Reacquisition of Nef-Mediated Tetherin Antagonism in a Single In Vivo Passage of HIV-1 through Its Original Chimpanzee Host

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SUMMARY

The interferon-induced host restriction factor tetherin poses a barrier for SIV transmission from primates to humans. After cross-species transmission, the chimpanzee precursor of pandemic HIV-1 switched from the accessory protein Nef to Vpu to effectively counteract human tetherin. As we report here, the experimental reintroduction of HIV-1 into its original chimpanzee host resulted in a virus that can use both Vpu and Nef to antagonize chimpanzee tetherin. Functional analyses demonstrated that alterations in and near the highly conserved ExxxLL motif in the C-terminal loop of Nef were critical for the reacquisition of antitetherin activity. Strikingly, just two amino acid changes allowed HIV-1 Nef to counteract chimpanzee tetherin and promote virus release. Our data demonstrate that primate lentiviruses can reacquire lost accessory gene functions during a single in vivo passage and suggest that other functional constraints keep Nef ready to regain antitetherin activity.

INTRODUCTION

As a consequence of retroviral threat over millions of years, humans have evolved antiviral factors that act at different stages of the viral life cycle: TRIM5α proteins, which inactivate incoming viral capsids; cytidine deaminases (e.g., APOBEC3G), which inhibit reverse transcription and introduce hypermutations into the viral genome; SAM domain- and HD domain-containing protein 1 (SAMHD1), which restricts HIV-1 infection of myeloid cells; and tetherin (also known as CD317 or BST2), which suppresses virion release from infected cells (reviewed in Malim and Emerman, 2008; Kirchhoff, 2010; Laguette and Benkirane, 2012). These antiviral factors are under selection pressure for diversification because they are engaged in an "arms race" with pathogens which themselves are adapting and evading their

activity (Meyerson and Sawyer, 2011). As a consequence, they act in a species-specific manner and provide protection against cross-species transmissions. However, these protective barriers can fail, as exemplified by the AIDS pandemic. One reason for this failure is that "modern" primate lentiviruses, i.e., SIV and HIV-1, have evolved effective countermeasures against the restriction factors of their respective hosts. These viral factors, which include Vif, Vpr, Vpu, Vpx, and Nef, are not essential for replication in some cell lines but required for efficient viral replication and spread in vivo.

Recent studies provided surprising details into the zoonotic origins of HIV-1 groups M, N, O, and P and the adaptive changes that followed during their replication in humans. It is now established that SIVcpz, the direct precursor of pandemic HIV-1, represents a recombinant of precursors of viruses nowadays found in red-capped mangabeys and *Cercopithecus* monkeys (Bailes et al., 2003). This chimeric virus received a *vpu* gene from the precursor of SIVgsn/mus/mon and a *nef* gene from the progenitor of SIVrcm and was thus equipped with two potential tetherin antagonists (Sauter et al., 2009). During adaptation in chimpanzees, Nef but not Vpu evolved to counteract tetherin. Nef was maintained as the tetherin antagonist after transmission of SIVcpz to gorillas, most likely because chimpanzee and gorilla tetherins are highly similar. However, this was not the case after zoonotic transmissions of SIVcpz and SIVgor to humans because the human tetherin ortholog contains a deletion that renders it resistant to Nef (Jia et al., 2009; Lim et al., 2010; Sauter et al., 2009; Zhang et al., 2009). The precursor of pandemic HIV-1 group M cleared this hurdle by switching from Nef to Vpu to counteract tetherin in the new human host (Sauter et al., 2009). In contrast, the Vpu and Nef proteins of nonpandemic HIV-1 group O and P strains remained poor tetherin antagonists, and group N Vpus gained some antitetherin activity but lost their capacity to degrade CD4 (Sauter et al., 2009; Sauter et al., 2011; Yang et al., 2011). Thus, tetherin poses a barrier for SIV transmission from primates to humans and only HIV-1 group M has fully cleared this hurdle.

Based on the above, it seems clear that switches between Nef- and Vpu-mediated tetherin antagonism preceded the emergence of pandemic HIV-1 and that the reacquisition of

a once lost Vpu function may have been a prerequisite for the effective spread of the AIDS pandemic. Currently, it is unknown how rapidly accessory primate lentiviral proteins can regain lost activities against host restriction factors. To address this, we examined how HIV-1 evolved to antagonize tetherin after experimental infections of chimpanzees, which were conducted over 10 years ago in the context of vaccine and pathogenesis studies (Novembre et al., 1997; Mwaengo and Novembre, 1998). We found that two closely related molecular clones of chimpanzee adapted HIV-1 regained Nef-mediated antitetherin activity. This came as a surprise, as both viruses also maintained their Vpu-mediated counteraction of tetherin. Most strikingly, we could demonstrate that as few as two amino acid changes were sufficient to render HIV-1 Nef active against chimpanzee tetherin. Thus, after a century of replicating in humans, HIV-1 Nef reacquired antitetherin activity within a single passage in its original host.

RESULTS

A Chimpanzee-Adapted HIV-1 Strain Utilizes Both Vpu and Nef to Antagonize Tetherin

To examine how Vpu and Nef function evolved after reintroduction of HIV-1 into its original chimpanzee (CPZ) host, we took advantage of two molecular clones (JC16 and NC7) derived from chimpanzees that were experimentally infected with HIV-1 in the mid 1980s (Novembre et al., 1997; Mwaengo and Novembre, 1998). JC16 was derived from CPZ C499, which was initially infected with the SF2 (also called ARV-2) isolate of HIV-1 in 1985 and subsequently inoculated with a CPZ-passaged LAV strain amplified in human peripheral blood mononuclear cells (PBMCs) (Fultz et al., 1987) in 1986 (Figure 1A). Notably, a molecular clone of SF2 contains a defective *vpu* gene, suggesting that this HIV-1 strain may not encode a functional tetherin antagonist. Cloning of JC16 was performed by PCR amplification of proviral sequences from a coculture of C499-derived PBMCs with cells from an uninfected CPZ (Mwaengo and Novembre, 1998). JC16 was obtained about 10 years after infection when C499 had developed severe CD4+ T cell depletion and AIDS (Novembre et al., 1997). NC7 was cloned from a second CPZ (C455) 1 month after transfusion with blood from C499.

Phylogenetic analyses showed that JC16 represents a recombinant between the LAV and SF2 strains. Most parts of the JC16 genome clustered with LAV, except for (1) the *vpr* gene, (2) a short stretch of 228 nucleotides encoding portions of the gp41 transmembrane, Tat and Rev proteins, and (3) most of the *nef* gene and the LTR region (Figure 1B). We found that the JC16 and NC7 Vpu amino acid sequences are identical (Figure 1C) and those of Nef differ only by a single substitution in the N terminus (Figure 1D). However, the CPZ-adapted Vpu and Nef sequences showed multiple differences from those of molecular clones derived from the parental SF2 and LAV isolates. Notably, the AxxxAxxxA motif that is conserved in HIV-1 Vpus and is critical for counteraction of human (HU) tetherin (Vigan and Neil, 2010; Skasko et al., 2012) was mutated to AxxxTxxxA (Figure 1C). The three A residues have been shown to interact directly with the transmembrane region of tetherin (Skasko et al., 2012). Thus, a V30M substitution in the CPZ

tetherin ortholog may have selected for the A15T adaptation in Vpu. The JC16 Nef exhibited multiple changes compared to the parental LAV and SF2 Nef proteins, but most of them clustered near the N terminus and in the C loop of Nef known to be critical for the interaction with adaptor protein (AP) complexes (Figure 1D). Although the chimpanzee-derived HIV-1 Vpu and Nef sequences varied substantially from those of the input HIV-1 strains, most functional domains, such as the β-TrCP-binding motif (DSGxxS) in Vpu and the acidic domain and the P(xxP)$_3$ and ExxxLL motifs in Nef, were conserved.

To determine how Vpu and Nef function evolved during readaptation of HIV-1 to chimpanzees, we cotransfected 293T cells with vectors coexpressing Vpu or Nef and eGFP together with constructs expressing HU or CPZ derived CD4. Flow cytometric analyses showed that the JC16 Vpu and Nef proteins reduced HU and CPZ CD4 cell surface expression by more than 80% and were thus fully active (Figures 2A and 2B). The JC16 Nef also downmodulated class I MHC and (weakly) CD28 cell surface expression in PBMCs (Figures S1A and S1B available online). Analyses of Vpu and Nef proteins containing a C-terminal AU-1 tag confirmed these results and showed that all constructs expressed proteins of the expected size (Figure S1C).

To test whether the CPZ-adapted HIV-1 Vpu maintained its capability to counteract tetherin, we first examined its effect on the cell surface expression levels of HU and CPZ tetherin. The JC16, LAV and NL4-3 Vpus reduced the levels of HU and CPZ tetherin surface expression by about 40% to 50%, whereas the SIVcpz GAB1 Vpu was inactive (Figures 2C and S1D). In contrast, the JC16 and GAB1 Nefs efficiently reduced the surface levels of CPZ but not of HU tetherin, whereas the LAV and SF2 Nefs generally had only modest effects (Figures 2D and S1D). Thus, the CPZ-adapted HIV-1 JC16 Nef showed a functional phenotype similar to SIVcpz Nefs and distinct from HIV-1 Nefs. Next, we measured infectious virus yields from 293T cells after cotransfection of a ΔVpuΔNef HIV-1 NL4-3 proviral vector with fixed quantities of Vpu and Nef constructs and different doses of tetherin expression plasmid. Our data showed that the JC16 Vpu maintained the ability to antagonize tetherin, being even slightly more effective against the CPZ than against the HU ortholog (Figure 2E). As mentioned above, this may be due to an A15T change in the TMD of the JC16 Vpu. While the antitetherin activity of the JC16 Vpu was as expected, it came as a surprise that the JC16 Nef also counteracted CPZ tetherin (Figures 2F and S1E).

Finally, we used proviral constructs to test the function of JC16 Nef and Vpu. The analysis of *nef*-defective JC16 constructs with or without a functional *vpu* gene confirmed that this CPZ-adapted HIV-1 strain uses both Vpu and Nef to antagonize CPZ tetherin (Figure 2G). In contrast, the CPZ-adapted HIV-1 JC16 strain did not efficiently counteract HU tetherin. We also examined whether the JC16 Nef targets the same residues as Nefs from wild-type SIVcpz strains. In agreement with previous studies (Jia et al., 2009; McNatt et al., 2009; Sauter et al., 2009), introduction of five amino acids (D$_{15}$IWKK$_{19}$) that are missing in the N-terminal part of HU tetherin restored its sensitivity to the JC16 Nef (Figure 2H). Thus, CPZ passage restored the capability of this HIV-1 Nef to target the region missing in the HU tetherin ortholog.
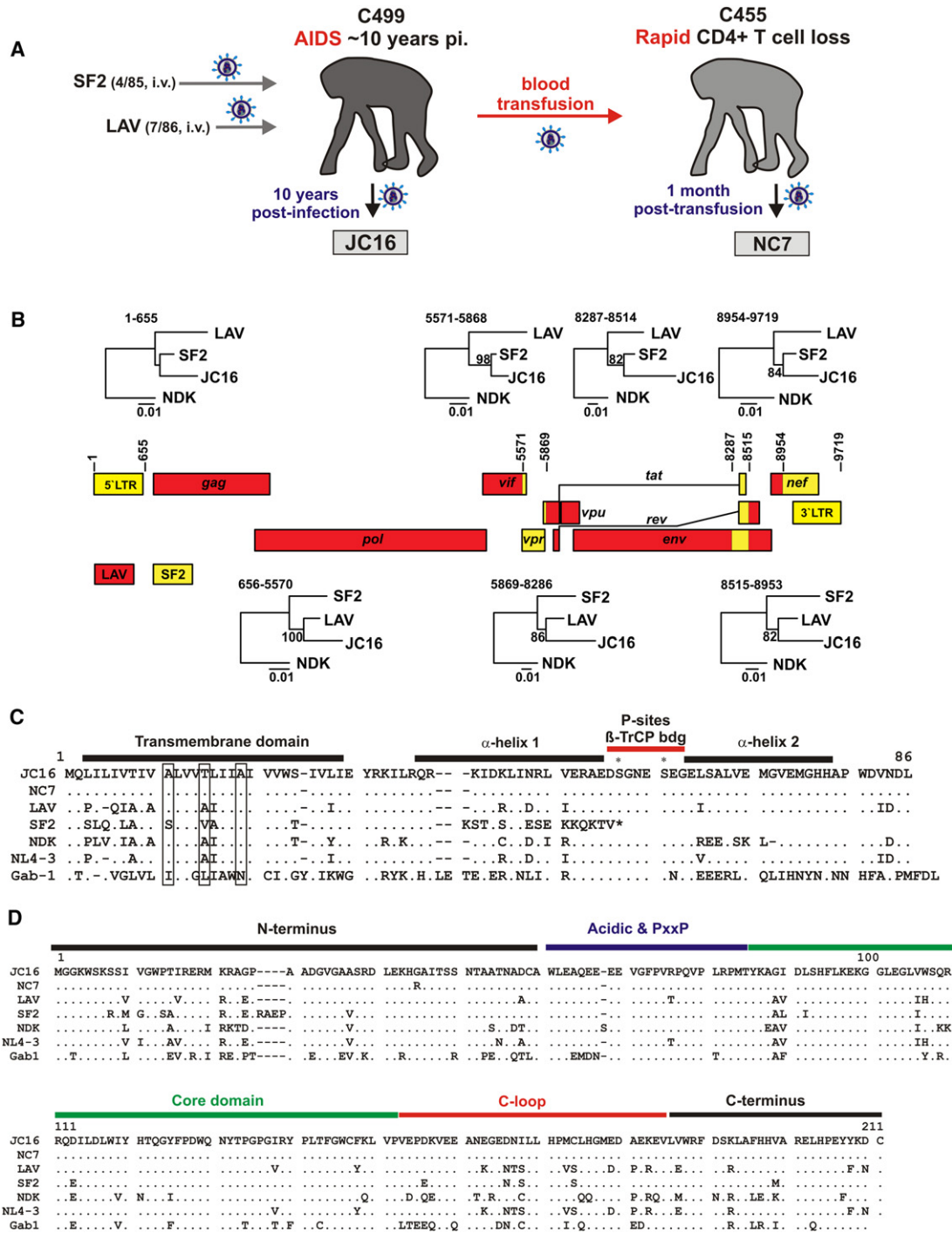
Figure 1. Origin and Properties of the CPZ-Adapted HIV-1 Clones JC16 and NC7

(A) Overview of the derivation of the CPZ-adapted HIV-1 JC16 and NC7 molecular clones. The numbers in parentheses give month and year of intravenous (i.v.) infection. See the main text for details.

(B) Generation of the recombinant JC16 genome from LAV and SF2 parental viruses. Open reading frames are indicated as boxes with HXB2 coordinates indicating inferred breakpoints (Korber et al., 1998). Neighbor-joining phylogenetic trees for the SF2 and LAV derived segments are shown above and below the recombination scheme, respectively. Bootstrap support ≥70% is shown above branches. Scale bars indicate genetic distance of 0.01.

(C and D) Alignment of Vpu (C) and Nef (D) amino acid sequences. The JC16 Vpu or Nef sequences are shown on top for comparison. The three Ala-residues involved in tetherin interaction in the hydrophobic transmembrane (TM) domain, the two helical regions, and the β-TrCP-binding motif containing two phosphorylation sites are indicated in (C). The myristoylated N terminus, the acidic region and the P(xxP)₃ motif, and the conserved core domain, the flexible C loop region, and the C terminus in the JC16 Nef are indicated in (D). The colored bars indicate the regions used in the generation of chimeras between the JC16 and LAV Nefs (see Figure 3A). Dashes indicate gaps introduced to optimize the alignment.
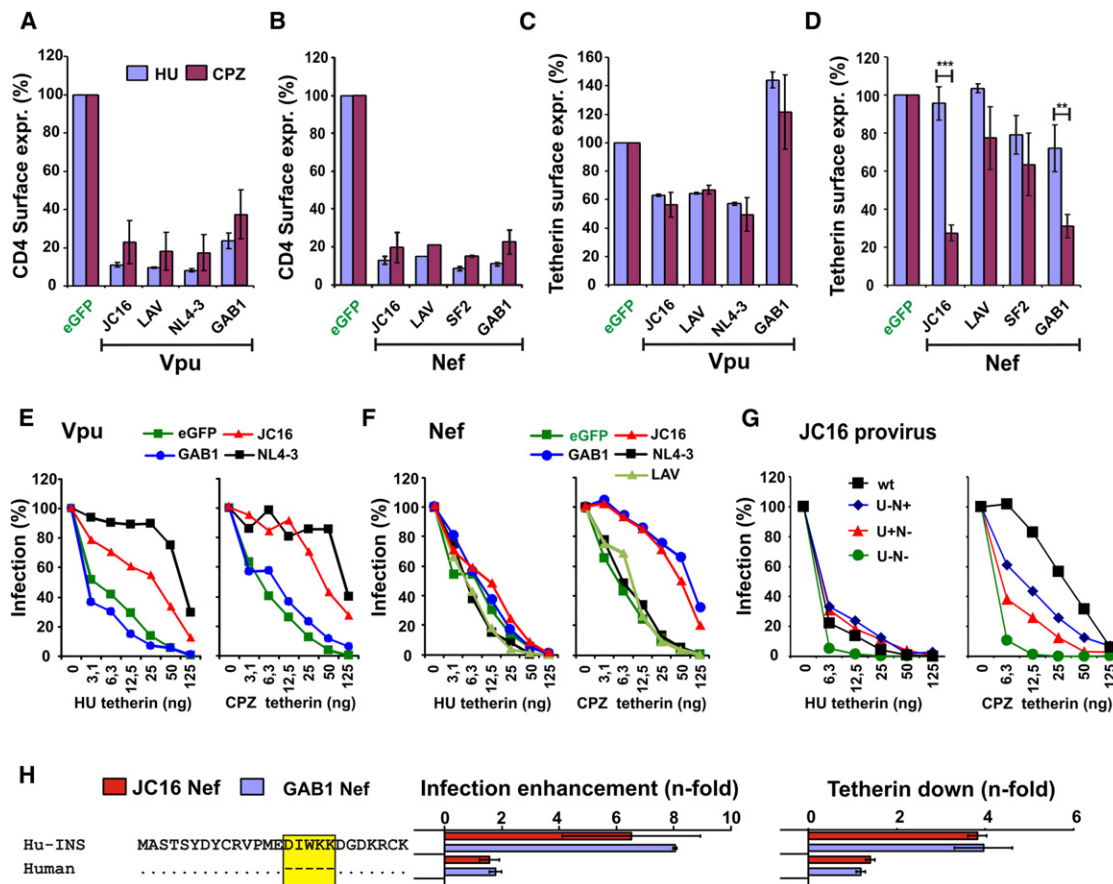
Figure 2. Functional Activity of Vpu and Nef Proteins of a CPZ-Adapted HIV-1 Strain

(A–D) Levels of CD4 (A and B) and tetherin (C and D) cell surface expression in the presence of Vpu or Nef relative to levels measured in cells transfected with the GFP control vector (100%). Values are averages (±SD) from three experiments. **p < 0.01, ***p < 0.001.

(E and F) Infectious virus yield from 293T cells cotransfected with the HIV-1 NL4-3 ΔVpuΔNef construct and vectors expressing the indicated *vpu* (E) or *nef* (F) alleles in combination with various quantities of plasmids expressing HU or CPZ tetherin.

(E–G) Panels give average values derived from triplicate infections of TZM-bl indicator cells relative to those obtained in the absence of tetherin expression vector (100%). All results were verified by p24 ELISA.

(G) The JC16 clone uses both Nef and Vpu to antagonize tetherin. Virus release from 293T cells after transfection with 2.5 μg proviral JC16 WT construct or mutants lacking *nef* (U+N–), *vpu* (U–N+), or both (U–N–) and varying amounts of plasmids expressing HU or CPZ tetherin.

(H) The JC16 Nef targets amino acid residues that are missing in the human tetherin ortholog. 293T cells were transfected with plasmids expressing HU tetherin or a variant in which the N-terminal deletion was restored (HU-INS). The middle panel shows the enhancement of infectious virus release relative to the eGFP only control derived from triplicate infections of TZM-bl indicator cells. The right panel shows the reduction of the surface expression levels of the respective tetherin variant in the presence of JC16 or GAB1 Nefs relative to those measured in cells transfected with the GFP only vector. Values are averages (±SD) from three independent experiments. The deletion in HU tetherin is highlighted by a yellow box.

See also Figure S1.

Two Amino Acid Changes Confer Antitetherin Activity to a Current HIV-1 Nef

To map the determinants of antitetherin activity in Nef, we generated a set of recombinants between the active JC16 Nef and the inactive LAV Nef (Figure 3A). Analysis of the recombinant Nefs showed that downmodulation of tetherin and enhancement of virion release was critically dependent on the presence of the C-terminal flexible loop (C loop) of JC16 (Figure 3A). Next, we swapped only the C loops of HIV-1 and SIVcpz Nefs (Figure 3B) and found that the presence of the JC16 C-loop (LAV-JC C loop) increased the antitetherin activity of LAV Nef, while insertion of the LAV loop (JC-LAV C loop) reduced the functional activity of JC16 Nef (Figure 3C). Further analyses demonstrated that the

central ten amino acids of the C loop (named Ccen) play a key role in Nef-mediated anti-tetherin activity (Figures S2A and S2B). Examination of single to triple amino acid substitutions demonstrated that changes of the variable residues within the otherwise conserved E/DxxxLL motif (DNI161-163NTS) as well as changes of MC168/169VS (named MC/VS) and a single C169S substitution downstream of this motif severely impaired the capability of the JC16 Nef to counteract CPZ tetherin (Figures 3D and S2C). Notably, none of these changes affected other Nef activities, such as the downmodulation of CD4, CD28, CD25, MHC-I, or CXCR4 (examples shown in Figure S2D).

We next examined the frequency of amino acid (aa) residues in the E/DxxxLL motif and the four C-terminally adjacent residues
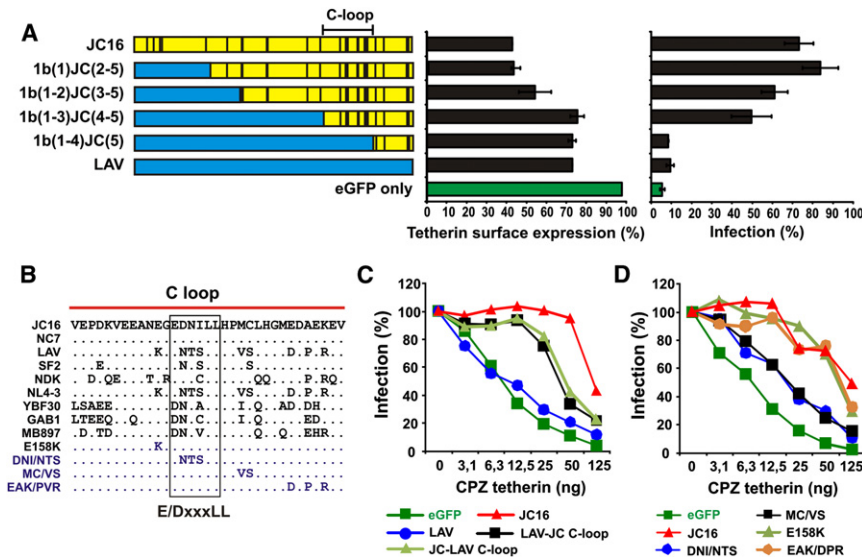
Figure 3. Mapping Residues Critical for the Gain of Antitetherin Activity by HIV-1 Nef

(A) The left panel indicates the chimeras between the JC16 (yellow) and LAV (blue) Nefs analyzed. Amino acid differences are indicated by vertical dashes. The effect of various Nefs on expression levels of CPZ tetherin (middle) or infectious virus release in the presence of CPZ tetherin (right) are shown as average values ± SD from triplicate experiments.

(B) Alignment of HIV-1 and SIVcpz C-loop regions of Nef. The JC16 C loop sequence is shown on top for comparison. The E/DxxxLL motif and specific mutants analyzed in (D) are indicated.

(C and D) Effect of Nefs differing in their C loop sequences (C) or specific amino acids (D) on infectious virus release in the presence of CPZ tetherin. "LAV-JC C-loop" represents the LAV Nef containing the C loop of the JC16 Nef and, vice versa, "JC-LAV C-loop" represents the JC16 Nef containing the C loop of the LAV Nef. The mutant JC16 Nefs contain changes of E158K, DNS161-163NTS, MC169/169VS, and EAK174/176/178DPR as shown in (B). Numbers give amino acid positions in the JC16 Nef. Curves represent the average infection values (n = 3) relative to those obtained in the absence of tetherin.

See also Figure S2.

in SIVcpz and HIV-1 Nef proteins. Overall, this 10 aa region is well conserved. However, SIVcpz Nefs usually contained an Ile (163I) just next to the dileucine motif, whereas HIV-1 Nefs contained a Ser or Cys residue at this position (Figure 4A). Furthermore, an adjacent Cys (169C) residue was highly conserved in SIVcpz Nefs, whereas HIV-1 M Nefs frequently encoded Ser or Asn. Strikingly, these residues were not only critical for anti-tetherin activity of the JC16 Nef, but the combination of the S163I and S169C changes (SF2-IC) also rendered the HIV-1 SF2 Nef fully active against CPZ tetherin (Figure 4B). Similarly, SF2 Nef proteins containing 163C or 163V residues, which are present in about 50% of all SIVcpz Nefs (Figure 4A), together with the S169C substitution (SF2-CC, SF2-VC) also antagonized CPZ tetherin (Figures S3A and S3B). None of these mutations in the SF2 Nef had significant effect on the CD4 downmodulation function (Figure S3C). Examination of the localization of these residues in the SF2 Nef crystal structure, which includes the entire C-terminal loop (Horenkamp et al., 2011), showed that despite their close physical proximity the critical 163I and 169C residues and the ExxxLL AP interaction site form distinct surfaces (Figures 4C and 4D). Moreover, the interaction site of Nef with CD4 (CAWL motif) is spatially well separated from the ExxxLL motif (Figure 4C). Altogether, these results suggest that residues 163I and 169C are specifically involved in the interaction of Nef with tetherin.

A minority of HIV-1 *nef* alleles encode a cystein at position 169, just like the vast majority of SIVcpz Nefs (Figure 4A). To examine whether a subset of contemporary HIV-1 Nefs is active against CPZ tetherin, we selected 12 previously described patient-derived *nef* alleles (Specht et al., 2010) that encoded 169C either together with 163S (as in most HIV-1 Nefs) or with 163C (as in some SIVcpz Nefs) (Figure S3D). All these HIV-1 *nef* alleles were substantially less potent in antagonizing CPZ tetherin

than the CPZ-adapted JC16 Nef (Figure S3E). However, HIV-1 *nef* alleles encoding 163C/169C were somewhat more active in promoting virus release in the presence of CPZ tetherin than those encoding 163S/169C (Figure S3F). Thus, some contemporary HIV-1 Nefs that contain the same residues as SIVcpz Nefs at amino acid positions 163 and 169 exhibit a modest activity against CPZ tetherin.

DISCUSSION

High mutation rates are a key feature of primate lentiviruses and allow them to efficiently adapt to their respective host environments. One might expect that this high adaptability comes at a cost in that viral genes which function in a species-specific manner should rapidly accumulate disruptive mutations. Surprisingly, however, we found that HIV-1 reacquired the ability to use its Nef protein to counteract CPZ tetherin within a single passage after reintroduction into its original chimpanzee host. Moreover, just two amino acid changes (S163I and S169C) in the C loop of HIV-1 Nef conferred full antitetherin activity (Figure 4). This is quite striking given that the chimpanzee-human transmission event of SIVcpz that resulted in the emergence of pandemic HIV-1 group M strains, which led to the disruption of the Nef mediated antitetherin activity, is estimated to have occurred a century ago (Korber et al., 2000; Worobey et al., 2008).

To our current knowledge both Nef and Vpu lost and regained antitetherin activity several times during the cross-species transmission events that preceded the emergence of AIDS. Nef is well known for its multifunctionality and most of its activities, such as downmodulation of CD4 and MHC-I or enhancement of viral infectivity, are species-independent (reviewed in Kirchhoff et al., 2008). Similarly, Vpu not only antagonizes tetherin in a
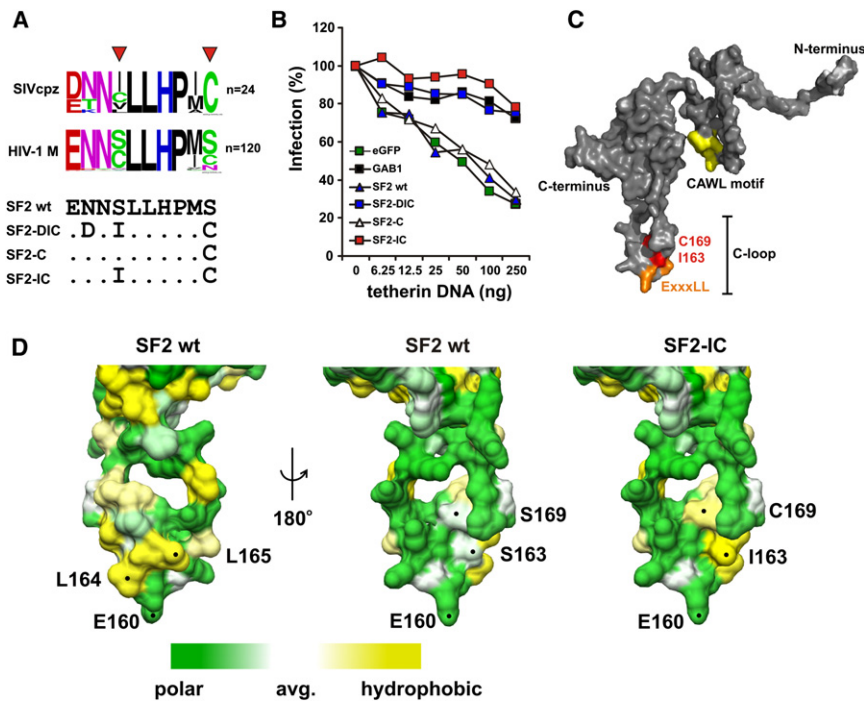
Figure 4. Two Amino Acid Changes Confer Antitetherin Activity to the HIV-1 SF2 Nef

(A) The upper panel gives frequency plots of amino acid residues in and next to the ExxxLL motif of HIV-1 M and SIVcpz Nef proteins, and the lower panel shows the mutations in the SF2 Nef analyzed in (B). The triangles indicate residues critical for anti-tetherin activity.

(B) Infectious virus yield from 293T cells co-transfected with the NL4-3 ΔVpuΔNef construct, vectors expressing the indicated *nef* alleles, and various quantities of plasmid expressing CPZ tetherin. The SF2 Nef mutants contain changes of NSS161/163/169DIC (SF2-DIC), S169C (SF2-C), and SS163/169IC (SF2-IC). All values represent averages of three measurements and are shown relative to those obtained in the absence of tetherin expression vector (100%). Results were confirmed in two independent experiments and by measuring p24 release.

(C) Structure of the HIV-1 Nef protein. The I163 and C169 (red) residues critical for tetherin antagonism and the acidic residue and two leucines (orange) in the E/DxxxLL motif involved in the interaction with AP complexes and the CAWL motif (yellow) involved in CD4 binding are indicated.

(D) Localization of residues critical for tetherin antagonism and AP interaction (all indicated by dots) and electrostatic surface potential of the C-terminal flexible loop of the SF2 Nef. Changes of S163I and S169C that are critical for the antitetherin activity of Nef generate a hydrophobic groove in the otherwise largely polar C loop.
See also Figure S3.

species-specific manner but also degrades CD4 independently of the host species (Sauter et al., 2009). Thus, even when Nef or Vpu lose a host-specific function after a cross-species transmission event, the preservation of the remaining activities will ensure the maintenance of genes that also remain predisposed to quickly regain the disrupted functions. It is thus tempting to speculate that a common byproduct of the multifunctionality of lentiviral accessory genes is an increased potential of these viruses to overcome species barriers. In fact, recent evidence suggests that Vpr and Vpx are also multifunctional (Planelles and Barker, 2010) and that the accessory viral proteins may generally interact with multiple cellular factors (Jäger et al., 2012).

Our finding that after reintroduction into its original chimpanzee host, HIV-1 not only evolved Nef-mediated activity against CPZ tetherin, but also maintained Vpu as a tetherin antagonist, adds to the evidence that tetherin antagonism is advantageous for viral spread in vivo (Liberatore and Bieniasz, 2011; Serra-Moreno et al., 2011). Perhaps more importantly, the present data are derived from an animal model that represents the closest approximation of AIDS in humans, i.e., infection of our closest relative, the chimpanzee, with pandemic HIV-1 group M strains. In comparison, previous in vivo results were obtained either in mouse models that required IFN induction to demonstrate definitive effects of tetherin or the SIVmac/macaque model (Liberatore and Bieniasz, 2011; Serra-Moreno et al., 2011).

The finding that the CPZ-adapted HIV-1 strain maintained two tetherin antagonists was unexpected since most primate lentiviruses use Nef, Vpu, or Env to antagonize this restriction factor. Chimpanzee C499 was productively infected with two different HIV-1 isolates, i.e., ARV-2 (SF2) in 1985 and LAV in 1986. The SF2 molecular clone, which is derived from ARV-2, encodes a grossly truncated Vpu (Figure 1C). Thus, it is tempting to speculate that the SF2 strain initially evolved Nef as an antagonist of CPZ tetherin because it lacks a functional Vpu. Later, LAV recombined with this virus, and the resulting CPZ-adapted JC16 strain received the critical region of the *nef* gene from SF2 (Figure 1B). Importantly, JC16 was particularly fit to replicate in chimpanzees since C499 ultimately developed high viral loads and AIDS. Furthermore, transfusion of its blood into a second ape (C455) was associated with high plasma viral loads and a rapid and sustained decline of CD4+ T cells (Novembre et al., 1997). Similarly, virus derived from C455 (termed NC) was pathogenic in two additional chimpanzees, whereas other HIV-1-infected chimpanzees did not show signs of disease progression (Novembre et al., 2001; O'Neil et al., 2000). Thus, the CPZ-adapted HIV-1 JC and NC strains may be particularly pathogenic in chimpanzees and it is tempting to speculate that the evolution of two effective tetherin antagonists may contribute to their virulence.

In summary, we show here that just two amino acid changes in the C loop of Nef are sufficient to fully restore the activity of a contemporary HIV-1 group M strain against the tetherin ortholog of its original chimpanzee host. Nef seems to be highly prone to regain antitetherin activity because it uses similar domains and mechanisms to down-modulate CD4 and to antagonize tetherin. In comparison, the functional constraints on Vpu may be less stringent because multiple changes in the TM region of SIVcpz Vpus seem to be required to confer activity against HU Tetherin (Lim et al., 2010). This may help to explain why

tetherin antagonism by the HIV-1 Nef protein was restored within a single ape passage, whereas HIV-1 group O and P Vpus have thus far been unable to gain antitetherin activity in humans (Sauter et al., 2009; Sauter et al., 2011; Yang et al., 2011). It will be of interest to further elucidate whether viral proteins that counteract antiviral factors in a species-specific manner have also commonly evolved functions or interactions that are species independent to avoid their complete inactivation and elimination after cross-species transmission.

EXPERIMENTAL PROCEDURES

JC16 and NC7 Molecular Clones

Generation of the JC16 and NC7 infectious molecular clones was performed as described previously (Mwaengo and Novembre, 1998). Splice overlap extension PCR was used to generate *vpu*- and/or *nef*-defective mutants. See the Supplemental Experimental Procedures for details.

Expression Vectors

Bicistronic CMV-based pCGCG expression vectors coexpressing *vpu*, *nef*, CD4, or tetherin and eGFP or DsRed2 have been described previously (Sauter et al., 2009). Splice overlap extension PCR with primers introducing XbaI and MluI restriction sites flanking the reading frames was used to generate chimeric *nef* alleles and *nef* mutants. PCR fragments were purified from agarose gels and inserted into the pCGCG vector using standard cloning techniques. All PCR-derived inserts were sequenced to confirm their accuracy.

Recombination Analysis of JC16

The recombination breakpoints of JC16 were determined as described in the Supplemental Experimental Procedures.

Cell Culture and Transfections

Cells were cultured and transfected as described in the Supplemental Experimental Procedures.

Flow Cytometric Analysis

To determine the effect of Vpu and Nef on CD4 and tetherin cell surface expression, 293T cells were transfected by the calcium phosphate method with 1 μg CD4 or tetherin expression vector coexpressing eGFP and 5 μg pCGCG eGFP/Vpu or Nef constructs expressing eGFP alone or together with Vpu or Nef. Two days after transfection, CD4 or tetherin expression was examined by FACS analysis. See the Supplemental Experimental Procedures for details.

Tetherin Antagonism

The capability of Vpu and Nef to antagonize tetherin was determined essentially as described previously (Sauter et al., 2009). For details, see the Supplemental Experimental Procedures.

Western Blot

Expression of Vpu, Nef and tetherin was examined as described in the Supplemental Experimental Procedures.

Sequence Analyses

Vpu or Nef amino acid sequences were aligned using multiple sequence alignment with hierarchical clustering (http://multalin.toulouse.inra.fr/). Vpu and Nefs sequences were drawn from the HIV Sequence Database (http://www.hiv.lanl.gov).

Nef Model Building

Mutations of S163C and S169I were inserted into the SF2 Nef structure as specified in the Supplemental Experimental Procedures.

Statistical Analysis

All statistical calculations were performed with a two-tailed unpaired Students t test using Graph Pad Prism Version 5.0. p values < 0.05 were considered significant. Correlations were calculated with the linear regression module.

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