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Article

Nef Proteins from Simian Immunodeficiency Viruses Are Tetherin Antagonists

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SUMMARY

The tetherin/BST2/CD317 protein blocks the release of HIV-1 and other enveloped viruses by inducing tethering of nascent particles to infected cell surfaces. The HIV-1 Vpu protein antagonizes the antiviral activity of human but not monkey tetherins and many simian immunodeficiency viruses (SIVs) do not encode Vpu. Here, we show that the apparently "missing" antitetherin activity in SIVs has been acquired by several SIV Nef proteins. Specifically, SIV_{MAC}/SIV_{SMM}, SIV_{AGM}, and SIV_{BLU} Nef proteins can suppress tetherin activity. Notably, tetherin antagonism by SIV Nef proteins is species specific, is genetically separable from other Nef activities, and is most evident with simian rather than human tetherin proteins. Accordingly, a critical determinant of sensitivity to SIV_{MAC} Nef in the tetherin cytoplasmic tail is variable in nonhuman primate tetherins and deleted in human tetherin, likely due to selective pressures imposed by viral antagonists, perhaps including Nef proteins.

INTRODUCTION

The tetherin protein (also known as BST2 or CD317) is a potent inhibitor of the release of enveloped viruses. It was recently identified as a factor in human cells that blocks release of HIV-1 particles from infected cells and is counteracted by the viral protein Vpu (Neil et al., 2008; Van Damme et al., 2008). Its precise mechanism of action is not well defined at present, but in cells constitutively expressing tetherin, protease-sensitive tethers retain fully formed and mature HIV-1 particles on the cell surface and tetherin colocalizes with puncta of Gag that likely represent nascent virions (Jouvenet et al., 2009; Neil et al., 2006, 2007, 2008). Recently, we and others have shown that human tetherin (hu-tetherin) has broad antiviral specificity and inhibits the release of particles assembled using structural proteins from all retroviruses tested, as well as filoviruses and arenaviruses (Jouvenet et al., 2009; Kaletsky et al., 2009; Sakuma et al., 2009).

The mechanism by which HIV-1 Vpu antagonizes hu-tetherin is not fully understood, but overexpressed HIV-1 Vpu reduces the overall levels of tetherin in cells and inhibits its appearance at the cell surface (Bartee et al., 2006; Van Damme et al., 2008). Furthermore, HIV-1 Vpu and hu-tetherin colocalize, and Vpu prevents the colocalization of hu-tetherin with nascent HIV-1 particles(Jouvenet et al., 2009; Neil et al., 2008). However, while tetherin proteins from nonhominid primates are potent inhibitors of HIV-1 particle release, they cannot be counteracted by HIV-1 Vpu (McNatt et al., 2009). Portions of primate tetherin genes, including sequences encoding the transmembrane domain that governs sensitivity to antagonism by Vpu, are unusually divergent and exhibit clear evidence of positive selection (McNatt et al., 2009). Thus, HIV-1 has apparently acquired a biological activity (i.e., Vpu), that has specifically evolved to antagonize the tetherin variant expressed in its host species.

Although hu-tetherin inhibits the release of particles assembled using a diverse array of retroviral structural proteins, only a subset of the primate lentiviruses encode Vpu. Thus, it seemed reasonable to suppose that SIVs have evolved alternative mechanisms to evade tetherin in their natural hosts. Indeed, earlier work indicated that the HIV-2 envelope protein could enhance particle release from cells that were subsequently shown to express hu-tetherin (Abada et al., 2005; Bour et al., 1996; Bour and Strebel, 1996; Varthakavi et al., 2003). Given this precedent, it was quite plausible that the envelope proteins of SIVs might have a similar function. Consistent with this idea, the Ebola virus envelope protein has recently been reported to be a tetherin antagonist (Kaletsky et al., 2009).

However, as reported herein, we found that the envelope protein of SIV_{MAC}, a macaque lentivirus that is closely related to HIV-2, did not antagonize macaque tetherin proteins. Rather, Nef proteins from SIV_{MAC} and several other SIVs antagonize primate tetherins. Notably, tetherin antagonism by SIV Nef proteins was species specific, and each SIV Nef was poorly active against human tetherin. Furthermore, the cytoplasmic tail of tetherin, which, like the transmembrane domain, has been evolving under positive selection in primates (McNatt et al., 2009), contains a discrete motif that is deleted in humans and variable in other primates and governs sensitivity to antagonism by SIV_{MAC} Nef. Thus, several primate lentiviruses that lack Vpu have acquired the ability to antagonize tetherin using their Nef proteins.

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RESULTS

Inhibition of SIV_{MAC} Particle Release by Tetherin Proteins

Hu-tetherin can inhibit the release of particles assembled using the structural proteins (Gag and/or GagPol) of a wide variety of retroviruses (Jouvenet et al., 2009), raising the question of how retroviruses that lack a Vpu gene are efficiently released from infected cells that might ordinarily express tetherin. Among the retroviruses previously tested for sensitivity to hu-tetherin were the primate lentiviruses, SIV_{MAC} and SIV_{AGM}Sab, neither of which encode a Vpu protein (Jouvenet et al., 2009). However, it has previously been shown that at least some strains of HIV-2, a virus that shares a recent common ancestor with SIV_{MAC}, encode an envelope protein that has Vpu-like activity (Abada et al., 2005; Bour et al., 1996; Bour and Strebel, 1996; Varthakavi et al., 2003). Therefore, we tested whether the release of particles generated by a full-length SIV_{MAC239} proviral construct could be inhibited by tetherin proteins found in macaque species, specifically two variants from rhesus macaques (rh-tetherin-1 and -2) and one from pig-tailed macaques (pgt-tetherin). Strikingly, each of the macaque tetherin proteins was a rather poor inhibitor of SIV_{MAC239} particle release (Figure 1A). This was the case whether or not the SIV_{MAC239} proviral DNA construct encoded a functional Env protein. Thus, these results suggested that SIV_{MAC239} might encode a tetherin antagonist other than the Env protein.

Next we coexpressed an SIV_{MAC239} provirus with varying amounts of human and macaque tetherin proteins. As previously shown for SIV_{MAC239} particles assembled using only the Gag and Pol proteins, the release of authentic SIV_{MAC239} virions appeared highly sensitive to hu-tetherin (Figures 1B and 1D). In contrast, tetherin proteins from macaques appeared to be significantly less effective inhibitors of SIV_{MAC239} virion release (Figures 1C, 1D, and S1).

These findings suggested the possibility that another SIV_{MAC} protein might be a macaque-tetherin-specific antagonist. The ability of Nef to regulate the levels of cell-surface proteins prompted us to examine whether it might fulfill this role. Strikingly, the release of virions generated by a proviral plasmid lacking Nef (SIV_{MAC239}[delNef]) appeared more sensitive to inhibition by macaque tetherins than SIV_{MAC239}(WT) (Figures 1C, 1D, and S1). In contrast, hu-tetherin inhibited SIV_{MAC239}(WT) release almost as well as it inhibited SIV_{MAC}(delNef) release (Figures 1B and 1D). Thus, Nef deletion specifically sensitized SIV_{MAC239} to inhibition by macaque tetherins.

Scanning electron microscopic analysis of cells cotransfected with plasmids expressing SIV_{MAC239} Gag and either rh-tetherin-1 or hu-tetherin revealed that either tetherin induced the accumulation of SIV_{MAC239} VLPs on the surface of cells (Figure 1E). Moreover, we have previously reported that HIV-1 Gag-GFP VLPs tethered to the plasma membrane by hu-tetherin can be internalized resulting in the accumulation of intracellular HIV-1 Gag-GFP (Neil et al., 2006, 2008), and similar findings were obtained with SIV_{MAC239} Gag-GFP and rh-tetherin-1 (Figure 1F), Specifically, in 293T cells stably expressing rh-tetherin-1, fluorescence microscopic analysis indicated that nearly half the cells transiently expressing SIV_{MAC239} Gag-GFP contained prominent intracellular accumulations of Gag-GFP (Figures 1F and 1G). Crucially, these accumulations decreased in frequency when increasing amounts of an SIV_{MAC239} Nef expression plasmid were cotransfected, or when cells that did not express tetherin were used (Figures 1F and 1G). Thus, macaque and human tetherin proteins block particle release in the same manner, and SIV_{MAC239} Nef antagonizes the tethering activity of macaque tetherin.

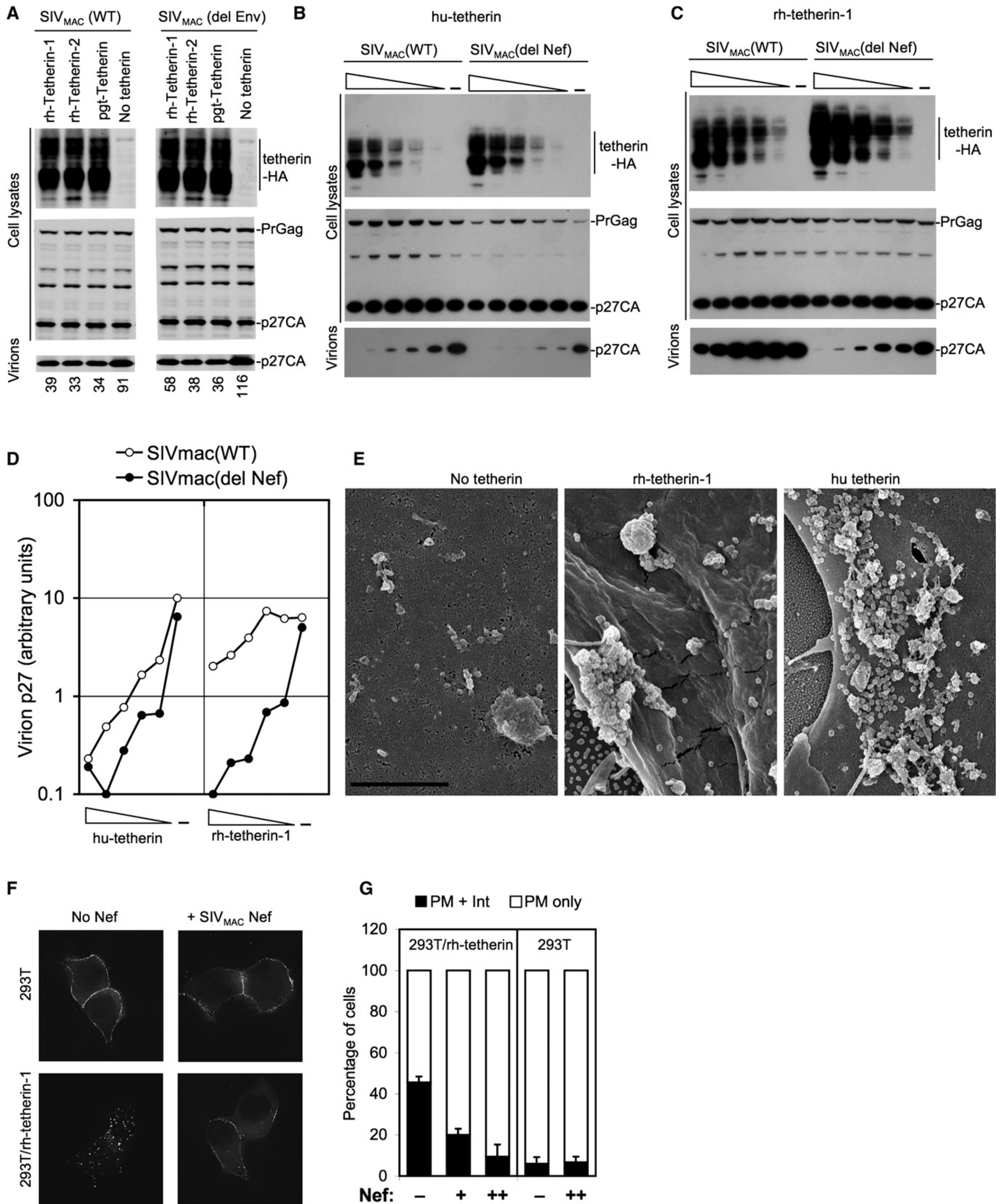
Nef Proteins from Divergent Primate Lentiviruses Antagonize Tetherin in a Species-Specific Manner

Vpu is present in only a minority of primate lentiviruses, while Nef is present in all primate lentiviruses isolated thus far. Therefore, to test whether Nef proteins from lentiviruses other than SIV_{MAC} might also be capable of counteracting tetherin, several of them were expressed in place of HIV-1 Nef in the context of a replication-competent HIV-1 proviral construct. The HIV-1 Vpu protein was inactivated in this construct, to test the effects of Nef on particle release in the absence of confounding factors. Proviral plasmids bearing the various Nef proteins were cotransfected with increasing amounts of plasmids expressing hu-, rh-1-, pgt-, and African green monkey (agm-) tetherin proteins. Western blot analyses indicated proviruses expressing the various Nef proteins generated HIV-1 virions with similar efficiency in the absence of tetherins (Figures 2A–2H).

The generation of extracellular virions by cells transfected with the parental proviral plasmid (which lacked both Nef and Vpu) was strongly inhibited by each of the four tetherins (Figures 2A and S2A), in the absence of effects on cell-associated Gag expression. A matched HIV-1 provirus, which lacked Nef but retained Vpu, was largely resistant to hu-tetherin. However, Vpu did not antagonize agm-tetherin, rh-tetherin, or pgt-tetherin (McNatt et al., 2009) (Figures 2B and S2B). A construct that lacked Vpu but retained HIV-1 Nef behaved essentially the same as the construct that lacked both Vpu and Nef (Figures 2C and S2C). Thus, HIV-1 Vpu specifically antagonized hu-tetherin but not monkey tetherins, while HIV-1 Nef did not antagonize any of the tetherin proteins.

Notably, replacement of HIV-1 Nef with SIV_{MAC} Nef resulted in substantial resistance to both pgt- and rh-tetherin (Figures 2D and S2D). Importantly, this effect was quite specific to the macaque tetherins, and SIV_{MAC} Nef only marginally enhanced particle release when inhibition was imposed by hu-tetherin or agm-tetherin. Conversely, SIV_{AGM}Sab Nef conferred a greater degree of resistance to agm-tetherin than did SIV_{MAC} Nef (Figures 2E and S2E), and SIV_{AGM}Sab Nef was also active against the macaque tetherins. However, SIV_{AGM}Sab Nef was similar to SIV_{MAC} Nef in that it was a poor antagonist of hu-tetherin (Figures 2E and S2E). Although this sample size is small, we note that in the two cases examined where the parental viruses lack a *vpu* gene (SIV_{MAC} and SIV_{AGM}Sab), the Nef proteins were able to antagonize the antiviral activity of tetherins found in the corresponding simian host species.

We also tested Nef proteins from three other SIVs, with the caveat that the SIV Nef proteins were not derived from the same host species as the tetherins. Nonetheless, an HIV-1 proviral construct expressing the Nef protein from SIV_{BLU} was largely resistant to pgt-tetherin and rh-tetherin (Figures 2F and S2F). Conversely, Nef proteins from SIV_{RCM} and SIV_{GSN} appeared largely unable to antagonize the tetherin proteins


A
SIV_MAC (WT)
SIV_MAC (del Env)
rh-Tetherin-1
rh-Tetherin-2
pgt-Tetherin
No tetherin
Cell lysates
Virions
tetherin -HA
-PrGag
-p27CA
39 33 34 91 58 38 36 116
B
hu-tetherin
SIV_MAC(WT)
SIV_MAC(del Nef)
C
rh-tetherin-1
D
SIVmac(WT)
SIVmac(del Nef)
Virion p27 (arbitrary units)
100
10
1
0.1
hu-tetherin
rh-tetherin-1
E
No tetherin
rh-tetherin-1
hu tetherin
F
No Nef
+ SIV_MAC Nef
293T
293T/rh-tetherin-1
G
PM + Int
PM only
293T/rh-tetherin
293T
Percentage of cells
Nef:

(Figures 2G, 2H, S2G, and S2H). However, in addition to the fact that Nef and tetherin proteins from nonorthologous species were tested, SIV_{GSN} naturally encodes a Vpu protein (Courgnaud et al., 2002), which potentially would obviate the requirement for this activity in Nef. An additional caveat with the aforementioned experiment is that the SIV Nef proteins were expressed in the Nef position of an HIV-1 provirus, and may be present at higher or lower levels than in their parental proviruses. Additionally, antibodies to the SIV_{RCM} and SIV_{GSN} proteins were not available, therefore we were unable to demonstrate that these Nef proteins were expressed, although we have previously shown that infection by nearly identical proviruses caused efficient CD4 downregulation (Schindler et al., 2006). Nonetheless, three of the five SIV Nef proteins tested in these assays exhibited apparent tetherin antagonizing activity. The sensitivity or otherwise of a given tetherin protein to antagonism by Nef did not correlate with its expression level (Figure S3), which was assessed under identical conditions to those used for the assays in Figures 2 and S2.

To exclude the possibility the aforementioned findings were the result of some unanticipated consequence of inserting foreign *nef* genes into the HIV-1 genome in *cis*, we determined whether Nef proteins, expressed in *trans*, would relieve tetherin imposed blocks. A Vpu and Nef-defective HIV-1 proviral plasmid was cotransfected with a fixed amount of each tetherin protein, and varying amounts of plasmids expressing the Nef proteins that exhibited apparent tetherin antagonizing activity (Figures 2 and S2). We also included HIV-1 Nef as a control, and an additional SIV_{AGM} Nef protein, from SIV_{AGM}Tan. As expected, western blot and infectivity assays revealed that particle release was strongly inhibited by each of the tetherin proteins, and coexpression of HIV-1 Nef in *trans* had little, if any, ability to relieve these tetherin-imposed blocks (Figures 3A and S4A). Conversely, SIV_{MAC} Nef relieved the inhibition of particle release imposed by macaque tetherins, but its effect on particle release in the presence of hu-tetherin or agm-tetherin was marginal (Figures 3B and S4B). Both SIV_{AGM}Sab and SIV_{AGM}Tan Nef restored particle release from cells expressing rh-, pgt-, or agm-tetherin but were less active in relieving the block imposed by hu-tetherin (Figures 3C, 3D, S4C, and S4D). Notably, these two SIV_{AGM}-derived proteins were the only Nef proteins that effectively relieved inhibition by agm-tetherin. Finally, SIV_{BLU} Nef efficiently antagonized the macaque tetherin proteins, but it also had some ability to enhance particle release in the presence of hu- and agm-tetherin (Figures 3E and S4E). However, effects on hu-tetherin and agm-tetherin were clearly reduced in magnitude as compared to effects on macaque tetherins. Overall, the effects of the various Nef proteins, expressed in *trans*, on HIV-1 particle release corroborated those obtained when they were expressed in *cis*. However, we were able to detect additional, albeit slight, anti-tetherin effects when Nef was expressed in *trans* (for example, SIV_{BLU} Nef slightly antagonized hu-tetherin [Figure 3E]). We suspect that the small differences in results obtained using the two assays reflect differences in the level of Nef expression, which is likely higher when Nef is expressed in *trans*. Notably, a modest degree of variation in the levels of expression of the various tetherin proteins (Figure S5), assessed under identical conditions to those used in the assays in Figures 3 and S4, did not correlate with the ability of a given tetherin protein to be antagonized by Nef. Moreover, the Nef proteins that did not antagonize tetherins (HIV-1 Nef) or Nef proteins that did antagonize monkey tetherins (SIV_{MAC239} Nef or SIV_{AGM}Tan Nef) did not appear to inhibit expression of any of the tetherin proteins (Figure S5).

The effects of Nef proteins on particle yield measured using infectious virion yield assays correlated well with those assessed using western blotting (Figure S4). However, in addition to their tetherin antagonizing effects, each Nef protein tested enhanced the intrinsic infectiousness of HIV-1 particles in the absence of tetherins. Nonetheless, the tetherin-independent effect on virion infectiousness was quite similar, and modest, irrespective of which Nef protein was expressed (Figure S4). Together, however, the compound effect of Nef proteins on particle release and infectiousness, resulted in quite large effects on overall infectious virion yield, approaching two orders of magnitude in some cases.

SIV Nef Proteins Can Enhance Virion Release from Simian Cells

If endogenously expressed monkey tetherins inhibit virion release and are antagonized by SIV Nef proteins, then Nef should enhance particle release from simian cells, particularly when

Figure 1. Effects of Env and Nef proteins on SIV_{MAC239} Particle Release

(A) Western blot analysis of wild-type (WT) and envelope deleted (del Env) SIV_{MAC239} particle release in the presence of tetherin-HA proteins from rhesus (rh) and pig-tailed macaques (pgt). Cells were transfected with 400 ng of proviral plasmid and 40 ng of each tetherin expression plasmid. Cell and virion lysates were probed with an anti-capsid monoclonal and/or rabbit anti-HA antibody, and signals detected using fluorescent secondary antibodies. The numbers below each lane indicate quantitation (LICOR) of the p27CA present in the virion pellet.

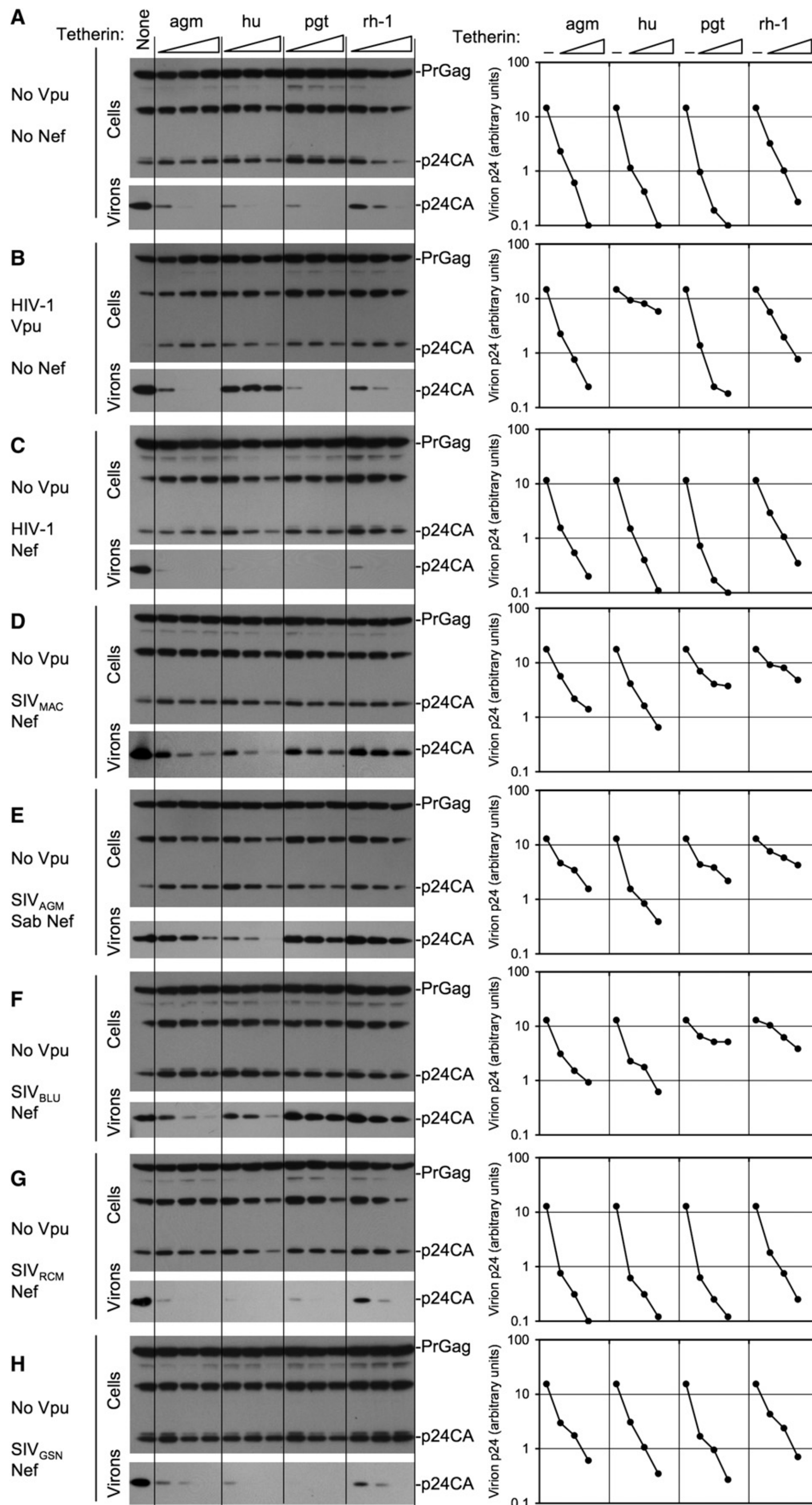
(B and C) Western blot analysis of wild-type (WT) and Nef-deleted (del Nef) SIV_{MAC239} particle release in the presence of the hu-tetherin (B) or rh-tetherin-1 (C) proteins. Cells were transfected with a fixed amount (400 ng) of proviral plasmid and varying amounts (50, 25, 12.5, 6.25, 3.12 or 0 ng) of each tetherin expression plasmid. Cell and virion lysates were probed with an anti-capsid and anti-HA epitope monoclonal antibodies, and signals detected using chemiluminescence reagents. The results shown are representative of at least three separate experiments.

(D) Quantitation of virion release inhibition by tetherin. P27CA in virion samples from (B) and (C) was measured using quantitative fluorescence-based western blotting (LICOR).

(E) Representative scanning EM images of cells transfected with pSIVGag-IRESGFP and either no tetherin, rh-tetherin-1, or hu-tetherin. Cells were selected for imaging based on similar levels of GFP fluorescence. Scale bar indicates 2 μm.

(F) Representative fluorescence micrographs of unmanipulated or rh-tetherin-1 expressing 293T cells transfected with plasmids expressing SIV_{MAC239} Gag-GFP fusion protein, in the presence or absence of SIV_{MAC239} Nef.

(G) Quantitation of the proportion of cells displaying both plasma membrane and internal (PM + Int) or plasma membrane only (PM) intense accumulations of SIV_{MAC239} Gag-GFP, under the same experimental conditions as in (F) (see F for examples). Cells were transfected with 0 (–), 0.8 (+), or 1.6 μg (++) of pCR3.1/Nef. Values represent the mean ± SD of triplicate determinations and are representative of two independent experiments. At least 100 individual cells were evaluated under each condition.


A
Tetherin:
None
agm
hu
pgt
rh-1
No Vpu
No Nef
Cells
Virons
PrGag
p24CA
Virion p24 (arbitrary units)
B
HIV-1 Vpu
No Nef
C
No Vpu
HIV-1 Nef
D
No Vpu
SIV MAC Nef
E
No Vpu
SIV AGM Sab Nef
F
No Vpu
SIV BLU Nef
G
No Vpu
SIV RCM Nef
H
No Vpu
SIV GSN Nef

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they are treated with IFNα, a known enhancer of tetherin expression. We first determined the effect of Nef on SIV_{MAC239} particle release from rhesus macaque 221 T-cells in single cycle replication assays, following infection with VSV-G-pseudotyped SIV_{MAC239}(WT) or SIV_{MAC239}(del Nef). Notably, 221 cells expressed tetherin in the absence of IFNα, and the levels of tetherin mRNA were modestly increased by IFNα treatement (Figure S6A). Western blotting analysis indicated that equivalently infected cells release fewer SIV_{MAC239}(del Nef) virions than SIV_{MAC239}(WT) virions, even though Gag was equally expressed (Figures 4A and S6B). This effect was exacerbated when the infected 221 cells were treated with IFNα. Notably, the discrepancy in particle release between SIV_{MAC239}(WT) and SIV_{MAC239}(del Nef) was not observed when human 293T cells were used in an otherwise identical single cycle replication assay (Figures 4A and S6C). Thus, stimulation of particle release by Nef was clearly host cell-type dependent. Notably, transmission electron microscopic analysis of 221 cells revealed very obvious accumulation of virions associated with the plasma membrane of cells when they were infected with SIV_{MAC239} (delNef), but not when they were infected with SIV_{MAC239}(WT) (Figure 4B). This phenotype was observed in otherwise untreated 221 cells but was especially striking when the infected 221 cells were treated with IFNα. Furthermore, these virions often appeared to be tethered together (Figure 4B; [iv] inset), Thus, the inefficient release of Nef-defective SIV_{MAC239} particles was accompanied by accumulation of virions at the plasma membrane, recapitulating previous observations of Vpu-defective HIV-1 virions in hu-tetherin expressing human cells.

We also examined whether SIV_{AGM}Sab Nef could enhance particle release from African green monkey COS-7 cells. In this case, VSV-G pseudotyped HIV-1 constructs encoding either no Nef, HIV-1 Nef, or SIV_{AGM}Sab Nef were used to infect COS-7 cells. Subsequent western blotting analysis indicated that SIV_{AGM}Sab Nef significantly increased the yield of HIV-1 particles from HIV-1 infected COS-7 cells, as compared to HIV-1 Nef or no Nef (Figures 4C and S6D). Again this effect was present in the absence of IFNα treatment, suggesting that COS-7 cells express some tetherin, but was exacerbated by IFNα. Overall, both SIV_{MAC239} and SIV_{AGM}Sab Nef proteins promoted particle release and alleviated the accumulation of tethered virus particles at the plasma membrane, in cells derived from relevant host species.

A Critical Determinant of Sensitivity to Antagonism by SIV_{MAC} Nef Is Variable in Monkey Tetherins and Deleted in Hu-Tetherin

The amino-terminus of tetherin is the only portion of the protein that is ordinarily exposed to the cell cytoplasm and was, therefore, the most likely determinant of sensitivity to antagonism by SIV Nef proteins. We constructed two plasmids expressing chimeric tetherins, in which the N termini of rh-tetherin and hu-tetherin (residues 1–21 and 1–26, respectively) were reciprocally exchanged, generating huCT-rh-tetherin and rhCT-hu-tetherin, respectively (Figure 5A). Each of these chimeric tetherin proteins inhibited the release of HIV-1 particles in the absence of Vpu or Nef (Figure 5B). Notably, hu-tetherin and the huCT-rh-tetherin chimera also inhibited the release of HIV-1 particles generated by proviral plasmids that encoded SIV_{MAC239} Nef in *cis*, assessed using both western blot or infectious virion assays (Figures 5B and 5C). Conversely, the rhCT-hu-tetherin protein, like rh-tetherin, did not efficiently inhibit the generation of HIV-1 particles generated in the presence of SIV_{MAC239} Nef. Similar experiments showed that the release of Nef-defective SIV_{MAC239} virions was inhibited by each of the intact and chimeric tetherins. Conversely, the intact, Nef-expressing SIV_{MAC239} construct was sensitive to inhibition by hu-tetherin and huCT-rh-tetherin, but was resistant to the rhCT-hu-tetherin and rh-tetherin proteins (Figures 5D and 5E). Thus, determinants in the cytoplasmic tails of rh- and hu-tetherins govern their sensitivity and resistance, respectively, to antagonism by SIV_{MAC} Nef.

The cytoplasmic tails of rh-tetherins differ from hu-tetherin in three short clusters of amino acids, including a 5 residue sequence that is deleted specifically in the human lineage. Therefore, we constructed three plasmids expressing hu-tetherin in which each variant amino acid cluster was substituted for its equivalent in rh-tetherin-1; (hu[PIL], hu[KM], and hu[GDIWK]; Figure 6A). Each of these tetherin proteins was expressed and inhibited the release of SIV_{MAC239}(del Nef) virions with approximately equal efficiency, as assessed by western blotting assays (Figures 6B and 6D). Moreover, hu(PIL) and hu(KM) tetherin proteins efficiently inhibited the release of SIV_{MAC239}(WT) virions and were, thus, apparently insensitive to antagonism by SIV_{MAC239} Nef (Figures 6C and 6D). Conversely, restoration of the ‘‘missing’’ 5 amino acid GDIWK motif in hu-tetherin conferred sensitivity to antagonism by SIV_{MAC239} Nef. Indeed, hu(GDIWK) was as sensitive to antagonism by SIV_{MAC239} Nef as was rhCT-hu protein that encoded the complete rh-tetherin-1 cytoplasmic tail (Figures 6C and 6D).

The cytoplasmic tail of tetherin has been evolving under positive selection, and the 5 residue motif that is deleted in humans contains codons that exhibit a high probability of being positively selected in other primate species (McNatt et al., 2009). Notably, rh-tetherin-1 and rh-tetherin-2 differ at one amino acid within this motif, encoding GDIWK and DDIWK, respectively (Figure S7A). Moreover, agm-tetherin, which is insensitive to antagonism by SIV_{MAC239} Nef (Figures 2 and 3), contains a single substitution relative to rh-tetherin-2 tetherin at this motif and encodes DDICK (Figure S7A). To further determine the role of this variable motif in determining susceptibility to antagonism by SIV_{MAC239} Nef, we generated plasmids expressing rh-tetherin lacking this

Figure 2. Effects of Nef Substitution on HIV-1 Particle Release in the Presence of Human and Monkey Tetherin Proteins

(A–H) Western blot analysis of HIV-1 particle release. A fixed amount (200 ng) of each proviral plasmid was cotransfected with empty vector (none) or with increasing amounts (3.12, 6.25, or 12.5ng, left to right) of African green monkey (agm), human (hu), pig-tailed macaque (pgt), or rhesus macaque (rh-1) tetherin-HA expression plasmids. Cell and virion lysates were probed with an anti-capsid monoclonal antibody. Proviral plasmids carrying neither Nef nor Vpu (A) or HIV-1 Vpu (B) were used as controls. Thereafter, proviral plasmids lacking Vpu, but encoding HIV-1 Nef (C), SIV_{MAC} Nef (D), SIV_{AGM}Sab Nef (E), SIV_{BLU} Nef (F), SIV_{RCM} Nef (G), or SIV_{GSN} Nef (H) were used. Samples derived from each viral construct were run on a single gel, and vertical lines are for visual guidance only. Left panels are film images following detection using chemiluminescence reagents; right panels show the results of p24CA quantitation in virion samples measured using quantitative fluorescence-based western blotting (LICOR). Results are from a single experiment and are representative of at least three separate experiments.

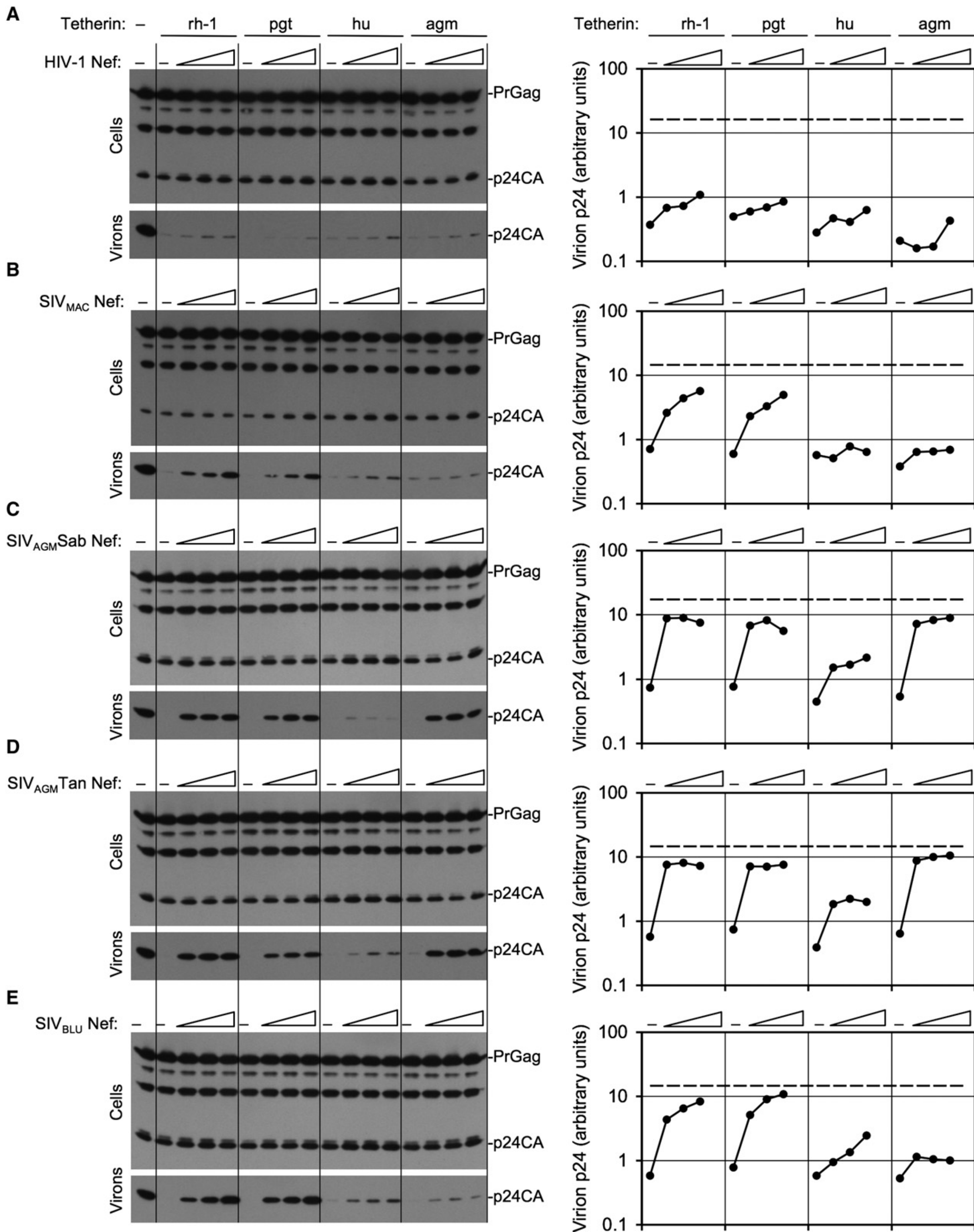

Figure 3. SIV Nef Proteins Expressed in *trans* Can Relieve Inhibition of HIV-1 Particle Release by Tetherins

(A–E) Western blot analysis of HIV-1 particle release. A fixed amount (200 ng) of each proviral plasmid was cotransfected with a fixed amount (12.5 ng) of rhesus macaque (rh-1), pig-tailed macaque (pgt), human (hu), or African green monkey (agm) tetherin-HA expression plasmids, along with varying amounts (0, 25, 50, and 100 ng) of HIV-1 Nef (A), SIV_{MAC} Nef (B), SIV_{AGM}Sab Nef (C), SIV_{AGM}Tan Nef (D), or SIV_{BLU} Nef (E) expression plasmids. Cell and virion lysates were probed with an anti-capsid monoclonal antibody. Samples derived using each Nef protein were run on a single gel; vertical lines are

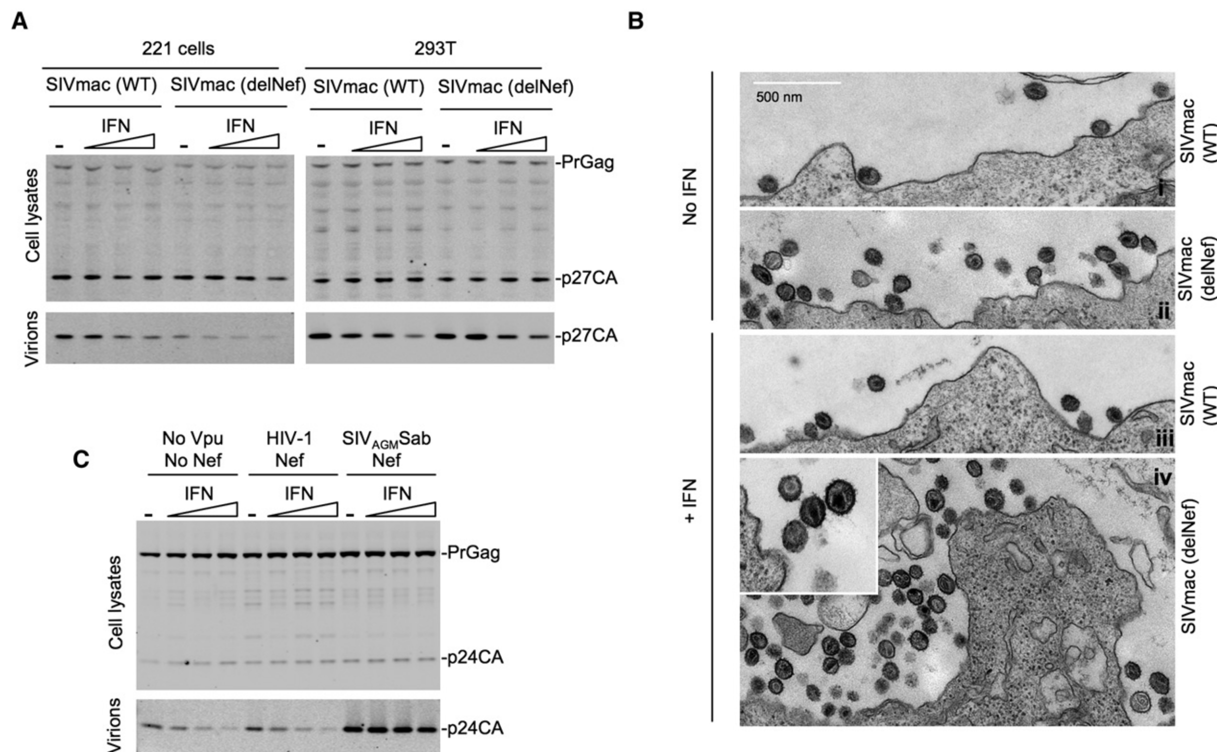

Figure 4. SIV Nef Proteins Can Enhance Virion Release and Relieve Virion Tethering in Simian Cells

(A) Rhesus macaque 221 cells and human 293T cells were infected with VSV-G pseudotyped SIV_{MAC239}(WT) or SIV_{MAC239}(del Nef), as indicated, at an MOI of 0.2 for 24 h, then washed and incubated with 0, 10, 100 or 1000 U/ml of IFNα for 24 hr. Cell and virion lysates were probed with anti-CA antibodies and signals revealed using fluorescence detection (LICOR; see Figures S6A and S6B for quantitation).

(B) Rhesus macaque 221 cells were infected with VSV-G pseudotyped SIV_{MAC239}(WT) (i and iii) or SIV_{MAC239}(del Nef) (ii and iv), as indicated, at an MOI of 2 for 24 hr, then washed and incubated in the absence (i and ii) or presence (iii and iv) of 1000 U/ml of IFNα for 24 hr. Cells were then fixed and examined using transmission electron microscopy. Scale bar = 500 nm. (iv) inset shows an expanded view of virions apparently tethered to each other.

(C) African green monkey COS-7 cells were infected with VSV-G pseudotyped HIV-1 encoding no Nef, HIV-1 Nef, or SIV_{AGM}Sab Nef, as indicated, at an MOI of 1 for 24 hr, then washed and incubated with 0, 10, 100, or 1000 U/ml of IFNα for 24 hr. Cell and virion lysates were probed with anti-CA antibodies and signals revealed using fluorescence detection (LICOR; see Figure S6C for quantitation of virion release). Results are representative of at least three independent experiments.

motif (rh[ΔGDIWK]), as well as single amino acid substitution mutants (W17C) of rh-tetherin-1 and rh-tetherin-2 (rh-1[GDICK] and rh-2[DDICK]). Notably, either the 5 amino acid deletion or the single amino acid substitution, in the context of either the rh-tetherin-1 or rh-tetherin-2, conferred resistance to antagonism by SIV_{MAC239} Nef (Figures S7B and S7C). Thus, naturally occurring deletions and substitutions within the same small motif, found in humans and African green monkeys, can confer resistance to antagonism by Nef proteins found in an SIV from another species.

Tetherin Antagonism Is Genetically Separable from Other SIV_{MAC239} Nef Activities

We next determined whether mutations that specifically abolish known biological activities of SIV_{MAC239} Nef affected its ability to antagonize tetherin. As is the case with most other biological activities of Nef proteins, mutation of the N-terminal myristate acceptor (G2A) abolished the ability of SIV_{MAC239}Nef to antagonize rh-tetherin (Figures 7A and 7B). Conversely, mutation of a single residue (Y223F) that abolishes the ability of SIV_{MAC239} Nef to downregulate MHC-I but leaves other activities of SIV_{MAC239} Nef, such as CD4 downregulation, intact (Schindler et al., 2004) had little effect on tetherin antagonizing activity. However, a series of three mutants that share the D204R substitution and have lost CD4 and CD28 downregulation activity, but maintain MHC-I downregulation activity (Schindler et al., 2004), were not effective rh-tetherin-1 antagonists (Figures 7A and 7B). Notably, none of these mutations had any effect on Nef expression levels (Schindler et al., 2004). Moreover, an analysis of five naturally occurring Nef proteins derived from the same lentivirus lineage as SIV_{MAC239} revealed clear differences in tetherin antagonizing activity (Figures 7C and 7D). Specifically, two SIV_{SMM}-derived Nef proteins were efficient rh-tetherin-1 antagonists, while two of three HIV-2 Nef proteins were devoid of tetherin-antagonizing activity and a third exhibited weak rh-tetherin-1-antagonizing activity. Because these Nef proteins all share the ability to downregulate CD4, MHC-I, and CD3 (Munch et al., 2005),

for visual guidance only. Left panels are film images following detection using chemiluminescence reagents; right panels show the results of p24CA quantitation in virion samples measured using quantitative fluorescence-based western blotting (LICOR). Results are from a single experiment and are representative of at least three separate experiments.

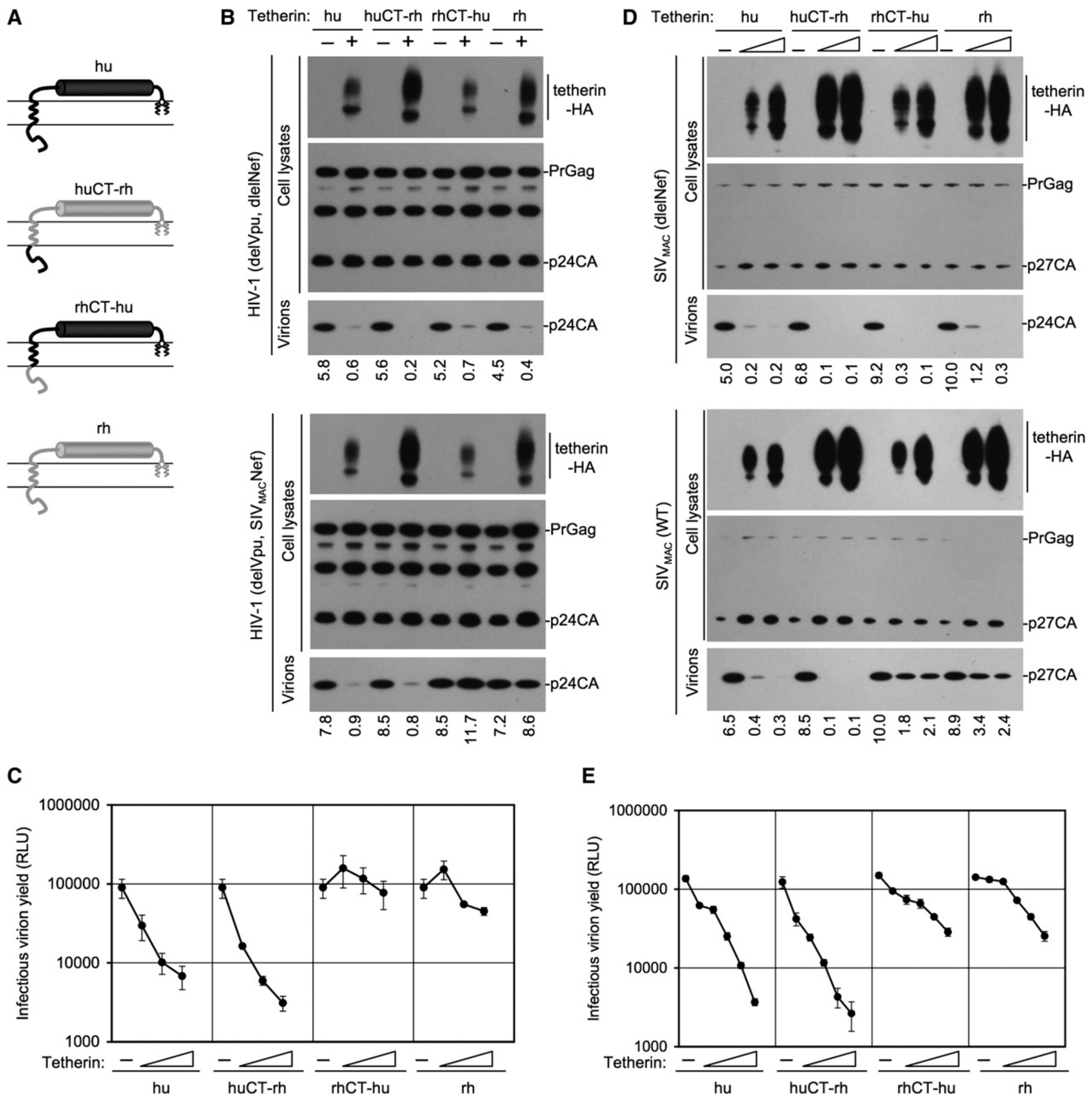

Figure 5. The Cytoplasmic Tail of Tetherin Governs Sensitivity to Antagonism by SIV_{MAC} Nef

(A) Schematic representation of the intact and chimeric tetherin proteins generated by exchange of sequences encoding the hu-tetherin and rh-tetherin cytoplasmic domains, generating huCT-rh-tetherin and rhCT-hu tetherin.

(B) A fixed amount of (500 ng) an HIV-1 proviral plasmid lacking both Vpu and Nef (upper panels) or lacking Vpu and encoding SIV_{MAC} Nef (lower panels) was transfected either alone (− lanes) or along with 50 ng of each intact or chimeric tetherin-HA expression plasmid depicted in (A) (+ lanes). The panels show western blots of cell and virion lysates probed with anti-capsid or anti-HA monoclonal antibodies, and detection with chemiluminescence based reagents. Numbers below each lane represent measurements of virion associated p24 measured using quantitative fluorescent western blotting (LICOR).

(C) An HIV-1 proviral plasmid carrying SIV_{MAC} Nef (500 ng) was cotransfected with increasing amounts (0, 50, 100, or 200 ng) of each tetherin expression plasmid depicted in (A). Infectious virion yield was measured as in Figure 3. Results are the mean ± SD of tripicate determinations from a single experiment and are representative of three independent experiments.

(D) Western blot analysis of SIV_{MAC239}(WT) and SIV_{MAC239} (delNef) particle release in the presence of the intact and chimeric tetherin-HA proteins. Cells were transfected with a fixed amount (500 ng) of each SIV_{MAC239} proviral plasmid and varying amounts (0, 50, and 100ng) of each tetherin expression plasmid. The panels show western blots of cell and virion lysates probed with anti-capsid or anti-HA monoclonal antibodies, and detection with chemiluminescence based reagents. Numbers below each lane represent measurements of virion associated p27 measured using quantitative fluorescent western blotting (LICOR).

(E) An SIV_{MAC239}(WT) proviral plasmid was cotransfected with increasing amounts (0, 13, 25, 50, 100, or 200 ng) of each intact or chimeric tetherin expression plasmid depicted in (A). Infectious virion yield in the culture supernatant was measured as in Figure 3. Results are the mean ± SD of tripicate determinations from a single experiment and are representative of three independent experiments.

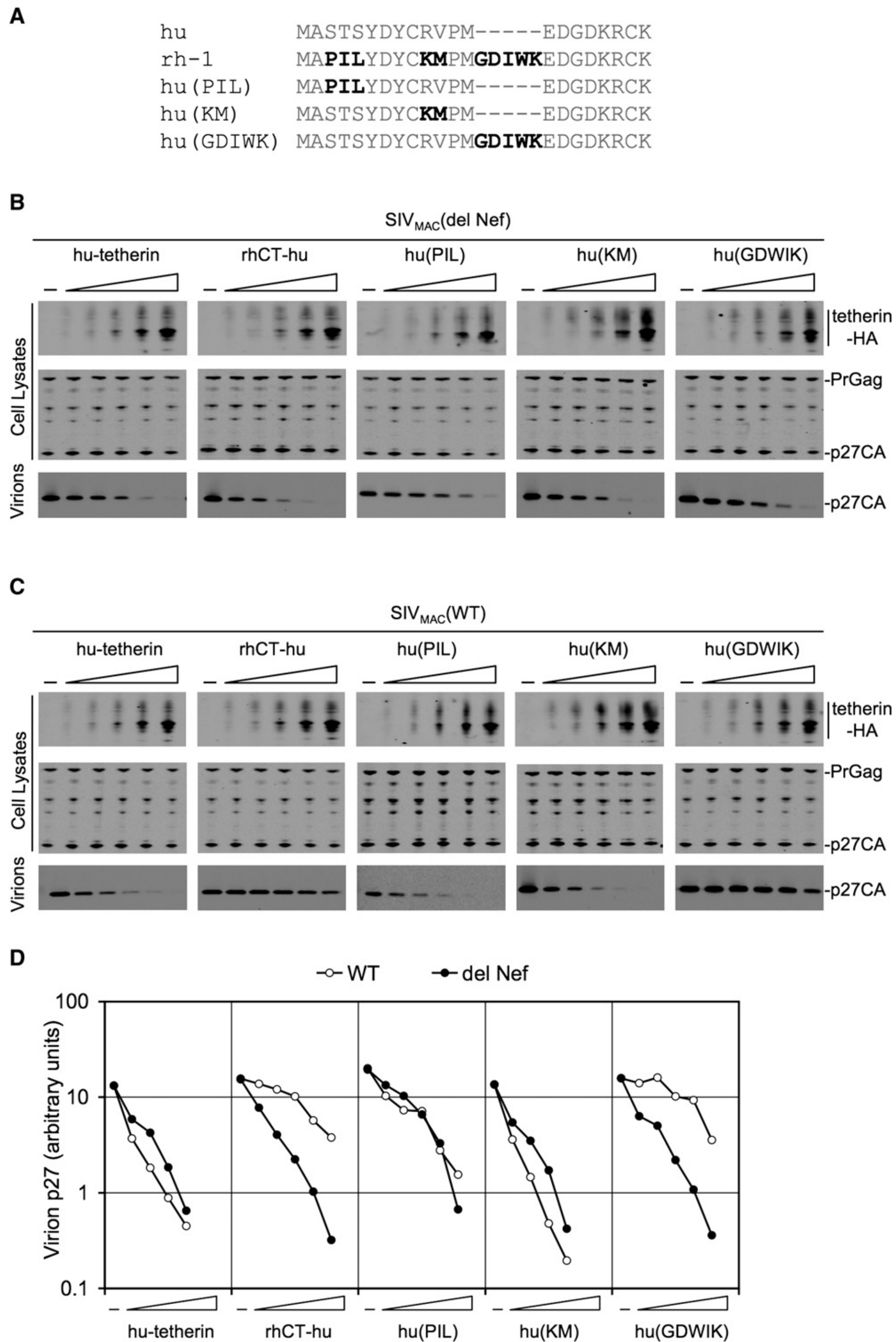

Figure 6. Insertion of a "Missing" Five Amino Acid Sequence in Hu-Tetherin Confers Sensitivity to Nef Antagonism

(A) Alignment of the cytoplasmic tails of hu-tetherin and rh-tetherin-1 N-terminal cytoplasmic tails with differences in rh-tetherin-1 highlighted. Also shown are the three mutant hu-tetherin constructs that were used.

(B) Western blot analysis of the expression of the mutant hu-tetherin proteins and inhibition of SIV_{MAC239}(del Nef) particle release. Cells were transfected with a fixed amount (500 ng) of proviral plasmid and 0, 12.5, 25, 50, 100 or 200 ng of each tetherin expression plasmid. Blots of cell lysates were simultaneously probed with rabbit anti-HA and (upper panels) and anti-CA monoclonal antibodies (middle panels). Lysates of pelleted virions were probed with anti-CA antibodies only. Signals were detected using fluorescent detection reagents (LICOR).

(C) Same as (B), except that SIV_{MAC239}(WT) was used in place of SIV_{MAC239}(del Nef).

(D) Quantitative analysis of virion associated p27CA, determined using the western blots shown in (B) for SIV_{MAC239}(del Nef) and (C) for SIV_{MAC239}(WT). Results are representative of at least three independent experiments.

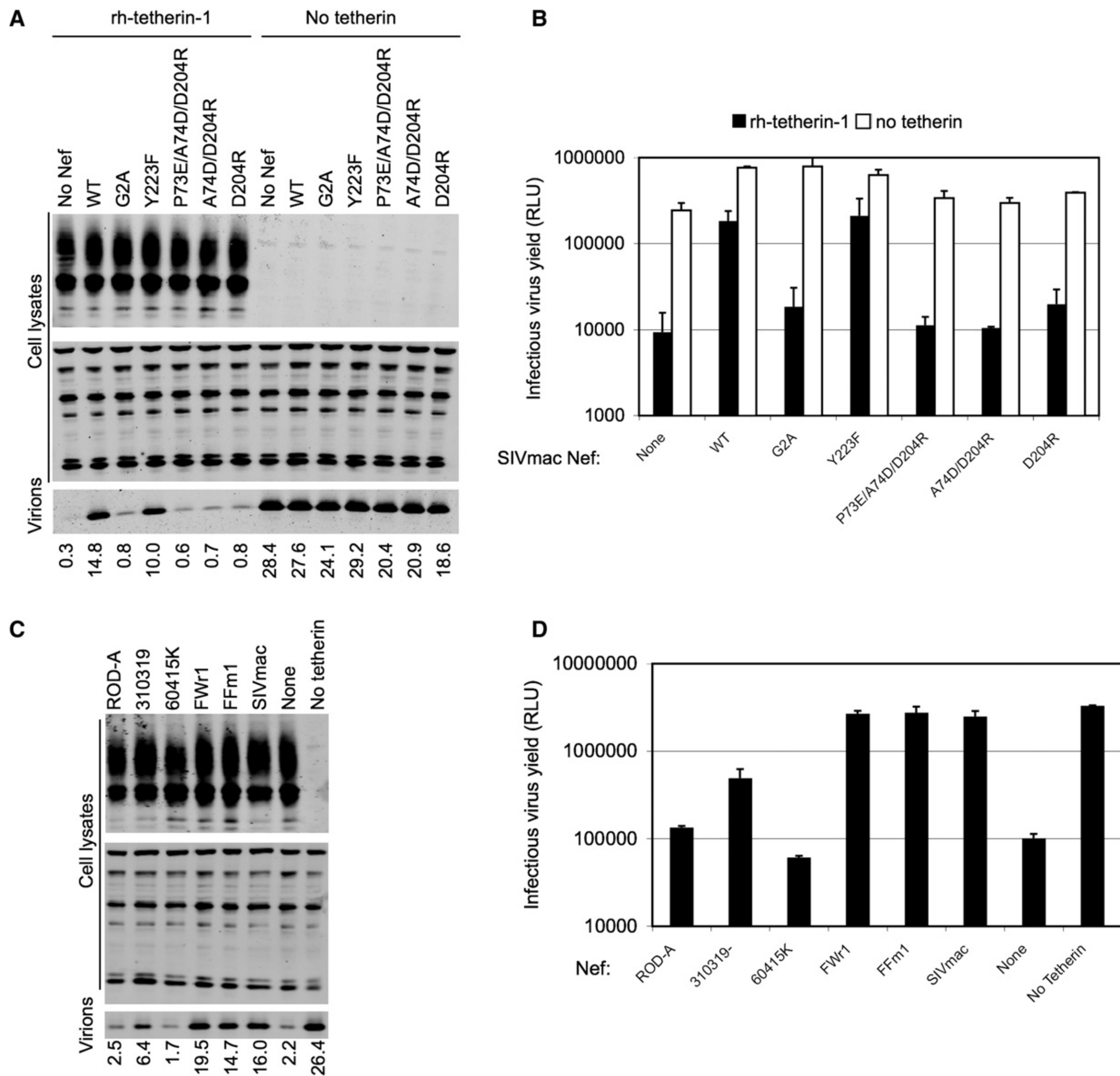

Figure 7. Effects of Introduced Mutations and Natural Variation in the SIV_{MAC}/SIV_{SMM}/HIV-2 Nef Proteins on Rh-Tetherin-1 Antagonism

(A) Cells were transfected with 400 ng of an HIV-1 proviral plasmid lacking Nef and Vpu, along with 25 ng of the rh-tetherin expression plasmid and 100 ng of each WT or mutant SIV_{MAC239} Nef expression plasmid. Blots of cell lysates were simultaneously probed with rabbit anti-HA (upper panels) and anti-CA monoclonal antibodies (middle panels). Lysates of pelleted virions were probed with anti-CA antibodies only (lower panels). Signals were detected using quantitative fluorescent western blotting (LICOR), and numbers below each lane represent measurements of virion associated p24.

(B) Cells were transfected as in (A) and the yield of infectious virions in the culture supernatant was measured using TZMbl indicator cells. Measurements are given in relative light units (RLU) following detection of β-galactosidase expression using a chemiluminescent assay. Results are the mean ± SD of tripicate determinations from a single experiment and are representative of two independent experiments.

(C) Cells were transfected with 400 ng of an HIV-1 proviral plasmid lacking Nef and Vpu, along with 25 ng of the rh-tetherin expression plasmid and 50 ng of each HIV-2, SIV_{SMM} or SIV_{MAC239} Nef expression plasmid. Blots were probed and analyzed as in (A) and numbers below each lane represent measurements of virion associated p24.

(D) Cells were transfected as in (C), and the yield of infectious virions in the culture supernatant was measured as in (B). Results are the mean ± SD of tripicate determinations from a single experiment and are representative of two independent experiments.

these data show that tetherin antagonism is genetically separable from several known biological activities of Nef, but may share some mechanistic properties with CD4 downregulation. Additionally, these data hint that natural variation in the ability of closely related Nef proteins to act as tetherin antagonists may be influenced by the host species in which the corresponding virus replicates.

DISCUSSION

Here we report that Nef proteins can enhance virion particle release via antagonism of tetherin proteins. Notably, this activity was not present in HIV-1 Nef, but five out of seven SIV Nef proteins tested, from five different SIV lineages, exhibited tetherin-antagonizing activity. Importantly, this Nef function exhibited

striking species specificity; none of the SIV Nef proteins were highly active against hu-tetherin, but in both cases where tetherins and Nef proteins were derived from the same host species (macaque and agm), efficient antagonism was observed.

Previously, we showed that the HIV-1 Vpu protein also antagonizes tetherins in a species-specific manner. Determinants of sensitivity to Vpu reside in the hu-tetherin transmembrane domain, which exhibits a strong signature of positive or diversifying selection, suggesting the possibility that Vpu, or a similar viral antagonist of tetherin, has imposed selective pressure on this portion of the tetherin gene. Notably, we also observed signatures of positive selection in sequences encoding the tetherin N-terminal cytoplasmic tail (McNatt et al., 2009). Obviously, given the finding that the cytoplasmic tail of tetherin can determine sensitivity or resistance to antagonism by SIV_{MAC} Nef, then Nef proteins are reasonable candidates for antagonists that impose diversifying selection pressure on this tetherin domain.

The mechanism by which SIV Nef proteins antagonize tetherins remains to be determined. Preliminary experiments did not reveal a clear effect of SIV_{MAC} or SIV_{BLU} Nef proteins on rh-tetherin cell-surface expression (data not shown). Nonetheless, HIV-1 and SIV Nef proteins downregulate several other cell surface proteins, most notably CD4 and MHC-I (Collins et al., 1998; Garcia and Miller, 1991; Schindler et al., 2006; Schwartz et al., 1996; Stumptner-Cuvelette et al., 2001), by recruiting these receptors to the endocytic machinery or by redirecting them to lysosomes (reviewed in Kirchhoff et al., 2008; Roeth and Collins, 2006). The HIV-1 Vpu protein excludes tetherin from sites of particle assembly at the plasma membrane and inhibits cell surface expression of tetherin (Jouvenet et al., 2009; Van Damme et al., 2008). However, further work will be required to determine precisely how antagonism of tetherin function by Nef and Vpu proteins is achieved.

The ability to antagonize tetherin has apparently arisen at least twice, perhaps three times during the evolution of primate lentiviruses, and strikingly the acquisition of this function has occurred in completely different ways, employing Nef, Vpu, and perhaps Env proteins. Of interest is the fact that both Nef and Vpu proteins, to varying degrees, remove or antagonize molecules (CD4 and tetherin) that are both capable of inhibiting the release of infectious viral particles from the cell surface (Lama et al., 1999; Ross et al., 1999). Moreover, both appear to be multifunctional proteins whose activities partially coincide. Overall these findings underscore the functional plasticity of primate lentiviral genomes in general, and *vpu* and *nef* genes in particular.

While the ability of Vpu and Nef proteins to antagonize tetherin is clearly demonstrable, it remains unproven that these activities are critical in vivo. SIV_{MAC239} chimeras that encode HIV-1 Nef, and therefore lack a predicted tetherin antagonist, are less pathogenic than intact SIV_{MAC239}, but are capable of causing simian AIDS in some animals (Alexander et al., 1999; Kirchhoff et al., 1999). Thus, tetherin antagonism might not be essential for pathogenesis. However, given the plasticity of Nef function it will be interesting to determine whether HIV-1 Nef can acquire tetherin antagonizing activity during SIV_{MAC239}/SHIV replication in vivo. Additionally, HIV-1 Vpu, despite lacking the ability to antagonize macaque tetherins, enhances replication in the context of SHIV infection of pig-tailed macaques (Stephens et al., 2002). Thus, other functions of Vpu and Nef may be critical in vivo. Nonetheless, the independent acquisition of tetherin-antagonizing activity that is specific to the host species of a given HIV/SIV by Nef and Vpu strongly suggests that these activities confer a selective advantage in vivo.

Finally, we note that three of the five known "nonessential" accessory genes in primate lentiviruses, namely *vif*, *vpu*,and *nef*, are now demonstrated to encode antagonists of innate or intrinsic immunity. Overall, these findings emphasize the extent to which intrinsic and innate host defenses are likely to have imposed evolutionary pressure on primate lentiviruses, influenced species tropism and provided the impetus for the acquisition of new genes and biological activities to enable immunodeficiency virus replication in an intrinsically hostile environment.

EXPERIMENTAL PROCEDURES

Plasmid Construction

Plasmids expressing the various HA-epitope-tagged tetherin proteins were constructed as previously described (Jouvenet et al., 2009; McNatt et al., 2009) using the cDNA sequences and oligonucleotides listed in Table S1. Two variants of rhesus macaque tetherin were used (rh-1 and rh-2) that differ at a single amino acid position in the cytoplasmic tail. No functional difference between the two variants was detected, and the variants were used interchangeably. Mutant human and rhesus tetherins were generated by PCR using long oligos directed to the 5′ end of the coding sequence (Table S1). To generate the SIV_{MAC} proviral clones used in this study, the full-length sequence of SIV_{MAC239} was assembled from plasmids p239SpSp5′ and p239SpE3′ (Kestler et al., 1990) (NIH AIDS Reagent Program) into a low copy number (pXF3) backbone. For the SIV_{MAC}(delNef) derivative of this clone, overlapping primers and PCR were used to introduce premature stop codon at codons 58 and 59 of Nef, immediately 3′ to the Env stop codon, using the primers listed in Table S2. HIV-1-based proviral plasmids expressing Nef proteins from various lentiviruses have been previously described (Munch et al., 2007; Schindler et al., 2006). To generate *vpu*-defective derivatives thereof, restriction fragments encompassing the *nef* region were cloned into pBRHIV-1NL4-3ΔVpu containing a deletion of nucleotides 3–120 of the vpu coding frame. For the experiments in which Nef proteins were expressed in *trans*, Nef coding sequences from HIV-1, SIV_{MAC239}, SIV_{BLU}, SIV_{AGM}Sab, and SIV_{AGM}Tan were amplified by PCR using primers introducing EcoRI and SalI sites at the 5′ end and 3′ end of the coding sequence respectively (Table S2). The PCR products were inserted into the pIRES2-GFP expression vector (Clontech). SIV_{MAC239} Nef was also inserted into pCR3.1. pCG-IRESGFP vectors expressing the SIV_{MAC239} mutant, HIV-2, and SIV_{SMM} Nef proteins used in Figure 7 have been described previously (Schindler et al., 2004).

Cell Lines and Transfection

293T and TZMbl cells (which express CD4 and CCR5 and contain a lacZ reporter gene under the control of an HIV-1 LTR) were maintained under standard conditions. All transfection experiments were performed in 293T cells, which do not constitutively express tetherin, that were seeded in 24-well plates at a concentration of 2.5×10^5 cells/well and transfected the following day using polyethylenimine (PolySciences). A 293T-derived cell line stably expressing rh-tetherin was derived by inserting the rh-tetherin-1 cDNA into a retroviral vector (LHCX) that was then used to transduce 293T cells, which were then selected in hygromycin.

To measure tetherin inhibition of SIV_{MAC239} particle release, cells were transfected with 400 ng of an SIV_{MAC239} proviral plasmid or the SIV_{MAC} (delNef) derivative, along with increasing amounts (3.12, 6.25, 12.5, 25, or 50 ng) of each tetherin-HA expression plasmid. In some experiments (Figure 5), the amount of SIV_{MAC} proviral plasmid DNA was increased to 500 ng and the amounts of tetherin plasmid were increased (to 0, 12.5, 25, 50, 100, 200 ng/well). For assays using the HIV-1 proviral plasmids encoding various SIV Nef proteins, cells were transfected with 200 ng of each proviral plasmid and varying amounts (0, 3.12, 6.25, or 12.5 ng) of each tetherin-HA plasmid. Alternatively, 500 ng of HIV-1 proviral plasmid and 0, 50, 100, and 200 ng of tetherin expression plasmid were used.

For assays in which Nef proteins were expressed in *trans*, cells were transfected with 400 ng of the HIV-1 proviral plasmid that lacked both Nef and Vpu, along with 25 ng of each tetherin-HA expression plasmid and varying amounts (0, 25, 50, or 100 ng) of each HIV-1 or SIV Nef expression plasmid. In all transfection experiments, the total amount of DNA was held constant within the experiment by supplementing the transfection with empty expression vector.

To generate the VSV-G pseudotyped viruses in Figure 4, 10 μg of proviral construct and 2 μg of VSV-G expression plasmid were used to transfect a 10 cm dish of 293T cells. Viral stocks were titered on TZMbl indicator cells.

Virus Release Assays and Infectivity Assays

At 48 hr posttransfection, virion containing culture supernatants were harvested, clarified by low-speed centrifugation and filtered (0.2 μm). Infectious virus release was determined by inoculating TZMbl indicator cells, plated the previous day in 96-well plates at 8×10^3 cells/well, with 50 μl of serially diluted supernatants. At 48 hr after infection, β-galactosidase activity was determined using GalactoStar reagent (Perkin Elmer). The remainder of the virion containing supernatant (750 μl) was layered onto 400 μl of 20% sucrose in PBS and centrifuged at 20,000 *g* for 2 hr at 4°C. Virion pellets and corresponding virion producing cells were dissolved in SDS-PAGE loading buffer. Virion and cell lysates were separated on 4%–12% acrylamide gels, blotted onto nitrocellulose membranes, and probed with anti-HIV-1-p24CA (183-H12-5C, which also recognizes SIV_{MAC239} p27CA) or anti-HA (Covance) monoclonal antibodies. Subsequently, blots were probed with anti-mouse HRP-conjugated goat secondary antibodies (Jackson), and proteins revealed using chemiluminescence detection reagents (Pierce). Alternatively, for quantitative western blotting, blots were simultaneously or individually probed with anti-CA and rabbit anti-HA antibodies, followed by goat anti-rabbit and anti-mouse antibodies conjugated to IRDye680 and IRDye800CW, respectively. Fluorescent signals were detected and quantitated using a LICOR Odyssey scanner.

Microscopy

Cells were seeded on 3.5 cm, glass-bottomed dishes coated with poly-L-lysine (Mattek). The following day, they were transfected with 1.6 μg of a mixture of plasmids expressing untagged SIV_{MAC239} Gag and SIV_{MAC239} Gag-GFP, along with 1.6 μg of empty pCR3.1 or pCR3.1/SIV_{MAC239} Nef, using Lipofectamine 2000. Cells were fixed 24 hr later and observed by deconvolution microscopy using an Olympus IX70-based Deltavision microscopy suite (Tokyo, Japan) as described previously (Neil et al., 2006). For transmission electron microscopy studies, 221 cells were fixed 48 hr postinfection, using 4% paraformaldehyde, for 10 min. Cells were then pelleted by centrifugation and processed for electron microscopy as described previously (Neil et al., 2006). Alternatively, HT1080 cells were cotransfected with 200 ng each of a plasmid expressing a codon optimized SIV Gag-IRES GFP cassette and either rh-tetherin-1 or hu-tetherin expression plasmids inspected by fluorescent and scanning electron microscopy using a Hitachi S4700 field emission SEM (University of Missouri Electron Microscopy Core Facility) as described previously (Zhadina et al., 2007).

SUPPLEMENTAL DATA

Supplemental Data include seven figures and two tables and can be found online at http://www.cell.com/cellhostandmicrobe/supplemental/S1931-3128(09)00175-9.

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