Influenza Virus Partially Counteracts a Restriction Imposed by Tetherin/BST-2

Bastien Mangeat¹,², Lorris Cavagliotti², Martin Lehmann², Gustavo Gers-Huber², Inderdeep Kaur², Yves Thomas³,⁴, Laurent Kaiser³,⁴,⁵, Vincent Piguet¹,²,*

¹Department of Dermatology and Wound Healing, Institute of Infection and Immunity, Cardiff University School of Medicine, Cardiff, Wales, United Kingdom.
²Dept. of Microbiology and Molecular Medicine, University of Geneva, Switzerland.
³Laboratory of Virology, ⁴Swiss National Reference Centre of Influenza, ⁵Division of Infectious Diseases, University Hospitals and Medical School of Geneva, University of Geneva, Geneva, Switzerland.

Running title: Potent tetherin-mediated inhibition of influenza virus

To whom correspondence should be addressed: Prof. Vincent Piguet, Department of Dermatology and Wound Healing, Institute of Infection and Immunity, Cardiff University and University Hospital of Wales, Heath Park, Cardiff, CF14 4XN, Wales, United Kingdom; Tel: +44 (0) 29 20 744721; Fax: +44 (0) 29 20 744312; E-mail: piguetv@cardiff.ac.uk

Keywords: BST-2; tetherin; CD317; influenza virus; restriction; HIV-1; neuraminidase; NS1; interferon

Background: The potency of the interferon-induced antiviral factor tetherin against influenza virus is unclear.

Results: Tetherin inhibits influenza virus, but this pathogen partially counteracts this defense through multiple mechanisms.

Conclusion: Tetherin takes part in the antiviral arsenal that protects humans against influenza virus infections.

Significance: We describe the antiviral activity of tetherin against influenza virus and how this pathogen partially escapes its action.

SUMMARY

Influenza virus infections lead to a burst of type-I interferon (IFN) in the human respiratory tract, which most probably accounts for a rapid control of the virus. While in mice IFN-induced Mx1 factor mediates a major part of this response, the situation is less clear in humans. Interestingly, a recently identified IFN-induced cellular protein, tetherin (also known as CD317, BST-2 or HM1.24), exerts potent antiviral activity against a broad range of retroviruses, as well as several other enveloped viruses, by impeding the release of newly generated viral particles from the cell surface. Here we show that influenza virus belongs to the targets of this potent antiviral factor. Ectopic expression of tetherin strongly inhibited fully replicative influenza virus. In addition, depleting endogenous tetherin increased viral production of influenza virions, both in cells constitutively expressing tetherin or upon its induction by IFN. We further demonstrate, by biochemical and morphological means, that tetherin exerts its antiviral action by tethering newly budded viral particles, a mechanism similar to the one that operates against HIV-1. In addition, we determined that the magnitude of tetherin antiviral activity is comparable or higher than the one of several previously identified anti-influenza cellular factors such as MxA, ADAR1, ISG15 and viperin. Finally, we demonstrate that influenza virus reduces the impact of tetherin-mediated restriction on its replication by several mechanisms. Firstly, the influenza virus NS1 protein impedes IFN-mediated tetherin induction. Secondly, influenza infection leads to a decrease of tetherin steady state levels, and the neuraminidase surface protein partly counteracts its activity. Overall, our study helps to delineate the intricate molecular battle taking place between influenza virus and its host cells.
viruses belonging to the family of orthomyxoviruses. They represent a major cause of morbidity and mortality in weakened individuals, as well as in young infants and in the elderly (1,2). Both the error-prone replication of their genome and its segmented organization, which favors the exchange of viral genes between different strains, support the appearance of antigenic variants capable of escaping immune responses present in a population, thus precluding long-term broad protection (3). The large animal reservoir of influenza viruses further increases the rate of inter-strain genetic shuffling. Thus, these viruses impact the human population both during epidemics or pandemics episodes, when influenza viruses with completely new antigenic properties can spread in the immunologically naïve human population (3). For these reasons, innate immunity plays a crucial role in the control of influenza infections. Accordingly, this viral infection triggers a burst of type-I IFN in the human respiratory tract (4-8), owing to its detection by the intracellular sensor RIG-I in infected cells (9), as well as by cell surface TLRs on patrolling immune cells (10). This IFN surge accounts for the control of the virus that usually occurs within few days, albeit through incompletely understood mechanisms (8,11,12). This burst of IFN is nevertheless delayed by the influenza virus NS1 protein, which inhibits IFN induction through several mechanisms (13-15). This delay probably allows the virus to replicate sufficiently to be transmitted to new hosts. In mice, the IFN-induced Mx1 factor is known to mediate a significant part of the IFN response (16,17). A series of other cellular factors endowed with anti-influenza potential has been uncovered recently, including viperin, ISG15, IFITM3 and ADAR1 (18-21). Notably, the relative contribution of these IFN-induced factors in the control of this virus in humans remains largely undetermined.

Importantly, tetherin constitutes another recently identified antiviral factor that acts as a powerful barrier to the replication of several viruses, including HIV-1 as well as several other non-related enveloped viruses, such as Ebola and Lassa viruses or the Kaposi-associated herpesvirus (22-27). Under basal conditions, tetherin is expressed in certain lymphocytes, plasmacytoid dendritic cells (pDCs) and myeloid cells (28-30). In addition, it is strongly up-regulated by IFN in numerous cell types, including epithelial cells (25,28). This protein is a heavily glycosylated type-II transmembrane protein with an unusual topology, which is otherwise only found in mammals in a minor but pathologically important topological variant of the prion protein (31,32). Tetherin is indeed linked to membranes both by its one-pass transmembrane domain and by a C-ter GPI anchor (Fig. 1A). This anti-viral factor localizes at the cell surface in lipid rafts, from where it continually recycles to the trans-Golgi network (33,34). Importantly, tetherin impedes the release of newly generated HIV-1 particles by direct incorporation into viral membranes, and formation of a molecular tether between the cell surface and the newly budded viral particles (34-39). The virions are then endocytosed and degraded in lysosomes (36). To escape this blockade, viruses often evolved specific counteraction mechanisms. HIV-1, for instance, encodes Vpu, which neutralizes tetherin by numerous mechanisms, including by directing its β-TrCP-mediated degradation (40,41). Other viruses, such as HIV-2 or Ebola, use their surface protein to counteract the antiviral factor (24,42,43). Finally, the importance of this restriction in the cellular antiviral arsenal is underscored by the apparent positive selection that tetherin undergoes, which is generally the hallmark of an ongoing molecular fight with pathogens (44).

It has been recently shown that tetherin was able to impede release of microvesicles formed by the influenza virus neuraminidase as well as virus-like particles (45,46). However, the significance of this observation was unclear since this antiviral potency did not extend to wild type viruses (46). Here we show that the range of restricted viruses actually extends to the complete wild type influenza virus. We further demonstrate that tetherin exerts its antiviral action by tethering newly budded viral particles. In addition, we show that the antiviral potential of tetherin is of comparable magnitude than other interferon-stimulated genes (ISGs) known to inhibit influenza virus, including MxA, viperin, IFITM3, ADAR1 and ISG15. Finally, we reveal a series of strategies used by influenza virus to attenuate the consequences of tetherin-mediated antiviral effect on its replication.
EXPERIMENTAL PROCEDURES

Plasmids and reagents  Expression plasmids for CMV promoter-driven untagged tetherin of human and murine origin were obtained from Origene (Rockville). Tetherin was subsequently sub-cloned using standard molecular biology procedures into a pEF1 or pCDNA3.1 backbone (both from Invitrogen), with a Flag or HA tag added in frame at its N-terminus. The tetherin triple cysteine mutant, 3C/A, in which the 3 lumenal cysteines are replaced by alanines, was created with the help of the QuickChange mutagenesis system (Stratagene). The tetherin construct harboring an internal HA tag, located between the coiled coil and GPI anchor domains, was a kind gift of Paul Bieniasz, as was its ΔGPI derivative and artificial tetherin (art-tetherin) construct. Plasmids for CMV promoter-driven expression of untagged ADAR1 and viperin were obtained from Origene (Rockville). Plasmids for CMV promoter-driven expression of untagged ISG15 and MxA were obtained from Open Biosystems. The ORFs of ADAR1, viperin, ISG15 and MxA were subsequently sub-cloned in pCDNA3.1 (Invitrogen) with an in-frame N-terminal Flag. The GFP expression plasmid was pEGFP.N1 (Clontech). The plasmid set used for A/Udorn/72 (H3N2) VLPs production, which was described previously (47), was a kind gift of Robert Lamb (Northwestern University). The plasmid set used for reverse-genetics-based H1N1 PR8 influenza virus production, which was described previously (48,49), was a kind gift of Peter Palese (Mount Sinai School of Medicine). The influenza virus ΔNS1 mutant was engineered, with the help of the QuickChange mutagenesis system (Stratagene), by creating a premature STOP codon after 12 amino acids in the NS1 ORF of the pDZ.NS genomic segment plasmid. Of note, this procedure did not modify the adjacent NS2 ORF. NS1 from the influenza strain WSN was expressed from the pCAGGS.NS1/WSN plasmid, under the control of the CMV promoter. The NS1 ORF from influenza strain Tx/91 was synthesized by Eurofins MWG Operon (Germany), and subsequently subcloned into the pCAGGS backbone. The Vpu-deficient HIV-1 expression vector (HIV-1 ΔVpu) was a kind gift of Didier Trono and is based on the pR9 proviral construct (50). The entire ORF of all these constructs was checked by sequencing, and corresponding maps are available upon request. Recombinant human IFN-α, TNF-alpha, Interferon-γ and IL-6 were obtained from Miltenyi Biotec (Germany).

Cells and transfections  293T, HeLa, A549 and MDCK cells were cultured following usual procedures. The transfection of these cells was performed either following a standard calcium-phosphate-based technique or with the help of the Fugene 6 reagent (Roche), according to manufacturer instructions.

Stable cell lines creation  To create MDCK and 293T cells stably expressing tetherin tagged with Flag or HA at its N-terminus, we introduced the relevant ORFs in the MLV-based retroviral vector pBabe-puro (obtained from Bob Weinberg through Addgene). Retroviral particles carrying these transgenes were then produced following standard procedures, with the addition of a Vpu-expressing plasmid to counteract the antiviral activity of tetherin in producer cells, and subsequently applied on MDCK and 293T cells. Transduced cells were selected with 4 μg/ml puromycin (Sigma) for 2 weeks. To create A549 cells stably expressing N-ter Flag-tagged tetherin, IFITM3, ISG15 or MxA, we introduced the relevant Flag-tagged ORF in the MLV-based retroviral vector pNG95 (a kind gift of Michael Malim). Retroviral particles carrying these transgenes were then produced following standard procedures, with the addition of a Vpu-expressing plasmid to counteract the antiviral activity of tetherin in producer cells, and applied on A549 cells. Transduced cells were subsequently selected with 700 μg/ml G418 (Sigma) for 2 weeks.

Influenza VLPs production and titration  A/Udorn/72 (H3N2) VLPs were produced essentially as previously described (47). Briefly, 293T were plated in 6-well dishes in complete DMEM medium and were transfected, in the presence or absence of tetherin, with the plasmids set required for VLPs production. This consists of 9 plasmids encoding CMV promoter-driven PB1, PB2, PA, NP, hemagglutinin, neuraminidase, M1, M2 and NS2, plus a plasmid expressing a genomic segment encoding a GFP reporter gene in negative orientation. After 12 hours of incubation, the cells were washed once with PBS, and cultured for 36 additional hours in serum-free Opti-MEM medium.
Viral supernatant was then collected and spun at 3,000 rpm for 3 minutes in a tabletop centrifuge to pellet contaminating cells. This cleared supernatant was then treated with 5 μg/ml of TPCK-treated trypsin (Sigma) to activate the viral hemagglutinin protein. The titration of these supernatants was performed as described (47). Briefly, 24 hours before viral titration, target 293T cells were transfected with plasmids expressing CMV promoter-driven influenza virus NP, PB1, PB2 and PA to allow for replication of the incoming genomic segment carrying the GFP reporter gene. Cells were then seeded in 48-well plates, to which VLPs were applied in serial dilutions. 48 hours later, titer was computed by scoring the numbers of green cells under a fluorescence microscope.

**Influenza virus production** Reverse-genetics-based PR8 influenza virus was produced by transfecting, in the presence or absence of tetherin, a set of 8 plasmids based on the pDZ backbone, each coding both for CMV-promoter driven viral protein in the positive sense, and for the corresponding genomic segments in the reverse orientation (49). Briefly, unless otherwise specified, 293T cells were transfected in 6-well dishes in complete DMEM medium. After 12 hours of incubation, cells were washed once with PBS, and cultured for 36 additional hours in serum-free Opti-MEM medium (Invitrogen). Viral supernatant was then collected and spun at 3,000 rpm for 3 minutes in a tabletop centrifuge to pellet contaminating cells. This cleared supernatant was then treated with 5 μg/ml of TPCK-treated trypsin (Sigma) to activate the viral hemagglutinin protein.

Influenza PR8 or A/Moscow/10/99 (H3N2) strains were amplified by infection of MDCK cells at a moi of 0.001, followed by culture for 72 hours in serum-free Opti-MEM supplemented with 1 μg/ml TPCK-treated trypsin. To generate influenza virus PR8 deleted for the NS1 gene, this virus was initially produced by the transfection in 293T of the reverse genetics PR8 system comprising a NS segment harboring a deleted NS1 ORF (but an unaffected NS2 ORF). This virus was subsequently amplified for 2 days in 7 days-old eggs, whose IFN system is still immature and therefore allow production of NS1-deficient viruses. The same overall procedure was performed in parallel for wild type PR8.

**Influenza virus titration** The titration of viral supernatants was performed by infecting MDCK cells plated in 48 well plates with serial dilutions of the viral supernatant. 20 hours later, cells were washed twice with PBS, fixed directly in the plate with 100% methanol at -20 degrees for 10 minutes, washed twice with PBS, and incubated for 30 minutes at room temperature in PBS 1% BSA. Infected cells were then revealed by immunofluorescent staining with a FITC-coupled anti-NP (#8257F from Millipore, at a 1/500th dilution in PBS) for 45 minutes at room temperature, followed by three PBS washes. Titer was computed by scoring the numbers of green cells under a fluorescence microscope.

**Influenza infections** Target cells, either MDCK, A549 or HeLa cells, were seeded in 6 well plates in complete DMEM medium. Virus, pre-activated with 5μg/ml TPCK-treated trypsin, was added at indicated moi. Approximately 14 hours later, cells were washed three times with PBS and incubated further for the relevant amount of time in serum-free Opti-MEM medium (Invitrogen). Viral supernatant was then collected and spun at 3,000 rpm for 3 minutes in a tabletop centrifuge to pellet contaminating cells. This cleared supernatant was then treated with 5 μg/ml of TPCK-treated trypsin (Sigma) to activate the hemagglutinin protein and the titration was performed as described before.

**HIV-1 production and infectivity titration** HIV-1 particles were produced by transient transfection of 293T cells with calcium-phosphate or Fugene (Roche). The supernatant of producer cells was collected 36 hours post-transfection. Viral titer was subsequently determined by applying filtered supernatant from producer cells on HeLa-CD4-LTR-LacZ indicator cells (51).

**Protein analysis** Cells were detached from dishes either by pipetting or by 10mM PBS-EDTA treatment, and subsequently lysed with RIPA buffer. Note that cells were never detached by trypsin treatment, to avoid cleavage of tetherin. Lysates were pre-cleared (13,000 rpm tabletop spin for 10 minutes), their protein content was quantified with the BCA kit (Thermo), and they were subjected to standard SDS-PAGE (extracts of duplicate samples were pooled for gel loading). Sizes of molecular weight markers are shown in kilodaltons in the figures. Unless otherwise
indicated, tetherin is detected with the rabbit antibody raised by Klaus Strebel (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) (52). All western blots of endogenous or tagged tetherin depict its glycosylated forms in the 28 to 37 kDa range, not its immature 20 kDa form. Depending on the experiments, the relative intensity of individual tetherin bands in the 28-37Kd range varies and we always depict the predominant species. When relevant, we additionally show the tetherin dimer around 60 kDa that resists denaturing and reducing conditions. Antibodies against the HA tag (clone 3F10, Roche), Flag tag (clone M2, Sigma), GFP (Miltenyi), PCNA (Oncogene Research Products), actin (Millipore), tubulin (Sigma), RIG-I (clone Alme-1, Alexis Biochemicals), Adar1 (clone D-8, Santa-Cruz), MxA (clone C-1, Santa-Cruz) and M1 (clone GA2B, Abcam) were of mouse origin. Antibodies against ezrin (Cell Signaling Technology), ISG15 (reference B-25, Santa-Cruz) and viperin (Abcam) were raised in rabbits. Gag p55 and p24 were detected with the mouse monoclonal antibody made by Bruce Chesebro and Kathy Wehrly (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) (53).

Protein analysis of influenza virions and VLPs
Viral supernatant was collected and spun at 3'000 rpm for 3 minutes in a tabletop centrifuge to pellet contaminating cells. Resulting supernatant was then ultracentrifugated, through a 20% sucrose cushion, at 175'000g for 90 minutes at 8 degrees. Pellets were resuspended in 1x sample loading buffer, and subsequently analyzed by western blotting using standard procedures. Quantification of M1 protein levels were performed by densitometry using Photoshop (Adobe).

Subtilisin treatment
293T cells were plated in 6-well dishes in complete DMEM medium and were transfected with the plasmids set required for influenza VLPs production in the presence or absence of tetherin, as described before. The ratio of tetherin compared to the sum of viral plasmids was 0.3. Six hours later, cells were washed and Opti-MEM medium was added. Thirty-six hours after transfection, viral-containing supernatants were collected and infectivity was titrated. Cells were then washed twice with PBS and incubated for 15 minutes with a buffer (10mM Tris-HCl, 150 mM NaCl, 1 mM CaCl2, pH 8) containing or not 100 µg/ml subtilisin A. The reaction was stopped by adding one volume of DMEM 10% FCS 5 mM PMSF. Cells that were not subtilisin-treated were then processed for western blotting as described above. In parallel, the viral supernatant, as well as supernatants from subtilisin and buffer treatments were cleared by spinning at 3000 rpm for 3 min, and one ml of each was ultracentrifugated through 20% sucrose, in SW55 Ti at 38 000 rpm for 75 minutes. Viral pellet were directly resuspended in 1x loading buffer and analyzed by standard western blotting.

Exogenous glycosidases treatment
MDCK cells stably expressing tetherin were washed once with PBS and then incubated with indicated glycosidases for 2 hours at 37° in serum-free Opti-MEM medium. Cellular extracts were subsequently analyzed by western blotting. Purified neuraminidase from Clostridium perfringens were obtained from Sigma (catalog numbers N2133 and N2876). PNGase F (Peptide: N-Glycosidase F) purified from Flavobacterium meningosepticum and recombinant Endo H (endoglycosidase H) were obtained from NEB.

Real Time PCR
Total RNA was extracted from cells with the help of the RNasy mini kit (Qiagen), including an on-column DNase treatment step. The integrity of the resulting RNAs was checked with a spectrophotometer. Then, they served as templates for the synthesis of cDNA by the Superscript II reverse transcriptase kit (Invitrogen), using random primers. The cDNAs were quantified by SYBR-green-based real-time PCR using JumpStart SYBR green Taq ReadyMix (Sigma), on a CFX96 cycler (Bio-Rad), with the following primers: tetherin (sense CTGCAACCACACTGTGATG, antisense ACGCGTCCCTGAAGCTTATG), TBP (sense GCCCGAAACGCCGAATATA, antisense: CGTGGCTCTCTTATCCTCATGA). The TBP quantification allowed normalization for the starting amount of RNA.

Electron microscopy
HIV-1 VLPs, devoid of accessory genes including vpu, were produced by transfection of the packaging construct psPAX2 in 293T cells, in the presence or absence of co-transfected untagged tetherin. The ratio of tetherin compared to this construct was 0.5. Transfected...
cells were washed once with PBS 14 hours after transfection and incubated for a further 22 hours. In parallel, MDCK stably expressing Flag-tetherin were infected with PR8 influenza virus at a moi of 2. Twelve hours later, infected cells were washed three times with PBS and incubated for a further 12 hours in serum-free Opti-MEM medium. All these cells were then washed twice with PBS, gently detached with 10mM PBS-EDTA, washed once with 0.9% NaCl, and finally resuspended in 0.1M Na-cacodylate solution containing 2% glutaraldehyde (EM grade). After 1 hour incubation at room temperature, cells were washed once with 0.1M Na-cacodylate, then resuspended in 0.1M Na-cacodylate and processed for embedding following standard procedures. Electron microscopy was performed with a Tecnai 20 microscope (FEI Company, Eindhoven, Netherlands).

**Flow cytometry** Cells were detached with 10mM PBS-EDTA, washed once with PBS, then fixed in PBS 1% PFA for 10 minutes at room temperature, washed once with PBS, once with 0.9% NaCl, and then stained with standard procedure with a monoclonal anti-tetherin antibody (kindly provided by Chugai Pharmaceutical, Japan) (54), followed by an anti-mouse Alexa 647 secondary antibody (Invitrogen). Cells were then analyzed with a FACSCalibur cytometer (BD Biosciences).

**RNA interference** To achieve transient downregulation of tetherin and MxA mRNAs, A549 cells were transfected using HiPerFect (Qiagen) with 20 nM of a siRNA pool specific for tetherin (#L-011817-00-0005, Dharmacon) or for MxA (#L-011735-00-0005, Dharmacon), or of a non-targeting siRNA (“Dharmacon siGenome Non-Targeting siRNA”). To generate cell lines stably downregulating tetherin and MxA, we obtained from Open Biosystems the pGIPZ lentiviral vectors expressing, under the control of the CMV promoter, a shRNAmir specific for tetherin (#RHS4430-99139775, targeted sequence AGATCAGACTTAAACCCATTAA), for MxA (#RHS4430-101103234, targeted sequence TCCAGATGGAACAGATTCT), and for a random control sequence (#RHS4346, targeted sequence ATCTCCTTGGGCAGAGATGAA). The production of lentiviral vector particles serving for the delivery of these shRNAmirs was done according to manufacturer instructions (which are standard procedures). Transduced cells were selected with 4 μg/ml puromycin (Sigma) for 2 weeks to generate a stable cell line.

**RESULTS**

**Tetherin inhibits influenza virus production**

We first addressed whether tetherin was able to impede the production of virus-like particles (VLPs), as previously reported (46). For that purpose, we used a system, derived from influenza A/Udorn/72 (H3N2) strain, that is devoid of the NS1 gene and relies on the expression of all other viral proteins from CMV promoter-driven plasmids (47). The negative-strand genomic RNA is synthesized from an additional plasmid and encodes a GFP reporter (47). We first co-transfected this viral system, in 293T cells, with Flag-tagged tetherin, which runs as a ladder of multiple bands due to its complex pattern of glycosylation. This confirmed that the presence of tetherin in producer cells potently decreases in a dose dependent manner the production of influenza-based VLPs, as scored by titrating the infectious output from these cells (Fig 1B). A western blot analysis of the M1 matrix protein in pelleted VLPs showed that this was paralleled by a decrease of the production of physical viral particles (Fig 1B). In contrast, in producer cells, neither the amount of M1 or GFP, which is expressed only after viral genomic replication, were affected by tetherin (Fig 1B). This indicates that the antiviral factor was impeding late steps of viral replication, but was not affecting transfection efficiency or genomic replication. These results were confirmed with an untagged version of human tetherin (as shown later in Fig. 3A). We next demonstrated that tetherin antiviral action extends to wild type influenza virus. Indeed, an untagged tetherin potently inhibited the release of a fully replication-competent influenza A/PR8/34 (H1N1) strain (thereafter abbreviated PR8) produced by a reverse-genetics system from 293T cells (48,49) (Fig. 1C). Tetherin-mediated inhibition of influenza viral titers was correlated with a decrease in the release of viral particles. Identical results were observed with a tagged version of tetherin (as shown later in Fig. 3B). Importantly, tetherin had no impact on viral protein expression of wild type influenza virus in producer cells, as
verified by western blot analysis of the M1 protein (Fig 1C). Finally, our observations were extended to the murine orthologue of tetherin, demonstrating that the anti-influenza activity is conserved across different mammalian species (Fig 1C).

We next investigated whether tetherin was also operative in the context of a genuine influenza virus replication cycle. For that, we generated MDCK cells stably expressing Flag-tagged human tetherin, and infected them with the influenza PR8 strain at a multiplicity of infection (moi) of 1. Forty-eight hours later, the infectious output present in the supernatant was titered, which revealed a strong reduction of infectious virus production in the presence of tetherin (Fig. 2A). Of note, we limited the assay to one round of replication, since multiple rounds of replication require proteolytic activation of influenza virus hemagglutinin by exogenously added trypsin, which would result in cleavage and inactivation of tetherin (34). In order to control that the affected step occurs late during the viral cycle, we monitored the viral protein M1 in cell extracts, which revealed that tetherin had no effect on its expression levels, strongly suggesting a mechanism of action late during the viral cycle (Fig. 2A). We confirmed these observations with tetherin stably expressed in A549 human respiratory epithelial cells (as shown later in Fig. 5B), as well as with the A/Moscow/10/99 (H3N2) strain (Fig. 2B). Interestingly, we also observed a significant reduction of tetherin signal as well as a shift in gel migration upon influenza infection, an observation that will be further investigated later in Fig. 7 and 8.

Finally, in order to analyze the antiviral potential of endogenous tetherin, we stably transduced HeLa cells, which naturally express moderate levels of this cellular factor, with lentiviral vectors expressing microRNA-adapted shRNA (shRNAmir) specifically targeting tetherin. The downregulation of tetherin in these cells led to an approximately 5 fold enhancement of influenza virus production when driven by a reverse genetics system (Fig. 2C), or during a genuine complete influenza virus replication cycle (Fig. 2D). This showed that not only ectopically expressed but also endogenous tetherin is able to restrict the production of infectious influenza virus. The relatively lower antiviral activity in these assays as compared to prior overexpression systems is most likely due to the relatively lower tetherin expression levels in HeLa cells when compared to overexpression systems (supplemental Fig. 1). We conclude that tetherin imposes a potent barrier to the replication of wild-type influenza virus.

Tetherin inhibits influenza virus by tethering newly budded viral particles to the cell surface

We next addressed whether the modalities of tetherin antiviral action against influenza virus were similar to the one operative against other viruses such as HIV-1, namely the tethering of newly budded viral particle to the cell surface through a bond formed by a tetherin dimer (34,35,38). As a first step towards this goal, we mutated the 3 luminal cysteines implicated in the strengthening of the tetherin dimer by disulfide bonds (Fig. 1A), yielding the 3C/A mutant. These changes significantly affected tetherin ability to inhibit influenza virus production (Fig. 3A). The release of influenza VLPs from 293T cells was indeed significantly more reduced by wild type tetherin than by its cysteine-mutated counterpart, both at the level of infectious output and physical particles output. The residual antiviral potential, which was also observed by others against HIV-1 (38), may be accounted for by the ability of tetherin to dimerize to some extent through its own cysteine bonds (35,55). Alternatively, the monomeric form of tetherin could also be endowed with some anti-influenza potential, as was suggested in the case of tetherin action against Lassa and Marburg viruses (27).

We then showed that tetherin GPI-anchor was crucial to inhibit influenza virus release both on full influenza virus PR8 reverse genetics system or on VLPs (Fig. 3B and supplemental Fig. 2 respectively), in good accordance with its proposed role in anchoring one end of the extended tetherin dimer in either the plasma or the viral membrane (Fig. 1A) (38). Indeed a AGPI tetherin construct was unable to restrict influenza viral particles production, even though it is known to be expressed at cell surface at normal levels (38). In parallel, we used a previously described “artificial tetherin”, which conserves overall
tetherin topology despite being composed of fragments from unrelated proteins (38). This chimera was able to inhibit influenza virus release in a significant fashion, both at the level of infectious and physical output (Fig. 3B and supplemental Fig. 2). Since this molecule has no other similarity to tetherin apart from its overall tether-like structure, it strongly suggested that the ability of tetherin to reduce influenza virus release was due to its tethering potential.

Next, we investigated whether treating tetherin-expressing producer cells with the subtilisin protease would result in the release of influenza virus into the supernatant, as expected if those were tethered on the cell surface. We initially produced influenza VLPs from 293T cells expressing untagged or tagged tetherin, which as expected led to a large decrease of viral release, as scored both by titration of infectivity and by western blot analysis of the M1 protein in viral pellets (Fig. 4A, upper part). Further incubating these cells with subtilisin resulted in a large additional release of influenza VLPs, while omitting the subtilisin in the incubation buffer abolished this phenomenon (Fig 4A, lower part). This experimental strategy, which was used previously on HIV-1 (25,34,38), therefore confirmed that tetherin traps virions at the cell surface through a protease-sensitive tether. Of note, the amount of M1 released by subtilisin treatment of tetherin-expressing cells is much lower than the total amount of M1 produced from tetherin-negative cells, most likely due to endocytosis and degradation of tethered particles, as was previously shown for HIV-1 particles (36).

Finally, electron microscopy analysis revealed that wild type PR8 virus strongly accumulated on the surface of MDCK cells stably expressing Flag-tetherin, but not on cells lacking the antiviral factor (Fig. 4B). These cells also contained endosomes-like structures filled with viral particles, the likely final outcome of their trapping at the cell surface (36). This overall pattern was very reminiscent of what we observed for HIV-1 ΔVpu in tetherin-expressing 293T cells (Fig. 4B) in agreement with others’ observations (25,36). Of note, for both HIV-1 and influenza virus, some virions were also present at the cell surface in the absence of tetherin. In the case of HIV, these viral particles are often still linked to the cytosol by a membranous canal, indicating that they are in the process of budding, which is confirmed by their immature morphology. In the case of influenza virus, these most likely represent virions that have not yet been released by neuraminidase from a post-budding hemagglutinin-mediated interaction with sialic acid, the cellular receptor for influenza virus. Overall, our results demonstrate that tetherin inhibits influenza virus replication by tethering newly formed viral particles on producer cells surface.

Relative potency of tetherin as compared to other antiviral factors

Next, we decided to assess the relative anti-influenza potency of tetherin as compared to other antiviral factors known to inhibit influenza virus. We first co-transfected 293T cells with the reverse-genetics-based influenza virus PR8 system and five untagged ISGs endowed with antiviral potency, namely tetherin, ADAR1, ISG15, MXA and viperin. Measuring the infectious output released from these cells revealed that tetherin and MxA were by far the most efficient anti-influenza factors in these settings (Fig. 5A). We then verified that the lack of activity of the other ISGs were not due to putative lower expression levels, which is difficult to assess while monitoring each ISG with a distinct antibody. Therefore, we generated Flag-tagged tetherin, ADAR1, ISG15, MXA and viperin, and performed the same analysis, with the same output results apart from a somewhat reduced activity of Mx, probably due to the tag (supplemental Fig. 3A and 3B). Of note, the relatively lower antiviral activity of viperin in our experiments as compared to a previous report is probably due to the species of origin of the viperin clone, which is human here but was murine in the previous report (19). Finally, to confirm these results, we generated A549 cells stably expressing these different Flag-tagged constructs, unless viperin and ADAR1 whose satisfactory expression level could not be obtained. Infecting these cells with wild type PR8 virus confirmed the potency of tetherin compared to other ISGs, as scored by titrating the infectious output from these cells after a whole replication cycle (Fig. 5B). The relatively low potency of MxA in these settings was again undoubtedly due to the presence of a tag. Longer exposure of the western blot for producer cells showed the
appearance of ISGylated proteins upon influenza infection. While we did not investigate the identity of these proteins, this result showed that our experimental system supports ISG15 addition to target proteins even though the whole ISGylation machinery was not co-transfected, in good accordance with an earlier report (56). In addition, these settings allowed us to test an additional newly identified anti-influenza factor, IFITM3, which specifically targets viral entry (Fig. 5B) (21). Cells stably expressing this antiviral factor imposed a very potent restriction to influenza virus multiplication, which occurred early during the replication cycle as shown by the absence of M1 signal in infected cells. Finally, this experiment confirmed that tetherin protein level is strongly diminished by influenza infection, which we previously observed in MDCK cells (Fig. 2A). Overall, these data indicate that tetherin antiviral potential compares favorably with the one of most previously described anti-influenza ISGs. Of note though, the apparent low antiviral activity of several of these antiviral factors may reflect the action of a putative unidentified viral counterstrike mechanisms specifically mounted against them.

To further assess the physiological relevance of tetherin antiviral potency, we knocked-down its expression in A549 cells, either transiently (Fig. 5C) or stably (supplemental Fig. 4), and monitored the consequence of this downregulation on the ability of IFN to inhibit influenza virus replication. These treatments provoked a significant, albeit modest, impairment of IFN ability to inhibit influenza virus replication. In parallel, downregulating the expression of MxA led to a similar effect. These relatively modest rescues of infectivity most likely stem from the large panel of antiviral factors that are induced by IFN, which reduces the impact of inhibiting their action individually. Interestingly, it appeared that IFN treatment very potently blocked expression of the M1 protein, pointing to a relatively early blockade to the infection, which would fit with an IFITM-mediated activity at viral entry.

**Partial escape of tetherin restriction through a multipronged mechanism**

After having ascertained that tetherin is a potent anti-influenza factor, we tested whether this virus had ways to modulate the potency of this restriction. We first noted that the cells used in vivo by influenza virus for its productive replication are thought to express only low tetherin levels under basal conditions (57), as illustrated by the absence of tetherin detection in the A549 respiratory epithelial cell line (Fig. 5A). A first mechanism may therefore be the avoidance of tetherin-expressing cells by the virus. Nevertheless, influenza infection initiates inflammation in vivo, which is triggered both through recognition of cytoplasmic viral genomes in infected cells, and through recognition of extracellular viral particles genomes by patrolling TLR-expressing immune cells such as pDCs and macrophages (9,10). This inflammatory milieu is known to induce tetherin expression (25,28,58), most likely through the paracrine action of cytokines. Here, we therefore characterized the effect of the main inflammatory cytokines on tetherin expression in A549 respiratory epithelial cells (Fig. 6A). As expected, tetherin was not detected in respiratory epithelial cells under basal conditions. However, while IL-6 and TNF-alpha had no effect, tetherin was significantly upregulated by both IFN-gamma and IFN-alpha. We therefore asked whether influenza virus possesses ways to handle this cellular defense. We first showed that the known ability of influenza virus NS1 protein to counteract IFN induction through RIG-I inhibition led to a blockade of tetherin upregulation (Fig. 6B). Indeed, infecting A549 cells with a wild type virus did not induce tetherin, while the infection with its counterpart deleted for the NS1 gene significantly induced the expression of tetherin, even though this virus did not lead to a detectable replication in these cells (as expected since the unrestricted induction of IFN leads to the upregulation of a series of antiviral factors). In addition, influenza strains, such as A/Texas/36/91 (H1N1) or A/WSN/33 (H1N1) (thereafter abbreviated Tx/91 and WSN, respectively), also possess the ability to inhibit cellular mRNA maturation through their NS1 protein, thereby inhibiting not only IFN induction but also the expression of ISGs in response to IFN present in the milieu (59,60). Accordingly, we showed that transfecting NS1 of Tx/91 or WSN in 293T cells potently inhibited tetherin upregulation in response to exogenously added IFN (Fig. 6C). Overall, these results indicate that influenza virus NS1 protein prevents tetherin induction by
impeding its IFN induction and/or by impeding its synthesis.

Since influenza virus delays the onset of inflammatory reactions but is unable to completely inhibit it, we reasoned that it is likely to encounter tetherin-expressing cells later during the infection. We therefore wondered whether influenza virus possesses means to replicate despite the presence of tetherin. In that respect, we previously observed that influenza infection led to the decrease of tetherin level in MDCK or A549 cells stably expressing this protein (Fig. 2A and 5B), which we confirmed in Fig. 7A. We often observed a similar decrease for other stably expressed proteins, such as ISG15, in addition to tetherin (for instance Fig. 7A), which indicates a broad mechanism of action. In that respect, we observed that the decrease of tetherin protein levels was paralleled by a decrease of its mRNA (Fig. 7B), pointing to a transcriptional mode of action. In conclusion, this reduction has the potential to diminish the activity of tetherin on newly produced viral particles.

Next, we also noticed that upon the presence of influenza virus, tetherin migration pattern systematically shifted to an apparent lower molecular weight (Fig. 2A, Fig. 7A, Fig. 7B, and data not shown). One possibility was that the neuraminidase protein was responsible for this phenomenon, through cleavage of sugars on the heavily glycosylated forms of tetherin. We could confirm this hypothesis by showing that treating MDCK cells stably expressing tetherin with purified exogenous neuraminidase induced a similar shift (Fig. 8A), whose pattern was distinct as compared to the one induced by other glycosidases (supplemental Fig. 5). In the same manner, co-transfecting neuraminidase with tetherin in 293T cells was sufficient to induce this shift (Fig. 8B, left part). We further asked whether this alteration of tetherin had functional consequences for the antiviral potential of the protein, which could be expected from the known importance of glycosylation for optimal antiviral activity against HIV-1 (38). For that, we first ectopically expressed the neuraminidase in 293T cells producing HIV-1 ΔVpu virus, and observed that this reduced the antiviral activity of tetherin against this virus, albeit in a modest fashion (Fig. 8B, right part). We then produced influenza virus particles either proficient or deficient in the neuraminidase protein and assessed whether their release was differentially affected by the presence of tetherin in producer cells. Again the presence of neuraminidase induced a shift in tetherin migration pattern (Fig. 8C, where tetherin shifts to a lower apparent molecular mass from lane 5 to lane 3), confirming a role for the neuraminidase in this shift. Of note, we had to remove the hemagglutinin in parallel since, in the absence of neuraminidase, this surface protein would stick to its receptor on producer cells and impede viral release. Importantly, influenza virus was significantly more sensitive to the inhibitory activity of tetherin when produced in the absence of neuraminidase, as illustrated by the stronger decrease of M1 in viral supernatant induced by the antiviral factor in the absence of neuraminidase (Fig. 8C). We therefore conclude that influenza virus uses its neuraminidase to prevent tetherin to reach full antiviral potency, possibly by mediating the deglycosylation of the antiviral cellular protein.

DISCUSSION

Tetherin has recently emerged as an important cellular defense against a series of enveloped viruses, including retroviruses, herpesviruses, rhabdoviruses, filoviruses and arenaviruses (22-27). Several of these viruses possess means to counteract this restriction, which permits their replication in tetherin-expressing cells. HIV-1, for instance uses its Vpu protein for that task (25). Of note, some viruses may encode for hidden tetherin antagonism functions, and for this reason appear to be insensitive to the antiviral action of tetherin.

It has been reported recently that tetherin inhibits the release of influenza-based virus-like particles (45,46), but that the wild type virus was not affected (46). He we confirm the initial observation of a potent antiviral action of tetherin against influenza VLPs. In addition, we show that this antiviral activity in fact also extends to wild type influenza virus. In particular, we show that overexpressed tetherin is able to inhibit this virus when produced both from a reverse-genetics transfection system or in the context of a real infection in MDCK or A549 cells. Moreover, influenza virus is also sensitive to the action of endogenous tetherin, as shown by the increase in
viral production in situations where tetherin expression is knocked-down. These data are in good agreement with older observations of poor viral production with a concomitant trapping of viral particles at the cell surface when influenza virus is produced from HeLa cells, which naturally express this antiviral factor (61). It is likely that the recently described absence of tetherin antiviral activity against wild type influenza virus stems from the low expression level of tetherin in stable cell lines used in this study (46). Importantly, we further determine that tetherin inhibits influenza virus by impeding its release from the surface of producer cells, a mechanism similar to the one at play against retroviruses (34,38). We indeed show that structural features required for the formation of tethers against HIV-1 are required in a similar fashion against influenza virus. Namely, dimerization of tetherin is required for optimal antiviral activity, while anchor to the membrane through a GPI moiety is crucial. In addition, replacing tetherin by an artificial construct harboring no homology with tetherin, apart from its overall structural shape is also efficient at restricting influenza virus production, strongly suggesting that the tether character of tetherin is crucial. We further show that the release of influenza viral particles can be restored by stripping the cell surface of tetherin-expressing cells with a protease, indicating that tetherin expression leads to the accumulation of virions on the cell surface after their budding, a conclusion that we confirmed by electron microscopy. We then compared the antiviral potential of tetherin with other known antiviral factors, which revealed that tetherin restriction magnitude lies in the same range as the one of two other well-known and potent antiviral factors, namely MxA and IFITM3. In addition, we showed that tetherin antiviral activity participates in the ability of type-I IFN to inhibit influenza virus replication to a similar extent than MxA.

We next investigated whether influenza virus is endowed with mechanisms to antagonize tetherin, which may mitigate the antiviral potency of tetherin against this pathogen and explain in part the previous discrepancy observed when using virus like particles or full length viruses (45,46). Surprisingly, we found that influenza virus evolved several additive strategies to reduce tetherin impact on its replication. We first showed that influenza virus uses its NS1 protein to impede IFN-mediated tetherin upregulation in epithelial cells. We then demonstrated that, when influenza virus encounters cells that already express tetherin, the infection significantly diminishes tetherin level, thereby potentially reducing its antiviral activity. The precise underlying mechanism nevertheless remains to be determined since an obvious culprit, NS1, is not endowed with the classical transcriptional inhibition through CPSF binding in the PR8 strain, which is used here (15,59). An additional role for influenza virus-induced shut-off of host cell translation can also be envisioned (62). Finally, we show through several experiments that influenza virus neuraminidase is endowed with a modest ability to neutralize tetherin potentially via the removal of glycosyl groups from this antiviral factor. This would be consistent with previous observations indicating an impairment of antiviral activity of tetherin upon mutation of its glycosylated residues (38). Such a reduced glycosylation may directly hinder tetherin antiviral activity, or may do so indirectly by hindering its proper migration to the cell surface or to viral budding zones.

These additive mechanisms that provide a partial protection to influenza virus may explain our observation that, in all the systems that we tested, the antiviral activity of tetherin is always significantly more potent against HIV-1 ΔVpu, devoid of mechanisms to counteract tetherin, than against wild type influenza virus (data not shown). It is of note, though, that we cannot exclude additional mechanisms of evasion of influenza virus from tetherin-mediated inhibition. For instance, the reported influenza virus M2-mediated downmodulation of the trafficking of glycosylated membrane proteins towards the apical side of polarized cells may lead to a decrease in the local concentration of tetherin at influenza virus budding zones (63). Alternatively, influenza virus may mechanically bud in a manner that is intrinsically less prone to infiltration by tetherin molecules, or may bud from microdomains where tetherin is only relatively less abundant. Finally, the relatively low rescue potential of neuraminidase, as compared for instance to Vpu, may be explained by the low dependency of the virus on this counteraction, since a significant part of its replication window in the host is performed in the absence of major inflammation response,
hence in the absence of tetherin, contrary to HIV-1 for instance, the replication of which strictly occurs in tetherin-expressing cells.

From our studies, we can elaborate on the interplay that takes place in vivo between influenza virus and tetherin. During the initial phase of the infection, the virus multiplies without encountering tetherin. The antiviral factor is indeed only lowly expressed in respiratory epithelial cells under basal conditions (57). Moreover, at early time points the innate immune system, and hence tetherin, is not induced efficiently, most probably owing to the inhibitory effect of NS1 on RIG-I (13,64,65). Later, when viral titers increase, the amount of cytosolic PAMPs probably becomes too high for the triggering of RIG-I to be fully suppressed by NS1. In addition, viral particles are taken up by patrolling or resident immune cells, including dendritic cells and macrophages, which sense viral genomes through their TLRs (10). Both these mechanisms trigger the onset of an inflammatory response, as observed in vivo with a surge of cytokines, amongst which type-I IFN (4-8). This leads to the upregulation of tetherin in respiratory epithelial cells (4,6) amongst other cell types (58,66,67). The virus therefore faces a cellular milieu in newly encountered cells that is laden with antiviral factors, including tetherin, which initiates the decrease of viral replication (7). The specific role of tetherin in this phenomenon is not clear yet, but it is likely that the virus is potently inhibited by the combined action of several ISGs in addition to tetherin (68). Importantly, while the onset of the innate response is not able to fully clear the infection, it is sufficient to impede the expansion of the infection and to stop viral transmission to new hosts (13). The fact that influenza virus does not encode for a protein capable of potently counteracting these antiviral factors probably accounts for its inability to thrive through the onset of the innate immune response. Nevertheless the partial hindering of tetherin antiviral action by neuraminidase or its depletion upon infection (Fig. 7 and Fig. 8) may give some more time to the virus for its transmission, thereby contributing to tilt the balance in its favor. Finally, later, the adaptive immune system emerges and fully clears the infection.

Our work establishes tetherin as a bona fide antiviral factor against wild type influenza virus, at least in vitro. It further opens up avenues to investigate whether specific clinical isolates and influenza subtypes or strains harbor a differential sensitivity towards tetherin, which may contribute to differential virulence of these isolates in vivo. In addition, it will be useful to investigate how the known absence of tetherin from birds impacts on the replication and pathogenesis of influenza virus in these important animal reservoirs. In conclusion, our study helps to delineate the role of tetherin in the antiviral landscape that influenza virus faces during an acute infection, and brings new light on the underlying molecular determinants of influenza virus replication and pathogenesis. It also highlights the potential benefits of therapeutically targeting influenza virus neuraminidase and NS1, since inhibiting the function of either would result in an increase of tetherin-mediated inhibition of this pathogen.

REFERENCES
1. Lambert, L. C., and Fauci, A. S. (2010) N Engl J Med 363, 2036-2044
2. Neuzil, K. M., Zhu, Y., Griffin, M. R., Edwards, K. M., Thompson, J. M., Tollefson, S. J., and Wright, P. F. (2002) J Infect Dis 185, 147-152
3. Taubenberger, J. K., and Kash, J. C. (2010) Cell Host Microbe 7, 440-451
4. Hayashi, S., Jibiki, I., Asai, Y., Gon, Y., Kobayashi, T., Ichiwata, T., Shimizu, K., and Hashimoto, S. (2008) Respirology 13, 203-214
5. Xu, W., Zheng, S., Goggans, T. M., Kiser, P., Quinones-Mateu, M. E., Janocha, A. J., Comhain, S. A., Slee, R., Williams, B. R., and Erzurum, S. C. (2006) J Interferon Cytokine Res 26, 609-627
6. Tong, H. H., Long, J. P., Li, D., and DeMaria, T. F. (2004) Microb Pathog 37, 193-204
7. Kaiser, L., Fritz, R. S., Straus, S. E., Gubareva, L., and Hayden, F. G. (2001) J Med Virol 64, 262-268
8. Richman, D. D., Murphy, B. R., Baron, S., and Uhlendorf, C. (1976) J Clin Microbiol 3, 223-226
9. Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsuki, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K. J., Yamaguchi, O., Otsu, K., Tsujimura, T., Koh, C. S., Reis e Sousa, C., Matsuura, Y., Fujita, T., and Akira, S. (2006) Nature 441, 101-105
10. Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S., and Reis e Sousa, C. (2004) Science 303, 1529-1531
11. Garcia-Sastre, A., Durbin, R. K., Zheng, H., Palese, P., Gertner, R., Levy, D. E., and Durbin, J. E. (1998) J Virol 72, 8550-8558
12. Hayden, F. G., Fritz, R., Lobo, M. C., Alvord, W., Strober, W., and Straus, S. E. (1998) J Clin Invest 101, 643-649
13. Molteno, B., Lopez, C. B., Pazos, M., Becker, M. I., Hermesh, T., and Moran, T. M. (2009) J Immunol 183, 3569-3573
14. Wolff, T., and Ludwig, S. (2009) J Interferon Cytokine Res 29, 549-557
15. Hale, B. G., Randall, R. E., Ortin, J., and Jackson, D. (2008) J Gen Virol 89, 2359-2376
16. Arnheiter, H., Skutnz, S., Noteborn, M., Chang, S., and Meier, E. (1990) Cell 62, 51-61
17. Haller, O., Kochs, G., and Weber, F. (2007) Cytokine & growth factor reviews 18, 425-433
18. Ward, S. V., George, C. X., Welch, M. J., Liou, L. Y., Hahm, B., Lewicki, H., de la Torre, J. C., Samuel, C. E., and Oldstone, M. B. (2011) Proc Natl Acad Sci U S A 108, 331-336
19. Wang, X., Hinson, E. R., and Cresswell, P. (2007) Cell Host Microbe 2, 96-105
20. Lenschow, D. J., Lai, C., Frias-Staheli, N., Giannakopoulos, N. V., Lutz, A., Wolff, T., Osiak, A., Levine, B., Schmidt, R. E., Garcia-Sastre, A., Leib, D. A., Pekosz, A., Knobeloch, K. P., Horak, I., and Virgin, H. W. t. (2007) Proc Natl Acad Sci U S A 104, 1371-1376
21. Brass, A. L., Huang, I. C., Benita, Y., John, S. P., Krishnan, M. N., Feeley, E. M., Ryan, B. J., Weyer, J. L., van der Weyden, L., Fikrig, E., Adams, D. J., Xavier, R. J., Farzan, M., and Elledge, S. J. (2009) Cell 139, 1243-1254
22. Jouvenet, N., Neil, S. J., Zhadina, M., Zang, T., Kratovac, Z., Lee, Y., McNatt, M., Hatzioannou, T., and Bieniasz, P. D. (2009) J Virol 83, 1837-1844
23. Pardieu, C., Vigan, R., Wilson, S. J., Calvi, A., Zang, T., Bieniasz, P., Kellam, P., Towers, G. J., and Neil, S. J. (2010) PLoS Pathog 6, e1000843
24. Kaletsky, R. L., Francia, J. R., Agrawal-Gamse, C., and Bates, P. (2009) Proc Natl Acad Sci U S A 106, 2886-2891
25. Neil, S. J., Zang, T., and Bieniasz, P. D. (2008) Nature 451, 425-430
26. Van Damme, N., Goff, D., Katsura, C., Jorgenson, R. L., Mitchell, R., Johnson, M. C., Stephens, E. B., and Guatelli, J. (2008) Cell Host Microbe 3, 245-252
27. Sakuma, T., Noda, T., Urata, S., Kawaoaka, Y., and Yasuda, J. (2009) J Virol 83, 2382-2385
28. Blasius, A. L., Giurisato, E., Cella, M., Schreiber, R. D., Shaw, A. S., and Colonna, M. (2006) J Immunol 177, 3260-3265
29. Ishikawa, J., Kaisho, T., Tomizawa, H., Lee, B. O., Kobune, Y., Inazawa, J., Oritani, K., Itoh, M., Ochi, T., Ishihara, K., and et al. (1995) Genomics 26, 527-534
30. Vidal-Laliena, M., Romero, X., March, S., Requena, V., Petriz, J., and Engel, P. (2005) Cell Immunol 236, 6-16
31. Kupzig, S., Korolchuk, V., Rollason, R., Sugden, A., Wilde, A., and Banting, G. (2003) Traffic 4, 694-709
32. Malim, M. H., and Emerman, M. (2008) Cell Host Microbe 3, 388-398
33. Rollason, R., Korolchuk, V., Hamilton, C., Schu, P., and Banting, G. (2007) J Cell Sci 120, 3850-3858
34. Hammonds, J., Wang, J. J., Yi, H., and Spearman, P. (2010) PLoS Pathog 6, e1000749
35. Hinz, A., Miguet, N., Natrajgan, G., Usami, Y., Yamanaka, H., Renesto, P., Hartlieb, B., McCarthy, A. A., Simorre, J. P., Gottlinger, H., and Weissenhorn, W. (2010) Cell Host Microbe 7, 314-323
36. Neil, S. J., Sandrin, V., Sundquist, W. I., and Bieniasz, P. D. (2007) *Cell Host Microbe* **2**, 193-203
37. Habermann, A., Krijnse-Locker, J., Oberwinkler, H., Eckhardt, M., Homann, S., Andrew, A., Strebel, K., and Krausslich, H. G. (2010) *J Virol* **84**, 4646-4658
38. Perez-Caballero, D., Zang, T., Ebrahimi, A., McNatt, M. W., Gregory, D. A., Johnson, M. C., and Bieniasz, P. D. (2009) *Cell* **139**, 499-511
39. Lehmann, M., Rocha, S., Mangeat, B., Blanchet, F., Uji-i, H., Hofkens, J., and Pugnet, V. (2011) *PLoS Pathog* **7**, e1002456
40. Mangeat, B., Gers-Huber, G., Lehmann, M., Zufferey, M., Luban, J., and Pugnet, V. (2009) *PLoS Pathog* **5**, e1000574
41. Douglas, J. L., Gustin, J. K., Viswanathan, K., Mansouri, M., Moses, A. V., and Fruh, K. (2010) *PLoS Pathog* **6**, e1000913
42. Le Tortorec, A., and Neil, S. J. (2009) *J Virol* **83**, 11966-11978
43. Hauser, H., Lopez, L. A., Yang, S. J., Oldenburg, J. E., Exline, C. M., Guatelli, J. C., and Cannon, P. M. (2010) *Retrovirology* **7**, 51
44. McNatt, M. W., Zang, T., Hatziioannou, T., Bartlett, M., Fofana, I. B., Johnson, W. E., Neil, S. J., and Bieniasz, P. D. (2009) *PLoS Pathog* **5**, e1000300
45. Yondola, M. A., Fernandes, F., Belicha-Villanueva, A., Ucelini, M., Gao, Q., Carter, C., and Palese, P. (2011) *J Virol*
46. Watanabe, R., Leser, G. P., and Lamb, R. A. (2011) *Virology* **417**, 50-56
47. Chen, B. J., Leser, G. P., Morita, E., and Lamb, R. A. (2007) *J Virol* **81**, 7111-7123
48. Quinlivan, M., Zamarin, D., Garcia-Sastre, A., Cullinane, A., Chambers, T., and Palese, P. (2005) *J Virol* **79**, 8431-8439
49. Lowen, A. C., Steel, J., Mubareka, S., Carnero, E., Garcia-Sastre, A., and Palese, P. (2009) *J Virol* **83**, 2803-2818
50. Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M., and Trono, D. (1996) *Science* **272**, 263-267.
51. Charnaux, P., Mirambeau, G., Roux, P., Paulous, S., Buc, H., and Clavel, F. (1994) *J Mol Biol* **241**, 651-662
52. Miyagi, E., Andrew, A. J., Kao, S., and Strebel, K. (2009) *Proc Natl Acad Sci U S A* **106**, 2868-2873
53. Toohey, K., Wehrly, K., Nishio, J., Perryman, S., and Chesebro, B. (1995) *Virology* **213**, 70-79
54. Ozaki, S., Kosaka, M., Wakatsuki, S., Abe, M., Koishihara, Y., and Matsumoto, T. (1997) *Blood* **90**, 3179-3186
55. Hammonds, J., Ding, L., Chu, H., Geller, K., Robbins, A., Wang, J. J., Yi, H., and Spearman, P. (2012) *J Virol* **86**, 2259-2272
56. Okumura, A., Pitha, P. M., and Harty, R. N. (2008) *Proc Natl Acad Sci U S A* **105**, 3974-3979
57. Erikson, E., Adam, T., Schmidt, S., Lehmann-Koch, J., Over, B., Goffinet, C., Harter, C., Bekeredjian-Ding, I., Sertel, S., Lasitschka, F., and Keppler, O. T. (2011) *Proc Natl Acad Sci U S A* **108**, 13688-13693
58. Ramilo, O., Allman, W., Chung, W., Mejias, A., Ardura, M., Glaser, C., Wittkowski, K. M., Piqueras, B., Banchereau, J., Palucka, A. K., and Chaus-sabel, D. (2007) *Blood* **109**, 2066-2077
59. Kochs, G., Garcia-Sastre, A., and Martinez-Sobrido, L. (2007) *J Virol* **81**, 7011-7021
60. Chen, Z., Li, Y., and Krug, R. M. (1999) *EMBO J* **18**, 2273-2283
61. Caliguiri, L. A., and Holmes, K. V. (1979) *Virology* **92**, 15-30
62. Salvatore, M., Basler, C. F., Parisien, J. P., Horvath, C. M., Bourmakina, S., Zheng, H., Muster, T., Palese, P., and Garcia-Sastre, A. (2002) *J Virol* **76**, 1206-1212
63. Henkel, J. R., Gibson, G. A., Poland, P. A., Ellis, M. A., Hughey, R. P., and Weisz, O. A. (2000) *J Cell Biol* **148**, 495-504
Acknowledgements- We thank Florence Leuba for technical help, as well as Manel Essaidi, Carole Bampi and Michael Bel for helpful discussions. We also thank Jeremy Luban, Thomas Pertel, Didier Trono, Paul Bieniasz, and Chugai Pharmaceutical for the kind gift of reagents.

FOOTNOTES
This work was supported by a Swiss National Science Foundation grant to V.P. This work was also supported by Cardiff University School of Medicine to VP and BM.

The abbreviations used are: teth., tetherin; HIV-1, human immunodeficiency virus type 1; NA, neuraminidase; HA, hemagglutinin.
**FIGURE LEGENDS**

**Figure 1.** Tetherin inhibits production of influenza VLPs and of a reverse-genetics-based influenza virus. (A) Model of tetherin structure and mode of action. A model of tetherin structure is depicted, showing the cysteines disulfide bonds (cys-cys), coiled coil and GPI anchor (left part). The proposed mode of action of tetherin against retroviruses is depicted (right part). Which tetherin extremity penetrates the virion is still under investigation. (B) Tetherin inhibits influenza VLPs release. 293T cells were transfected in duplicate with the plasmids set generating influenza VLPs, in the absence or presence of increasing doses of a Flag-tagged human tetherin. The ratio of tetherin compared to the sum of viral plasmids was 0.15 and 0.3. The cellular levels of tetherin, GFP, influenza M1 and PCNA (as a loading control) were monitored by western blotting. In addition, a western blot analysis of the viral M1 protein was performed on pelleted viral particles from pooled duplicates (lower panel). In parallel, titer of the viral output was monitored in 293T cells owing to the genomic GFP reporter (upper panel). (C) Tetherin inhibits a reverse-genetics-based influenza virus. 293T cells were transfected in duplicate with the reverse-genetics plasmids set generating complete influenza PR8 virions, in the absence or presence of increasing doses of untagged human or murine tetherin. The ratio of tetherin compared to the sum of viral plasmids was 0.15 (a1, for amount 1) and 0.3 (a2, for amount 2). The cellular levels of tetherin, influenza M1 and ezrin (as a loading control) were monitored by western blotting. Of note, the anti-tetherin antibody does not recognize murine tetherin. Evenness of transfection efficiency was controlled by co-transfecting a GFP plasmid. In addition, for the highest dose of tetherin (d2), a western blot analysis of the M1 protein was performed on pelleted viral particles from pooled duplicate supernatants (lower panel). In parallel, titer of the viral output was scored by titration on MDCK cells (upper panel). Replacing the hemagglutinin segments by an empty plasmid (∆HA) served as a negative control for our immunofluorescence-based titration method. All sections of this figure are representative of at least 4 independent experiments performed in duplicate.

**Figure 2.** Influenza virus replication is restricted in tetherin-expressing cells. (A) Influenza virus replication is restricted in MDCK stably expressing tetherin. MDCK, stably expressing tetherin or not, were infected with PR8 influenza virus at a moi of 1. Forty-eight hours later, the cellular levels of tetherin, influenza M1 and actin (as a loading control) were monitored by western blotting (lower panel). Both parts of the western blot figure come from the same scan of the same blot. In parallel, titer of the viral output present in the supernatant was scored by titration on MDCK cells (upper panel). (B) Influenza virus H3N2 is restricted in cells stably expressing tetherin. 293T, stably expressing Flag-tagged tetherin or not, were infected with A/Moscow/10/99 (H3N2) influenza at a moi of 0.5. Forty-eight hours later, the cellular levels of tetherin, influenza M1 and PCNA (as a loading control) were monitored by western blotting (lower panel). In parallel, titer of the viral output present in the supernatant was scored by titration on MDCK cells (upper panel). (C) Endogenous tetherin inhibits production of a reverse-genetics-based influenza virus system. HeLa cells, stably expressing a shRNAmir specific for tetherin (teth.) or its non-silencing counterpart (ctrl), were transfected in duplicate with the reverse-genetics plasmids set generating complete influenza PR8 virions. Forty-eight hours later, a western blot analysis allowed monitoring the cellular levels of tetherin, PCNA (as a loading control) and influenza M1, which served as a control for the evenness of transfection and lack of adverse effect of tetherin on viral proteins expression (lower panel). In parallel, titer of the viral output was scored by titration on MDCK cells (upper panel). (D) Endogenous tetherin restricts replication of influenza virus. HeLa cells, stably expressing a shRNAmir specific for tetherin (teth.) or its non-silencing counterpart (ctrl), were infected in duplicate with PR8 influenza virus at a moi of 2 or 20. Forty-eight hours after infection, a western blot analysis allowed monitoring the cellular levels of tetherin and actin (as a loading control) (left panel). In parallel, titer of the viral output present was scored by titration on MDCK cells (right panel). The consecutive sections of this figure are representative of 3, 2, 2 and 2 independent experiments performed in duplicate, respectively.
Figure 3. Determining tetherin structural features that mediate influenza virus restriction. (A) Dimerization of tetherin is required for full activity against influenza virus. 293T cells were transfected in duplicate with the plasmids set generating influenza VLPs, in the absence or presence of increasing doses of untagged tetherin or its counterpart mutated for the cysteines mediating dimerization (3C/A). The ratio of tetherin compared to the sum of viral plasmids was 0.075, 0.15 and 0.3. Forty-eight hours later, we monitored by western blotting the cellular levels of tetherin, actin (as a loading control) and influenza M1, which served as a control for the evenness of transfection and lack of adverse effect of tetherin on viral proteins expression (lower panel). In addition, a western blot analysis of the viral M1 protein was performed on pelleted viral particles from pooled duplicates (lower panel), and titer of the viral output was monitored in 293T cells owing to the genomic GFP reporter (upper panel). (B) The overall tether structure of tetherin is sufficient for its anti-influenza activity. 293T cells were transfected in duplicate with the reverse-genetics plasmids set generating complete influenza PR8 virions. In addition, cells were co-transfected with increasing doses of either a tetherin plasmid harboring an internal HA tag, its ΔGPI counterpart, or an artificial tetherin tagged in the same manner. The ratio of tetherin compared to the sum of viral plasmids was 0.2 and 0.5. Forty-eight hours later, we monitored by western blotting the cellular levels of tetherin, actin (as a loading control) and influenza M1, which served as a control for the evenness of transfection and lack of adverse effect of tetherin on viral proteins expression (lower panel). In addition, a western blot analysis of the viral M1 protein was performed on pelleted viral particles from pooled duplicates (lower panel), and titer of the viral output was assessed by titration in MDCK cells (upper panel). The titer of the virus produced in the absence of tetherin was given the arbitrary score of 100%. The western blot figure was assembled from cropped areas of the same scan of the same blot. Both sections of this figure are representative of three independent duplicate experiments.

Figure 4. Tetherin inhibits influenza virus release by trapping newly formed virions at the plasma membrane. (A) Biochemical evidence of tetherin-induced influenza virions trapping. 293T cells were transfected in duplicate with the plasmids set generating influenza VLPs, in the absence or presence of human tetherin either untagged or tagged at its N-ter with HA or Flag (as indicated between brackets). Thirty-six hours after transfection, supernatants were collected, infectivity was titrated and content in M1 protein was monitored by western blotting analysis (upper part). Thereafter, producer cells were incubated with a buffer containing or not subtilisin A, and resulting supernatants were collected (labeled “VLPs released by subtilisin” and “VLPs released by buffer” respectively). The VLPs content of these supernatants was monitored by western blotting analysis of the M1 protein in viral pellets. In parallel, we monitored by western blotting the cellular levels of tetherin, actin (as a loading control) and influenza M1, which served as a control for the evenness of transfection and lack of adverse effect of tetherin on viral proteins expression (lower panel). The western blot figure was assembled from cropped areas of the same scan of the same blot. (B) Electron microscopy reveals tethering of influenza virions. MDCK cells stably expressing or not Flag-tagged tetherin were infected with PR8 influenza virus (upper panel). In parallel, 293T cells were transfected with plasmids allowing for HIV-1 VLPs production, in the presence or absence of tetherin (lower panel). Twenty-four hours after infection and 36 hours after transfection, the presence of influenza virions or HIV-1 VLPs tethered at the cell surface was monitored by electron microscopy. The arrow indicates endosomal-like structure filled with viral particles. The results are representative of results obtained from both samples of duplicate experiments.

Figure 5. Relative potency of tetherin as compared to other ISGs. (A) Tetherin restriction potential compared to other ISGs against influenza virus production from a reverse genetics system. 293T cells were transfected in duplicate with the reverse-genetics plasmids set generating complete influenza virions. In addition, cells were co-transfected with indicated untagged antiviral factors, with a ratio of 0.4 compared to the sum of viral plasmids. Forty-eight hours later, the cellular levels of indicated ISGs (detected with their cognate antibodies), influenza M1 and actin (as a loading control) were monitored by western blotting (lower panel). Evenness of transfection was controlled by co-transfecting GFP and by...
monitoring the viral M1 protein. In parallel, titer of the viral output was determined by titration in MDCK cells (upper panel). (B) Tetherin restriction potential compared to other ISGs against influenza infection of A549 respiratory epithelial cells. A549 cells, stably expressing Flag-tagged versions of indicated antiviral factors, were infected in duplicate with PR8 influenza virus at a moi of 2. Forty hours later, the cellular levels of indicated ISGs (detected with an anti-Flag antibody) and actin (as a loading control) were monitored by western blotting (lower panel). The level of infection of these cells was assessed by western blotting of M1. In parallel, titer of the viral output present in the supernatant was scored by titration on MDCK cells (upper panel). (C) Contribution of tetherin restriction to the IFN-mediated anti-influenza activity. A549 cells were transfected with 20nM of siRNA pools against either tetherin or MxA. Eighteen hours post-transfection, relevant cells were treated with 3’000 and 10’000 U/ml of IFN-alpha (indicated by growing triangle). Twelve hours later, relevant cells were treated one more time in the same manner, and immediately infected with influenza virus PR8 at a moi of 1. Eight hours later, cells were extensively washed. After further 20 hours, viral supernatant was collected and titered on MDCK cells. In parallel, the cellular levels of tetherin, Mx, M1 and actin were monitored by western blotting (lower panel). The asterisk indicates a non-specific band. All three sections of this figure are representative of 2 independent experiments performed in duplicate.

Figure 6. Influenza virus inhibits IFN-mediated tetherin induction and reduces tetherin levels. (A) Tetherin is upregulated by certain cytokines in respiratory epithelial cells. A549 cells were treated in duplicate either with 1000 U/ml IFN-alpha, or 50ng/ml IL-6, or 500 U/ml TNF-alpha, or 5 ng/ml IFN-gamma. Twenty-four hours later, western blotting analysis of duplicate cell extracts was performed to monitor tetherin induction as well as PCNA (as a loading control). Analyzing TNF-alpha-induced IκB degradation served as a positive control for the biological activity of this cytokine. (B) Influenza PR8 NS1 prevents tetherin induction in infected A549 cells. A549 cells were infected with either influenza PR8 ΔNS1 at a moi of 0.5 or an equivalent volume of wild type (wt) PR8 influenza virus, and washed twice with PBS 5 hours later. Twenty-four hours later, western blotting analysis of cell extracts was performed to monitor influenza M1, actin (as a loading control), and the induction of tetherin (indicated by asterisks) and RIG-I. This figure section is representative of 2 independent experiments. (C) NS1 proteins from WSN and Tx/91 influenza strains prevent tetherin induction in response to IFN treatment. 293T cells were co-transfected with the indicated NS1-expressing plasmids and/or a GFP construct. Twenty-four after transfection, indicated cells were treated with 1000 U/ml of IFN-alpha. After 12 hours of incubation, cell surface tetherin was labeled and cells were analyzed by flow cytometry. Monitoring GFP fluorescence (y axis) served as a surrogate for tracking NS1-expressing cells. The NS1-mediated inhibition of tetherin upregulation is revealed by the disappearance of tetherin-positive cells in the upper-right corner of the plots upon NS1 expression.

Figure 7. Influenza infection reduces tetherin levels. (A) Influenza PR8 infection leads to downregulation of tetherin protein level. A549 cells stably expressing Flag-tagged tetherin or ISG15 were infected with PR8 influenza virus at a moi of 2 and 20 (indicated by the growing triangle). Forty-eight hours later, western blotting analysis of cell extracts was performed to monitor influenza M1, actin (as a loading control), and tetherin. ISG15. (B) Influenza PR8 infection leads to downregulation of tetherin RNA level. A549 cells stably expressing Flag-tagged tetherin were infected with PR8 influenza virus at a moi of 2. Thirty-six hours later, western blotting analysis of cell extracts was performed to monitor influenza M1, tubulin (as a loading control) and tetherin (left panel). In parallel, total RNA was extracted and tetherin mRNA level was measured by real-time PCR after conversion in cDNA (right panel). Both sections of this figure are representative of 2 independent experiments performed in duplicate.

Figure 8. Influenza virus neuraminidase offers partial protection against tetherin. (A) Exogenous neuraminidase treatment induces a shift in tetherin migration. MDCK stably expressing Flag-tagged tetherin were treated with 500 mU/ml neuraminidase (NA, purified from Clostridium perfringens, Sigma
(N2133) for 2 hours at 37°C. Cellular extracts were subsequently analyzed by western blotting to monitor tetherin migration pattern. Equal loading was controlled by detecting actin. (B) Influenza virus neuraminidase reduces tetherin anti-HIV-1 activity. 293T cells were transfected in duplicate with the HIV-1 ΔVpu proviral plasmid, in the absence or presence of untagged tetherin. The ratio of tetherin compared to the proviral plasmid was 0.25. Thirty-six hours later, we monitored by western blotting the cellular levels of tetherin, tubulin (as a loading control) and HIV-1 p55gag (left panel). Evenness of transfection efficiency was controlled by co-transfecting a GFP plasmid. In parallel, titer of the viral output was scored by titration on indicator HeLa cells (right panel). This figure section is representative of 4 independent duplicate experiments. (C) The absence of neuraminidase potentiates the antiviral activity of tetherin. 293T cells were transfected in duplicate with the reverse-genetics plasmids set generating complete influenza PR8 virions, or with this set minus the hemagglutinin and neuraminidase plasmids (ΔHA/ΔNA). In addition, increasing doses of untagged tetherin was co-transfected. The ratio of tetherin compared to the sum of viral plasmids was 0.2 and 0.6. Forty-eight hours later, we monitored by western blotting the cellular levels of tetherin, actin (as a loading control) and influenza M1. In addition, viral production was measured by western blotting analysis of the viral M1 protein in pelleted viral particles, for which we depict an additional short exposure time (indicated by an asterisk) to allow easier assessment of protein levels. The level of M1 protein in viral pellets was quantified by densitometry, and a blot was generated from the results of two independent experiments (right panel). The values obtained in the absence of tetherin were given the arbitrary values of 100%.
Figure 3

A

B

Infectious units per ml

% of infectious output

tetherin-HA

- - - -
tetherin-ΔGPI-HA

- - - -
art-tetherin-HA

- - - -
virions

M1

M1

M1

M1

-36

-36

-22

-22

actin

actin

actin

actin

-36

-36

-22

-22

cells
tetherin
cells
tetherin

Figure 8

(A) Western blot analysis of NA treatment and tetherin expression. NA treatment was performed with or without (+ or -) treatment. Tetherin and actin were assessed.

(B) Western blot analysis of influenza NA, tetherin, and HIV-1 ΔVpu expression. The levels of p55 Gag, tetherin, and tubulin were measured. The graph on the right shows the number of infectious units per ml.

(C) Western blot analysis of influenza wt and ΔNA/ΔHA virions and cells. Tetherin and M1 expression were assessed. The graph on the right shows the percentage of M1 in virions.
Influenza virus partially counteracts a restriction imposed by tetherin/BST-2
Bastien Mangeat, Lorris Cavagliotti, Martin Lehmann, Gustavo Gers-Huber, Inderdeep Kaur, Yves Thomas, Laurent Kaiser and Vincent Piguet

J. Biol. Chem. published online April 6, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M111.319996

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2012/04/06/M111.319996.DC1