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Influenza virus is not restricted by tetherin whereas influenza VLP production is restricted by tetherin

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ABSTRACT

Tetherin (ST2/CD317) is a cellular protein that restricts the release from cells of some enveloped viruses including HIV-1. To examine if influenza virus is affected by tetherin, MDCK cells constitutively expressing human tetherin and control MDCK cells were infected with influenza virus. No difference was observed in infectious titers, at 24 h or 48 h post-infection. In contrast, tetherin expression inhibited influenza virus-like particle (VLP) release into the media. Expression of the HIV protein Vpu overcame the tetherin block of influenza virus VLPs. A human tetherin mutant that lacks a C-terminal GPI anchor attachment signal (tetherin-ΔGPI) was constructed to test if this mutant could be incorporated into the released virus or VLP particles. Whereas tetherin-ΔGPI was incorporated into influenza VLPs it was not incorporated into influenza virions. Taken together these data suggest that influenza virions may contain a tetherin antagonist.

Introduction

To combat viral infections cells have developed a variety of strategies to restrict virus infections at various points in their life cycles. Tetherin (also known as CD317/BST-2/HN1.24) is an interferon-inducible integral membrane protein that contributes to the establishment of the anti-viral state; however, there is a basal constitutive level of expression in many cell types (reviewed in Evans et al., 2010).

Tetherin is a type II integral membrane protein with a cytoplasmic N-terminus and an extracellularly localized C-terminus that is post-translationally modified by addition of a glycosylphosphatidylinositol (GPI) membrane anchor. Thus, the tetherin molecule is anchored in the membrane at both of its termini. Tetherin is expressed at the plasma membrane and is localized to lipid rafts (Kupzig et al., 2003). Tetherin is a homodimer that is disulfide-linked through three extracellular cysteine residues. The ectodomain is also glycosylated by two N-linked carbohydrate chains that are heterogeneously modified (possibly by polylactosaminoglycan) that cause tetherin to migrate on SDS-PAGE as a smear of 28–45 KDa (Perez-Caballero et al., 2009).

The first enveloped virus shown to be restricted in its release from infected cells by tetherin was human immunodeficiency virus (HIV-1) (Neil et al., 2008; Van Damme et al., 2008). More recently tetherin has been shown to have a broad activity against diverse families of enveloped viruses including human immunodeficiency virus 2 (HIV-2), simian immunodeficiency virus (SIV), Ebola virus and Marburg virus, Lassa fever virus, vesicular stomatitis virus and Kaposi's sarcoma herpes virus (KSVH) (Jouvenet et al., 2009; Kaletsky et al., 2009; Radoshitzky et al., 2010; Sakuma et al., 2009; Weidner et al., 2010). Many viruses can overcome restriction of budding by tetherin using diverse viral proteins: Vpu for HIV-1; Env for HIV-2; Env/Nef interplay for SIV, GP for Ebola virus and protein K5 of KSHV (Gupta et al., 2009; Jia et al., 2009; Kaletsky et al., 2009; Le Tortorec and Neil, 2009; Mansouri et al., 2009; Neil et al., 2008; Van Damme et al., 2008). Vpu is thought to antagonize tetherin by removing it from the sites of virus assembly through internalization and proteasomal degradation (reviewed in Evans et al., 2010).

Many of the studies performed to analyze the role of tetherin in restricting the release of an enveloped virus have been performed using virus-like particles (VLPs). Recently it has been observed that whereas the release of Ebola virus VLPs are restricted by tetherin, infectious Ebola virus is not restricted by tetherin (Radoshitzky et al., 2010). This suggests that Ebola virions contain a tetherin antagonist probably excluding tetherin from the virions.

Tetherin is a lipid raft-associated apically-expressed membrane protein (Kupzig et al., 2003) and as influenza virus utilizes lipid rafts as a budding platform (Takeda et al., 2003) it was of interest to examine the effect of tetherin on influenza virus budding and on the budding of influenza VLPs. We found that whereas tetherin expression did not affect influenza virus budding, influenza VLP budding was restricted.

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Results

Influenza virus growth is not restricted by expression of tetherin

An MDCK cell line that constitutively expresses a N-terminally HA-tagged tetherin protein was generated. On SDS-PAGE, tetherin migrated heterogeneously (Fig. 1a), due to carbohydrate modification (Perez-Caballero et al., 2009), which we speculate is due to addition of polylactosaminoglycan. MDCK and MDCK-tetherin cells were infected with influenza virus A/Udorn/72 and A/WSN/33 at a multiplicity of infection of 1 plaque forming units (PFU)/cell and at 24 h and 48 h post-infection (p.i.) the infectivity of the released virus was determined. The virus titers were found to be very similar whether the virus was grown in MDCK or MDCK-tetherin cells (Fig. 1b). Analysis of the accumulation of virus-specific polypeptides in infected cells and in released virions at 24 h p.i. showed that they were comparable when influenza A/Udorn/72 virus was used to infect MDCK or MDCK-tetherin cells (Fig. 1c).

To compare influenza virus to another virus, vesicular stomatitis disease virus (VSV), that had been shown to be restricted by tetherin expression (Weidner et al., 2010) we used a trans-complementation strategy (Pawliczek and Crump, 2009). This was done to circumvent the viral titer derived from infected cells that were not transfected with DNA, due to the difficulty of being unable to transfect all the cells in a culture but being able to infect all the cells with virus. We used two defective viruses, influenza A/Udorn/M2S71 (Chen et al., 2008) and VSV/ΔG/GFP (Takada et al., 1997), a VSV that expresses green fluorescent protein in place of the G gene. Influenza A/Udorn/M2S71 was complemented by expression of M2 protein and VSV/ΔG/GFP was complemented by expression of G protein. 293 T cells were transfected with cDNA expressing tetherin and M2 or tetherin and G and 24 h later cells were infected with influenza A/Udorn/M2S71 or VSV/ΔG/GFP, respectively. For influenza virus, titers were measured by plaque assay on M2CK cells (Chen et al., 2007) and for VSV titers were determined by flow cytometry (Watanabe and Lamb, 2010). It was found that tetherin expression reduced VSV yield by 6-fold

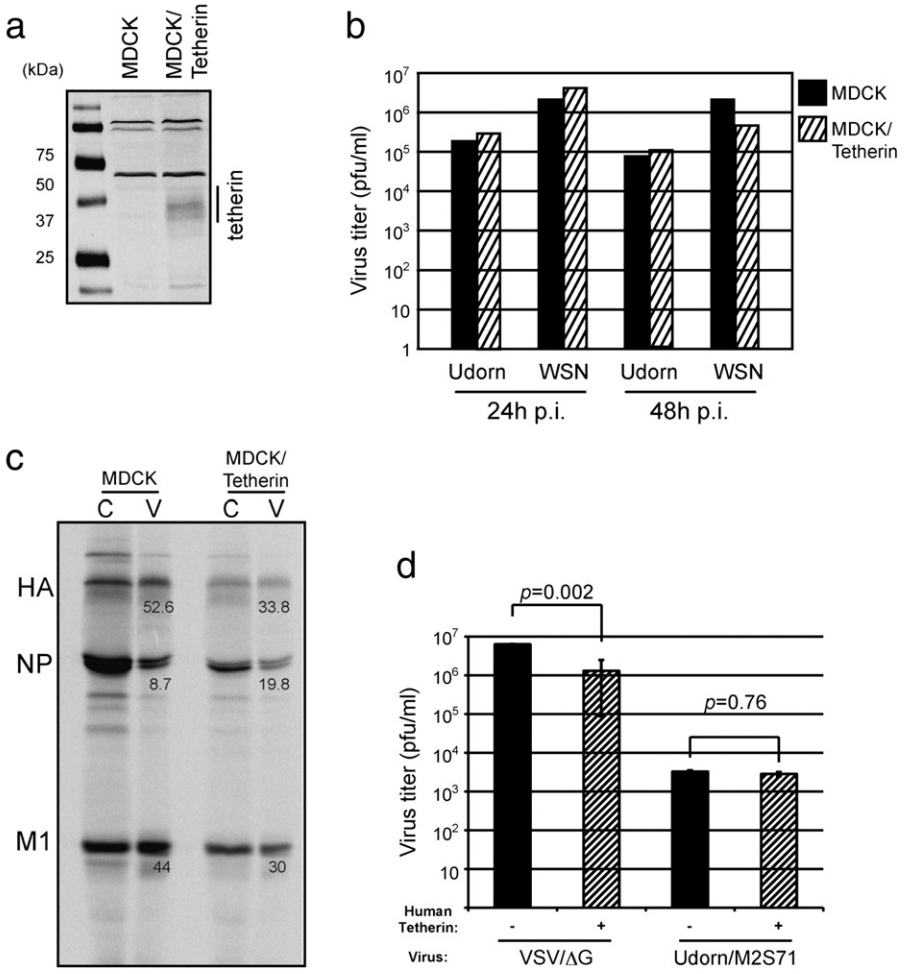

Fig. 1. Human tetherin expressed in MDCK cells does not restrict influenza virus budding. (a) Constitutive expression of HA-tagged Hu tetherin in MDCK cells. Cells were lysed in SDS-lysis buffer and polypeptides separated by SDS-PAGE followed by immunoblotting with anti-HA Ab to detect the HA-tagged tetherin. A bar indicates the heterogeneously migrating glycosylated tetherin species. (b) Infectious titer at 24 and 48 h p.i. of influenza virus (A/Udorn/72 and A/WSN/33) grown in MDCK or MDCK/tetherin cells. (c) Budding efficiency of wt Udorn from MDCK or MDCK/tetherin cells at 24 h p.i.. Infected cells were labeled with ^{35}[S] Trans-label and influenza virus-specific polypeptides in both cells and virions were immunoprecipitated using goat anti-Ud. C = intracellular polypetides; V = polypeptides released into media as virus. Numbers below bands indicate quantification of the released viral polypeptide as a percentage of total cell + virus. (d) Effect of tetherin on influenza virus and VSV budding using a trans-complementation assay. 293 T cells were plated onto gelatin-coated 6-well plate at a density of 0.3×10^6/well the day before transfection. 293 T cells were transfected with 0.5 μg plasmid DNA pCAGGS-M2 or pCAGGS-VSV G and pCAGGS tetherin. After 24 h incubation, cells were infected with influenza virus A/Udorn/M2S71 or VSV/ΔG/GFP/VSV G at MOI = 3.3. Culture supernatant was harvested at 24 h (A/Udorn/M2S71) or 7 h (VSV/ΔG) p.i. and the infectious titer was determined as described below. The infectious titer of influenza virus A/Udorn/M2S71 was determined by plaque assay using M2CK cells as described previously (Chen et al., 2007). To determine the infectious unit of VSV/ΔG/GFP, BHK cells were infected with serially diluted virus. Cells were harvested at 24 h p.i. and GFP (+) cells were detected by flow cytometry (FACSCalliber Becton Dickinson, Franklin Lakes, NJ). VSV infectious units were calculated based on the percentage of GFP (+) cells in the population.

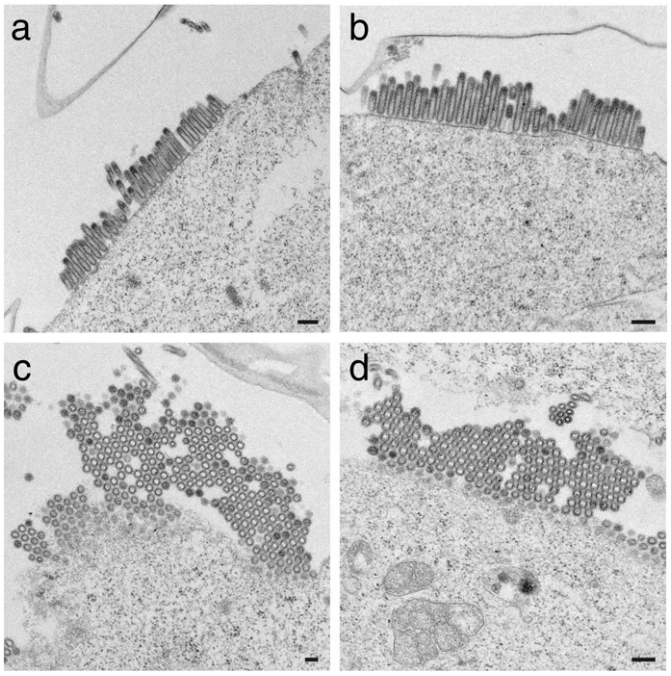

Fig. 2. Electron micrographs of influenza virus in 293 T cells with or without tetherin expression at 12 h p.i. (A) and (C) Thin sections of negative stained control infected 293 T cells. (A and B) Longitudinal section (C and D) tangential section. (B) and (D) 293 T cells infected with influenza virus (A/Udorn/72) and expressing human tetherin. 293 T cells were transfected with human tetherin and 24 h post-transfection were infected with influenza virus (A/Udorn/72). Cells were processed for electron microscopy at 12 h p.i. as described previously (Leser and Lamb, 2005).

($p = 0002$), whereas influenza virus titers were not affected ($p = 0.76$).

To examine the morphology of budding influenza virus we chose to use 293 T cells because >90% transfection efficiency could be achieved in these cells as determined by flow cytometry after transfection of a plasmid expressing green fluorescent protein. At 24 h post-transfection with and without tetherin, 293 T cells were infected with influenza A/Udorn/72 virus and at 12 h p.i. the cells were processed for thin sectioning and electron microscopy. Over 50 cells were examined and they all appeared indistinguishable in that in both longitudinal and horizontal sections tetherin expression did not alter budding morphology (Fig. 2).

Tetherin expression restricts the budding of influenza virus VLPs

It has been found for Ebola virus that tetherin expression does not restrict virus budding but it does restrict the release of VLPs from cells (Radoshitzky et al., 2010). Previously we have shown that influenza virus forms VLPs on expression of PB1, PB2, PA, NP, HA, NA, M1 and M2 (Chen et al., 2007). We tested the effect of co-expression of varying amounts of tetherin with the VLP complete plasmid set. It was found that increasing levels of tetherin expression decreased the yield of released VLPs (Fig. 3a). However, VLPs were not found at the plasma membrane with sufficient abundance to make a meaningful examination of budding with and without tetherin expression by EM analysis.

Tetherin expression at the cell surface is antagonized by expression of HIV Vpu, most likely due to Vpu causing internalization and degradation of tetherin (Iwabu et al., 2009; Neil et al., 2008; Van Damme et al., 2008). To test if influenza VLP budding restriction was sensitive to expression of Vpu, VLPs were formed in the presence of tetherin, with or without Vpu expression. As shown in Fig. 3b, Vpu antagonizes tetherin restriction of budding and VLP release was restored to levels similar to that found without tetherin expression.

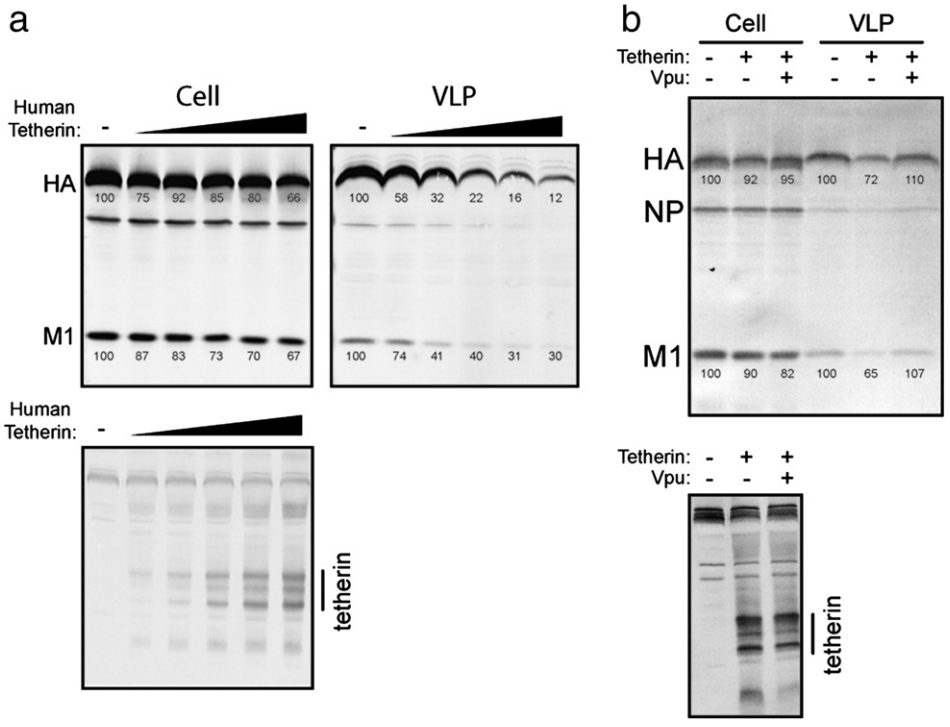

Fig. 3. Tetherin expression restricts the budding of influenza VLPs. (a) Dose-dependent restriction of VLP budding by human tetherin expression. 293 T cells were transfected with a constant amount of plasmid DNAs for VLP production and increasing amounts of human tetherin expression vector. The culture supernatant was harvested at 48 h post-transfection and VLPs collected by ultracentrifugation. Upper panel: immunoblot of influenza virus-specific polypeptides using anti-Udorn sera. Bottom panel: immunoblot using HA 12CA5 MAb to detect HA-tagged human tetherin. (b) Co-expression of Vpu overcomes tetherin restriction of VLP budding. 293 T cells were transfected with the plasmid DNAs for VLP production, and tetherin and Vpu plasmid cotransfected as indicated. Upper and lower panels in (b) are as in (a).

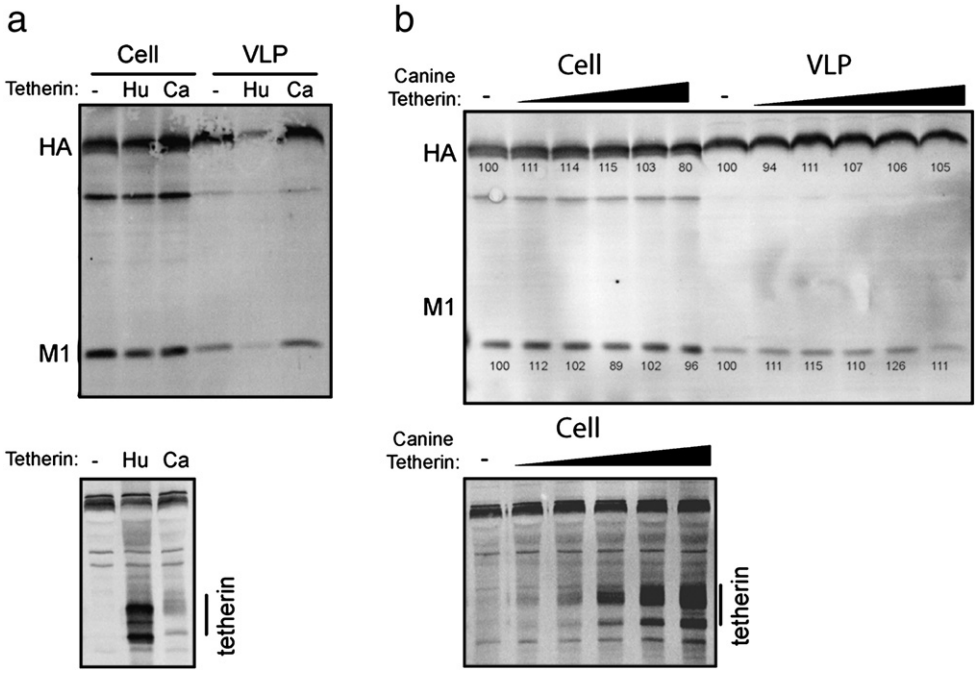

Fig. 4. Canine tetherin does not restrict influenza VLP production in MDCK cells. A full-length cDNA clone to canine tetherin was obtained by RT-PCR of RNA from MDCK cells and engineered to have a HA-tag. (a) The effect of expression of canine (Ca) tetherin and human (Hu) tetherin on VLP formation. Upper panel: Immunoblot of cell and VLP polypeptides. Lower panel: Immunoblot of HA-tagged Hu and Ca tetherin. (b) The effect of increasing amounts of Ca tetherin expression on VLP formation. Upper and lower panels as in (a).

Canine tetherin does not restrict influenza virus VLP release

Influenza virus replicates to high titer in MDCK cells (Choppin, 1969). Thus, we were interested in determining if over-expression of canine tetherin restricted influenza VLP release. Canine BST-2 (tetherin) (GenBank XM_860510.1) has the same three domains as human tetherin, the three cysteine residues involved in disulfide bond formation and two sites for N-linked glycosylation are conserved. Overall, there is 40% amino acid identity. A cDNA expressing canine tetherin was isolated using MDCK cell RNA and appropriate oligonucleotide primers. Whereas expression of human tetherin restricts influenza virus VLP release, expression of canine tetherin did not restrict VLP release (Fig. 4a). Increasing levels of canine expression had no effect on the amount of VLP release (Fig. 4b).

Human tetherin ectodomain is important for restriction of influenza VLPs

To examine the region of the human tetherin molecule important for influenza VLP restriction we expressed human/canine chimeric tetherin molecules between the N-terminal cytoplasmic region (cyt), the transmembrane domain (TM) and the ectodomain (ecto) (Fig. 5a). The four chimeric molecules were all expressed and formed disulfide-linked dimers (Fig. 5b). It was found that canine tetherin expression restricted the release of HIV VLPs (Fig. 5c) and that all the chimeric molecules restricted HIV VLP release. In contrast only chimeric tetherin molecules that contained the human tetherin ectodomain were capable of restricting influenza VLP release (Fig. 5d). However, it was noted that the chimeric molecules gave intermediate results between those observed for canine or human tetherin (Fig. 5e).

Human tetherin is incorporated into VLPs but not influenza virions

To investigate the reason influenza VLPs are restricted by tetherin whereas influenza virions are not being restricted by tetherin, we examined the possibility that tetherin was incorporated into VLPs but not into virions. To prevent tetherin linking one VLP to another we used a construct originally designed for studies with HIV-1 in which the C-terminus of human tetherin lacks 20 amino acids to avoid addition of a C-terminal GPI anchor (ΔGPI tetherin) (Perez-Caballero et al., 2009). 293 T cells were either transfected with the VLP plasmid set and ΔGPI tetherin or were transfected with ΔGPI tetherin and 24 h later infected with influenza A/Udorn/72 as described above. Released VLPs and influenza virions were analyzed by immunoblotting for ΔGPI tetherin incorporation. As shown in Fig. 6 ΔGPI tetherin was expressed in cells (Fig. 6a) and showed three–four species (Perez-Caballero et al., 2009). When VLPs and virions were examined it was found that ΔGPI tetherin was incorporated into VLPs but in contrast ΔGPI tetherin was barely detectable in virions.

Discussion

Since it was first recognized that tetherin restricted the release of HIV-1 from cells (Neil et al., 2008; Van Damme et al., 2008) it has been discovered that the release of several enveloped viruses is restricted by tetherin, including HIV-2, SIV, Lassa fever virus, KSHV and VLPs of Ebola virus (Jia et al., 2009; Jouvenet et al., 2009; Kaletsky et al., 2009; Radoshitzky et al., 2010; Sakuma et al., 2009; Weidner et al., 2010). A common feature of many enveloped viruses that are restricted by tetherin is that they bud from lipid rafts (Bavari et al., 2002; Lindwasser and Resh, 2001; Nguyen and Hildreth, 2000; Ono and Freed, 2001). This observation is consistent with the fact that tetherin is localized to lipid raft domains in the plasma membrane. As influenza virus is known to use lipid rafts as a scaffold for budding (Scheiffele et al., 1999; Takeda et al., 2003) we investigated the effect of over-expression of tetherin on influenza virus release from cells. Data from both cells constitutively expressing tetherin and from cells transiently expressing tetherin indicated that tetherin does not restrict influenza virus release. However, in contrast when influenza VLPs were generated by expressing PB1, PB2, PA, NP, M1, NEP (NS2), M2, HA and NA i.e. all of the virus encoded proteins except NS1, VLP release was restricted by tetherin expression. These data are related to the finding that release of NA-containing vesicles (VLPs that budded due to NA expression) were restricted in budding by the expression of tetherin (Yondola et al., 2011). Analysis of the protein composition of

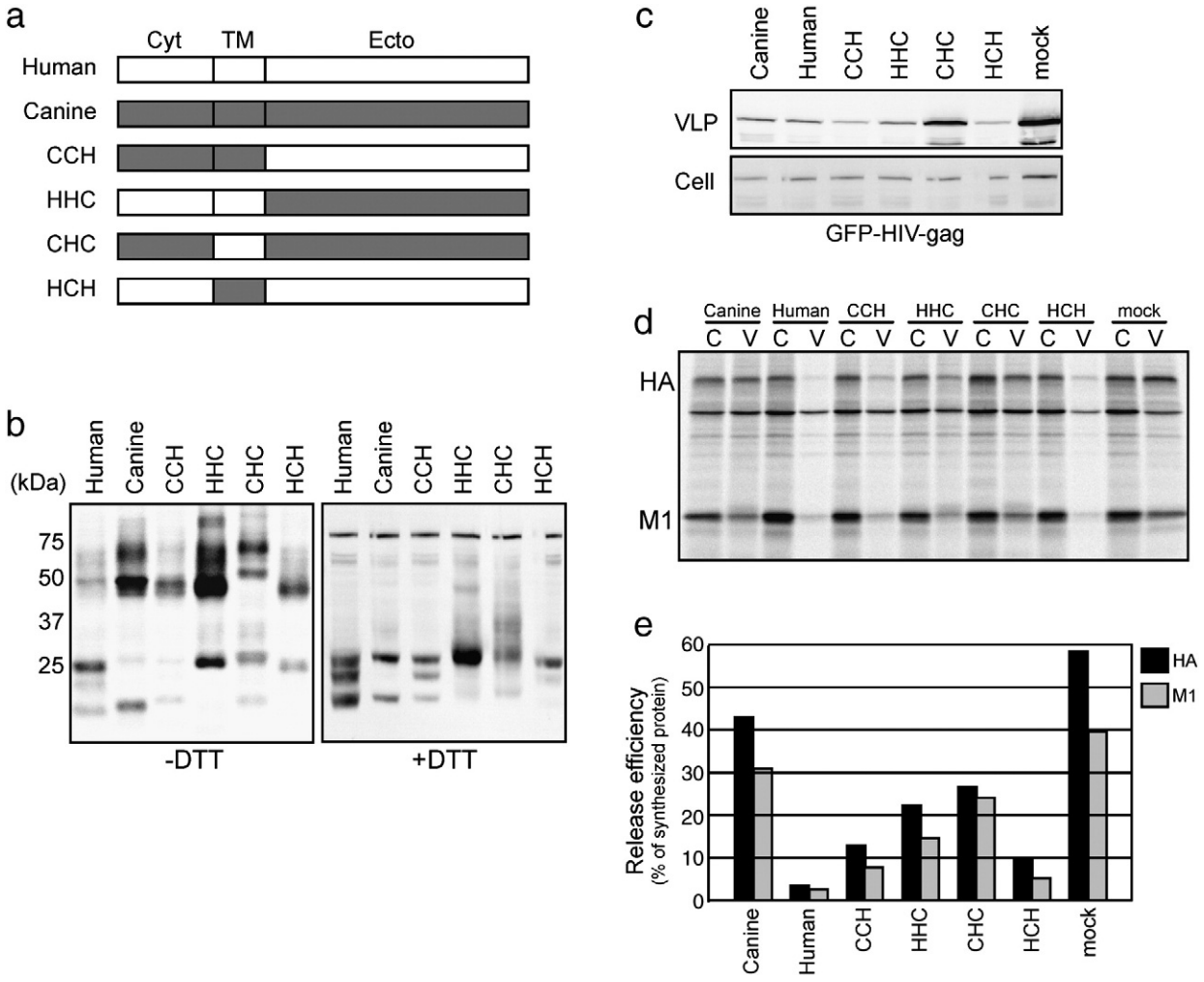

Fig. 5. The inhibition of VLP release is specified by the tetherin ectodomain. (a) Schematic diagram for Hu and Ca chimeric tetherins. Tetherin was divided into three parts (Cyt: cytoplasmic domain, TM: transmembrane domain, and ecto: ectodomain). Hu and Ca tetherin components are represented as open and shaded boxes, respectively. The chimeric molecules are designated H or C for the origin of the domain, in order Cyt, TM and ecto. (b) Dimer formation of chimeric tetherins. 293 T cells transfected with expression plasmids for tetherin chimeras were lysed into SDS-PAGE sample buffer with or without DTT. The expressed protein was visualized by immunoblotting with anti-FLAG Ab. Nomenclature of chimeras follows Hu or Ca origin of Cyt, TM or ecto. (c) Inhibition for HIV-VLP release by both human and canine tetherin. HIV-VLP was produced in 293 T cells as described (Schmitt et al., 2005) in the presence of Ca or Hu tetherin or the Ca/Hu chimeric molecules. Released HIV-VLPs were harvested by ultracentrifugation through 30% sucrose cushion (upper panel). Released and intracellular HIV-VLPs were detected by immunoblotting using p24 antibody. (d and e) Analysis of the region of tetherin conferring restriction of influenza VLP release. 293 T cells were transfected with plasmids for VLP formation and Ca tetherin, Hu tetherin or the Ca/Hu chimeric tetherin constructs. VLP release was examined as described in the legend to Fig. 2.

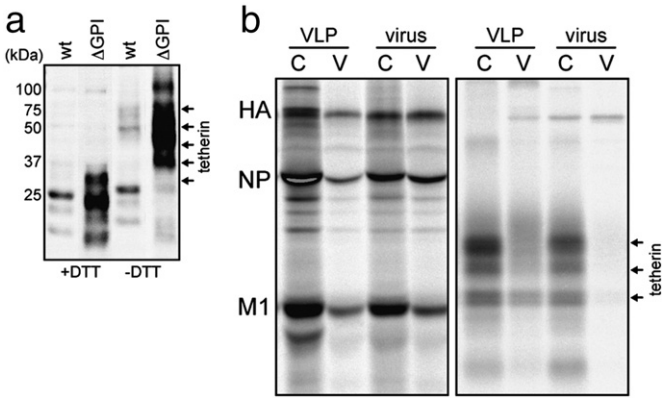

Fig. 6. Influenza virus excludes human tetherin from virions. (a) The expression and dimer formation of human tetherin mutant lacking addition of GPI (ΔGPI). The sample was prepared in the presence or absence of DTT and tetherin expression was detected by immunoblotting with anti-FLAG antibody. (b) Tetherin incorporation into released VLPs and influenza virions. VLP and virus released from ΔGPI tetherin expressing 293 T cells were harvested after ^{35}[S] radio labeling. Virus proteins and tetherin were immunoprecipitated by anti-Udorn and anti-FLAG antibodies, respectively, and polypeptides analyzed by SDS-PAGE.

influenza virus particles and VLPs derived from cells expressing ΔGPI tetherin provided further evidence for the difference between influenza virus and VLPs. When ΔGPI tetherin, that can only be anchored in cells via its TM domain, was expressed it was incorporated into VLPs but not incorporated into influenza virus particles. This provides a simple explanation for tetherin restricting VLP release but not release of virions.

Tetherin restriction of VLPs but not virions is very similar to the report that tetherin restricts the release of Ebola VLPs but not Ebola virus (Radoshitzky et al., 2010). These data suggest that both influenza virus and Ebola virus infections antagonize either tetherin function or tetherin expression. For influenza virus the big difference between VLP and virus production is the lack of expression of NS1 in the VLP system. NS1 down-regulates the host innate immune system by impairing interferon (INF) production and NS1 interferes with the establishment of the INF-induced anti-viral state (reviewed in Ehrhardt et al., 2010; Hale et al., 2008). As tetherin expression is induced by INF treatment (Kawai et al., 2008) it is likely that NS1 expression down-regulates tetherin expression. However, it is not simple to test this hypothesis in cells expressing the DNA-dependent RNA polymerase II transcripts for VLP formation as another function of

NS1 is to inhibit polyadenylation of host mRNAs, which in turn affects transport of mRNAs out of the nucleus (Nemeroff et al., 1998). However, the activities of NS1 on tetherin expression cannot be the only processes involved in giving rise to the difference between VLP release and influenza virus particle release, because ΔGPI tetherin was expressed in influenza virus-infected cells but was poorly incorporated into virions. We speculate that the tetherin TM domain is forced out of the budding patch, the budozone, by the high levels of expression of the HA and NA TM domains.

In our study a separate finding of some interest is that canine tetherin did not restrict influenza VLP release. Analysis using the canine/human chimeric tetherin molecules, indicates that the main determinant in restriction of influenza VLPs is the human ectodomain. For HIV-1 analysis of chimeric tetherin constructed from structurally related elements from very different proteins led to the finding that the overall topology/structural elements of tetherin were the critical determinants for HIV-1 restriction (Perez-Caballero et al., 2009). Although canine and human tetherin show 40% sequence identity, it may be that specific ectodomain sequence differences are important for influenza VLP release restriction. It has also been observed that whereas dimerization of tetherin is required for HIV restriction (Perez-Caballero et al., 2009), it is not important for tetherin restriction of Lassa fever virus and Marburg VLPs. Thus, there may be differences in the precise mechanism by which tetherin interacts with various VLPs.

Although tetherin may not be an important host restriction factor for influenza virus, other host restriction factors such as viperin (Wang et al., 2007), IFITM III (Brass et al., 2009), and ISG15 (Zhao et al., 2010) do restrict influenza virus. These findings combined with the discovery of other host restriction factors against other viruses, such as APOBEC3 (Sheehy et al., 2003) and TRIM5α (Stremlau et al., 2004) for HIV-1, suggest that some viruses may encounter several host restriction factors.

Materials and methods

Cells, viruses, and antibodies

293 T (human embryonic kidney) and MDCK (Madin-Darby canine kidney) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). MDCK cells stably expressing influenza virus M2 protein (M2CK cells) (Chen et al., 2008) were maintained in DMEM supplemented with 10% FBS, 200 μg/ml Geneticin (G418; InvivoGen, San Diego, CA), and 2 mM amantadine (Sigma-Aldrich [Sigma], St. Louis, MO).

To establish MDCK cells constitutively expressing human tetherin, pCR/HA-tetherin transfected MDCK cells were selected with Geneticin (G418) (2 mg/ml) and cloned by limiting dilution method. Resistant clones were examined for tetherin expression by immunoblotting and clones expressing the highest levels of tetherin were selected. The resulting MDCK-tetherin cells were maintained in DMEM supplemented with 10% FBS and G418 (200 μg/ml).

Influenza virus A/Udorn/72 and A/WSN/33 were amplified in the allantoic cavity of embryonated chicken eggs and infectious titers determined by plaque assays on MDCK cells. Recombinant influenza virus A/Udorn/72 possessing an M2 ion channel protein lacking 27 C-terminal amino acid residues (Udorn/M2S71) was described previously and propagated in M2CK cells (Chen et al., 2008). VSV possessing the GFP gene in place of its G protein gene (VSV/ΔG/GFP) was a kind gift from Dr. J. Rose (Yale University) and was propagated in BHK-21 cells transiently-expressing VSV-G protein by transfection of pCAGGS-VSVG.

Goat serum raised to purified influenza A/Udorn/72 (goat anti-Ud) was used to detect HA, NP, and M1 proteins in immunoblotting and immunoprecipitation. Mouse anti-Flag monoclonal antibody (Flag-M2, Sigma) and anti-HA monoclonal antibody (12CA5) were used to detect Flag-tagged or HA-tagged tetherin proteins, respectively, in both immunoblotting and immunoprecipitation.

Plasmids

A full-length human tetherin cDNA was amplified with PCR by using pCR/HA-tetherin vector (a gift from Dr. P. Bieniasz) as a template. An open reading frame encoding canine tetherin was amplified by RT-PCR using whole RNA extract from MDCK cells as a template and specific primers using the reported canine BST-2 protein sequence (GenBank XM_860510). Chimeric tetherins were constructed by using 4-primer PCR. All fragments were designed to carry a Flag tag sequence at the 5′-terminus and DNAs were cloned into the pCAGGS expression vector. The nucleotide sequences of all constructs were confirmed using an Avant-3100 DNA Sequencer (Applied Biosystems). Expression plasmids used for influenza VLP production were described previously (Chen et al., 2008).

VLP production

Influenza VLP, were produced as described previously (Chen et al., 2008) with slight modifications. Briefly, 293 T cells were split into 6-well plates at a density of 0.3×10^6/well on the day before transfection. The plasmid set for VLP production was transfected with or without tetherin expression plasmids using Lipofectamine and PLUS reagent (Invitrogen, Carlsbad, CA). Transfection mixtures were replaced with DMEM supplemented with 10% FBS, nonessential amino acids, and sodium pyruvate at 4 h post-transfection and incubated for a further 48 h. The supernatant was collected and concentrated by ultracentrifugation (Beckman, Ti70.1 rotor, 45,000 rpm, 2 h), lysed with protein loading buffer (PLB) and subjected to SDS-PAGE followed by immunoblotting with goat anti-Udorn to detect the released VLP proteins. The cells were lysed into PLB and examined for protein expression by immunoblotting. For the quantitative VLP budding assay, the transfected cells were starved at 24 h post-transfection in DMEM deficient in methionine and cysteine. The cells were labeled with 50 μCi/well of ^{35}S-Trans label and incubated for a further 20 h. The released VLPs were collected by ultracentrifugation and lysed with RIPA buffer. Cells were also lysed with RIPA buffer and the debris was removed by centrifugation. Each fraction was subjected to immuneprecipitation using goat anti-Udorn and protein G Sepharose to precipitate viral proteins. Proteins were analyzed by SDS-PAGE on a 15% polyacrylamide gel. Radiolabeled proteins were detected by using a Fuji BioImager FLA-5100 and quantified by MultiGauge v3.0 software (Fuji Medical Systems, Stanford, CT).

Trans-complementation and virus titration

To avoid the problems associated with being unable to transfect all the cells in a culture but being able to infect all of the cells we used cells infected with a replication defective virus and trans-complemented the defective gene by transfection of the cDNA encoding the wt protein. This approach was established for herpes virus to limit virus replication only to the cells expressing the proteins of interest after plasmid transfection (Pawliczek and Crump, 2009). Replication incompetent influenza Udorn/M2S71 or VSV/ΔG/GFP were used in combination with the co-transfection of pCAGGS-M2 or pCAGGS-VSVG, respectively. In brief, 293 T cells were split onto gelatin-coated 6-well plates at a density of 0.3×10^6/well on the day before transfection. Cells were transfected with plasmid DNA and after 24 h incubation, cells were infected with Udorn/M2S71 or VSV/ΔG/GFP at MOI = 0.33 or 3.3, respectively. Culture supernatant was harvested at 24 h (Udorn/M2S71) or 7 h (VSV/DG) post infection (p.i.) and the infectious titer of the complemented virus was determined as described below. The infectious titer of influenza Udorn/M2S71 was

determined by plaque assay using M2CK cells as described previously (Chen et al., 2007). To determine the infectious unit of VSV/ΔG/GFP, BHK cells were infected with serially diluted virus. Cells were harvested at 24 h p.i. and GFP (+) cells were detected using a FACSCalliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Infectious units were calculated based on the percentage of GFP (+) cells in the population.

Tetherin incorporation assay

The incorporation efficiency of tetherin into the released particle was obtained by comparing the intracellular to released protein ratio for both tetherin and viral proteins. Briefly, 293 T cells were split into 6 cm dishes at a density of 1×10^6/dish the day before transfection. The cells were transfected with pCAGGS/Hu tetherinΔGPI alone or in combination with the plasmid set for VLP production. The cells transfected with pCAGGS/Hu tetherinΔGPI alone were infected with wild type influenza A/Udorn/72 at a MOI = 3 at the day after transfection. The cells were labeled with 100 μCi of Promix at 6 h p.i. (or 24 h post-transfection for VLP) and incubated for 20 h. Viral proteins and tetherin were immunoprecipitated from both the released particle fraction and the cellular fraction as described above. The anti-Flag M2 monoclonal antibody was used for immunoprecipitation of the flag-tagged tetherin.

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