morphology in multiple other organs, including brain, liver, kidney, bladder, skin, muscle, and pancreas. In contrast, Mm8494 remained clinically healthy throughout the 80-week observation period. Post mortem examination at euthanization revealed no pathological abnormalities except a mild hyperplasia of the lymph nodes and the splenic white pulp. The remaining animal, Mm8495, showed declining $CD4^+$ T-cell counts by 16 wpi, mild lymphadenopathy by 24 wpi, and splenomegaly by 28 wpi. This animal had to be euthanized at 46 wpi because of severe disease. Histopathologic examination revealed SIV-associated lymphoid hyperplasia with progression to depletion, a chronic active gastroenteritis with opportunistic infections, and a moderate interstitial pneumonia.

Thus, the four animals (Mm8151, Mm8155, Mm8493, and Mm8495) with high viral loads developed AIDS and died within the 80 to 84 weeks of observation as a result of simian AIDS. Two macaques, Mm8003 and Mm8494, with intermediate viral loads remained clinically healthy with relatively stable $CD4^+$ T-cell counts throughout the same period.

Changes in Nef are selected in vivo. Sequence analysis of PCR fragments amplified from PBMC, plasma RNA, and positive bulk cocultivations revealed that the increase in viral loads in animals infected with $SIVmac239_{(EDR)}$ coincided with consistent selection of changes at and in the vicinity of the mutated residues in Nef. The reversion of the D204R mutation was detected at 2 wpi in Mm8151 and Mm8155 and at 4 wpi in Mm8003 (Table 1). In contrast, no reversion was seen in three animals infected with the second construct in which the nonmutated D204 codon in the 5′ LTR was deleted. The rapid emergence of the D204R reversion in animals infected with the first construct likely reflects recombination between the mu-

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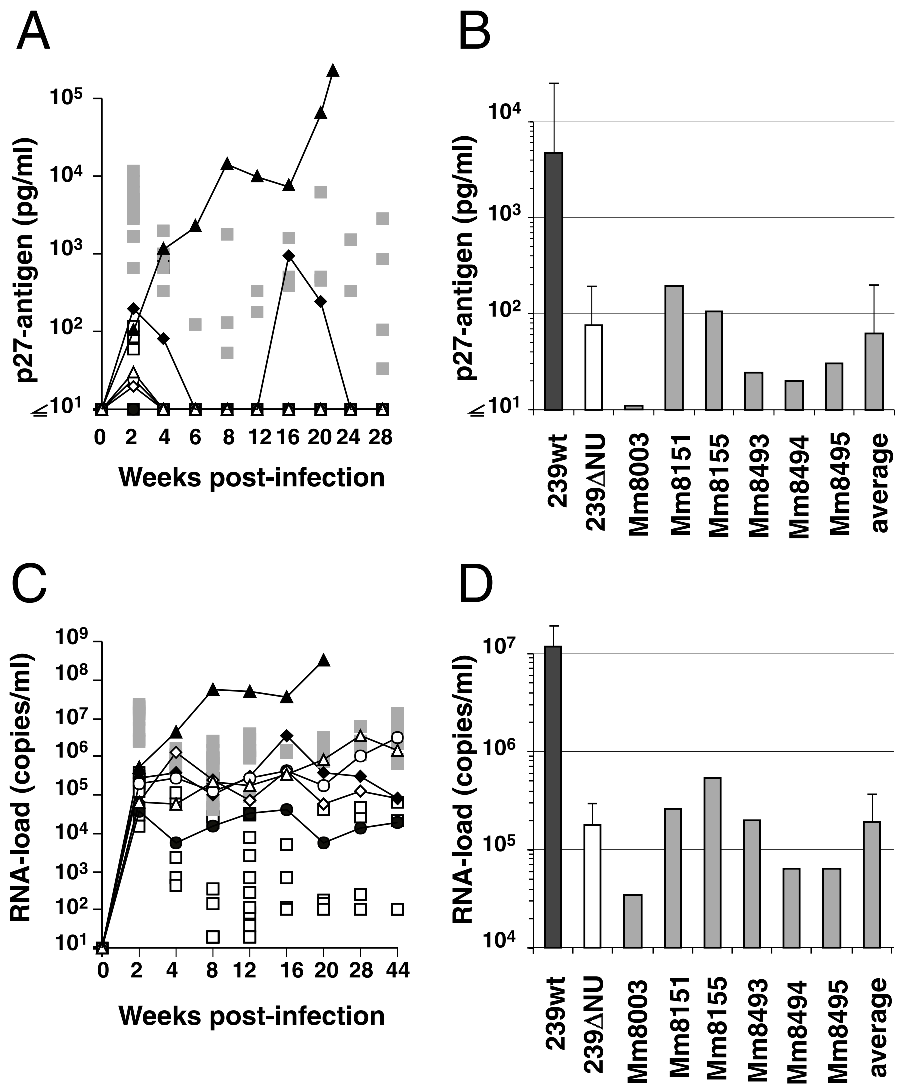

FIG. 3. Replication of the SIVmac239 EDR Nef variants in rhesus macaques. Three macaques, Mm8003 (●), Mm8151 (◆), and Mm8155 (▲), were infected with $SIV239_{(EDR)}$, and three animals, Mm8493 (○), Mm8494 (◇), and Mm8495 (△), were infected with $SIV\Delta US239_{(EDR)}$. (A) Levels of p27 plasma antigenemia. The limit of detection is approximately 20 pg/ml. In animals infected with SIVmac239ΔNU, p27 antigen was below the detection limit at all time points after 2 wpi. For comparison, values obtained from four animals infected with SIVmac239ΔNU (□) and from 12 macaques inoculated with 239 *nef*(open) virus SIVmac32H/1XC (■) are shown. Each symbol represents the result of a single determination from a single animal at a given time point. (B) Histograms showing p27 antigenemia at 2 wpi. The mean p27 values for control viruses at 2 wpi are 67 (±39) pg/ml for SIVmac239ΔNU, $n = 4$, compared to wild-type 4,689 (±2,928) pg/ml, $n = 12$. The mean p27 level for the six experimental animals is 63 (±69) pg/ml. (C) Viral RNA loads. For comparison, values obtained from 10 animals infected with SIVmac239ΔNU and from seven animals inoculated with *nef*(open) SIVmac32H/1XC are shown. (D) Histogram showing viral RNA loads at 2 wpi. The mean RNA load values for control viruses are 1.8 × 105 ($\pm 1.7 \times 10^5$) for SIVmac239ΔNU, $n = 10$, compared to 239wt 1×10^7 ($\pm 5 \times 10^6$), $n = 7$. The mean RNA load for the six experimental animals at 2 wpi is 1.9×10^5 ($\pm 1.7 \times 10^5$).

tated 3′ and nonmutated 5′ LTRs present in the proviral clone. Interestingly, there was selection of a serine at position 204 in the majority of sequences from Mm8493 and Mm8495 after 28 wpi. This suggested that serine could functionally substitute for the aspartic acid usually found at this position in 239-Nef.

No rapid reversion at mutated codons 73 and 74 was detected in any of the six animals. A single nucleotide change predicting a change of P73E to lysine, however, was detected in five animals (Table 1). In Mm8155, Mm8493, and Mm8495, this lysine-73 predominated until death from AIDS, whereas forms containing the original proline came to predominate later in infection in Mm8003 and Mm8151. We also found that the wild-type asparagine codon at position 72 of the *nef* ORF, which was not mutated, was replaced by an aspartic acid in sequences from the same five animals. This change always coexisted with the P73E→K change on the same molecule (data not shown). While no reversion of the mutated codon 74 was observed, later in infection A74D→G or A74D→N changes were detected. This weak selective pressure for changes at position 74 is not surprising, because the A74D substitution had little effect on Nef functions in vitro (Fig. 1 and 2). Nevertheless, selective pressure for changes at each of the three mutated positions was consistently observed in five of the animals infected with SIV containing $239_{(EDR)}$-*nef*. Importantly, in animal Mm8494, which maintained low cell-associated viral loads (data not shown) and did not progress to AIDS, we observed no reversions throughout the observation period of 84 weeks. While the inability to detect reversions in this animal

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TABLE 1. Amino acid changes at positions 72, 73, 74, and 204 selected in vivo[a]

Mutations:	AAC <u>CC</u>A G<u>C</u>T → AAC <u>GA</u>A G<u>A</u>C	<u>GAT</u> → <u>CGC</u>
Amino acid residue:	N <u>P</u> <u>A</u> → N <u>E</u> <u>D</u>	<u>D</u> → <u>R</u>
Amino acid position:	72 73 74	204

w	Mm8003 72	Mm8003 73	Mm8003 74	Mm8003 204	Mm8151 72	Mm8151 73	Mm8151 74	Mm8151 204	Mm8155 72	Mm8155 73	Mm8155 74	Mm8155 204
0	N_{100}	E_{100}	D_{100}	R_{100}	N_{100}	E_{100}	D_{100}	R_{100}	N_{100}	E_{100}	D_{100}	R_{100}
1	-	-	-	-	-	-	-	-	-	K_{15}	-	-
2	-	-	-	-	-	-	-	D_{90}	-	K_{30}	-	D_{80}
4	-	K_{10}	-	D_{70}	-	-	-	$D_{>95}$	D_{50}	K_{70}	-	$D_{>95}$
6	-	K_{30}	-	D_{90}	-	K_{10}	-	$D_{>95}$	$D_{>90}$	$K_{>90}$	-	$D_{>95}$
6*	-	K_{50}	-	$D_{>95}$	-	K_{50}	-	$D_{>95}$	$D_{>95}$	$K_{>95}$	-	$D_{>95}$
8	-	K_{90}	-	$D_{>95}$	D_{20}	K_{50}	-	$D_{>95}$	$D_{>95}$	$K_{>95}$	-	$D_{>95}$
12	D_{50}	K_{90}	-	$D_{>95}$	D_{10}	P_{50}	-	$D_{>95}$	$D_{>95}$	$K_{>95}$	N_{10}	$D_{>95}$
16	D_{90}	K_{90}	-	$D_{>95}$	-	P_{80}	G_{50}	$D_{>95}$	$D_{>95}$	$K_{>95}$	N_{30}	$D_{>95}$
32	$D_{>90}$	$K_{>90}$	G_{30}	$D_{>95}$	-	$P_{>80}$	$G_{>90}$	$D_{>95}$	$D_{>95}$	$K_{>95}$	N_{20}	$D_{>95}$
64	$D_{>90}$	$P_{>80}$	-	$D_{>95}$	-	$P_{>80}$	G_{50}					

w	Mm8493 72	Mm8493 73	Mm8493 74	Mm8493 204	Mm8494 72	Mm8494 73	Mm8494 74	Mm8494 204	Mm8495 72	Mm8495 73	Mm8495 74	Mm8495 204
0	N_{100}	E_{100}	D_{100}	R_{100}	N_{100}	E_{100}	D_{100}	R_{100}	N_{100}	E_{100}	D_{100}	R_{100}
6*	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-
20	-	K_{50}	-	-	-	-	-	-	$D_{>90}$	$K_{>90}$	-	S_{80}
24	-	K_{70}	-	-	-	-	-	-	$D_{>90}$	$K_{>90}$	-	$S_{>90}$
28	$D_{>90}$	$K_{>90}$	N_{50}	$S_{>90}$	-	-	-	-	$D_{>90}$	$K_{>90}$	N_{50}	$S_{>90}$
32	$D_{>90}$	$K_{>90}$	$N_{>90}$	$S_{>90}$	-	-	-	-	$D_{>90}$	$K_{>90}$	N_{60}	D_{60}
36	$D_{>90}$	$K_{>90}$	$N_{>90}$	$S_{>90}$	-	-	-	-	$D_{>90}$	$K_{>90}$	$N_{>90}$	$D_{>90}$
40	$D_{>90}$	$K_{>90}$	$N_{>90}$	$S_{>90}$	-	-	-	-	$D_{>90}$	$K_{>90}$	$N_{>90}$	$D_{>90}$
44	$D_{>90}$	$K_{>90}$	$N_{>90}$	$S_{>90}$	-	-	-	-	$D_{>90}$	$K_{>90}$	$N_{>90}$	$D_{>90}$

[a] Amino acid sequences at positions 72, 73, 74, and 204 in Nef, derived from DNA population sequencing, are shown for each of the macaques at the indicated weeks postinfection (w). DNA was obtained from bulk cocultivations of CEMx174 cells with rPBMC collected at the indicated time points or from lymph node biopsy (*). Similar results were obtained with DNA isolated directly from rPBMC and with genomic viral RNA amplified by reverse transcription-PCR. The results were also confirmed by sequence analysis of 131 single PCR clones. The percentage of sequences that encode a given residue is indicated as a subscript. Dashes indicate identity to the sequence of the input mutant. For animal Mm8155, the last sequence was obtained at the time of death (21 wpi).

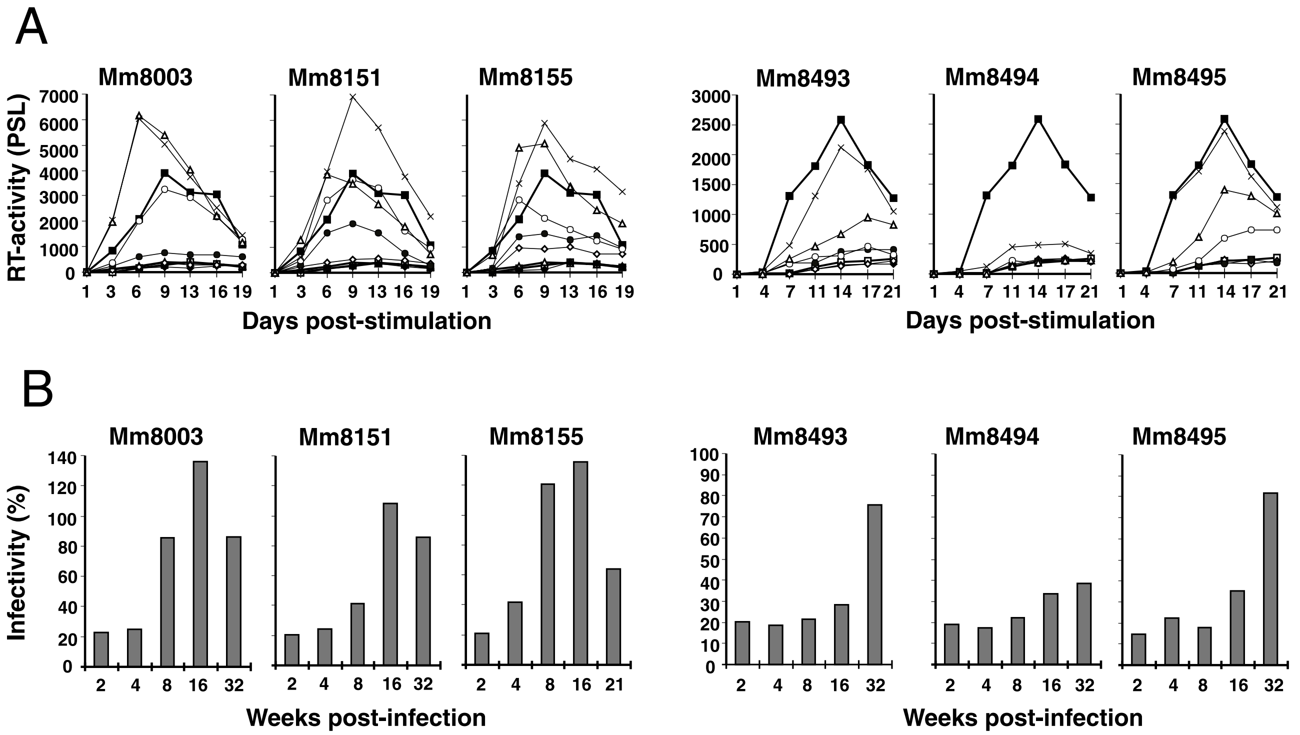

FIG. 4. Replication and infectivity of reisolates. (A) Ability of virus reisolated from rPBMC obtained at 1 wpi (◆), 2 wpi (◇), 4 wpi (●), 8 wpi (○), 16 wpi (△), and 32 wpi (×) to replicate in cultured rPBMC. 239wt (■) and SIVΔNU (□) replication in aliquots of the same rPBMC cultures is shown on each panel for comparison. Reverse transcriptase (RT) activity of supernatants was quantitated as described in the legend to Fig. 2A. (B) Infectivity of reisolates in sMAGI cells. The sMAGI indicator cells were infected with stocks of virus containing 100 ng of p27 antigen produced by cocultivation of rhesus macaque PBMC with CEMx174 cells. The values are shown as a percentage of 239wt activity and are the averages of four independent measurements.

may be the consequence and not the cause of lower levels of replication, this result supports our previous observations from long-term nonprogressors of HIV-1 infection, which showed that nonprogression can be associated with point mutations that disrupt the ability of Nef to downregulate CD4 and enhance viral replication in vitro (31).

Amino acid changes selected in vivo restore 239-Nef function. The emergence of amino acid changes in Nef in five of the six infected macaques coincided with an enhanced infectivity in sMAGI cells and with enhanced replication in PBMC of SIV reisolated from these animals (Table 1 and Fig. 4). In contrast, SIV reisolated from the remaining animal, Mm8494, in which no reversions were detected, showed inefficient replication and low infectivity. To confirm that the enhanced replication of reisolated virus resulted from the observed changes at positions 72, 73, and 204 in 239-Nef rather than from alterations elsewhere in the viral genome, we engineered the observed changes onto the 239wt provirus and tested their effect on SIV replication in vitro. The D204R→D and D204R→S changes in $239_{(EDR)}$-Nef alone did not completely restore SIV replication (Fig. 5A) or infectivity (Fig. 5B). However, the additional P73E→K substitution and a third N72→D change sequentially restored functional activity in both assays to levels observed with wild-type 239-Nef. Similar results were obtained with *nef* alleles containing these changes derived from the infected animals (data not shown). As shown in Fig. 5C, these changes also restored the ability of $239_{(EDR)}$-Nef to downregulate CD4 expression. Thus, P73E→K and N72→D P73E→K changes can functionally replace P73 and A74 and the D204R→S change can replace D204 to enhance SIV replication in rPBMC and in sMAGI cells and to downregulate CD4. The efficient selection of second-site compensatory changes in the surfaces disrupted by P73E, A74D, and D204R is strong evidence that these surfaces and their functions are important for SIV replication in vivo.

DISCUSSION

Biochemical and cell-based studies indicate that Nef has multiple functions and that these functions are performed through multiple independent interactions with the host cell signal transduction and protein sorting machinery (29, 36, 45). This study indicates that a 239-Nef mutation which disrupts the interactions of Nef required for the downregulation of CD4 expression and for enhanced SIV replication in vitro also disrupts SIV replication in rhesus macaques. Not only were viral loads low early in macaques infected with the SIV containing the $239_{(EDR)}$-Nef variant, but there was also a strong selective pressure for revertants and second-site mutations which restored Nef function. The selection of these changes was associated with rises in viral loads, and virus recovered from these animals possessed virologic properties similar to those of wild-type SIV. While the EDR mutation disrupts other Nef functions, such as downregulation of CD28 cell surface expression (T. Swigut and J. Skowronski, unpublished results), we conclude that the surfaces of Nef required to downregulate CD4 and to enhance SIV replication in vitro are critical for Nef function in vivo.

The observation that amino acid changes selected in vivo that restore CD4 downregulation also enhance SIV infectivity in sMAGI cells and SIV replication induced from rPBMC suggests that common molecular interactions of 239-Nef with cellular factors may underlie these three functions (10, 29). However, it remains possible that these three effects are not

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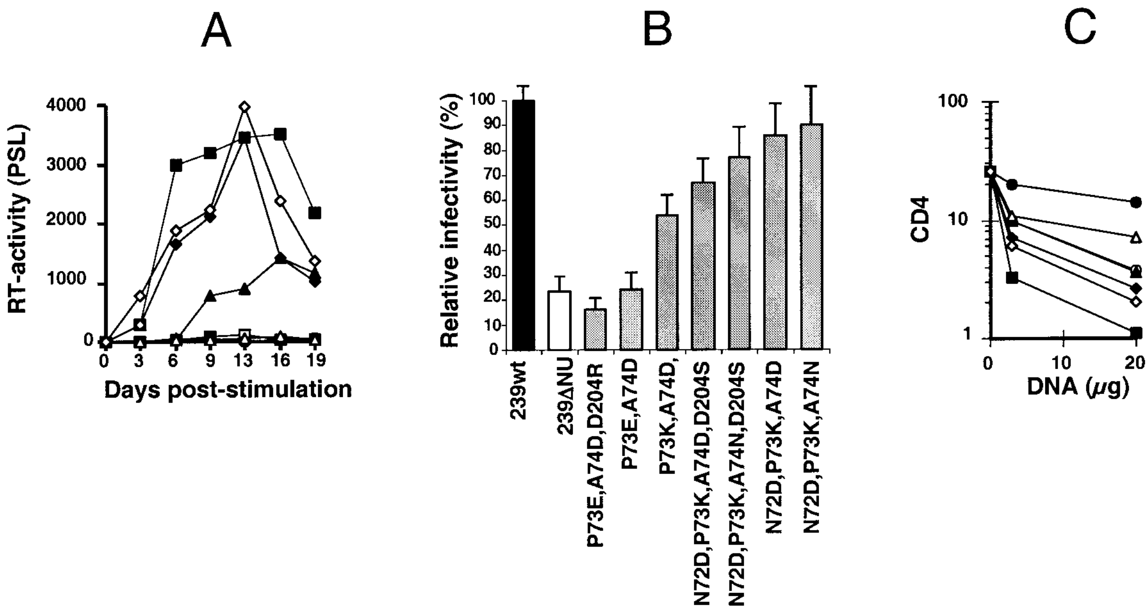

FIG. 5. Amino acid changes selected in vivo restore Nef function. (A) Replication (reverse transcriptase [RT] activity) of SIV containing P73K and A74D (▲), P73E, A74D, and D204R (●), P73E and A74D (△), N72D, A73K, and A74D (◆), or N72D, A73K, and A74N (◇) changes in Nef as well as control wild-type SIVmac239 (■) and 239ΔNU (□) viruses in rPBMC. (B) Infectivity in sMAGI cells. (C) Effect of Nef variants with A74D (▲), P73K and A74D (△), P73E, A74D, and D204R (●), P74K (○), N72D, P73K, and D74D (◆), and D204S (◇) changes and of a control 239-Nef (■) on CD4 surface expression, determined as described in the legend to Fig. 1A.

mediated by common molecular interactions but merely map to overlapping surfaces in the 239-Nef protein. If mutations that separate CD4 downregulation from the positive effect of Nef on SIV replication are indeed identified, they can be used to probe the relative contribution of these effects to SIV virulence. A previous study showed that Nef enhances virion infectivity even in cells lacking CD4 (1), suggesting that there may be multiple components to the effect of Nef on infectivity, including CD4-dependent and CD4-independent effects. The EDR mutation may disrupt the CD4-dependent component, which has been revealed recently by observations that CD4 expression on the cell surface inhibits both the infectivity of HIV particles by reducing virion Env incorporation and the release of HIV-1 progeny virions from producer cells, and that these effects can be overcome by expression of Nef (27, 38).

Nef downregulates class I MHC complexes from the cell surfaces and thereby can protect infected cells from detection and lysis by cytotoxic T lymphocytes (8, 9, 32, 43). Notably, the EDR mutation does not affect the ability of 239-Nef to downregulate surface expression of class I MHC complexes. Since this mutation disrupts SIV replication early in infection, the downregulation of class I MHC complexes from the surface of infected cells cannot be the only mechanism by which 239-Nef enhances SIV loads in vivo. The downregulation of class I MHC is likely to be important after the first 10 to 14 days of infection, when the host cytotoxic T-cell response is known to be critical for controlling viral loads (26, 32). Therefore, the ability of Nef to downregulate class I MHC and the ability of Nef to downregulate CD4 may be complementary functions that allow Nef to enhance the replication and persistence of immunodeficiency viruses, and our data clearly show that class I MHC downregulation is not sufficient for the positive effect of 239-Nef on SIV virulence.

It now becomes clear that Nef has multiple functions which are selected independently. Therefore, it is likely that their combination is important for maximal enhancement of SIV-HIV replication and persistence in the host. This possibility has strong implications for the development of pharmaceutical agents that would disrupt Nef function. While our data suggest that the identification of drugs that can disrupt CD4 downregulation will be efficacious in inhibiting viral replication in vivo, it will also be important to identify and target individual molecular interactions of Nef that are critical for multiple independent Nef functions. One such candidate interaction is membrane association of Nef, mediated by posttranslational N-terminal myristoylation of the Nef proteins (18), which has been shown to be required for all known functions of Nef proteins. A similar strategy that disrupts the membrane attachment of the Ras oncoprotein by interfering with its posttranslational C-terminal farnesylation has been successfully used to prevent Ras-mediated cellular transformation (25).

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Disrupting Surfaces of Nef Required for Downregulation of CD4 and for Enhancement of Virion Infectivity Attenuates Simian Immunodeficiency Virus Replication In Vivo

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Volume 74, no. 21, p. 9836–9844, 2000. Page 9843: The Acknowledgments should appear as shown below.

ACKNOWLEDGMENTS

We thank Marion Hamacher, Mandy Krumbiegel, Nadim Shohdy, Pat Burfeind, and Maria Coronesi for excellent technical assistance; members of the Skowronski laboratory, Winship Herr, and Klaus Uberla for critical reading of the manuscript; and Bernhard Fleckenstein for support. We also thank Julie Overbaugh and Bryce Chackerian for sMAGI cells.

This work was supported by the Sander-Stiftung, BMBF grant 01Ki9478 (F.K), Sonderforschungsbereich 466 (F.K.), PHS AI-42561 (J.S.), and The Council for Tobacco Research USA grant 4688 (J.S.).