Broad-Spectrum Inhibition of Retroviral and Filoviral Particle Release by Tetherin[▽]

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The expression of many putative antiviral genes is upregulated when cells encounter type I interferon (IFN), but the actual mechanisms by which many IFN-induced gene products inhibit virus replication are poorly understood. A recently identified IFN-induced antiretroviral protein, termed tetherin (previously known as BST-2 or CD317), blocks the release of nascent human immunodeficiency virus type 1 (HIV-1) particles from infected cells, and an HIV-1 accessory protein, Vpu, acts as a viral antagonist of tetherin. Here, we show that tetherin is capable of blocking not only the release of HIV-1 particles but also the release of particles assembled using the major structural proteins of a variety of prototype retroviruses, including members of the alpharetrovirus, betaretrovirus, deltaretrovirus, lentivirus, and spumaretrovirus families. Moreover, we show that the release of particles assembled using filovirus matrix proteins from Marburg virus and Ebola virus is also sensitive to inhibition by tetherin. These findings indicate that tetherin is a broadly specific inhibitor of enveloped particle release, and therefore, inhibition is unlikely to require specific interactions with viral proteins. Nonetheless, tetherin colocalized with nascent virus-like particles generated by several retroviral and filoviral structural proteins, indicating that it is present at, or recruited to, sites of particle assembly. Overall, tetherin is potentially active against many enveloped viruses and likely to be an important component of the antiviral innate immune defense.

Evolution has endowed eukaryotic cells with a variety of molecules and activities that are capable of inhibiting the replication of the viruses that parasitize them. Among these molecules are constitutively expressed, specific inhibitors of virus replication such as the APOBEC3 proteins (4, 32). In addition, components of the type I interferon (IFN)-induced innate immune system can directly inhibit the replication of viruses (29, 33). In turn, viruses have evolved antagonists that inhibit the IFN response or directly counteract cellular antiviral functions (such as lentivirus Vif proteins that neutralize APOBEC3 proteins) (4, 32).

While the expression of many genes is known to be upregulated when cells encounter type I IFN, the actual mechanisms by which IFN-induced gene products inhibit virus replication are known for only a few of them. We and others recently identified an IFN-induced antiretroviral protein, termed tetherin, that appears mechanistically unique in that it blocks the release of nascent virions from human immunodeficiency virus type 1 (HIV-1)-infected cells (22, 23, 36). Notably, an HIV-1 accessory protein, Vpu, acts as a viral antagonist of tetherin. Specifically, Vpu colocalizes with tetherin and causes its downregulation from the cell surface (23, 36). Thus, the existence of tetherin explains the previously observed requirement for Vpu during HIV-1 particle release from certain cells, particularly those that have been exposed to type I IFN (11, 14, 22, 34, 37).

Tetherin is a membrane protein with unusual topology in that it harbors a transmembrane anchor near it N terminus and a putative glycophosphatidyl-inositol lipid anchor at its C terminus (15). It appears to induce the formation of, and may indeed be a physical component of, protease-sensitive tethers that retain HIV-1 virions on infected cell surfaces (21, 23). Importantly, the HIV-1 particles whose retention is induced by tetherin are fully formed and mature and have independent lipid bilayers that are discontinuous with cell membranes (21, 23). Thus, tetherin seems to act after the formation of virus particles and prevents their dissemination to uninfected cells, apparently by causing the adherence of virion and cell membranes.

The promiscuous expression of tetherin upon the exposure of cells to IFN (5, 23) suggests that it might be part of a broader innate immune defense that limits the replication of perhaps many enveloped viruses. Here, we investigate this possibility and show that tetherin is capable of blocking the release of particles assembled using the major structural proteins of a wide variety of retroviruses, including prototype members of the alpharetrovirus, betaretrovirus, deltaretrovirus, lentivirus, and spumaretrovirus genera. In addition, we show that the release of particles assembled using filovirus matrix proteins from Marburg virus (Mv) and Ebola virus (Eb) is also sensitive to inhibition by tetherin. These findings indicate that tetherin is a very broadly specific inhibitor of particle release from the plasma membrane and is therefore unlikely to require specific interactions with viral proteins to be effective. Thus, tetherin is potentially an effective component of the innate immune defense against many enveloped viruses and could provide an impetus for the evolution of tetherin antagonists such as Vpu.

MATERIALS AND METHODS

Plasmid derivation. Several Gag and GagPol proteins were expressed using pCRV1, a previously described hybrid expression vector (38) that is derived from

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▽ Published ahead of print on 26 November 2008.

pCR3.1 (Invitrogen) and from a highly deleted HIV-1 provirus. The unmodified pCRV-1 expresses HIV-1 Tat, Rev, and Vpu and contains *cis*-acting Tat and Rev response elements; transgenes are inserted into a multiple-cloning site that is positioned in a location normally occupied by the *gag*, *pol*, *vif*, and *vpr* gene, encoding portions of the HIV genome, which are deleted in pCRV1. A derivative of pCRV1 lacking the *vpu* gene (pCRV1delVpu) was generated. HIV-1 GagPol, SIVmac239 Gag-Pol, SIV$_{AGM}$Sab1-A Gag-Pol, Rous sarcoma virus (RSV) Gag, and feline immunodeficiency virus Gag were amplified from proviral plasmids and inserted as EcoRI-NotI fragments into pCRV1 and pCRV1/delVpu. Other plasmids expressing retroviral Gag/GagPol proteins that were used included pEIAV-Gag-Pol (20), pCRV1/ConGag-Pol (expressing human endogenous retrovirus K$_{CON}$ [HERV-K$_{CON}$] Gag-PR-Pol) (17), pCMVHT1 (expressing human T-lymphotrophic virus type 1 [HTLV-1] GagPol) (7), pCAG/PFV-Gag (expressing wild-type [WT] prototypic foamy virus [PFV] Gag) pCAG/PFV-Lck-Gag (expressing retargeted PFV Gag) (39), and pSARM4 (a Mason-Pfizer monkey virus [MPMV] proviral plasmid) (1).

Plasmids expressing codon-optimized HIV-1 Gag and Gag-green fluorescent protein (GFP), namely, pCR3.1/HIV-Gag and pCR3.1/HIV-Gag-GFP, were previously described (25). Similar plasmids expressing codon-optimized SIV$_{MAC}$ Gag/Gag-GFP proteins (pCR3.1/SIVGag and pCR3.1/SIVGag-GFP) and equine infectious anemia virus (EIAV) Gag/Gag-GFP proteins (pCR3.1/EIAVGag and pCR3.1/EIAVGag-GFP) were generated by inserting codon-optimized versions of each Gag gene into pCR3.1 and pCR3.1/GFP. pMLVGag-GFP and pMLVGag-HA, expressing murine leukemia virus (MLV) Gag with GFP or hemagglutinin (HA) fused at its C terminus, were previously described (21), as was pCRV1/MPMVGag-GFP (9).

Plasmids expressing the Vp40 matrix protein of Eb with either a myc tag or GFP fused to its amino terminus were previously described (19). The matrix protein of Mv (strain Musoke) was amplified from pCAG/MvVp40 and inserted as an EcoRI-XhoI fragment into pCR3.1/GFP to generate Mv Vp40 with GFP fused to its N terminus.

Plasmids expressing HIV-1 Vpu, namely, pCR3.1/Vpu, were previously described (21). pCR3.1-Tetherin-HA was generated by inserting an NheI restriction site at nucleotide position 463 of the tetherin gene. Thereafter, oligonucleotides encoding an HA epitope tag were inserted into the NheI site. An LHCX (Invitrogen)-based retroviral vector expressing tetherin-HA was derived from pCR3.1-Tetherin-HA.

Cells and transfection. 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. 293T cell-derived cell lines stably expressing tetherin-HA were generated by retroviral transduction. A clone was selected for the immunofluorescence experiments. 293T cells were transfected using polyethylenimine (PolySciences).

Virus release assays and Western blot analysis. Virus-like particle (VLP) release assays were performed by transfecting 293T cells as described above. Depending on their levels of expression and VLP release efficiencies, these assays were conducted using 24-well, 12-well, or 6-well plates or 10-cm culture dishes. The amounts of plasmids expressing viral structural proteins and tetherin-HA and Vpu were varied accordingly, in approximate proportion to the numbers of cells transfected.

For HIV-1 and SIV$_{AGM}$Sab VLP release assays, 1×10^5 cells in 24-well plates were transfected with 200 ng of pCRV1 or pCRV1-delVpu-based Gag-Pol expression plasmids, along with 0, 25, 50, or 100 ng of pCR3.1/Tetherin-HA. EIAV and feline immunodeficiency virus (FIV) VLP release assays were carried out using the same format, by transfecting cells with pCRV1-FIVGag-2xmyc-delVpu and pEIAV-Gag-Pol, respectively, in the presence or absence of 200 ng of pCR3.1/Vpu.

SIV$_{MAC}$ VLP release assays were carried out by transfecting approximately 2.5×10^5 cells in 12-well plates with 400 ng of pCRV1/SIVmac-Gag-Pol or pCRV1/SIVmac-Gag-Pol-delVpu along with 0, 50, 100, or 200 ng of pCR3.1/Tetherin-HA. Eb VLP and MPMV release assays were carried out using the same format, by transfecting cells with pCRV1/mycEbVp40 or pSARM4, in the presence or absence of 400 ng of pCR3.1/Vpu.

Mv VLP release assays were done by transfecting approximately 5×10^5 cells in a six-well plates with 800 ng of pCR3.1/GFP-MvVp40 or, along with 0, 100, 200, or 400 ng of pCR3.1/Tetherin-HA, in the presence or absence of pCR3.1/Vpu. HERV-K particles were generated in the same format, by transfecting cells with 1.8 μg of CCGBX, 0.5 μg of pCRV1/ConGag-PR-Polm, and 0.5 μg of pCR3.1/K-Rev.

For RSV, PFV, and HTLV-1 VLP release assays, approximately 3×10^6 cells in 10-cm dishes were transfected with 2 μg of pCRV1/RSVGag-2xmyc-ΔVpu, pCAG/PFV-Gag, pCAG/PFV-Lck-Gag, or pCMVHT1 along with 0, 1, 2, or 4 μg of pCR3.1/Tetherin-HA in the presence or absence of pcDNAI/Vpu.

Cells and extracellular virus particles were harvested as described previously

(21). Virion and cell lysates were separated on 4 to 12% acrylamide gels, and proteins were probed with various antibodies: anti-myc (9E10), anti-HIV-1 p24CA (183-H12-5C), anti-EIAV equine serum, anti-HERVK CA serum (18), anti-MPMV CA rabbit serum (28), anti-PFV human serum, anti-HTLV-1 p19 (Zeptometrix, Buffalo, NY), and anti-GFP (Roche). Subsequently, blots were probed with species-specific horseradish peroxidase-conjugated goat secondary antibodies. Semiquantitative analysis of Western blots was carried out by scanning using an Alpha Innotech imaging system and rendering the blots as TIFF files. Band intensities (given in arbitrary units) associated with released VLPs, at each amount of transfected tetherin expression plasmid, were quantitated using Image J software (W. S. Rasband, U.S. National Institutes of Health, Bethesda, MD [http://rsb.info.nih.gov/ij/], 1997 to 2008). Histograms of pixel intensity for each lane were generated using the plot lane function in the Gels Analysis toolbox, and the area under the curve of the histogram peak was then calculated.

Microscopic analyses. In experiments where fluorescent microscopy was performed, cells were plated on poly-D-lysine-coated glass-bottomed dishes (Mattek) prior to transfection. For HIV-1, SIV$_{MAC}$, EIAV, and MLV, plasmids expressing unfused Gag and Gag-fluorescent fusion proteins were combined at a 5:1 ratio before transfection. For MPMV, the Gag-GFP plasmid was combined at a 5:1 ratio with full-length proviral plasmid. For filoviruses, GFP-Mv Vp40 and GFP-Eb Vp40 were combined with untagged or myc-tagged Vp40, respectively, at a 5:1 ratio. Cells were transfected as described above, fixed 18 to 20 h after transfection, and then stained using anti-HA (Covance) and anti-mouse immunoglobulin G Alexafluor 594 (Molecular Probes) antibodies. Nuclei were stained with Hoechst 33258 stain. Fluorescent imaging of fixed cells was done using the Olympus IX70-based Deltavision microscopy suite. Quantification of colocalization was performed using MetaMorph software (Molecular Devices). Specifically, regions of 10 by 10 pixels were drawn around retrovirus VLPs or along Vp40-induced filovirus filaments, and fluorescent intensities in both the GFP channel (green) and the tetherin channel (red) were quantitated. The average intensity in each channel was recorded and transferred to Excel for correlation analysis (see Fig. 6). For each virus, more than 300 VLPs from three to five cells were analyzed.

RESULTS

To analyze the ability of tetherin to inhibit the release of a variety of retroviruses, we used transient transfection assays in cells that do not ordinarily express tetherin, namely, 293T cells. VLPs were generated by transfecting cells with plasmids expressing viral structural proteins, specifically retroviral Gag and/or GagPol, or filoviral matrix proteins. In most cases, particle release was assessed using Western blot assays with antibodies raised against the viral capsid or matrix proteins. However, in some cases where anti-Gag antibodies were not available, Gag proteins appended at their C terminus with an epitope tag (myc) were used. Plasmids expressing the viral proteins were transfected in 293T cells along with small, increasing amounts of a plasmid encoding an epitope-tagged version of tetherin in which an HA tag was inserted between the extracellular coiled-coiled domain and the glycosylphosphatidylinositol anchor. This tetherin variant is indistinguishable from untagged tetherin in terms of its ability to inhibit HIV-1 particle release (data not shown). In addition, we examined whether the previously reported ability of Vpu to enhance the release of diverse retrovirus particles (11) required the presence of tetherin and whether the ability of Vpu to reverse the inhibitory effect of tetherin was dependent on the source of the viral proteins used to generate the particles. Notably, epitope-tagged tetherin was also indistinguishable from untagged tetherin in terms of the ability of Vpu to antagonize its inhibition of HIV-1 particle release (data not shown).

Tetherin inhibits the release of diverse lentiviruses. We first compared the effects of tetherin and Vpu on the release of VLPs generated by the expression of primate lentivirus
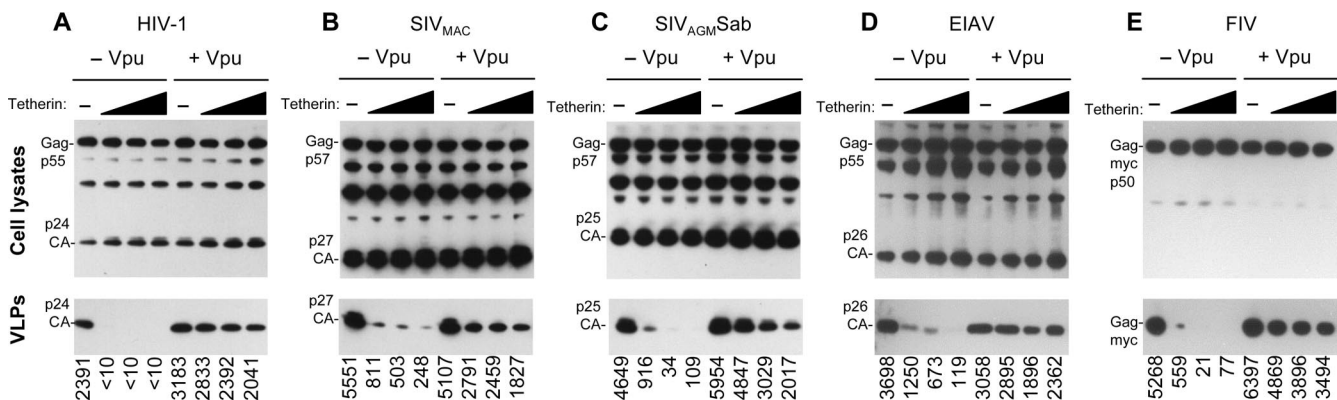
FIG. 1. Tetherin inhibits lentivirus VLP release. Shown is a Western blot analysis of 293T cells and the corresponding VLPs after coexpression of lentivirus Gag-Pol (A to D) or myc-tagged Gag (E) protein in the absence or presence of Vpu and various amounts of tetherin-HA. HIV-1 (A), SIV$_{MAC}$ Gag (B), and SIV$_{AGM}$Sab (C) proteins were revealed using an anti-HIV-1 p24CA antibody. EIAV Gag (D) was revealed with anti-EIAV serum. FIV Gag (E) was revealed with anti-myc antibodies. Numbers below each lane indicate values obtained upon densitometric scanning of VLP blots.

(HIV-1, SIV$_{MAC}$, or SIV$_{AGM}$Sab) GagPol proteins. The yield of VLPs generated by these proteins was unaffected by the presence or absence of Vpu in tetherin-negative 293T cells (Fig. 1A to C). However, the expression of tetherin dramatically inhibited the release of each of these primate lentiviruses without affecting viral protein expression (Fig. 1A to C). Moreover, the reduction in the yield of VLPs was almost completely abolished when Vpu was coexpressed, again in the absence of effects on viral protein expression. Tetherin also inhibited the release of VLPs generated by the expression of EIAV GagPol proteins or by the expression of a myc epitope-tagged FIV Gag protein (Fig. 1D and E). In both cases, the inhibitory effect of tetherin was reversed by the expression of Vpu. Thus, tetherin inhibits the release of a variety of lentiviruses in a manner that can be reversed by the HIV-1 Vpu protein.

Tetherin inhibits the release of alpharetroviruses, betaretroviruses, and deltaretroviruses. Next, we examined whether tetherin could inhibit the release of particles generated by proteins from other retroviruses that share little or no se-

quence homology with lentivirus Gag or Pol proteins. RSV, a prototype alpharetrovirus, resembles lentiviruses in that it assembles at the cell plasma membrane, and the expression of RSV Gag alone is sufficient to induce VLP assembly and budding. Notably, the generation of extracellular RSV VLPs was dramatically reduced by tetherin expression without effects on RSV Gag expression (Fig. 2A). Vpu did not affect the yield of RSV VLPs in the absence of tetherin, but the inhibitory effect of tetherin was abolished when Vpu was coexpressed (Fig. 2A). Therefore, tetherin has the ability to inhibit a prototype alpharetrovirus, and Vpu can counteract this effect.

Betaretroviruses often assemble complete immature particles within the cell cytoplasm prior to envelopment. Thereafter, Env expression can be required for the delivery of viral particles to cell membranes. For example, MPMV, a well-characterized prototype betaretrovirus, assembles as nonenveloped particles in a juxtanuclear location before movement to, and envelopment by, the plasma membrane (31). Therefore, the efficient generation of MPMV particles requires the ex-
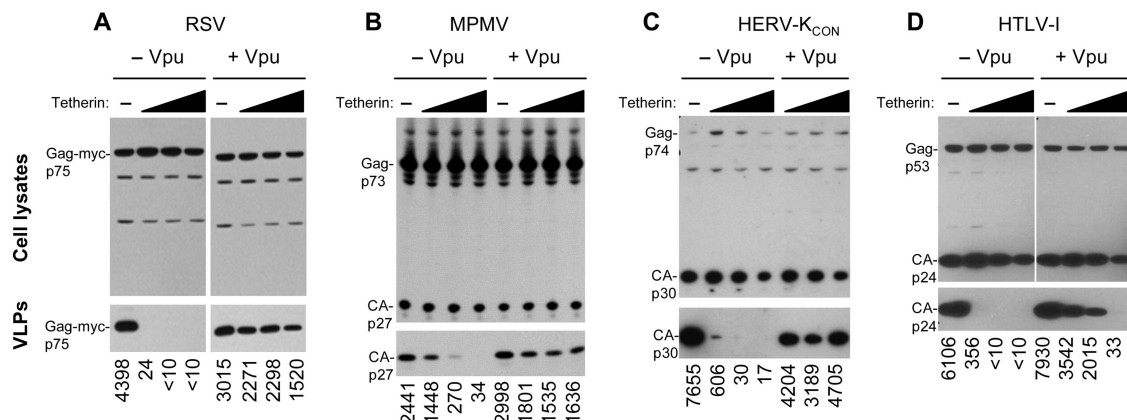

FIG. 2. Tetherin inhibits alpharetrovirus, betaretrovirus, and deltaretrovirus release. Shown is a Western blot analysis of 293T cells and corresponding VLPs after the coexpression of Gag-myc or Gag-Pol proteins in the presence or absence of Vpu and various amounts of tetherin. RSV Gag (A) was revealed with anti-myc antibodies, MPMV Gag (B) and HERV-K Gag (C) were revealed using anti-CA antisera, and HTLV-1 Gag (D) was revealed with an anti-HTLV-1 p19 antibody. Numbers below each lane indicate values obtained upon densitometric scanning of VLP blots.
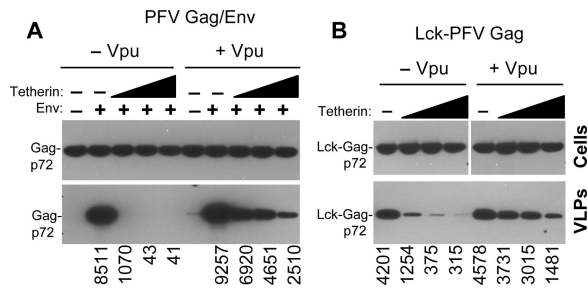
FIG. 3. Tetherin inhibits spumaretrovirus VLP release. Shown is a Western blot analysis of 293T cells and the corresponding VLPs after the expression of WT PFV Gag (A), either alone or along with a PFV Env, or retargeted Lck-PFV-Gag (B) in the absence or presence of Vpu and various amounts of tetherin-HA. PFV Gag was revealed with anti-PFV human serum. Numbers below each lane indicate values obtained upon densitometric scanning of VLP blots.
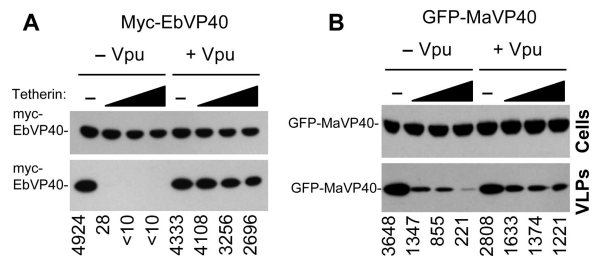

FIG. 4. Tetherin inhibits filovirus release. Shown is a Western blot analysis of 293T cells and the corresponding VLPs after the coexpression of the filovirus matrix protein Vp40 in the absence or presence of Vpu and various amounts of tetherin-HA. myc-Eb Vp40 (A) was revealed with anti-myc antibodies, while GFP-Mv Vp40 (B) was revealed with anti-GFP antibodies. Numbers below each lane indicate values obtained upon densitometric scanning of VLP blots.

pression of the viral envelope protein. In contrast, HERV-K, a recently reconstituted endogenous retrovirus that is related to betaretroviruses, appears to assemble at the plasma membrane (8, 17). Notably, the yields of both MPMV virions and HERV-K VLP viruses were reduced by tetherin expression without effects on Gag expression (Fig. 2B and C). Moreover, the tetherin-dependent reduction in the release of VLPs was reversed when Vpu was coexpressed (Fig. 2B and C), even though Vpu had no effect on particle release in the absence of tetherin. Curiously, MPMV release appeared to be less affected by low levels of tetherin than the other retroviruses tested, although it was clearly inhibited at higher levels of tetherin expression (Fig. 2B).

HTLV-1 is a prototype member of the deltaretrovirus genus, which comprises a group of complex retroviruses that assemble at the plasma membrane. Western blot analysis of 293T cells transfected with HTLV-1 Gag-Pol expression plasmids revealed that tetherin expression dramatically inhibited the release of HTLV-1 particles without affecting viral protein expression (Fig. 2D). Moreover, the yield of VLPs was at least partly restored by Vpu coexpression except when large amounts of tetherin were used (Fig. 2D). Overall, tetherin inhibited the release of all prototype members of the *Orthoretrovirinae* subfamily of retroviruses that were tested, and in each case, the effect was antagonized by the coexpression of Vpu.

Tetherin inhibits spumaretrovirus release. Spumaviruses, or foamy viruses, form a divergent subfamily of the retroviruses and share some features in common with hepadnaviruses rather than orthoretroviruses (27). Their morphogenesis pathway differs from that of most retroviruses in that they assemble as complete capsids at a pericentriolar region of the cytoplasm, and Env mediates transport to the plasma membrane, envelopment, and particle release. The requirement for Env during particle release is very stringent in the case of PFV (26), but this requirement can be bypassed by appending the PFV Gag N terminus with a plasma membrane-targeting signal, such as the Lck N terminus (10, 39). Such modified PFV Gag proteins (e.g., Lck-Gag) can behave more like conventional retroviral Gag proteins in that they can assemble at the plasma membrane and be released as VLPs in the absence of other viral proteins (39). Thus, to examine the effects of tetherin on PFV VLP release, cells were transfected with PFV Gag expression

plasmids in the presence or absence of plasmids expressing PFV Env, tetherin, and/or Vpu (Fig. 3A). Extracellular VLPs were released only when Env was coexpressed with WT PFV Gag (Fig. 3A), and the Env-dependent release of PFV VLPs was completely inhibited by small amounts of coexpressed tetherin (Fig. 3A). The release of Lck-retargeted PFV Gag appeared to be marginally less affected by tetherin than its WT counterpart (Fig. 3B). However, the release of both types of VLPs was clearly inhibited by tetherin, and thus, the activity of tetherin was largely independent of the mechanism by which PFV Gag was targeted to membranes. Moreover, as was the case with the orthoretrovirus VLPs, the ability of tetherin to block particle release was inhibited when Vpu was coexpressed in *trans* (Fig. 3A and B), and Vpu had no effect on particle release in the absence of tetherin.

Tetherin inhibits the release of filovirus VLPs. The family *Filoviridae* includes Eb and Mv. These negative-sense RNA viruses encode seven proteins that form filamentous particles, but the major structural protein, the matrix protein (Vp40), is the only component necessary for the assembly and budding of VLPs (35). Western blot analysis of 293T cells expressing myc-tagged Eb Vp40 or GFP-tagged Mv Vp40 revealed that the production of filovirus VLPs was unaffected by Vpu expression in the absence of tetherin (Fig. 4A and B). However, the release of VLPs generated by the Vp40 proteins of both filoviruses was inhibited by the expression of tetherin in the absence of effects on Vp40 expression (Fig. 4A and B). In the case of Eb Vp40, tetherin almost completely blocked VLP release. Finally, as was the case with retroviruses, Vpu had no effect on particle release in the absence of tetherin, while the activity of tetherin was inhibited when Vpu was coexpressed (Fig. 4A and B). These data indicate that tetherin is broadly specific in terms of the inhibition of VLP release, suggesting that it targets some component that is shared by all retrovirus and filovirus VLPs.

Localization of nascent retrovirus and filovirus VLPs and tetherin at the plasma membrane. Given these findings, we next determined whether tetherin could localize to putative sites of VLP assembly even when VLPs were assembled using very divergent viral structural proteins. To facilitate this, we generated a 293T cell line stably expressing uniform levels of epitope-tagged tetherin. Thereafter, these cells were transfected with various available plasmids expressing fluorescently
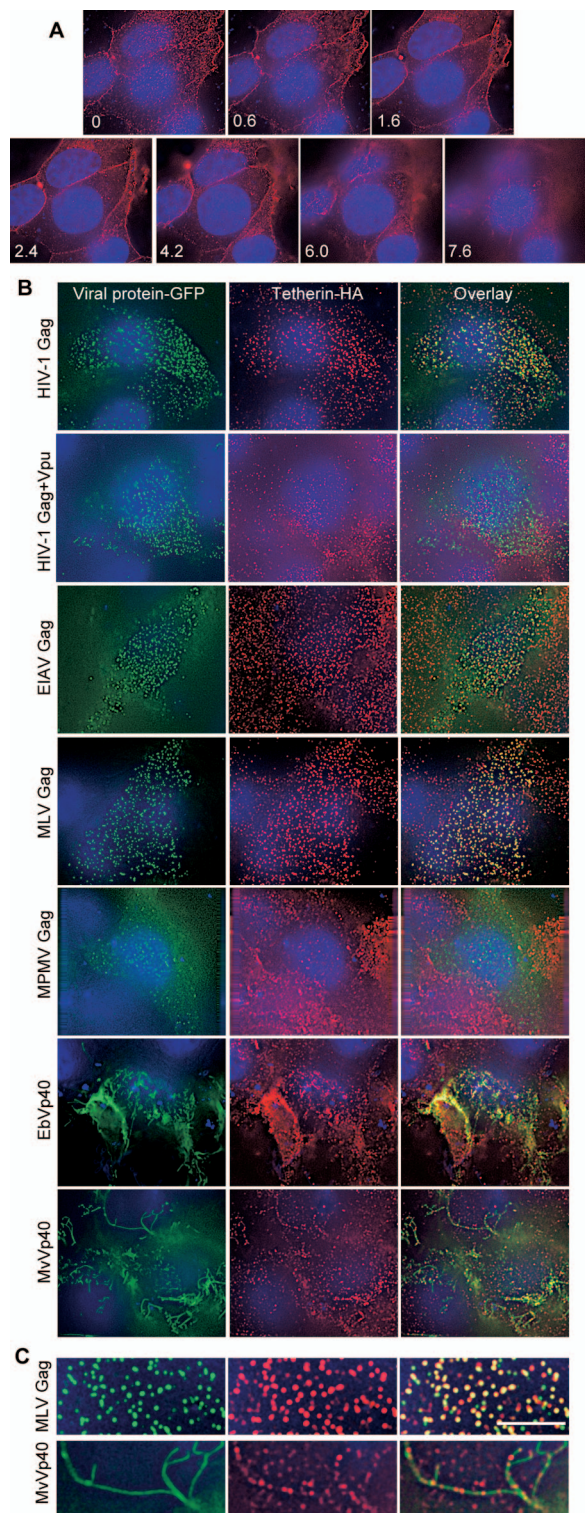
FIG. 5. Localization of nascent retrovirus and filovirus VLPs and tetherin at the plasma membrane. (A) Images showing a series of deconvolved optical sections through 293T cells stably expressing tetherin-HA and stained with an anti-HA antibody (red). Numbers in each panel indicate the z-axis distance (in μm) from the cell coverslip interface. (B) 293T cells stably expressing tetherin-HA (red) were transfected with various viral structural proteins, as indicated, fused to GFP (green) and fixed. Nuclei were stained with Hoechst stain (blue). Single deconvolved optical sections acquired at the cell-coverslip interface are shown.

tagged viral Gag or matrix proteins (in the presence of excess untagged viral protein), and colocalization analyses were performed (Fig. 5 and 6). In either the presence or the absence of viral proteins, stably expressed tetherin-HA was detected primarily at the plasma membrane, with some accumulations at intracellular sites (Fig. 5A). Analysis of cells expressing GFP-tagged versions of HIV-1, SIV$_{MAC}$, and EIAV Gag proteins revealed, as expected, characteristic puncta of Gag-GFP. Notably, tetherin colocalized extensively with these Gag-GFP puncta at the plasma membrane, and the intensity of tetherin staining correlated with the intensity of viral protein fluorescence (Fig. 5 and 6). In addition, plasma membrane-localized puncta of MLV Gag-GFP, a gammaretrovirus whose release is inhibited by tetherin (23), also colocalized particularly well with tetherin (Fig. 5B and C). No obvious effect on tetherin distribution was noticeable in cells expressing these Gag proteins. Based on previous analyses, these Gag-GFP puncta likely represent nascent VLPs (13, 16), and the colocalization of tetherin with them raises the possibility that tetherin is directly incorporated into virions. Alternatively, it is also possible that the sites at which tetherin and Gag-GFP colocalize represent sites of simultaneous VLP and tetherin endocytosis. Nonetheless, the fact that Vpu coexpression substantially inhibited tetherin colocalization with HIV-1 Gag-GFP (Fig. 5B and 6) suggests that colocalization is important for tetherin function. In contrast to the other retroviruses examined, colocalization of tetherin with MMPV Gag-GFP was not evident, and there was no correlation between tetherin and MPMV Gag-GFP signals (Fig. 5B and 6). Interestingly, in cells transfected with the MPMV proviral plasmids, the expression of tetherin appeared to be reduced (Fig. 5B). In cells expressing GFP-Eb Vp40 or GFP-Mv Vp40, filamentous accumulations of the respective proteins, which likely represent nascent VLPs, were often decorated with puncta of tetherin (Fig. 5B and C). In these cases, tetherin localization did appear to be altered to at least partly mimic that of Vp40, particularly where large accumulations of the Vp40 protein were observed (Fig. 5B). Overall, tetherin colocalized with a variety of VLPs at the plasma membrane.

DISCUSSION

While many cells do not constitutively express tetherin, its expression can be induced by IFN-α (5, 23), and as such, tetherin is likely part of a general IFN-induced antiviral response. Many viral infections could potentially trigger tetherin expression, which could in turn inhibit virus dissemination. Here, we show that tetherin is capable of inhibiting the release of a variety of VLPs assembled using the structural proteins of retroviruses and filoviruses. While we have obviously not surveyed all enveloped viruses, the viral proteins used herein included those that have no sequence homology to one another, are from different virus families, are targeted to cell membranes in different ways, and exhibit different morphogen-

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(C) Expanded view of a portion of the images shown in A exemplifying the localization of tetherin puncta with MLV Gag-GFP puncta and Mv Vp40 filaments. Scale bars indicate 10 μm (A) and 4 μm (B).
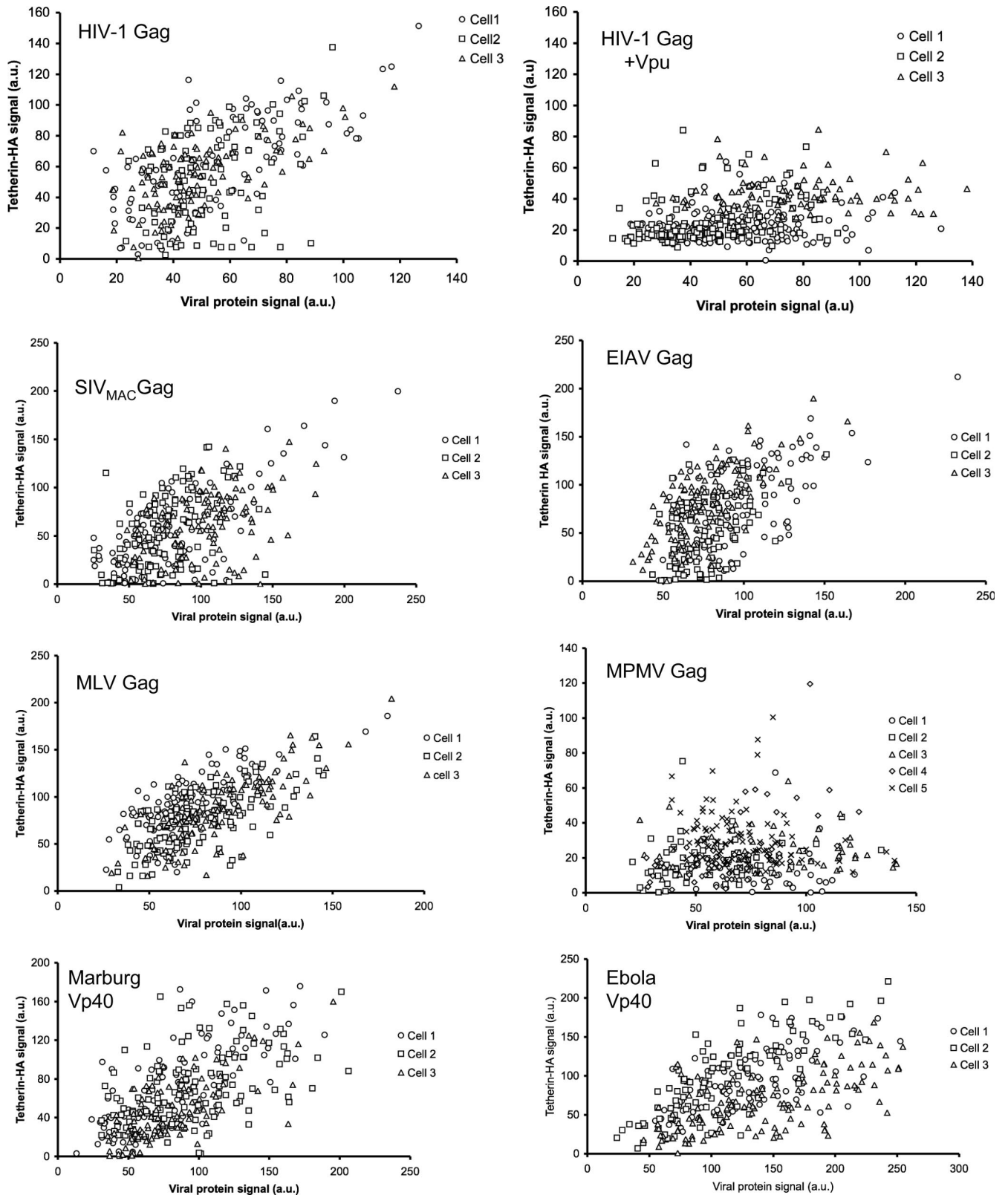
FIG. 6. Quantitative analysis of colocalization between tetherin and nascent VLPs. Images were acquired at the focal plane representing the cell-coverslip interface. For each viral protein-GFP fusion, 10 pixel-square regions encompassing puncta or filaments of viral protein-GFP fluorescence were selected for three to five viral protein-GFP-expressing cells. The mean fluorescence intensities associated with viral protein-GFP (x axis) and tetherin (y axis) in each region are plotted. a.u., arbitrary units.

esis pathways. The fact that the release of all VLPs examined was inhibited, at least to some extent, suggests that tetherin might be a very broad antiviral factor that restricts the spread of many envelope viruses. In particular, these findings suggest that tetherin functions by targeting a component of retroviruses and filoviruses that is shared among them even in the absence of viral protein sequence homology. This idea, coupled with the fact that the tetherin protein essentially consists of two membrane anchors linked by a predicted coiled coil, makes attractive the notion that tetherin restricts particle release by "cross-linking" the viral and host lipid bilayers after budding. The striking colocalization of tetherin with VLPs at the plasma membrane is also consistent with such an idea.

The inhibition of virion release by tetherin did not lead to a dramatic accumulation of cell-associated viral proteins. The reasons for this are not clear but suggest that the VLPs that are retained by tetherin are destroyed at rate that exceeds or is not greatly different from that of their synthesis. This would likely be through endocytosis, which we and others have previously shown to be the fate of Vpu-defective HIV-1 (12, 21), followed by lysosomal degradation. Moreover, it may simply be the case that only a fraction of the viral protein that is synthesized is actually released as particles. Thus, the amount of viral protein that is observed in cell lysates would be determined by its intrinsic turnover rate rather than particle release versus retention.

Many enveloped viruses that bud through the plasma membrane might have been placed under evolutionary pressure to avoid or antagonize tetherin activity, as does HIV-1 by expressing Vpu. In this study, we deliberately generated VLPs using minimal viral components wherever possible to avoid the potentially confounding variable of undiscovered antagonists that might obscure tetherin activity. Nonetheless, our findings beg the question of how viruses that are intrinsically sensitive to tetherin might avoid its activity. There is at least one other example of a potential tetherin antagonist, namely, the K5 protein of Kaposi's sarcoma-associated herpesvirus, which is able to reduce steady-state levels of tetherin when overexpressed (2). Thus, it is conceivable that accessory proteins of unknown function that are encoded by several complex retroviruses could include those that exhibit antitetherin activity. Additionally, some reports suggest that certain Env proteins, particularly those from HIV-2 and certain HIV-1 strains, exhibit Vpu-like activity (6, 30) and could, therefore, represent tetherin antagonists. Since Env proteins are, by definition, membrane proteins, one could envisage that other viral Env proteins could possess such activities. Among the VLPs tested, MPMV was the only one for which a full-length viral genome was used to generate VLPs because previous reports indicated that Env is required for the transport of capsids to the plasma membrane for envelopment (31). Curiously, particles generated by this construct appeared to be somewhat less sensitive to low levels of tetherin than those generated by Gag proteins of other retroviruses. Whether this is an intrinsic property of MPMV particles or is due to the presence of a tetherin antagonist in MPMV (e.g., Env) is unknown. However, we did notice that levels of tetherin expression appeared to be reduced in cells transfected with a mixture of MPMV proviral and Gag-GFP expression plasmids, hinting at the latter possibility.

Tetherin could also be avoided by viruses in the absence of

a direct antagonism of its function. Viruses that do not induce a strong IFN response or attenuate the IFN response through the action of viral inhibitors may not require a tetherin antagonist, particularly if they replicate in cells or tissues that do not ordinarily express tetherin. Another possible way for enveloped viruses to avoid tetherin would be to bud through membranes, or membrane domains, from which tetherin is absent. Tetherin harbors a glycosylphosphatidylinositol anchor and may therefore be concentrated into putative cholesterol-rich domains at the plasma membrane (15). Several enveloped viruses, including Eb and HIV-1, have been reported to assemble at cholesterol-rich plasma membrane domains (3, 24), and colocalization analysis of most nascent retrovirus and filovirus VLPs with tetherin revealed a good correlation between the presence of viral protein and tetherin signals at the plasma membrane. Thus, if it is indeed the case that particular membrane microdomains are selected by viruses for budding, then tetherin's localization might facilitate encounters with nascent virions. Accordingly, the intrinsic properties of viral structural proteins, in particular, the specific membrane domains to which they are targeted, might influence tetherin sensitivity. Nevertheless, these data show that tetherin can act in a very broadly specific way, as is characteristic of innate immune effector activities. An understanding of precisely how tetherin and its antagonists function could therefore provide therapeutic opportunities for a variety of viral infections.

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