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Immunoelectron Microscopic Evidence for Tetherin/BST2 as the Physical Bridge between HIV-1 Virions and the Plasma Membrane

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Abstract

Tetherin/BST2 was identified in 2008 as the cellular factor responsible for restricting HIV-1 replication at a very late stage in the lifecycle. Tetherin acts to retain virion particles on the plasma membrane after budding has been completed. Infected cells that express large amounts of tetherin display large strings of HIV virions that remain attached to the plasma membrane. Vpu is an HIV-1 accessory protein that specifically counteracts the restriction to virus release contributed by tetherin. Tetherin is an unusual Type II transmembrane protein that contains a GPI anchor at its C-terminus and is found in lipid rafts. The leading model for the mechanism of action of tetherin is that it functions as a direct physical tether bridging virions and the plasma membrane. However, evidence that tetherin functions as a physical tether has thus far been indirect. Here we demonstrate by biochemical and immunoelectron microscopic methods that endogenous tetherin is present on the viral particle and forms a bridge between virion particles and the plasma membrane. Endogenous tetherin was found on HIV particles that were released by partial proteolytic digestion. Immunoelectron microscopy performed on HIV-infected T cells demonstrated that tetherin forms an apparent physical link between virions and connects patches of virions to the plasma membrane. Linear filamentous strands that were highly enriched in tetherin bridged the space between some virions. We conclude that tetherin is the physical tether linking HIV-1 virions and the plasma membrane. The presence of filaments with which multiple molecules of tetherin interact in connecting virion particles is strongly suggested by the morphologic evidence.


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Introduction

HIV interacts with a series of host proteins that facilitate its replication in cell. One of the clearest examples of this dependence on host machinery is the interaction between the p6 region of the HIV-1 Gag protein and components of the cellular ESCRT machinery that are required for viral budding [1,2]. Conversely, some host cell factors act to limit viral replication, and are collectively known as host restriction factors. Host cell restriction factors have been identified that target specific steps in the human immunodeficiency virus type 1 (HIV-1) lifecycle, including APOBEC3G [3,4,5], Trim5α [6,7], and recently tetherin [8,9]. These innate cellular defenses are constitutively expressed by host cells and can be upregulated in response to viral infection through the expression of type 1 interferons (IFNs). Viruses in turn have evolved to express adaptor molecules that counteract important host cell restrictions, as illustrated by the Vif protein of HIV, which enhances the proteasomal degradation of APOBEC3G, and the Vpu protein, which relieves the host restriction imposed by tetherin. HIV-1 Vpu is a 16-kDa type 1 integral membrane protein [10,11]. Vpu operates as a multifunctional adaptor protein causing surface down-regulation and proteasomal degradation of CD4 in infected T lymphocytes [12,13,14] and enhancing viral particle release [15,16]. These two activities are separable, mapping to distinct structural domains and occurring in different subcellular compartments [17,18]. The particle release activity of Vpu was noted long ago to be cell-type dependent [8,19,20,21]. The presence of a host restriction factor acting at the level of particle release was suggested several years ago by experiments in which heterokaryons between restrictive and permissive cell lines exhibited a dominant restriction to particle release that was relieved by Vpu [16]. Recently the host cell restriction factor inhibiting particle release in the absence of Vpu was identified as bone marrow stromal cell antigen 2 (BST-2), also known as HM1.24, CD317, or tetherin [8].

Tetherin is a 28- to 36-kDa, type II integral membrane glycoprotein. The atypical topology of tetherin is comprised of a short N-terminal cytoplasmic tail, a single transmembrane...
spanning region, and a glycosyl-phosphatidylinositol (GPI) anchor at its C-terminus. Tetherin's subcellular distribution is punctate on the plasma membrane and is also found on intracellular endosomal membranes, particularly the trans-Golgi network [22,23]. Tetherin expression is constitutive in restrictive human cell lines, including HeLa, H9, Jurkat, Molt4, primary T lymphocytes, and primary macrophages, and is absent in cells that are permissive for particle release, such as 293T, HOS, and HT1080 [8]. Tetherin expression is IFN-inducible, and IFN treatment of permissive human cell lines results in a restrictive, Vpu-dependent particle release phenotype. Tetherin overexpression results in a dramatic accumulation of virion particles attached to the plasma membrane of cells infected with vpu-deficient viruses. Virions are often found clustered at focal areas on the plasma membrane, and appear to be attached in long linear arrays extending from these plasma membrane foci [8,15]. As the name implies, tetherin is presumed to provide a physical tether between the plasma membrane and retained virions. However, the mechanism by which tetherin attaches nascent, mature HIV-1 virions to the plasma membrane remains to be fully elucidated. Tetherin and virions appear to colocalize by confocal microscopy at the particle budding site in some reports [8,9,24], but not in others [25]. Immunoelectron microscopy of HA-tetherin failed to demonstrate concentration of the protein at particle budding sites [25], and no definitive immunoelectron microscopic analysis of endogenous tetherin at the particle budding site has yet been reported. Furthermore, tetherin was recently found to be lacking on virions released from HeLa cells by shearing, suggesting that perhaps tetherin is not acting directly as a physical tether at all [26].

In this study, we provide evidence that tetherin is the physical linkage responsible for attachment of nascent HIV-1 virions to the plasma membrane. Partial protease stripping experiments utilizing both over-expressed and endogenous tetherin sources demonstrated incorporation into HIV-1 virions. In the absence of Vpu, tetherin was enriched on filamentous structures connecting virions to the plasma membrane, and was present between chains of tethered viral particles present in focal accumulations in infected cells. These results confirm tetherin’s role in the physical attachment of virions to the plasma membrane and to each other in the absence of Vpu.
This band was substantially smaller than the full-length HA- representing a uniform cleavage product with a protected HA tag. HA-tetherin on this blot formed a single dominant band at 13 Kd, retroviral particle density enriched in Gag proteins (Fig. 2B). Incorporate tetherin as evidenced by detection at a typical

Figure 1. Interferon-α induces the expression of tetherin in A3.01 and 293T cells. (A) A3.01 cells were treated with 3000 U/ml of IFN-α for 24 hours (lane 2) and compared with untreated cells (lane 1). Cell lysates were harvested and analyzed by Western blotting using a rabbit anti-tetherin polyclonal antiserum. Detection was performed via infrared detection on a LiCor Odyssey instrument. (B) 293T cells were treated overnight with 5000 U/ml of IFN-α (solid line) and the cell surface density of tetherin was then analyzed by flow cytometry. Unstimulated 293T cells (filled line) and a secondary antibody alone sample (dotted line) were analyzed as controls. (C) A3.01 cells were treated overnight with 3000 U/ml IFN-α. Solid unfilled line represents post-IFN cell surface labeling with anti-tetherin antiserum. doi:10.1371/journal.ppat.1000749.g001

incorporate tetherin as evidenced by detection at a typical retroviral particle density enriched in Gag proteins (Fig. 2B). HA-tetherin on this blot formed a single dominant band at 15 Kd, representing a uniform cleavage product with a protected HA tag. This band was substantially smaller than the full-length HA-tetherin observed in untreated cell lysates (Fig. 2A). This is in fact the size that would be expected following cleavage at the predicted subtilisin cleavage site following residue 67 (motif RNVTH, residues 64–68 of tetherin). Recognizing that over-expression of a membrane protein could result in its incorporation into virions in a non-physiologic manner, we next sought to detect endogenous tetherin on released virions from infected T cells. A3.01 cells were infected with VSV-G-pseudotyped NL4.3/Udel, cultured for 48 h, incubated with IFN-α for an additional 24 hours, and then subjected to gentle proteolytic digestion using TPCK-treated trypsin. Protease stripped supernatants were again concentrated and separated on a linear sucrose gradient. Endogenous tetherin was enriched in the peak viral fraction and co-sedimented precisely with NL4.3/Udel virions (Fig. 2C). Interestingly, two major species of tetherin were detected on virions by Western blotting. The presence of both forms was consistent with Western blot analysis performed on IFN-α stimulated A3.01 cell lysates (Fig. 1A), and suggests that the gentle trypsin treatment released virions without cleaving all of the full-length, virion-associated tetherin. These data demonstrate that tetherin is incorporated onto virions, and would be consistent with the proposed role of tetherin in physically linking particles to the plasma membrane and to each other. We next examined wildtype particles for the presence of tetherin. IFN-stimulated A3.01 cells were infected with wildtype NL4.3, and particle gradients performed as for NL4.3/ Udel. No tetherin was detected in the peak fractions from NL4.3 virions, indicating that they failed to incorporate endogenous tetherin due to the influence of Vpu (Fig. 2C). NL4.3-infected cells stripped with TPCK-treated trypsin released low amounts of virions and also failed to concentrate tetherin (data not shown). The incorporation of endogenous tetherin on NL4.3/Udel virions released by gentle protease digestion, and not wildtype NL4.3 virions, is consistent with a physical tethering of HIV virions that is overcome by Vpu.

Tetherin is Concentrated along Filamentous Connections between HIV-1 Virions

The results above are supportive of the idea that tetherin is present on particles and functions as a physical tether. However, we thought that more direct evidence would require demonstration of tetherin at the budding site and within virion-virion connections in the patches of tethered virions frequently seen when restrictive cells are infected with vpu-deficient HIV-1. We therefore performed immunoelectron microscopic analysis of tetherin in A3.01 cells under a variety of conditions, including unstimulated cells, cells that had been subjected to IFN-α stimulation, and following infection with NL4.3/Udel. Several techniques were pursued in order to achieve specific staining. We first performed immunostaining with polyclonal anti-tetherin antisera prior to embedding in cells that had been lightly fixed with paraformaldehyde. Cells were then extensively washed and exposed to secondary antibody conjugated to 6 nm gold particles, followed by fixation, embedding, sectioning, and examination by transmission electron microscopy. IFN stimulated A3.01 cells maintained typical T-lymphocytic morphology (Fig. 3A). Tetherin staining in uninfected cells was not diffuse, but was detected at focal membrane projections and small pits along the plasma membrane (Fig. 1B and 1C). Detection of tetherin by immunogold labeling in this manner in unstimulated cells was minimal (less than 0.1% of cells examined, Fig. S1A and S2A), while specific labeling was observed on 3.5% of IFN-stimulated cells (Fig. S2A). Infection of IFN-stimulated A3.01 cells with NL4.3/Udel resulted in vast focal accumulations of mature virions attached to the plasma membrane (Fig. 3D). Strikingly, within the tethered patches of virions, filamentous structures
connecting mature virions exhibited positive tetherin immuno-gold labeling (Fig. 3E and F, arrows). In each case, the immunogold beads appeared to be arrayed upon a filamentous, electron-dense substrate. The apparent filaments were sometimes noted to link multiple virions together (Fig. 3F). We note that the length of the linearly-arrayed tetherin in these micrographs appears inconsistent with a simple protein bridge, but could represent tetherin on an extended membranous projection (see Discussion).

Tetherin localizes to HIV-1 budding sites on the plasma membrane of infected cells

In order to generate robust tetherin immunogold labeling, A3.01 cells in the experiments above were only slightly fixed, causing aberrant virion morphology. In an attempt to improve virion morphology while addressing the same question, IFN-stimulated, NL4.3/Udel infected A3.01 cells were treated with indinavir to inhibit particle maturation, then stained prior to complete fixation and embedding as before. We reasoned that

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**Figure 2.** Tetherin is incorporated into virions released by protease treatment of NL4.3/Udel infected cells. 293T cells stably expressing an N-terminal, HA-tagged tetherin were transfected with NLUdel as described in Materials and Methods. (A) Cell lysate of 293T cells expressing HA-tetherin (+) or parental 293T cells (−), probed with anti-HA antibody. (B) At two days post-transfection, cell monolayers were digested with 5 ug/ml of subtilisin for one hour at 37°C. Supernatants were concentrated through a sucrose cushion and pelleted material was then layered onto a linear sucrose gradient. Fractions were analyzed by Western blotting using an anti-p24 monoclonal antibody (top blot) and anti-tetherin polyclonal antisera (bottom blot). (C) A3.01 cells were infected with VSV-G pseudotyped NLUdel and treated with 3000 U/ml of IFN-α at 3 days post-infection. The following day, IFN-stimulated cells were treated with 3 μg/ml TPCK-trypsin for 4 hours at 37°C. Supernatants were then analyzed as in (A). Density of peak gradient fraction is indicated by arrow. (D) NL4.3 virions released from IFN-stimulated A3.01 cells were harvested and analyzed by gradient centrifugation (without protease treatment).

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Tetherin is the physical link between linear chains of HIV-1 particles

We demonstrate above that tetherin is present in concentrated fashion at focal sites of particle budding. We next asked how the long strings of virions observed in many studies of vpu-deficient virions might form. Fig. 5 presents a series of micrographs in which immunogold staining was observed between virions within apparent chains or strings. Most common were the clustered immunogold beads between immature particles as shown in Fig. 5A and 5B. A second pattern was similar to the filamentous structures already described (Fig. 5C and 5D). The stacked, parallel appearance in some sections was suggestive to us of a helical filament cut in cross section (Fig. 5C), while in others a more linear extended connection was apparent (Fig. 5D). Tetherin staining appeared to be present within chains extending several microns away from the plasma membrane, and was not observed more than 50 nm away from particles (illustrated by Fig. 5F). To demonstrate that the presence of tetherin between virions was not due to aggregates or non-specific sticking of the anti-rabbit conjugated 6nm gold beads, we substituted 10nm protein A coated gold beads. Chains of NL4.3/Udel virions demonstrated 10nm gold beads connecting the virions to each other and to the plasma membrane.
membrane, confirming the previous findings (Fig. 5I). In this experiment, extended filaments were not readily apparent, but rather the protein A gold was found directly between adjacent particles.

To further confirm the specificity of the observed labeling, identical experiments were performed in infected cells in the absence of labeling with the primary antibody. Strings of immunogold particles were not observed in over 50 sections examined from cells infected with NL4.3/Udel either with (Fig. S1D–F) or with indinavir (Fig. S1G). Individual gold particles were sometimes observed on the cell surface in these experiments, but at a much reduced frequency (detailed in Fig. S2A).

Figure 4. Immunoelectron micrographic analysis of tetherin of NL4.3/Udel-infected A3.01 cells treated with indinavir; labeling at site of particle budding. (A) Low magnification image of infected cells exhibiting accumulation of immature tethered virions, bar = 2μm. (B) Immunogold labeling of tetherin localized to particle budding sites on the surface of IFN-α stimulated cells. Bar = 100nm. (C) Higher power view from plasma membrane region exhibited in (B); bar = 50nm. Tetherin-positive filamentous structures are shown connecting immature particle to the plasma membrane. (D) Immunogold labeling of plasma membrane region exhibiting accumulation of tethered immature virions and significant labeling of membrane budding site. Bar = 100nm. (E) Higher power view of membrane proximal region from (D); bar = 50nm. HIV-1 virions attached to the plasma membrane exhibit significant tetherin immunolabeling. (F) Tetherin immunolabeling at virion budding locations along plasma membrane. Arrow indicates cluster of tetherin immunolabeling on particle membrane, at “open” end of virion shell. Bar = 100nm. (G) Higher magnification view of tetherin-positive staining at particle budding site. Bar = 50nm. (H) Additional view of tetherin immunolabeling on “open end” of immature virion. (I) Immunolabeling of tetherin associated with virion lipid envelope, bar = 50nm.

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Cryosectioning and immunogold labeling confirms direct tetherin association with particles

A second technique to establish the relationship between tetherin and virion budding sites and particles was desirable. We continued this examination with A3.01 cells that had been infected with NL4.3/Udel and IFN-stimulated, but performed cryosectioning followed by immunolabeling with the identical polyclonal anti-tetherin sera and secondary goat anti-rabbit antibody conjugated to gold. Staining was observed predominantly on the plasma membrane (PM, Fig. 6A) but also in apparent intracellular vesicles (V, Fig. 6A). A low level of apparent background staining was seen outside of cells (Fig. 6A). Importantly, the majority of virion particles observed were labeled with gold particles, and overall staining outside of 50 nm from particles was rare (Fig. 6B, 6C). We counted the number of gold beads associated with 100 consecutive virus particles labeled in the presence or absence of primary antibody. Background in the absence of primary antibody was exceedingly low (Fig. S1H and S1I), and the mean number of...
gold beads per virion stained with the anti-tetherin antibody was 9.3±6 (Fig. S2B), while the mean number of gold beads was 0.2±6 in control experiments omitting the primary antibody. Tetherin staining was specifically located at the base of viral particles bridging the virion membrane and plasma membrane, similar to what had been observed with the pre-embedding labeling technique (Fig. 6D). We slightly altered the fixation technique and repeated the cryosectioning and immunostaining experiment, (Fig. 6E and 6F). With this alteration in methods, very little background staining was observed, and 91% of gold beads were found to be located within 50nm of visible particles. The number of gold beads per virion in this experiment was 3.2±2.2, while using pre-immune sera the mean number of gold beads per virion (within 50nm) was 0.2±0.2 (Fig. S3, panels A–C). Taken together, these data are supportive of the role of tetherin as a physical link between the plasma membrane and virions, and suggest a potential role for a filamentous connection or substrate on which tetherin concentrates that contributes to the tethering phenomenon.

**Discussion**

Tetherin is a restriction factor that restricts the release of retroviruses and filoviruses [27,28,29] by inducing their accumulation at the plasma membrane of infected cells. Vpu overcomes this restriction for HIV, while HIV-2 and SIVs have evolved distinct mechanisms to counteract the block. In the case of HIV-2 Rod10 isolate, the envelope glycoprotein counteracts tetherin, while Nef fulfills this function for a number of SIV strains [30,31]. The mechanism by which Vpu lifts the restriction to particle release imposed by tetherin remains under study. Downregulation of cell surface tetherin was observed initially by the Guatelli laboratory [9], providing a conceptually simple model that remains under study. Downregulation of surface tetherin has not been universally observed despite the clear relief of restriction by Vpu [26]. Evidence for Vpu-mediated degradation of tetherin by proteasomal [25,32] or lysosomal [24] pathways has been presented. The potency of the block to particle release induced by tetherin makes it an exciting area of investigation, both for its ability to enlighten understanding of intrinsic host restriction mechanisms and for its therapeutic implications.

The work presented here addressed a simple question: is tetherin the physical connection that links retained virions to the plasma membrane and to each other? Surprisingly, this had not yet been established, and some data to the contrary has been presented. During final revision of this manuscript, however, a paper strongly supporting tetherin as the physical link between virions and the plasma membrane was published [33]. In this paper, Perez-Caballero and coworkers demonstrate that tetherin is directly incorporated onto the membrane of HIV-1 particles. Our
data agree with this finding, and support a model in which tetherin is present in the correct location to act as a physical tether. Tetherin is present at the budding site, it links particles together in long chains or strings, and it is present on released virion particles. These results certainly imply that removal of tetherin from the site of budding should remove the restriction, supporting the contention that cell surface downregulation of tetherin should correlate with relief of restriction. Results here were performed with endogenous tetherin in an infected T cell line, chosen because it is a prototypical cell line exhibiting a Vpu-responsive (tetherin-mediated) restriction to particle release. It will be important to repeat these experiments in infected primary T cells and macrophages, but we anticipate that the presence of tetherin at the site of particle retention will be similar.

In some of our experiments, an extended filamentous electron-dense structure formed a bridge between retained virions. These filamentous structures demonstrated strong immunolabeling with tetherin. The length and intensity of labeling of these filaments makes it clear that multiple tetherin molecules are present, and that they cannot be tetherin dimers alone. The distance covered by these linear structures is too great to represent a protein bridge formed by tetherin-tetherin interactions. It is possible that membrane extensions bearing a high concentration of tetherin formed these apparent filaments. We observed these structures more consistently using the pre-embedding labeling technique than in cryosections, but failed to observe them at all in the absence of primary antibody or when pre-immune sera was employed. Thus we think they are specific structures worthy of additional investigation. Tetherin is intimately associated with the actin cytoskeleton [34], and focal areas of intense cortical actin were apparent underlying focal collections of tetherin in some sections (not shown). It is possible that actin or actin-associated proteins also contribute to the linear and perhaps helical filamentous connections we observed that were studded with tetherin.

In summary, tetherin was specifically associated with particle budding sites and was found between virions in chains. These data support a physical role for tetherin in retaining virion particles in restrictive cells.

Materials and Methods

Ethics statement

Animals for production of antisera were housed and handled at Cocalico Biologicals, Inc., Reamstown, PA. All animals were handled in strict accordance with good animal practice in accordance with NIH’s Office of Laboratory Animal Welfare as reviewed by the Institutional Animal Care and Use Committee (IACUC) at Cocalico Biologicals (Animal Welfare Assurance number A3669-01).

Cell lines and plasmids

293T cells (obtained from the American Type Culture Collection) were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (PS). A3.01 cells (a gift from Klaus Strebel, NIH) were propagated in RPMI-1640 supplemented with 10% FBS, 2mM L-glutamine, and PS. A HA-tetherin cell line stably expressing an N-terminal, HA-tagged tetherin was provided by Klaus Strebel, NIH) were propagated in RPMI-1640 supplemented with 10% FBS, 2mM L-glutamine, and PS. A HA-tetherin cell line stably expressing an N-terminal, HA-tagged tetherin was obtained from the American Type Culture Collection (ATCC). The HA-Tetherin sequence was amplified by PCR cloning and inserted into pCMV-HA (Clontech). The HA-Tetherin sequence was amplified by PCR cloning and inserted into the Age1-Pac1 sites of pCMV-IA-HA (Clontech). The HA-Tetherin sequence was amplified by PCR cloning and inserted into the Age1-Pac1 sites of the retroviral vector pQCXIP (Clontech) to generate pQCXIP-HA-tetherin.

Retroviral stock production

HIV-1 viral stocks were generated by Eugene HD (Roche Diagnostics) co-transfection of 293T cells with the molecular clone, pNL4-3/Udel, and the vesicular stomatitis virus glycoprotein G (VSV-G) [35]. pCMV-HA-Tetherin is a plasmid encoding an N-terminal, HA-tagged tetherin obtained by PCR cloning of tetherin cDNA into the SalI-Xhol sites of pCMV-IA-HA (Clontech). The HA-Tetherin sequence was amplified by PCR cloning and inserted into the Age1-Pac1 sites of the retroviral vector pQCXIP (Clontech) to generate pQCXIP-HA-tetherin.

Generation of tetherin antisera

Anti-tetherin antiserum was elicited in rabbits at Cocalico Biologicals, Inc. (Reamstown, PA, USA) using a recombinant GST-tagged tetherin fusion protein. The tetherin fragment was composed of the entire ectodomain spanning amino acids 43–179 inserted into vector pGEX6p-1 (GE LifeSciences). The tetherin fusion construct was concentrated from clarified bacterial sonicates using glutathione sepharose 4B beads. The GST-tag was excised with PreScission protease (GE Life Sciences) and the supernatants were then further purified using cation-exchange chromatography (HiTrap Q HP, GE Life Sciences) and the supernatants were harvested and stored at −80 °C.

Flow cytometry

5×10⁶ 293T cells were propagated overnight in 10cm² dishes; A3.01 cells were maintained in suspension culture. The cells were incubated for 24 h in the presence of 5000 U/ml IFN-α. On the following day the cell monolayers (293T) was washed twice with pre-warmed PBS and cells detached using 3 ml of Versene (0.2 g/l EDTA-4Na in PBS; Invitrogen) per dish. Cells were then pelleted and washed repeatedly with ice-cold PBS. Cells were resuspended in 2% BSA-PBS and allowed to incubate on ice for 10 min prior to the addition of primary antibody (rabbit α-tetherin) for 1 h on ice. The cells were washed twice with 2% BSA-PBS followed by the addition of Alexa Fluor 633-conjugated anti-rabbit IgG secondary antibody (Molecular Probes; Invitrogen) diluted to 1 μg/ml in 2% BSA-PBS. The mixture was allowed to incubate in the dark for 30 min on ice. The stained cells were analyzed using a FACS Canto (BD Biosciences) flow cytometer. Subsequent data analyses were performed using FlowJo 7.2.4 (Tree Star).
Cellular protease stripping experiments and sucrose density gradient analysis

In order to determine the presence of over-expressed tetherin on HIV-1 virions, 5 × 10⁶ HA-tetherin cells were plated per 10 cm² dish. The following day, the cells were transfected with Fugene HD using 3 µg pNL4-3/Udel per dish. At 48 hours post-transfection, the cells were washed twice with phosphate-buffered saline (PBS; pH 7.4). Cell monolayers were then incubated for 30 min at 37°C in 3.5 ml of Tris-Cl (pH 8.0), 150 mM NaCl, 5 mM CaCl₂, and 5 µg/ml sodium butyrate (Sigma Aldrich). The digest was stopped by the addition of 3.5 ml of PBS-containing growth media plus protease inhibitors. Cellular supernatants were clarified by low-speed centrifugation and then purified by ultracentrifugation through a 20% sucrose cushion (100,000 g for 3 hours, 4°C). Pellets were then resuspended in 1 ml of PBS and overlaid onto linear 20–60% sucrose gradients. Ultracentrifugation of gradients was performed overnight at 100,000 × g at 4°C. Equal 900 µl fractions were collected and density determined using a refractometer. Samples were diluted in PBS and concentrated using a micro Centrifuge (2 hours at 100,000 g; 4°C). Pellets were subsequently resuspended in 1 × SDS-PAGE load buffer containing 100 mM DTT. Analysis of fractionated material was performed by Western blotting using anti-p24 hybridoma 183-H12-5C (obtained from Bruce Chesebro and Hardy Chen through the NIH AIDS Research Reference and Reagent Program) supernatants (1:1000) and rabbit anti-tetherin polyclonal antisera (1:2000).

Endogenous tetherin incorporation into HIV-1 virions was assessed by infecting A3.01 cells with VSV-G pseudotyped NL4.3/Udel, 1.0 × 10⁶ A3.01 cells were infected with VSV-G pseudotyped NL4.3/Udel at an MOI of 0.75. The cells were cultured for 3 days prior to the addition of 3000 U/ml IFN-γ. On day 4 post-infection, the cells were pelleted at 350 × g for 5 minutes at room temperature and washed twice with 10 ml PBST. The cells were resuspended in 5 ml serum-free growth media supplemented with 3 µg/ml TPCA-treated tryptic and incubated for 3 hours at 37°C. The protease activity of the supernatant was ablated by the addition of 5 ml of PBS-containing growth media plus protease inhibitors. The subsequent analysis of cellular digests was performed as described above.

Electron microscopy

A3.01 cells were infected with VSV-G pseudotyped NL4.3/Udel and stimulated with IFN-γ as described above. A subset of cell cultures were treated for 24 h prior to harvest with 5 µM indinavir to inhibit proteolytic particle maturation and assist in maintenance of virion morphology. At 48 hours, cells samples were fixed, sectioned, and stained for examination by transmission electron microscopy as previously described [37]. Pre-embedding immunogold labeling of samples for tetherin was performed by gentle fixation in 4% paraformaldehyde for 20 min at room temperature (RT). The cells were then extensively washed with 1% BSA-PBS and blocked with 1% gelatin-PBS for 30 min at RT. Primary antibody (rabbit anti-tetherin antisera) was diluted to 1:300 in 1% gelatin-PBS and incubated with cells for 1.5 hours at RT with gentle agitation. The cells were then washed extensively, fixed with 2.5% glutaraldehyde for 1 hour at RT, and postfixed with 1% osmium tetroxide at 4°C for 1 hour. Dehydration with ethanol was performed and the cells were embedded in Eponate-12 resin. Regular 70 nm ultra-thin sections were produced, double stained with uranyl acetate and lead citrate, and observed under a Hitachi H7500 transmission electron microscope at 75 KV. Infected and control A3.01 cells were also analyzed by immuno-EM techniques on ultra-thin cryosections. Cryosections were generated by fixing cells in freshly made 4% paraformaldehyde in PBS for 20 minutes at RT. Tissue blocks were embedded in 10% gelatin. In a variation of this procedure, an additional fixation step with 0.7% gluteraldehyde for 40 minutes was performed. The cells were then washed extensively with PBS prior to incubation with 2.3 M sucrose, or alternatively 30% polyethylene glycol (PEG) as a cryoprotectant. The cell pellets were frozen using liquid nitrogen under controlled conditions (cryogen) and sectioned using a cryo-ultra microtome generating 70 nm cryosections. The sections were thawed on nickel grids with formvar supporting membranes prior to antibody labeling. The samples were blocked using 1% BSA/1% gelatin in PBS for 1 h at RT. Glycine was then used to block any free aldehyde groups present. The samples were incubated in 5 µg/ml of primary rabbit anti-tetherin antibody in 1% BSA/1% gelatin overnight at 4°C. The sections were washed extensively prior to the addition of secondary goat anti-rabbit IgG conjugated with 6 nm gold beads for 1 hour at RT. The sections were then air dried in a film of methylcellulose with 1% uranyl acetate prior to observation under a Hitachi H7500 transmission electron microscope. Control experiments included unlabeled cells, cells labeled in the absence of IFN stimulation, uninfected cells either treated or untreated with IFN, and samples from each experimental group in which the primary antibody was omitted during the immunolabeling procedure. A minimum of 25 cells were examined for each experimental arm and control when recording labeling positivity. A total of 70 cells and their associated viruses were examined to derive the mean number of immunogold particles present per virion (counting as positive those gold particles within 50nm of the virion) in the cryosection-immuno-labeling experiments.

Supporting Information

Figure S1 Micrographs from control experiments. All cells are A3.01 cells. (A) Untreated cells demonstrate lack of background surface labeling; secondary antibody only. Bar = 200nm. (B) Tetherin labeling in uninfected cells that were not treated with IFN. Rare collections of focal staining (arrow) were noted; quantitation included in Fig. S2. Bar = 200nm. (C) Tetherin labeling in IFN-stimulated, uninfected cells. Focal collections of immunostaining were much more common (see Fig. S2); representative focal staining shown. Bar = 100nm. (D) Infected cells, no IFN treatment, no primary antibody. No significant staining noted. Bar = 200nm. (E) Infected cells, no IFN treatment, primary and secondary antibody staining performed. Occasional focal staining noted (arrow). Bar = 200nm. (F) IFN-stimulated, infected cells, no primary antibody. No apparent immunolabeling observed. Bar = 200nm. (G) Indinavir-treated, infected cells, immunostaining in absence of primary antibody; only very rare gold beads noted. Bar = 200nm. (H) Cryosectioned cells stained without primary antibody; particles fail to label with secondary antibody conjugated to gold. Bar = 200nm. (I) Cryosectioned cells, intracellular vesicle with particles, no primary antibody used. A single gold particle noted away from virion (arrow). Bar = 200nm. Found at: doi:10.1371/journal.ppat.1000749.s001 (8.79 MB TIF)

Figure S2 Quantitation of positive immunolabeling. (A) Quantitation of positive signal with pre-embedding labeling technique. 50 cells were counted for each experimental group. Shown is the percentage of cells labeled with 6nm gold particles. Normal bar represents unstimulated cells. Note that NL4.3/Udel (NLUdel)-infected cells following IFN stimulation demonstrated significant levels of specific staining. (B) Number of particle-associated gold
beads in cryosection/immunolabeling experiments. 70 cells and all associated particles were counted in the case of the intact labeling protocol; 25 cells and associated particles were assessed for the control (no primary antibody) group. Shown is the mean ± SD number of gold particles overlying or within 50nm of virion particles. (C) IFN stimulation of A3.01 results in restriction of particle release. Shown are particles released from A3.01 cells infected with VSV-G-pseudotyped NL4.3 or NLUdel in the absence (−) or presence (+) of 3000 U/ml of IFN-α. Where present, the attached particles did demonstrate tetherin immunostaining on the outer surface, shown by pre-embedding labeling with anti-tetherin antiserum and 6nm anti-rabbit immunogold. Bar = 100nm. Shown at: doi:10.1371/journal.ppat.1000749.s003 (4.04 MB TIF)

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Author Contributions

Conceived and performed the experiments: JH JJW PS.Performed the experiments: JH JJW HY. Analyzed the data: JJW PS. Contributed reagents/materials/analysis tools: PS. Wrote the paper: PS.