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Interaction between Nef and INI1/SMARCB1 augments replicability of HIV-1 in resting human peripheral blood mononuclear cells

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Abstract

A central feature of HIV-1 infection is the inability of entering virus to integrate into chromosomes of resting T lymphocytes unless they are mitogenically activated. In contrast, SIVpbj1.9 replicates in initially resting T lymphocytes by activating infected cells. Previous reports have shown that a difference in Nef-mediated T cell activation between HIV-1 and SIVpbj1.9 plays a critical role in the differing abilities of these viruses to replicate in resting lymphocytes. However, the molecular details of these differences are still unclear. Here, we show that infection with a chimeric virus, HSIVnef, which harbors the 5' 308 nucleotides of SIVpbj1.9 *nef* in place of the 5' 221 nucleotides of HIV-1 *nef* in the HIV-1 proviral backbone, resulted in integration of the provirus into host chromosomes without mitogenic activation and thereby replication in resting human PBMCs (hPBMCs). These results indicate that Nef is an essential viral determinant for the integration of provirus into host chromosomes in resting T cells. Using the yeast two-hybrid system, we identified integrase interactor-1 (INI1/SMARCB1) as a cellular factor that is involved in the integration process via interaction with Nef. Although INI1 interacted with both SIVpbj1.9 and HIV-1 Nefs, SIVpbj1.9 Nef, but not HIV-1 Nef, enhanced proviral integration into host DNA. Furthermore, mutational analysis revealed that the basic-amino-acid-rich amino-terminal domain in SIVpbj1.9 Nef is crucial for interaction with INI1 and virus replication in resting hPBMCs. Taken together, these data indicate that Nef is a critical viral protein for incorporating nascent proviral DNA into host chromosomes in resting PBMCs and that this occurs through interaction with INI1. This elucidates the basis for replication of the integrated provirus when the host cell is in a resting state.

Keywords

HIV-1; SIVpbj1.9; Nef; INI1/SMARCB1; integrase; integration of provirus; resting T cells

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Introduction

It is known that HIV-1 successfully enters but fails to replicate in resting CD4⁺ T cells due to various host innate defense mechanisms acting prior to integration of the proviral DNA into host chromosomes [1,2]. These mechanisms include incomplete reverse transcription due to SAMHD1-mediated reduction of the dNTP pool [3], abortive single-strand DNA modification by cellular factors such as APOBEC3G, which counteracts the viral protein Vif [4,5,6,7], and impaired import of the preintegration complex (PIC) into the nucleus due to a shortage of ATP [8]. These observations are consistent with *in vivo* data showing that in the largely quiescent T lymphocytes in the peripheral circulation of HIV-1-infected humans, viral DNA is present predominantly in an extrachromosomal form [8,9]. However, upon stimulation with a mitogen such as phytohemagglutinin (PHA), productive infection proceeds [10,11,12]. Recent findings indicate that during T-cell activation, the viral integrase (INT) is phosphorylated by c-Jun N-terminal kinase (JNK) and becomes a substrate for Pin1 [13]. Pin1 subsequently stabilizes INT for efficient HIV-1 integration, leading to productive infection [13]. These results suggest that activation of resting T lymphocytes triggers intracellular signaling to enhance integration of provirus into host cell chromosomes.

SIVpbj1.9, a variant SIV from sooty mangabey monkeys, is known to induce in pig-tailed macaques (*Macaca nemestrina*) an acute syndrome characterized by bloody mucoid diarrhea and the death of most animals within 1 to 2 weeks [14,15]. Fultz reported that the extreme virulence of SIV_{smm}pbj14 and its replicative properties are related to its ability to activate resting T cells [16]. The ability to activate simian PBMCs (sPBMCs) to proliferate appeared a specific property of the acutely lethal variant, because incorporation of [³H]thymidine by PBMCs from native animals was observed only with SIV_{smm}pbj14 and not with other viruses [16]. The report also showed that this virus replicated in resting sPBMCs just as efficiently as in mitogen-stimulated PBMCs, irrespective of the addition of exogenous interleukin-2 (IL-2) [16]. This finding indicates that SIVpbj1.9 infection activates resting T cells for productive infection.

Among the viral proteins, Nef plays an essential role in activation of resting T cells and thereby virus replication in the cells. Du et al. demonstrated that SIVpbj1.9 Nef is the major viral determinant for activation of resting PBMCs and virus replication [17]. They further showed that substitution of two amino acids, RQ, with YE to create a YxxL motif in SIVmac239 enabled the virus to replicate in resting PBMCs and to cause acute disease in macaque monkeys [17], similar to the pathogenesis of SIVpbj1.9. Similar results were obtained using a recombinant SHIV containing SIVpbj1.9 *nef*, which activated lymphocytes and replicated productively in resting PBMCs [17,18]. Our data also indicated that SIVpbj1.9 Nef significantly enhanced interleukin-2 (IL-2) promoter activity, while that of HIV-1 did not [19]. This was corroborated by the observation that SIVpbj1.9 Nef induced production of IL-2 even in the absence of activation with CD3 and/or CD28 antibodies, whereas HIV-1 Nef activated T cells only upon stimulation of the cells with CD3 and/or CD28 antibodies [19], consistent with other reports [18,20,21,22]. However, the following important questions remain: 1) Is T-cell activation by SIVpbj1.9 Nef sufficient for virus replication in resting CD4⁺ T cells? 2) If so, how does T-cell activation by SIVpbj1.9 Nef

induce integration of the proviral DNA into host chromosomes for productive virus infection in resting T cells? Addressing these questions will bring insights into the role of Nef in activation and replication of the primate immunodeficiency viruses in resting PBMCs.

In this study, we employed the yeast two-hybrid system to define the roles of SIVpbj1.9 Nef in replication of HIV-1 in resting CD4⁺ T cells and to elucidate the cellular determinants that are critical for these processes. Several cellular proteins were identified as interacting with the Nefs, and among them, INI1 was chosen for study as a potential candidate for enabling differential Nef function in HIV-1 replication in resting T cells. INI, also known as SMARCB1, is a core subunit of SWI/SNF chromatin remodeling complexes and functions as a tumor suppressor [23]. It has been reported that the INI1 protein plays an important role in the insertion of HIV-1 proviral DNA into host chromosomal targets through its direct interaction with the viral protein INT [24], and mitogenic cell activation promotes HIV-1 DNA integration into host chromosomes [10,11,12]. Since HIV-1 Nef activates resting T cells for extensive proliferation [19,20,21,22], our finding that the interaction of SIVpbj1.9 Nef and INI1 facilitates viral DNA integration will provide important clues for understanding the mechanism of the limited HIV-1 replication in resting T cells.

Materials and methods

Cells and viruses

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh whole blood and stimulated as described previously [25]. The CD4-positive T lymphoid cell lines CEMx174 and Jurkat were employed for viral DNA transfection and virus infection. The strain of HIV-1 used was HXB2, which contains a functionally complete viral genome, and SIVpbj1.9 was generated by transfection with pPBj1.9, a plasmid obtained from Dr. James I. Mullins through the NIH AIDS Research & Reference Reagent Program (Germantown, MD). The nef recombinant virus HSIVnef was generated by replacing the first 221 nucleotides of HXB2 nef with the corresponding region of SIVpbj1.9 nef (the first 308 nucleotides) as described elsewhere [19]. The site of substitution was located between an artificially introduced SmaI site at the env/nef boundary and the KpnI site within HXB2 nef.

Reverse transcriptase (RT) assay

Human PBMCs, Jurkat cells, and CEMx174 cells were infected with virus at 10,000 counts per minute (cpm) of RT activity per one million cells. To test virus replication in activated PBMCs, freshly isolated PBMCs were incubated with 5 µg of phytohemagglutinin (PHA) and 10 U of IL-2 per ml for 3 days before virus infection. After a 4- to 6-hour incubation with virus, cells were washed and resuspended with fresh medium. At every third or fourth day, half of the supernatant was collected and replaced with fresh medium. Virus replication was monitored by measuring the RT activity of pelleted virions from the culture supernatants [26]. Briefly, virus pellets were recovered from supernatants by centrifugation and were lysed with dissociation buffer. RT assays were performed in a reaction mixture of RT assay buffer [³H]-dTTP and poly(A)-dT15 (Boehringer Mannheim). After the reactions were complete, samples were absorbed on ion exchange cellulose paper (DE81, Millipore,

Bedford, MA). Following several washes with 2X SSC and 100% ethanol, radioactivity was measured by using a scintillation counter.

Alu-PCR and Southern hybridization

To detect integration of viral DNA into genomic DNA, human PBMCs were infected with HIV-1, HSIVnef, or SIVpbj1.9, and Alu-PCR was performed on the genomic DNA extracted from PBMCs infected for 24 h as described [27]. The primer sequences employed for the Alu-PCR were as follows: A primer, Alu5-TagA, was designed with the human Alu consensus sequence (CAGUGCCAAGUGUUUGCUGACG) and an artificial TagA sequence (CCAAAGUGCUGGGAUUACAG). The first-round primers were Alu5-TagA and 1U3-Ura (5'-CCUGGUGUGUAGUUCUGCUAAUCAG; 100-76) for HIV-1 and Alu5-TagA and PI-1U3-Ura (5'-CUCUUGCGGUUAGCCUUCUCUGCA; 124-101) for SIVpbj1.9. The second-round nested PCR primers were TagA (5'-CAGTGCCAAGTGTGCTGACGCCAAAG) and INT-2U3 (5'-AATCAGGGAAGTAGCCTTGTGTGTG; 81-56) for HIV-1 and TagA and PI-2U3 (5'-CCTCTTCCTTTGACAAGCC TGACT; 100-77) for SIVpbj1.9. PCR products were analyzed by Southern hybridization with a γ -[³²P]ATP-labeled H-probe (5'-TGGAAGGGCTAATTCACTCCCAACGAAGACAAGATATCCT; 1-40) for HIV-1, and S-probe (5'-CTGGCATGGAAGTTTGATCCAAAGTTAGC TTATAACTATA; 1-40) for SIVpbj1.9 according to the manufacturer's protocol (Amersham Pharmacia Biotech).

Yeast two-hybrid assay

The yeast two-hybrid assay was performed according to the manufacturer's protocol (Clontech, Palo Alto, CA). Briefly, HIV-1 and SIVpbj1.9 *nef* genes in a pLexA-binding domain (BD) fusion vector (His⁺) and a Jurkat cDNA library expressed in a pB42-activation domain (AD) fusion vector (Trp⁺) were introduced into yeast strain EGY48 by cotransformation, and positive colonies were screened twice to eliminate false positives [24]. pB42AD-cDNA plasmids were then recovered from positive colonies, sequenced and introduced into EGY48/p8op-lacZ/nef by transformation to confirm the interaction with HIV-1 and SIVpbj1.9 Nefs.

Mammalian two-hybrid assay

Except for the cells, the mammalian two-hybrid assay was performed essentially the same as the yeast two-hybrid assay. Briefly, *nef* expressers in a pM-BD fusion vector (Clontech) and INI1 in a pVP16AD fusion vector were introduced by cotransfection into NIH 3T3 cells with a reporter gene, pG5CAT, and pCMV- β -gal to control for transfection efficiency. Three days after transfection, chloramphenicol acetyltransferase (CAT) enzymatic activity was measured as per the manufacturer's protocol (Clontech).

Protein purification and glutathione-S-transferase (GST) pull-down assay

Full-length INI1, HIV-1 *nef*, and SIVpbj1.9 *nef* in a pGEX-5X GST-fusion vector and His-tagged HIV-1 INT were purified from overnight culture of *E. coli* BL21 transformed with each plasmid, using glutathione Sepharose beads (Amersham Pharmacia Biotech) and Ni-NTA agarose beads (QIAGEN, Valencia, CA), respectively. The HIV-1-INT-expressing

plasmid, pINS.D.His.Sol, was obtained from Dr. Robert Craigie through the NIH AIDS Research & Reference Reagent Program. HA-tagged INI1 in pB42AD was expressed in yeast, and yeast lysate was obtained using Y-PER yeast cell lysis buffer (Pierce, Rockford, IL). For the GST pull-down assay, protein-bound glutathione Sepharose beads (Amersham Pharmacia Biotech) were incubated with yeast lysate and/or His-tagged protein for 1 h in binding buffer, 40 mM Tris, pH 8.2, 150 mM NaCl, 0.1% NP-40, and 5 mM EDTA, and complexes were analyzed by immunoblotting with anti-HA (BabCo, Richmond, CA) and anti-penta His (QIAGEN) mouse antibodies and anti-GST goat antibody (Amersham Pharmacia Biotech).

β-galactosidase (β-gal) assay

Yeast strain EGY48/p8op-lacZ was cotransformed with wild-type *nef* in pLexA and with INI1 in pB42AD. Following selection from nutrition-deficient media, transformed colonies were cultured in liquid medium until log phase, measured at 600 nm. To determine the binding affinity of Nef with INI1, β-gal activity in the transformed yeast was quantitated as per the manufacturer's protocol (Clontech). The units of β-gal activity were calculated by the following equation: Miller units = $(A_{420} \times 1000) / (A_{600} \times \text{time}_{\text{min}} \times \text{volume}_{\text{ml}})$.

Integration assay

The *in vitro* DNA strand transfer assay was performed as described [24]. Briefly, HIV-1 INT substrate U5.5 (5'-GGATCCGGAAAATCTCTAGCA) was labeled with 50 μCi of [γ -³²P] ATP incubated with 10 U of T4 polynucleotide kinase (New England Biolabs). After kinase inactivation at 70°C, U5.5 was annealed with U5.4 (ACTGCTAGAGATTTTCCGGATCC). Annealed double-stranded DNA was purified using a NucTrap probe purification column (Stratagene). Different amounts of proteins, GST-Nefs, GST, and GST-INI1 were added to the integration reaction mixture consisting of purified HIV-1 integrase, DNA substrate, and integration buffer (20 mM HEPES, pH 7.5, 10 mM MnCl₂, 10 mM dithiothreitol, 0.05% NP-40). After a 1-h incubation, an equal volume of loading buffer (98% deionized formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to stop the reaction. The samples were analyzed by 20% polyacrylamide urea gel electrophoresis and autoradiography.

Results

INI1 interacts with the HIV-1 and SIVpbj1.9 Nefs

To define differential functions of HIV-1 and SIVpbj1.9 Nefs for viral replication in resting T cells, host factors bound to HIV-1 and SIVpbj1.9 Nefs were screened using the yeast two-hybrid assay. Our result showed that the reporter gene *lacZ* was expressed, producing a blue color on the plate, when INI1 in the AD fusion vector (AD) was expressed together with HIV-1 *nef* (H-*nef*) or SIVpbj1.9 *nef* (S-*nef*) in the BD fusion vector (BD) in the presence (Fig. 1A, left), but not in the absence (Fig. 1A, right) of galactose, demonstrating interactions between INI1 and both HIV-1 Nef (H-Nef) and SIVpbj1.9 Nef (S-Nef) protein. In contrast, expression of INI1 alone (LexA + INI1) or Nef alone (H-*nef* or S-*nef* + B42AD) did not induce *lacZ* expression. Further, expression of the reporter *lacZ* was not observed when Nefs and unrelated palmitoyl transferase (PalT) (H-*nef* + PalT and S-*nef* + PalT) or

INI1 and HIV-1 vif (H-vif) (H-vif + INI1) were co-expressed (Fig. 1A), indicating that interactions between INI1 and Nef were specific. A quantifiable β -gal assay further demonstrated the specificity of the interaction between Nef and INI1, as shown in Figure 1B, wherein only negligible amount of β -gal activity was detected from interactions between Nef or INI with the unrelated proteins HIV-1 Vif and PalT (Fig. 1B).

To verify the interaction of INI1 with the HIV-1 and SIVpbj1.9 Nefs in mammalian cells, a mammalian two-hybrid assay was performed [28]. Co-transfection with the INI1 expressor along with either the HIV-1- or SIVpbj1.9-*nef* expressor resulted in strong CAT enzymatic activity, while little activity was detected in samples transfected with a single *nef*-expressor (Fig. 2A). These data confirmed that both HIV-1 and SIVpbj1.9 Nefs specifically bind INI1 in NIH 3T3 cells. Consistent with these results, we found that both HIV-1 and SIVpbj1.9 Nefs interacted with a GST-INI1 fusion protein using the GST pull-down assay and Western blotting analysis (Fig. 2B). Taken together, these results indicate that the HIV-1 and SIVpbj1.9 Nefs directly bind to INI1 in both mammalian and yeast cells.

HIV-1 and SIVpbj1.9 Nefs displayed differential proviral DNA integration activity

To examine whether the observed physical interactions of HIV-1 and SIVpbj1.9 Nefs with INI1 affect proviral DNA integration into host chromosomes, an *in vitro* DNA strand transfer assay reflecting the efficiency of proviral DNA integration into host chromosomes [24] was performed with purified 6His-tagged HIV-1 integrase (INT), GST-INI1, and GST-H-Nef or GST-S-Nef proteins. As shown previously [24], INT and/or INI1 significantly augmented DNA strand transfer (Fig. 3A and C, GST). While the change of HIV-1 Nef-mediated DNA strand transfer activity in the presence of INI1 was nominal (Fig. 3A, H-Nef), SIVpbj1.9 Nef dramatically increased DNA strand transfer activity with increasing concentration of INI1 (Fig. 3A, S-Nef). These results suggest that SIVpbj1.9 Nef, but not HIV-1 Nef, might facilitate viral DNA integration into target DNA. SIVpbj1.9 Nef alone significantly increased the integration activity in a dose-dependent fashion, which was further slightly enhanced in the presence of INI1 (Fig. 3B, S-Nef), while HIV-1 Nef-mediated augmentation of the integration activity was minimal even at the highest dose of HIV-1 Nef protein (Fig. 3B, H-Nef), indicating that SIVpbj1.9 Nef alone can induce proviral DNA integration into host chromosomes. Moreover, SIVpbj1.9 Nef-mediated integration was significantly increased by addition of INI1 (Fig. 3A, S-Nef) in a dose-dependent manner, and it was further enhanced by increasing the amount of INT (Fig. 3C, S-Nef). By contrast, the increased level of HIV-1-Nef-mediated DNA strand transfer was significantly lower than that with SIVpbj1.9 Nef in the presence of INI1 (Fig. 3A) or of INI1 and INT (Fig. 3C). Taken together, these data imply that SIVpbj1.9 Nef interactions with INI1 and/or INT could enhance proviral DNA integration into the target host chromosomes, while HIV-1 Nef interactions with INI1 and/or INT could not.

The N-terminal region of SIVpbj1.9 Nef is sufficient for virus integration into the host chromosomes of resting hPBMCs

Previous studies have shown that newly reverse-transcribed HIV-1 DNAs accumulate as extrachromosomal DNA in resting T cells, causing abortive viral replication [8,9]. These results suggest that proviral DNA integration may be one of the most critical steps that limit

HIV-1 replication in resting T cells. As SIVpbj1.9 Nef, but not HIV-1 Nef, enhances DNA strand transfer, as shown above (Fig. 3), we hypothesized that HIV-1 and SIVpbj1.9 Nefs differentially affect viral DNA integration into host chromosomes. To investigate this possibility, resting and PHA-activated hPBMCs were infected with equivalent amounts of HIV-1 or SIVpbj1.9 for 24 h, and integration of the HIV-1 and SIVpbj1.9 DNAs into the host chromosomes was detected, using Alu-PCR followed by Southern blot analysis with HIV-1 and SIVpbj1.9 LTR-specific probes. Our results showed that SIVpbj1.9 provirus was present in the host chromosomes of both resting and activated hPBMCs, while HIV-1 provirus was detected only in activated hPBMCs (Fig. 4).

Du et al. previously showed that the N-terminal YxxL motif of SIVpbj1.9 was sufficient for SIVmac239 replication in resting PBMCs [17]. To determine whether the N-terminal region of SIVpbj1.9 is sufficient for HIV-1 integration into host chromosomes, we previously generated a chimeric virus, termed HSIVnef, by replacing the first 221 nucleotides of HXB2 nef with the corresponding region of SIVpbj1.9 nef [19]. Using HSIVnef, we found that the virus harboring the N-terminal region of SIVpbj1.9 Nef in place of the corresponding the region of HIV-1 is efficiently integrated into the host genome in resting hPBMCs as well as PHA-activated hPBMCs (Fig. 4). These results indicate that replacement of HIV-1 Nef with the corresponding N-terminal fragment of SIVpbj1.9 Nef is sufficient for HIV-1 DNA to integrate into host chromosomes of resting hPBMCs, suggesting that the first 221 nucleotides of SIVpbj1.9 Nef contain the necessary elements required for the incorporation of viral DNA into the host chromosome.

Substituting the N-terminal region of the HIV-1 Nef with SIVpbj1.9 Nef is sufficient for HIV-1 replication in resting hPBMCs

To determine whether HSIVnef containing the N-terminal region of SIVpbj1.9 Nef is sufficient for viral replication in resting hPBMCs, we transfected CEMx174 cells with HIV-1, HSIVnef, and SIBpbj1.9 DNAs and measured viral replication using an RT assay. As expected, all of the viruses replicated efficiently, while SIVpbj1.9 showed a slightly delayed onset of replication (data not shown). Moreover, analysis of viral proteins from the transfected cells and virion particles in culture supernatant using Western blot showed that introduction of the N-terminal fragment of SIVpbj1.9 nef did not impair HIV-1 protein expression and processing (data not shown). We further tested replication of HIV-1, HSIVnef, and SIVpbj1.9 in CEMx174 (Fig. 5A) or Jurkat T cell lines (Fig. 5B) using cell-free progeny viruses produced from transfected CEMx174 cells. HIV-1 and HSIVnef robustly replicate in both cell lines. However, SIVpbj1.9 replication was not detected in Jurkat T-cells, which is consistent with previous reports [29,30]. These data indicate that the progeny viruses of HSIVnef were intact and infectious.

Next, we tested replication of HIV-1, HSIVnef, and SIVpbj1.9 in resting and PHA-activated hPBMCs. An equivalent amount (10,000 cpm of RT activity/ 1×10^6 cells) of each virus was used for inoculation, and virus replication was monitored as described previously [26]. All of these viruses replicated efficiently in the activated hPBMCs (Fig. 5C). Interestingly, however, both chimeric HSIVnef and SIVpbj1.9 replicated in resting hPBMCs, while HIV-1

did not (Fig. 5D). These results suggest that the N-terminal region of SIVpbj1.9 Nef is sufficient for HIV-1 replication in resting hPBMCs.

The amino-terminal basic-amino-acid-rich motif of SIVpbj1.9 Nef is essential for interaction with INI1, DNA strand transfer, and replication in resting hPBMCs

To further dissect the Nef domains in the N-terminal region that are important for replication of HIV-1 in resting hPBMCs, deletion mutants of the SIVpbj1.9 nef gene were generated (Fig. 6A). We first examined the effect of these mutations on interactions with INI1 and DNA strand transfer activity. Consistent with the results in Fig. 1, the reporter gene *LacZ* was expressed when INI1 in the AD fusion vector (AD) was expressed together with SIVpbj1.9 nef (S-nef) in the BD fusion vector (BD) (Fig. 6B). Interestingly, INI interaction with SIVpbj1.9 Nef was not affected by deletions in the N-terminal YxxL motifs, which are essential for activation of T cells [17], or in the PxxPxxP region, which is critical for signaling cascades [31,32,33]. In contrast, deletions of the basic-amino-acid-rich N- (N-term) or C-terminal region (C-term) eliminated interactions between Nef and INI1 (Fig. 6B), indicating that these two sites were essential for the interactions. Next, we investigated the significance of N- or C-term-mediated interaction with INI1 on proviral DNA integration into host chromosomes, using an *in vitro* DNA strand transfer assay as described above. While the SIVpbj1.9 Nef SH2(1+2) mutant did not significantly affect DNA strand transfer activity compared with S-nef, the N-term or C-term mutants significantly reduced wild-type SIVpbj1.9 Nef (S-nef)-mediated DNA strand transfer activity (Fig. 6C). These results suggest that the N- and C-termini of SIVpbj1.9 Nef are critical for interactions with INI1 as well as integration of proviral DNA into host chromosomes. Next, to determine the role of these motifs for HSIVnef virus replication in resting hPBMCs, we introduced mutations substitution of the second nucleotide G with A (HSIVnef-G/A) to remove myristoylation and deletions of SH2(1+2) (HSIVnef-SH2(1+2)) and N-term (HSIVnef-N-term), as shown above, in the recombinant nef gene of the HSIVnef genome. C-term was not introduced into the nef gene of the HSIVnef genome, since the site overlaps with the 3' LTR, which is required for virus replication. Recombinant and mutant virions were produced by transfecting CEMx174 cells with these viral genome constructs. An equivalent amount of each virus was used for inoculation, and the ability of these viruses to replicate was examined in resting and PHA-activated hPBMCs. Our results showed that HSIVnef and HSIVnef-SH2(1+2) replicated robustly in activated hPBMC, while replication of HSIVnef-G/A and HSIVnef-N-term was significantly inhibited and delayed (Fig. 6D-1). Furthermore, deletion of the myristoylation motif or the basic-amino-acid-rich N-terminal domain completely eliminated the ability of HSIVnef to replicate, whereas HSIVnef-SH2(1+2) replicated as efficiently as HSIVnef in resting hPBMCs (Fig. 6D-2). Taken together, these mutational analyses indicate that the N-terminal basic-amino-acid-rich domain in the recombinant Nef is essential for interaction with INI1, DNA strand transfer, and replication in resting hPBMCs.

Discussion

In this study, we address the functional differences of HIV-1 and SIVpbj1.9 Nefs on viral DNA integration into host chromosomes for productive infection in resting T lymphocytes.

Our data show that the N-terminal region of SIVpbj1.9 Nef is sufficient for HIV-1 replication in resting hPBMCs and that this replication is associated with increased integration of the HIV-1 DNA into host chromosomes. To our knowledge, this is the first demonstration that Nefs of primate immunodeficiency viruses play different roles in viral DNA integration and replication.

Previous studies indicate that quiescent human peripheral blood T lymphocytes (PBL) do not support efficient replication of HIV-1 by restricting the preintegration step of the virus life cycle [11–13,34–38]. However, activation of resting T cells overcomes these impediments so that viral DNA replicates efficiently. HIV-1 Nef is known to activate T cells through signaling cascades that are almost identical to those involved in TCR-mediated T cell activation [39]. However, Nef alone is not sufficient for productive infection of HIV-1 in resting T cells. Thus, it is important to understand the mechanism by which interactions between Nef and host factors contribute to virus replication.

Unlike the Nefs of other lentiviruses, SIVpbj1.9 Nef has a strong stimulatory effect on IL-2 production and proliferation of host T lymphocytes, which is essential for viral replication, even in resting T cells [17]. In contrast, HIV-1 Nef is not sufficient to activate resting T cells. IL-2 production increases significantly in Jurkat T cells or primary CD4⁺ cells expressing HIV-1 Nef only when TCR/CD3 and CD28 co-receptor are stimulated by antibodies or ligands [19,20,21] or when the T cells are exposed to mitogens that bypass surface receptors [40]. Our previous data showed that recombinant HSIVnef Nef enhanced IL-2-promoter-mediated activity and IL-2 secretion, whereas HIV-1 Nef did not [19]. Interestingly, the same recombinant HSIVnef provirus successfully integrated into a host chromosome and replicated in resting PBMCs (Fig. 2). Our findings suggest that Nef-mediated T cell activation contributes to the replication of the virus in the resting T cells by enhancing integration of extrachromosomal viral DNA into the host genome.

Our findings indicate that, independently or coordinated with upregulation of integration of viral DNA into host chromosomes by Nef-mediated T cell activation, Nef can promote integration by interacting with INI1, although the precise molecular mechanism is unclear at present. However, the binding of Nef with INI1 is remarkable in light of the known distribution of these proteins in different subcellular compartments: HIV-1 Nef is known to be expressed in the cytoplasmic membrane (myristoylated form) or in the cytosol (non-myristoylated form) [41,42], while INI1 is expressed in the nucleus [43]. This raises a mechanistic question on how Nef in the cytoplasm can interact with INI1 in the nucleus to enhance integration of viral DNA into host chromosomes. According to Turelli et al. [44], the incoming retroviral PICs trigger the exportin-mediated cytoplasmic export of the SWI/SNF component INI1, also known as SMARCB1, and of the nuclear body constituent PML, and the HIV genome associates with these proteins before nuclear localization. Previous reports have shown that Nef-deficient virus particles are impaired for reverse transcription in target cells [45–47], indicating that Nef plays a critical role in the process of synthesis of proviral DNA. Since Nef is incorporated into virion particles [48], these reports suggest that the Nef protein, after the virus enters the host cell, can increase the amount of proviral DNA by increasing reverse transcription and/or triggering PICs by interacting with

INI1 to modulate nuclear localization, which collectively contributes to enhancement of viral DNA integration.

Interaction between Nef and INI1 in the cytoplasm could facilitate not only nuclear transport but also integration of the reverse-transcribed DNA into host chromosomes. It has been reported that INT interacts with INI1 [24], and concerted action of JNK and Pin1 increases INT stability, which is required for efficient HIV-1 integration and infection [13]. Since Nef activates JNK [49–53], which phosphorylates INT [13], and since our data indicate that infection with HSIVnef, but not HIV-1, results in efficient integration of proviral DNA into the host genome in resting hPBMCs (Fig. 4), the corresponding N-terminal fragment of SIVpbj1.9 Nef in HIV-1 is sufficient to increase INT stability by activating JNK. Furthermore, we also found that the strand transfer activity of HIV-1 INT was enhanced in the presence of Nefs and INI1, and the increased levels were especially pronounced with SIVpbj1.9 Nef (Fig. 3). In this respect, it would be of interest to examine whether phosphorylation of INT is facilitated in the complex of Nef/Int/INI1, thereby favoring integration of provirus into the host chromosome. Further studies would clarify the potential links between interaction of Nef/INI1 and integration of provirus into host chromosomes in resting T cells.

Our results strongly imply that SIVpbj1.9 Nef, but not HIV-1 Nef, facilitates proviral DNA integration into the chromosomes of resting T cells. Although in the *in vitro* DNA strand transfer assay, SIVpbj1.9 Nef increased INT activity less than twofold, this small change could overcome the threshold of proviral integration in resting PBMCs *in vivo*. Subsequent studies could focus on surveying different HIV strains that appear particularly virulent with regard to motif differences in the nef genes. Since our mutational analysis indicated that the N-terminal basic-amino-acid-rich domain in the recombinant Nef is essential for interaction with INI, DNA strand transfer, and replication in resting hPBMCs, we believe that identification of this motif in SIVpbj1.9 nef should bring useful insights for understanding roles of Nef in HIV-1-infection in resting T cells. In addition, recombinant viruses containing SIVpbj1.9nef, such as HSIVnef, could be used to generate novel animal models to study HIV-1 pathogenesis and develop preventive and therapeutic methods.

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References

1. Pierson TC, Zhou Y, Kieffer TL, Ruff CT, Buck C, et al. Molecular characterization of preintegration latency in human immunodeficiency virus type 1 infection. *J Virol.* 2002; 76:8518–8531. [PubMed: 12163571]
2. Strebel K, Luban J, Jeang KT. Human cellular restriction factors that target HIV-1 replication. *BMC Med.* 2009; 7:48. [PubMed: 19758442]
3. Gao WY, Cara A, Gallo RC, Lori F. Low levels of deoxynucleotides in peripheral blood lymphocytes: a strategy to inhibit human immunodeficiency virus type 1 replication. *Proc Natl Acad Sci U S A.* 1993; 90:8925–8928. [PubMed: 7692440]

4. Malim MH. APOBEC proteins and intrinsic resistance to HIV-1 infection. *Philos Trans R Soc Lond B Biol Sci.* 2009; 364:675–687. [PubMed: 19038776]
5. Goila-Gaur R, Khan MA, Miyagi E, Strebel K. Differential sensitivity of “old” versus “new” APOBEC3G to human immunodeficiency virus type 1 vif. *J Virol.* 2009; 83:1156–1160. [PubMed: 19004939]
6. Chiu YL, Soros VB, Kreisberg JF, Stopak K, Yonemoto W, et al. Cellular APOBEC3G restricts HIV-1 infection in resting CD4+ T cells. *Nature.* 2005; 435:108–114. [PubMed: 15829920]
7. Goila-Gaur R, Strebel K. HIV-1 Vif, APOBEC, and intrinsic immunity. *Retrovirology.* 2008; 5:51. [PubMed: 18577210]
8. Bukrinsky MI, Stanwick TL, Dempsey MP, Stevenson M. Quiescent T lymphocytes as an inducible virus reservoir in HIV-1 infection. *Science.* 1991; 254:423–427. [PubMed: 1925601]
9. Bukrinsky MI, Sharova N, Dempsey MP, Stanwick TL, Bukrinskaya AG, et al. Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. *Proc Natl Acad Sci U S A.* 1992; 89:6580–6584. [PubMed: 1631159]
10. Spina CA, Guatelli JC, Richman DD. Establishment of a stable, inducible form of human immunodeficiency virus type 1 DNA in quiescent CD4 lymphocytes in vitro. *J Virol.* 1995; 69:2977–2988. [PubMed: 7707524]
11. Stevenson M, Stanwick TL, Dempsey MP, Lamonica CA. HIV-1 replication is controlled at the level of T cell activation and proviral integration. *EMBO J.* 1990; 9:1551–1560. [PubMed: 2184033]
12. Zack JA, Arrigo SJ, Weitsman SR, Go AS, Haislip A, et al. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell.* 1990; 61:213–222. [PubMed: 2331748]
13. Manganaro L, Lusic M, Gutierrez MI, Cereseto A, Del Sal G, et al. Concerted action of cellular JNK and Pin1 restricts HIV-1 genome integration to activated CD4+ T lymphocytes. *Nat Med.* 2010; 16:329–333. [PubMed: 20173753]
14. Dewhurst S, Embretson JE, Anderson DC, Mullins JI, Fultz PN. Sequence analysis and acute pathogenicity of molecularly cloned SIVSMM-PBj14. *Nature.* 1990; 345:636–640. [PubMed: 1971917]
15. Fultz PN, McClure HM, Anderson DC, Switzer WM. Identification and biologic characterization of an acutely lethal variant of simian immunodeficiency virus from sooty mangabeys (SIV/SMM). *AIDS Res Hum Retroviruses.* 1989; 5:397–409. [PubMed: 2765298]
16. Fultz PN. Replication of an acutely lethal simian immunodeficiency virus activates and induces proliferation of lymphocytes. *J Virol.* 1991; 65:4902–4909. [PubMed: 1870205]
17. Du Z, Lang SM, Sasseville VG, Lackner AA, Ilyinskii PO, et al. Identification of a nef allele that causes lymphocyte activation and acute disease in macaque monkeys. *Cell.* 1995; 82:665–674. [PubMed: 7664345]
18. Stephens EB, Mukherjee S, Liu ZQ, Sheffer D, Lamb-Wharton R, et al. Simian-human immunodeficiency virus (SHIV) containing the nef/long terminal repeat region of the highly virulent SIVsmmPBj14 causes PBj-like activation of cultured resting peripheral blood mononuclear cells, but the chimera showed no increase in virulence. *J Virol.* 1998; 72:5207–5214. [PubMed: 9573293]
19. Park IW, He JJ. HIV-1 Nef-mediated T-cell activation and chemotaxis are decoupled using a HIV-1/SIVpbj1.9. chimeric nef variant. *Arch Virol.* 2013; 158:845–852. [PubMed: 23224761]
20. Schibeci SD, Clegg AO, Biti RA, Sagawa K, Stewart GJ, et al. HIV-Nef enhances interleukin-2 production and phosphatidylinositol 3-kinase activity in a human T cell line. *AIDS.* 2000; 14:1701–1707. [PubMed: 10985305]
21. Schrager JA, Marsh JW. HIV-1 Nef increases T cell activation in a stimulus-dependent manner. *Proc Natl Acad Sci U S A.* 1999; 96:8167–8172. [PubMed: 10393966]
22. Wang JF, Park IW, Groopman JE. Stromal cell-derived factor-1alpha stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hematopoietic progenitor cells: roles of phosphoinositide-3 kinase and protein kinase C. *Blood.* 2000; 95:2505–2513. [PubMed: 10753828]

23. Margol AS, Judkins AR. Pathology and diagnosis of SMARCB1-deficient tumors. *Cancer Genet.* 2014 pii: S2210-7762(14)00155-0.
24. Kalpana GV, Marmon S, Wang W, Crabtree GR, Goff SP. Binding and stimulation of HIV-1 integrase by a human homolog of yeast transcription factor SNF5. *Science.* 1994; 266:2002-2006. [PubMed: 7801128]
25. Ertel W, Kremer JP, Kenney J, Steckholzer U, Jarrar D, et al. Downregulation of proinflammatory cytokine release in whole blood from septic patients. *Blood.* 1995; 85:1341-1347. [PubMed: 7858264]
26. Park IW, Myrick K, Sodroski J. Effects of vif mutations on cell-free infectivity and replication of simian immunodeficiency virus. *J Acquir Immune Defic Syndr.* 1994; 7:1228-1236. [PubMed: 7965633]
27. Wu P, Phillips MI, Bui J, Terwilliger EF. Adeno-associated virus vector-mediated transgene integration into neurons and other nondividing cell targets. *J Virol.* 1998; 72:5919-5926. [PubMed: 9621054]
28. Fagan R, Flint KJ, Jones N. Phosphorylation of E2F-1 modulates its interaction with the retinoblastoma gene product and the adenoviral E4 19 kDa protein. *Cell.* 1994; 78:799-811. [PubMed: 8087847]
29. Alkhatib G, Liao F, Berger EA, Farber JM, Peden KW. A few SIV co-receptor, STRL33. *Nature.* 1997; 388:238. [PubMed: 9230431]
30. Farzan M, Choe H, Martin K, Marcon L, Hofmann W, et al. Two orphan seven-transmembrane segment receptors which are expressed in CD4-positive cells support simian immunodeficiency virus infection. *J Exp Med.* 1997; 186:405-411. [PubMed: 9236192]
31. Manninen A, Huotari P, Hiipakka M, Renkema GH, Saksela K. Activation of NFAT-dependent gene expression by Nef: conservation among divergent Nef alleles, dependence on SH3 binding and membrane association, and cooperation with protein kinase C-theta. *J Virol.* 2001; 75:3034-3037. [PubMed: 11222731]
32. Moarefi I, LaFevre-Bernt M, Sicheri F, Huse M, Lee CH, Kuriyan J, Miller WT. Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement. *Nature.* 1997; 385:650-653. [PubMed: 9024665]
33. Saksela K, Cheng G, Baltimore D. Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. *EMBO J.* 1995; 14:484-491. [PubMed: 7859737]
34. Zhou Y, Zhang H, Siliciano JD, Siliciano RF. Kinetics of human immunodeficiency virus type 1 decay following entry into resting CD4+ T cells. *J Virol.* 2005; 79:2199-2210. [PubMed: 15681422]
35. Swiggard WJ, Baytop C, Yu JJ, Dai J, Li C, et al. Human immunodeficiency virus type 1 can establish latent infection in resting CD4+ T cells in the absence of activating stimuli. *J Virol.* 2005; 79:14179-14188. [PubMed: 16254353]
36. Cameron PU, Saleh S, Sallmann G, Solomon A, Wightman F, et al. Establishment of HIV-1 latency in resting CD4+ T cells depends on chemokine-induced changes in the actin cytoskeleton. *Proc Natl Acad Sci U S A.* 2010; 107:16934-16939. [PubMed: 20837531]
37. Colin L, Van Lint C. Molecular control of HIV-1 postintegration latency: implications for the development of new therapeutic strategies. *Retrovirology.* 2009; 6:111. [PubMed: 19961595]
38. Pierson TC, Kieffer TL, Ruff CT, Buck C, Gange SJ, et al. Intrinsic stability of episomal circles formed during human immunodeficiency virus type 1 replication. *J Virol.* 2002; 76:4138-4144. [PubMed: 11907256]
39. Simmons A, Aluvihare V, McMichael A. Nef triggers a transcriptional program in T cells imitating single-signal T cell activation and inducing HIV virulence mediators. *Immunity.* 2001; 14:763-777. [PubMed: 11420046]
40. Keppler OT, Tibroni N, Venzke S, Rauch S, Fackler OT. Modulation of specific surface receptors and activation sensitization in primary resting CD4+ T lymphocytes by the Nef protein of HIV-1. *J Leukoc Biol.* 2006; 79:616-627. [PubMed: 16365153]
41. Franchini G, Robert-Guroff M, Ghayeb J, Chang NT, Wong-Staal F. Cytoplasmic localization of the HTLV-III 3' orf protein in cultured T cells. *Virology.* 1986; 155:593-599. [PubMed: 3538645]

42. Yu G, Felsted RL. Effect of myristoylation on p27 nef subcellular distribution and suppression of HIV-LTR transcription. *Virology*. 1992; 187:46–55. [PubMed: 1736544]
43. Craig E, Zhang ZK, Davies KP, Kalpana GV. A masked NES in INI1/hSNF5 mediates hCRM1-dependent nuclear export: implications for tumorigenesis. *EMBO J*. 2002; 21:31–42. [PubMed: 11782423]
44. Turelli P, Doucas V, Craig E, Mangeat B, Klages N, et al. Cytoplasmic recruitment of INI1 and PML on incoming HIV preintegration complexes: interference with early steps of viral replication. *Mol Cell*. 2001; 7:1245–1254. [PubMed: 11430827]
45. Aiken C, Trono D. Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. *J Virol*. 1995; 69:5048–5056. [PubMed: 7541845]
46. Chowder MY, Pandori MW, Spina CA, Richman DD, Guatelli JC. The growth advantage conferred by HIV-1 nef is determined at the level of viral DNA formation and is independent of CD4 downregulation. *Virology*. 1995; 212:451–457. [PubMed: 7571414]
47. Schwartz O, Marechal V, Danos O, Heard JM. Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cell. *J Virol*. 1995; 69:4053–4059. [PubMed: 7539505]
48. Qi M, Aiken C. Nef enhances HIV-1 infectivity via association with the virus assembly complex. *Virology*. 2008; 373:287–297. [PubMed: 18191978]
49. Ma W, Mishra S, Gajananayaka N, Angel JB, Kumar A. HIV-1 Nef inhibits lipopolysaccharide-induced IL-12p40 expression by inhibiting JNK-activated NF-kappaB in human monocytic cells. *J Biol Chem*. 2009; 284:7578–7587. [PubMed: 19019824]
50. Mangino G, Percario ZA, Fiorucci G, Vaccari G, Manrique S, et al. In vitro treatment of human monocytes/macrophages with myristoylated recombinant Nef of human immunodeficiency virus type 1 leads to the activation of mitogen-activated protein kinases, IkappaB kinases, and interferon regulatory factor 3 and to the release of beta interferon. *J Virol*. 2007; 81:2777–2791. [PubMed: 17182689]
51. Lee SB, Park J, Jung JU, Chung J. Nef induces apoptosis by activating JNK signaling pathway and inhibits NF-kappaB-dependent immune responses in *Drosophila*. *J Cell Sci*. 2005; 118:1851–1859. [PubMed: 15827086]
52. Varin A, Manna SK, Quivy V, Decrion AZ, Van Lint C, et al. Exogenous Nef protein activates NF-kappa B, AP-1, and c-Jun N-terminal kinase and stimulates HIV transcription in promonocytic cells. Role in AIDS pathogenesis. *J Biol Chem*. 2003; 278:2219–2227. [PubMed: 12419805]
53. Schrager JA, Der Minassian V, Marsh JW. HIV Nef increases T cell ERK MAP kinase activity. *J Biol Chem*. 2002; 277:6137–6142. [PubMed: 11726657]

A

B

Fig. 1.

Specific interaction of INI1 with HIV-1 and SIVpbj1.9 Nefs. (A) Yeast two-hybrid analysis. Approximately 4×10^6 copies of cDNA from the Jurkat T-cell line were expressed with the B42 activation domain (AD) and tested for coactivation of the reporter gene, *lacZ*, together with a plasmid encoding the LexA-binding domain (BD)-nef fusion protein. AD fusion protein expression was analyzed as on (+ galactose, left panel) or off (- galactose, right panel) to examine specific interactions. HIV-1 vif and palmitoyl transferase (PalT) were used as negative controls. (B) β -galactosidase activity was measured to evaluate the specificity of the interaction between INI1 and HIV-1 and SIVpbj1.9 Nef using yeast two-hybrid analysis.

Fig. 2.

Interactions between INI1 and Nef. (A) Mammalian two-hybrid analysis. NIH 3T3 cells were transfected with the indicated plasmids, and CAT enzymatic activity in the transfected samples was measured. (B) GST pull-down assay. Complexes of GST-INI1 associated with HA-Nefs were isolated using glutathione Sepharose beads, and analyzed by immunoblotting with anti-HA and anti-GST antibodies. The protein bands corresponding to HIV-1 Nef (35 kD), SIVpbj1.9 Nef (41.5 kD), GST-INI1 (72 kD), and GST (28 kD) are indicated. The yeast lysate from the pB42AD transformant (AD) was used as a negative control.

Fig. 3.

The function of Nef in virus integration into host chromosomes. An *in vitro* DNA strand transfer assay was performed with increasing concentrations of (A) INI1 (0, 100, 200, 400, and 600 ng, sequentially), (B) Nefs (25, 75, 300, and 1500 ng, sequentially), or (C) HIV-1 Int (120, 300, and 600 ng, sequentially). The reaction samples were analyzed by 20% acrylamide urea gel electrophoresis and autoradiography. The band intensities were standardized on the basis of the intensity of the substrate band and are shown below each lane.

Fig. 4.

The function of Nef in provirus integration into the host chromosomes. Provirus integrated into the host chromosomal DNA was detected using modified Alu-PCR. Genomic DNAs were recovered from freshly isolated human PBMCs infected with the viruses. PCR products were analyzed by 2% agarose gel electrophoresis and by Southern hybridization.

Fig. 5. Replication of recombinant HSIVnef in resting human PBMCs. Kinetics of virus replication in CEMx174 cells (A), Jurkat cells (B), activated hPBMCs (C), and resting hPBMCs (D). hPBMCs (1×10^7) and CEMx174/Jurkat cells (1×10^6) were infected with 100,000 and 10,000 cpm, respectively, corresponding to the reverse transcriptase (RT) activity of each virus generated from transfected CEMx174.

A MGGVTSKKQRRRGGNLYERLLQARGETYGRLEGEYS
 Δ N-term Δ SH2(1+2) Δ SH2(3)
QSQDASGKGLSSLSCPEQKYCEGQFMNTPWRNPATERAKL
DYRQQNMDDVDSADLVGCPVSPRVPVRIMTYKLAIMSHFIK
 Δ SH3
EKGGLGIIYSDRRHKILDLYLEKEEGIIPDWQNNYTAGPGIRY
PMFFGWLWKLVPVNVSDEAQEDETHYLMHPAQT SQWDDPW
GEVLAWKFDPKLAYNYKAFVEHP EFGSQSGLSKEEVQRRLT
ARGLLKMADKKKTS
 Δ C-term

Fig. 6.

Effect of nef mutations on interaction with INI, integration activity, and replication in resting human PBMCs. (A) Each amino acid of SIVpbj1.9 Nef is indicated by a one-letter code, and deletions are underlined. (B) The β -galactosidase activity was measured to evaluate the specificity of the interaction between INI1 and mutant Nef of SIVpbj1.9, using yeast two-hybrid analysis, as described in Fig. 1. (C) An *in vitro* DNA strand transfer assay was performed with the indicated mutant Nef (1500 ng) in the absence (open bar) or presence of INI1 (600 ng) (closed bar), as described in the legend to Fig. 3 and in Materials and methods. The reaction samples were analyzed by 20% acrylamide urea gel electrophoresis and autoradiography, and the band intensities were standardized on the basis of the intensity of S-Nef in the presence of INI1. (D) The same amount of HSIVnef or the

indicated Nef mutant HSIV_{nef} was used to infect activated (D-1) or resting (D-2) hPBMCs, and the kinetics of replication of these viruses were determined by measuring RT activity in the culture supernatants as described above.