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Tetherin Sensitivity of Influenza A Viruses Is Strain Specific: Role of Hemagglutinin and Neuraminidase

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ABSTRACT

The expression of the antiviral host cell factor tetherin is induced by interferon and can inhibit the release of enveloped viruses from infected cells. The Vpu protein of HIV-1 antagonizes the antiviral activity of tetherin, and tetherin antagonists with Vpu-like activity have been identified in other viruses. In contrast, it is incompletely understood whether tetherin inhibits influenza A virus (FLUAV) release and whether FLUAV encodes tetherin antagonists. Here, we show that release of several laboratory-adapted FLUAV strains and a seasonal FLUAV strain is inhibited by tetherin, while pandemic FLUAV A/Hamburg/4/2009 is resistant. Studies with a virus-like particle system and analysis of reassortant viruses provided evidence that the viral hemagglutinin (HA) is an important determinant of tetherin antagonism but requires the presence of its cognate neuraminidase (NA) to inhibit tetherin. Finally, tetherin antagonism by FLUAV was dependent on the virion context, since retrovirus release from tetherin-positive cells was not rescued, and correlated with an HA- and NA-dependent reduction in tetherin expression. In sum, our study identifies HA and NA proteins of certain pandemic FLUAV as tetherin antagonists, which has important implications for understanding FLUAV pathogenesis.

IMPORTANCE

Influenza A virus (FLUAV) infection is responsible for substantial global morbidity and mortality, and understanding how the virus evades the immune defenses of the host may uncover novel targets for antiviral intervention. Tetherin is an antiviral effector molecule of the innate immune system which can contribute to control of viral invasion. However, it has been unclear whether FLUAV is inhibited by tetherin and whether these viruses encode tetherin-antagonizing proteins. Our observation that several pandemic FLUAV strains can counteract tetherin via their HA and NA proteins identifies these proteins as novel tetherin antagonists and indicates that HA/NA-dependent inactivation of innate defenses may contribute to the efficient spread of pandemic FLUAV.

The interferon (IFN) system is an integral part of innate immunity (1, 2). Sensors of the IFN system recognize pathogen-associated molecular patterns and induce signaling cascades which induce the production and release of IFN. Binding of IFN to cell surface receptors elicits signals which induce the expression of IFN-stimulated genes (ISGs), some of which have antiviral activity (3, 4). A recently described ISG with antiviral activity is tetherin (CD317, BST-2). Tetherin's antiviral activity was identified in the context of HIV-1 infection, and it was shown that the viral protein U (Vpu) can antagonize tetherin (5, 6). Subsequently, it was demonstrated that several viruses are sensitive to inhibition by tetherin and that some of these viruses encode tetherin antagonists (7). Finally, recent studies indicate that tetherin can limit viral replication in the host (8, 9), underlining that tetherin is an important component of the innate defenses against viral invasion and can force viruses to install countermeasures.

The particular membrane topology of tetherin is key to its antiviral activity: Tetherin has membrane anchors at its N and C termini, which enable the protein to insert simultaneously into the viral envelope and the plasma membrane. As a consequence, tetherin forms a physical connector between virus and host cell which impedes the release of progeny virions into the extracellular space (10, 11). Tetherin exerts its antiviral activity at the plasma membrane, and most but not all virally encoded tetherin antagonists inactivate tetherin by reducing tetherin levels at the plasma membrane (7). For instance, the Vpu protein of HIV-1 targets tetherin for degradation in endosomes/lysosomes (6, 7, 12–14) and interferes with transport of tetherin to the cell surface (6; reviewed in reference 7).

Influenza A viruses (FLUAV) cause annual epidemics (seasonal FLUAV) and intermittent pandemics (pandemic FLUAV) which are associated with substantial morbidity and mortality

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(15). FLUAV are released from the plasma membranes of infected cells (16), the site where tetherin unfolds its antiviral activity, and thus either should be inhibited by tetherin or should encode tetherin antagonists. However, the role of tetherin in FLUAV infection has not been well defined. Initial studies indicated that FLUAV is only inefficiently inhibited by tetherin or is completely tetherin insensitive (17–19), while release of FLUAV-like particles is inhibited by tetherin (18). In contrast, subsequent analyses demonstrated appreciable inhibition of FLUAV release by tetherin (20–22). In addition, evidence for a tetherin-antagonizing activity of certain neuraminidase (NA) proteins was reported (20, 23), but the antagonism is believed to be relatively inefficient (21). The surface proteins of several viruses can antagonize tetherin (24–29), but so far whether combinations of hemagglutinin (HA) and NA can inhibit tetherin has not been examined systematically.

Here, we demonstrate in a FLUAV-based virus-like particle (VLP) system that the HA of the pandemic 1918 influenza virus rescues its corresponding NA from inhibition by tetherin, while the HA of the related, laboratory-adapted WSN virus fails to do so. Furthermore, we show that the HA and NA proteins of several pandemic FLUAV can rescue an A/PR/8/1934 reassortant virus from inhibition by tetherin. Finally, we demonstrate that tetherin antagonism by HA and NA is dependent on the viral budding system and may involve reduction of tetherin expression.

MATERIALS AND METHODS

Cell culture. HEK 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Pan Biotech) supplemented with 10% fetal calf serum (Biochrom) and 1% penicillin-streptomycin (Pan Biotech) in a humidified atmosphere with 5% CO_2 at 37°C. MDCK and NCI-H358 cells (30) were cultured under the same conditions using minimum essential medium (MEM) (Gibco) or Roswell Park Memorial Institute (RPMI) 1640 medium (Pan Biotech), respectively, supplemented with 10% fetal calf serum and penicillin-streptomycin.

Plasmids. The plasmids encoding HIV-1 Vpu (31), human tetherin (32), 1918 HA (A/South Carolina/1/1918)/), 1918 NA (A/Brevig Mission/1/1918) with and without a C-terminal V5 tag (33), WSN HA (A/WSN/1933), WSN NA (A/WSN/1933) (33), and HIV-1 p96ZM651gag-opt (34) were described previously. Expression plasmids for WSN HA and NA with C-terminal V5-antigenic tags were constructed by PCR using the primers 5′-GCGAATTCGCATGAATCCAAACCAGAAAATAATAACCATTGG-3′ and 3′-GCCTCGAGGCCTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCCTTGTCAATGG-5′ (WSN NA) and 5′-GCGGGTACCACCATGAAGGCAAAACTACTGGTCCTGTTATATGCATTTGTAGCTACAGATGCAGACACAATATGTATAGGCTACC-3′ and 3′-GCCTCGAGGCTCACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCGATGCATATTCTGCACTGCAAAGACCCATT-5′ (WSN HA). PCR-generated sequences were cloned into pCAGGS via EcoRI/XhoI (WSN NA-V5) and KpnI/XhoI (WSN HA-V5). For the generation of a retroviral vector encoding tetherin, the tetherin sequence was PCR amplified using primers hTetherin-5B (5′-GGGATCCACCATGGCATCTACTTCGTATG-3′) and hTetherin-3X (5′-GGCTCGAGTCACTGCAGCAGAGCGCTGAG-3′) and cloned into pQCXIP-mcs using BamHI and XhoI. pQCXIP-mcs is a modified version of pQCXIP (Clontech) in which the original polylinker has been replaced by a linker containing restriction sites for NotI, BamHI, AgeI, HpaI, MluI, XhoI, NruI, and EcoRI, employing the oligonucleotides linkQCXIPr (5′-CGGAATTCTCGCGACTCGAGACGCGTGTTAACACCGGTGGATCCGCGGCCGCGG-3′) and linkQCXIPf (5′-CCGCGGCCGCGGATCCACCGGTGTTAACACGCGTCTCGAGTCGCGAGAATTCCG-3′). The integrity of all PCR-amplified sequences was verified by automated sequencing.

Immunoblotting. For analysis of protein expression by Western blotting, samples were separated by 12.5% SDS-PAGE and subsequently transferred onto nitrocellulose membranes (Hartenstein) (0.2 μm). Membranes were blocked with 5% milk powder–phosphate-buffered saline (PBS) plus 0.1% Triton X-100 (PBS-T). Incubation with the first antibody was performed overnight at 4°C, while the secondary antibody was applied at room temperature (RT) for 1 h. Subsequently, membranes were washed three times for 5 min with PBS-T. A commercially available ECL kit (ECL Prime Western blotting detection reagents; GE Healthcare) was used to detect bound antibodies, and signals were visualized by employing the imaging system ChemoCam with the ChemoStarProfessional software (Intas). For quantification of signal intensity, the ImageJ program was used (35).

Antibodies. For protein detection by Western blotting, the following antibodies were used: mouse anti-V5 antibody (Invitrogen) at a 1:2,500 dilution, rabbit anti-β-actin antibody (Abnova) at a 1:1,000 dilution, rabbit anti-tetherin antibody (NIH AIDS Research and Reference Reagent Program) at a dilution of 1:1,000, rabbit anti-tetherin antibody (Abcam) at a dilution of 1:1,000, anti-p24 hybridoma cell culture supernatant (183-H12-5C) for detection of Gag at a dilution of 1:500, goat anti-FLUAV (Chemicon) at a dilution of 1:500, and rabbit anti-H3 (Immune Technology Corp) at a dilution of 1:1,000. As secondary antibodies for Western blotting, horseradish peroxidase (HRP)-coupled antibodies against mouse, goat, or rabbit (Dianova) were used at a 1:10,000 dilution. For focus formation assay, FLUAV was detected with a goat anti-FLUAV antibody (Virostat) at a 1:1,000 dilution. The anti-goat–HRP antibody (Dianova) used for immunoblotting served also as the secondary antibody in the focus formation assay but at a dilution of 1:1,000.

Recombinant viruses. The PR8-derived 6 + 2 reassortant virus expressing the HA and NA surface glycoproteins of A/California/7/2009 (H1N1pdm) was generated through reverse genetic methods by transfection of RNA polymerase I-based expression vectors as described elsewhere (36). The HA protein of the PR8 × 2009 HA/NA reassortant expresses at HA position 225 a D-to-G substitution, which is a natural variation occurring in H1N1pdm strains. The PR8 × 1968HA/NA (X-31 virus) is a reassortant virus containing the HA and NA segments of A/Aichi/2/1968 (H3N2) while the remaining segments stem from A/PR/8/1934 (H1N1) (37).

Virus production in hen's eggs. A/PR/8/1934 (H1N1), A/WSN/1933 (H1N1), A/Panama/2007/1999 (H3N2), PR8 × 2009 HA/NA, and PR8 × 1968 HA/NA were propagated in the chorio-allantoic cavities of 10-day-old embryonated hen eggs (Valo BioMedia GmbH, Germany) for 48 h at 37°C. A/Panama/2007/99 (H3N2) was reconstituted from an 8-plasmid system (38) before amplification in eggs.

Production of virus-like particles. For production of FLUAV-like particles, 293T cells were seeded in 6-well plates at a density of 2.8×10^5 cells/well and cotransfected with a plasmid encoding NA and either a plasmid encoding HA or empty plasmid in conjunction with a plasmid encoding tetherin or empty control plasmid. For production of HIV-like particles, 293T cells were seeded in 6-well or 12-well plates and cotransfected with a plasmid encoding codon-optimized Gag and either HA- or NA-encoding plasmids or both plasmids in the presence or absence of a tetherin-encoding plasmid or an empty control plasmid. At 12 to 16 h posttransfection, the medium was changed, and at 40 h posttransfection, 2 ml supernatant per well of a 6-well plate or 1 ml of supernatant per well of a 12-well plate was harvested and cleared from debris by centrifugation at 1,500 × *g* and 4°C for 5 min. Subsequently, 1.4 ml/0.7 ml (for 6-well/12-well plates) supernatant was loaded onto a 20% sucrose cushion in TNE buffer and centrifuged at 17,000 × *g* for 2 h at 4°C. After discarding the supernatant, the pelleted VLPs were mixed with 30 μl 2× SDS-containing lysis buffer (50 mM Tris [pH 6.8], 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.1% bromphenol blue, 1 mM EDTA) and boiled for 20 min at 95°C. Cell lysates were collected in 200 μl/100 μl (for 6-well/12-well plates) 2× SDS lysis buffer/well and also boiled for 30 min at 95°C. Finally, all samples were subjected to immunoblot analysis.

Infection of tetherin-expressing cells with FLUAV. For infection experiments, 293T cells were seeded in 6-well plates at a density of 2.8×10^5 cells/well. After 24 h, the cells were transfected with either a constant amount (6 μg) or increasing amounts (0.5, 1.0, 2.0, 4.0, or 6.0 μg) of tetherin expression plasmid, angiotensin-converting enzyme 2 (ACE2) encoding plasmid, or empty plasmid as control, employing the calcium phosphate transfection method. The transfection medium was replaced by fresh culture medium after an overnight incubation. At 24 h posttransfection, the culture medium was removed and the cells were washed gently with PBS and then incubated with Dulbecco's PBS (DPBS) supplemented with Ca^{2+}, Mg^{2+}, and 0.2% bovine serum albumin (BSA) and containing A/PR/8/1934 (H1N1) at a multiplicity of infection (MOI) of 0.1 or 0.01, A/Panama/2007/1999 (H3N2) at an MOI of 0.3, A/WSN/1933 (H1N1) at an MOI of 0.01, A/Hamburg/4/2009 (H1N1) at an MOI of 0.3, PR8 × 2009 HA/NA at an MOI of 0.01, or PR8 × 1968 HA/NA at an MOI of 0.01. After 1 h of incubation at 37°C, the infection medium was removed, the cells were gently washed with PBS, and fresh infection medium (DMEM supplemented with 0.2% bovine serum albumin and 1% penicillin-streptomycin) was added. Finally, the culture supernatants were collected at 24 h postinfection and cleared from debris by centrifugation, and the amount of infectious units present in the supernatants was determined by focus formation assay. To assess tetherin's antiviral activity in MDCK cells, cells were seeded in 12-well plates at a density of 1.4×10^5 cells/well. After 24 h of incubation, the cells were transduced with a murine leukemia virus (MLV) vector encoding human tetherin or transduced with an empty control vector, as described previously (39). At 24 h postransduction, the cells were infected with A/PR/8/1934 (H1N1) or A/Hamburg/4/2009 (H1N1) at an MOI of 0.01, 0.1, or 1.0 and processed as described above.

Knockdown of tetherin expression in NCI-H358 cells and FLUAV infection. The NCI-H358 cells were seeded in 12-well plates at a density of 1.1×10^5 cells/well. After 24 h, the cells were transfected with 1 μg of small interfering RNA (siRNA) specific for tetherin or with scrambled, nonsense siRNA as a control (Santa Cruz Biotechnology), using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. The transfection medium was replaced by fresh RPMI medium at 36 h posttransfection. At 48 h posttransfection, either the cells were infected as described below or tetherin expression was examined by Western blotting. For this, the cells were gently washed with PBS, collected in 100 μl of 2× SDS lysis buffer/well, and boiled for 30 min at 95°C. Finally, all samples were subjected to immunoblot analysis, as described above.

For FLUAV infection, the culture medium was removed, and the cells were gently washed with prewarmed DPBS with Ca^{2+}, Mg^{2+}, and 0.2% BSA and then incubated with DPBS containing Ca^{2+}, Mg^{2+}, BSA, and A/PR/8/1934 (H1N1) at an MOI of 0.1, A/Hamburg/4/2009 (H1N1) at an MOI of 0.3, PR8 × 2009 HA/NA at an MOI of 0.1, or PR8 × 1968 HA/NA at an MOI of 0.1. After 1 h of incubation at 37°C, the medium containing viruses was removed, the cells were gently washed with prewarmed DPBS with Ca^{2+}, Mg^{2+}, and BSA, and fresh infection medium was added. The virus titers in the culture supernatant were determined at 24 h postinfection by focus formation assay.

Impact of FLUAV infection on tetherin-mediated inhibition of the release of HIV-like particles. To monitor the impact of FLUAV on tetherin-mediated restriction of release of HIV Gag-based VLPs, 293T cells were transfected to produce VLPs in the presence and absence of tetherin, as described above. At 16 h posttransfection, the cells were infected with A/Hamburg/4/2009 (H1N1) at an MOI of 1 and with PR8 × 1968 HA/NA at an MOI of 0.1 or 1, and the cultures were processed as described above. Release of Gag-VLPs in the culture supernatants and expression of Gag, tetherin, and FLUAV proteins in cell lysates were determined at 24 h postinfection by immunoblotting, as described above.

Impact of FLUAV infection on tetherin expression. In order to determine the impact of FLUAV infection on tetherin expression, 293T cells were transiently transfected with 2 μg of plasmid encoding tetherin or with empty plasmid as a control and subsequently infected with A/PR/8/

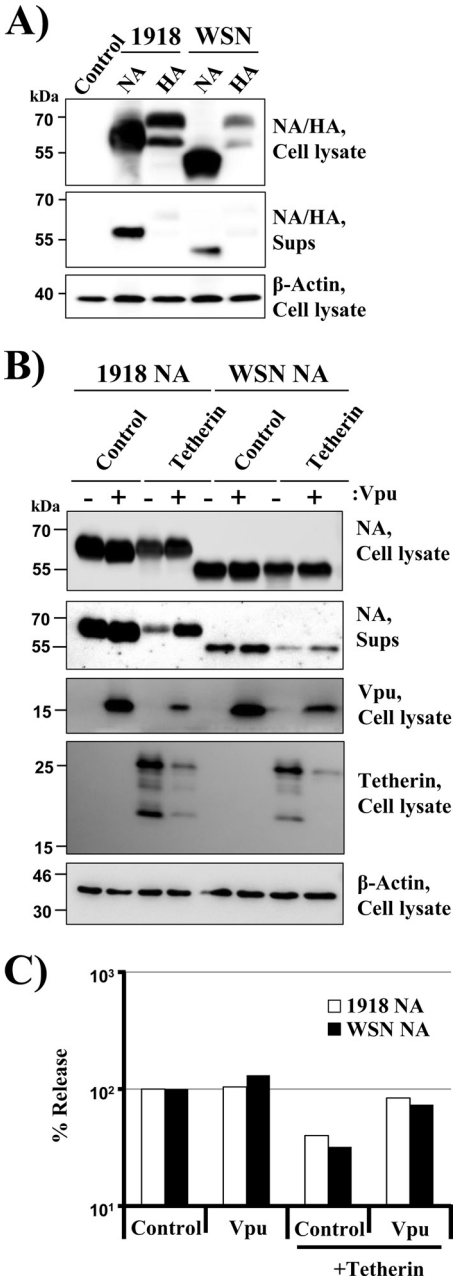

FIG 1 Tetherin inhibits release of neuraminidase-based FLUAV-like particles. (A) 293T cells were transfected with expression plasmids encoding the indicated NA and HA proteins with a C-terminal V5 antigenic tag or transfected with empty plasmid (control). At 40 h posttransfection, HA and NA expression in cell lysates and supernatants was detected via Western blotting, using a V5-specific antibody. Tetherin and Vpu expression was visualized using antibodies raised against these proteins. Detection of β-actin expression in cell lysates served as loading control. Similar results were obtained in at least four independent experiments. (B) The experiment was carried out as described for panel A, but cells were additionally cotransfected with an empty plasmid (control) or tetherin-encoding plasmid. In addition, a Vpu-encoding plasmid was cotransfected as indicated. The results of a representative experiment are shown and were confirmed in six separate experiments. (C) The signal intensities measured for panel B were quantified using ImageJ software. The NA signal present in supernatants was normalized to NA in cell lysates, and normalized NA signals in supernatants measured in the absence of tetherin and Vpu were set as 100%. 1918, pandemic 1918 FLUAV; WSN, A/WSN/1933; HA, hemagglutinin; NA, neuraminidase; Sups, supernatants.

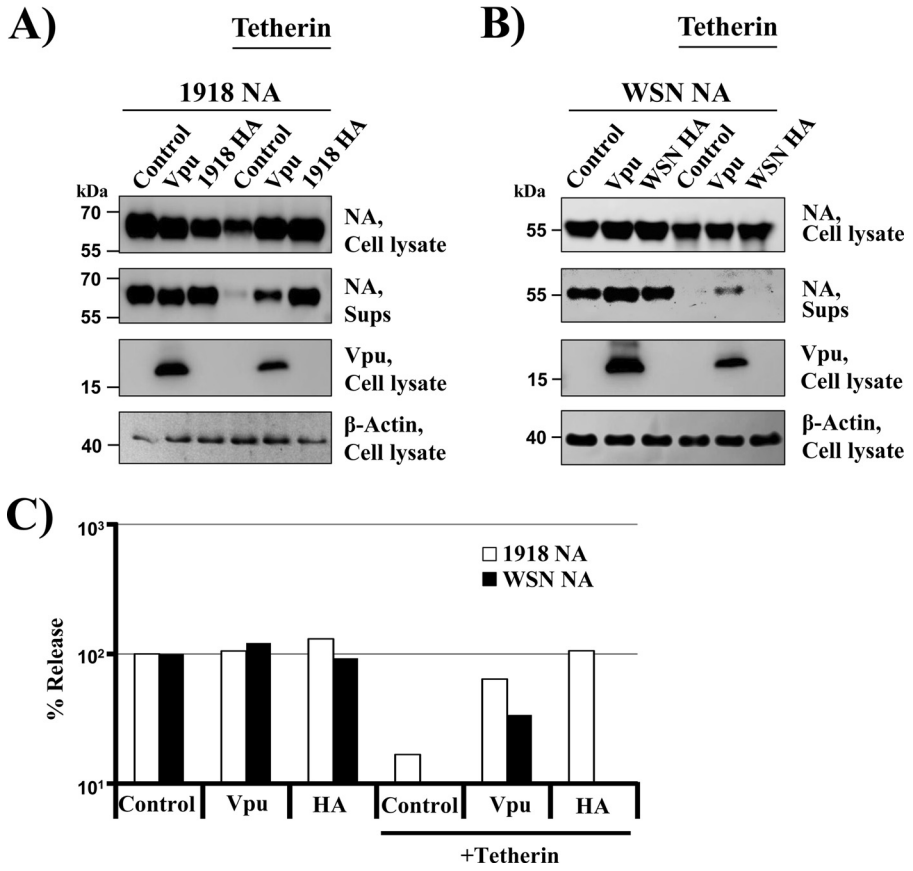

FIG 2 The hemagglutinin of pandemic 1918 influenza virus antagonizes tetherin. (A and B) 293T cells were cotransfected with expression plasmids encoding 1918 NA (A) or WSN NA (B) and empty plasmid or tetherin-encoding plasmid. In addition, cells were transfected with plasmids encoding the indicated HA proteins or Vpu or were transfected with an empty plasmid (control). At 40 h posttransfection, NA expression in cell lysates and supernatants was detected via Western blotting, using a V5-specific antibody. In addition, expression of Vpu in cell lysates was monitored. Detection of β-actin expression in cell lysates served as loading control. The results of representative experiments are shown and were confirmed in at least five (A) and three (B) separate experiments. (C) The signal intensities measured for panels A and B were quantified using ImageJ software. The NA signal present in supernatants was normalized to NA in cell lysates, and normalized NA signals in supernatants measured in the absence of tetherin and a tetherin antagonist were set as 100%.

1934 (H1N1) at an MOI of 0.1, A/Hamburg/4/2009 (H1N1) at an MOI of 0.3, PR8 × 2009 HA/NA at an MOI of 0.1, or PR8 × 1968 HA/NA at an MOI of 0.1, as described above. At 24 h postinfection, the cells were harvested for Western blot analysis of tetherin expression, as described above. Finally, Western blot images were analyzed with ImageJ software to quantify tetherin expression.

Focus formation assay. The amount of infectious units within the culture supernatants was determined by focus formation assay, as described previously (19, 40). In brief, serial 2-, 5-, or 10-fold dilutions of samples were prepared in infection medium (DMEM with 1% penicillin-streptomycin and 0.1% BSA) and were titrated on MDCK cells. After 1 h of incubation, the medium was replaced with infection medium containing an Avicel overlay and 2.5 μg/ml N-acetylated trypsin (Sigma). After an incubation period of 24 h, the cells were fixed with 4% formalin in PBS and incubated for 1 h with anti-FLUAV polyclonal goat antibody (Virostat). Subsequently, the cells were washed, incubated for 1 h with anti-goat–HRP antibodies (Dianova), washed, and incubated for 10 min with True Blue substrate (KPL). Finally, foci were counted, and viral titers were calculated as focus formation units (FFU) per ml of culture supernatant.

Statistical analysis. Results were analyzed for statistical significance using the Student *t* test in the QuickCalcs GraphPad software (nonsignificant [NS], $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$).

RESULTS

Tetherin inhibits release of neuraminidase-based virus-like particles. The glycoproteins of several viruses were identified as tetherin antagonists (24–29). In order to address whether the NA and HA proteins of FLUAV exert tetherin-antagonizing activity, we employed an NA-based VLP system, which was previously used to analyze release restriction by tetherin (23). For this, the NA proteins of the pandemic virus A/Brevig Mission/1/1918 (abbreviated 1918) and the related, laboratory-adapted A/WSN/1933 (abbreviated WSN) were equipped with a V5 antigenic tag and transiently expressed in 293T cells, followed by detection of NA expression in cell lysates and supernatants. The corresponding HA proteins, also harboring a V5 tag, were analyzed in parallel. Both NA proteins were comparably expressed in transfected cells and facilitated release of VLPs, but release was more efficient from cells expressing 1918 NA than for those expressing WSN NA (Fig. 1A). Expression of 1918 HA in cell lysates was higher than expression of WSN HA, and none of the HA proteins tested was efficiently released in the extracellular space, which may be due to lack of budding capacity and/or retention of HA-based VLPs at the cell surface, as a consequence of HA binding to cellular factors modi-

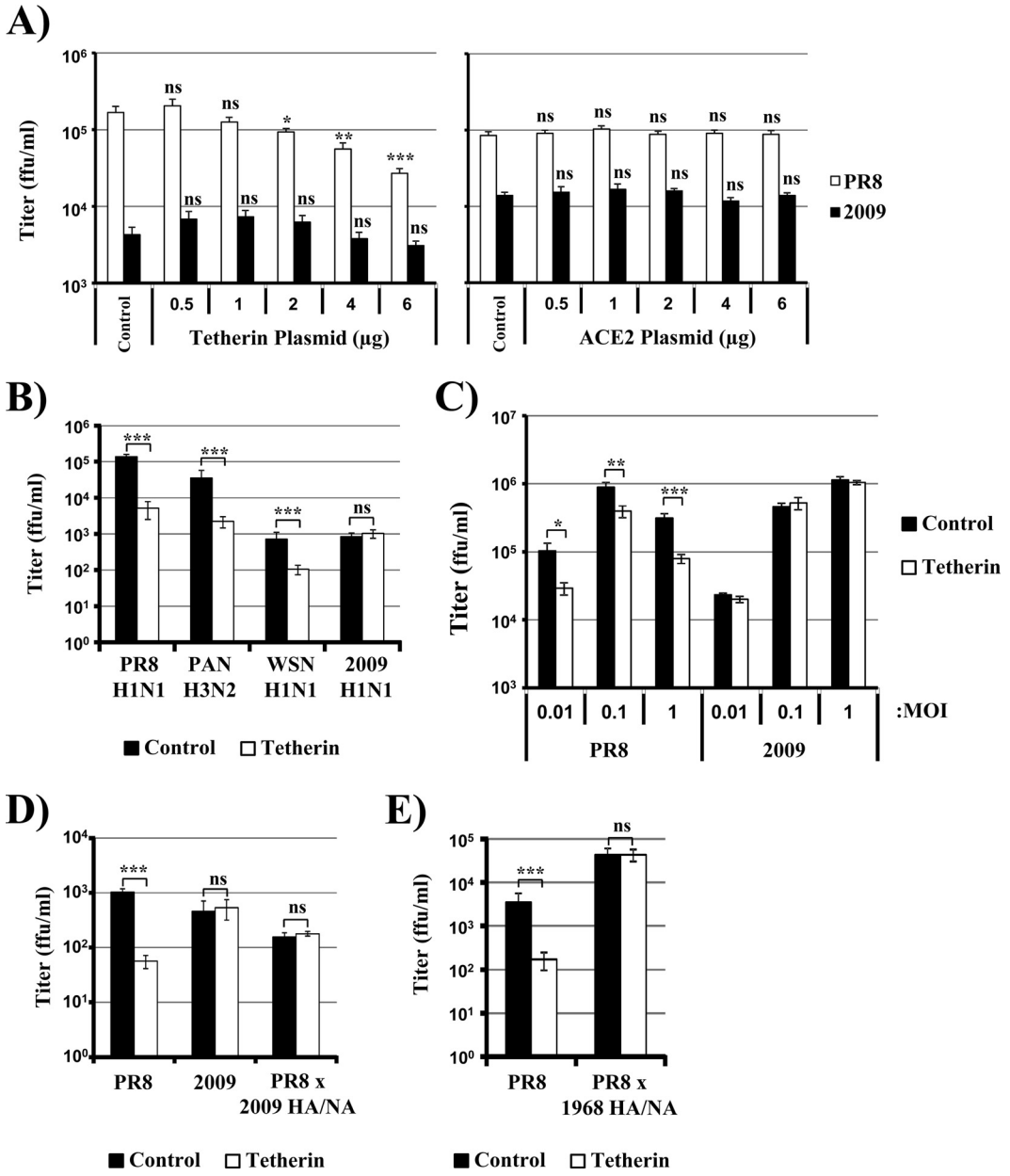

FIG 3 Neuraminidase and hemagglutinin of pandemic FLUAV confer resistance to tetherin in transfected cells. (A) Empty plasmid (control) or the indicated amounts of tetherin (left panel)- or angiotensin-converting enzyme 2 (ACE2) (right panel)-encoding plasmids were transfected into 293T cells, and the cells were infected with A/PR/8/1934 (H1N1) (abbreviated PR8) or A/Hamburg/4/2009 (H1N1) (abbreviated 2009) at MOIs of 0.1 and 0.3, respectively. At 1 h postinfection, the input viruses were removed by washing, and at 24 h postinfection, the supernatants were harvested and titers determined using the focus formation assay. The results of a single, representative experiment performed with triplicate samples are shown and were confirmed in three separate experiments. The statistical significance of differences between infection of control cells and tetherin- or ACE2-expressing cells was assessed employing Student's *t* test. (B) 293T cells transfected with a plasmid encoding tetherin or transfected with empty plasmid (control) were infected with laboratory-adapted FLUAV PR8 and A/WSN/1933 (H1N1) (abbreviated WSN), seasonal FLUAV A/Panama/2007/1999 (H3N2) (abbreviated PAN), or pandemic FLUAV 2009 at an MOI of 0.1 (PR8), 0.3 (PAN and 2009), or 0.01 (WSN) for 1 h. Subsequently, cells were processed and viral titers determined as described for panel A. The results of a single experiment performed with quintuplicate samples are shown. Error bars indicate standard deviations (SD). Similar results were obtained in two separate experiments. (C) MDCK cells transduced to express tetherin or control transduced were infected with PR8 or 2009 at an MOI of 0.01, 0.1, or 1.0 and processed as described for panel B. The results of a single experiment performed with triplicate samples are shown and were confirmed in three separate experiments. Error bars indicate SD. (D and E) Infection was carried out as described for panel B, using wt PR8 and reassortant PR8 viruses equipped with HA and NA of 2009 (PR8 × 2009 HA/NA) (D) or A/Aichi/2/1968 (H3N2) (PR8 × 1968 HA/NA) (E) at an MOI of 0.01. The results of single, representative experiments performed with sextuplicate samples are shown and were confirmed in a separate experiment. FFU, focus-forming units/ml; MOI, multiplicity of infection.

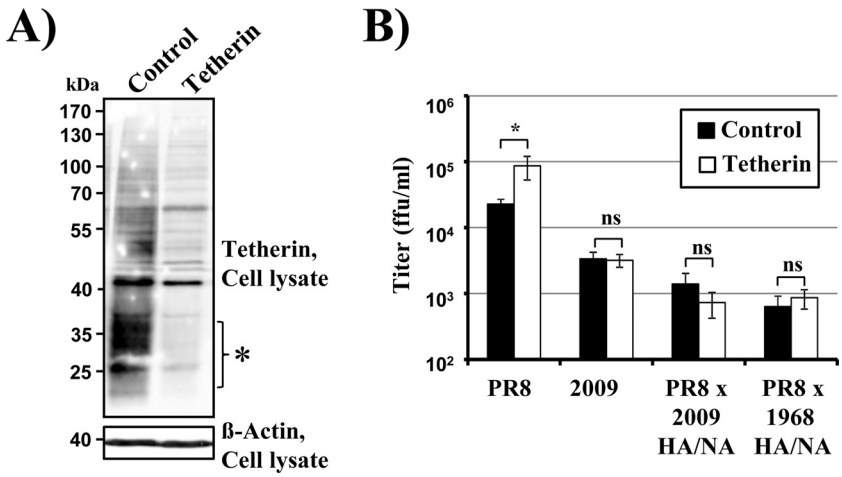

FIG 4 Neuraminidase and hemagglutinin of pandemic FLUAV confer resistance to endogenous tetherin in NCI-H358 cells. (A) NCI-H358 cells were transfected with scrambled siRNA (control) or tetherin-specific siRNA, and tetherin expression was assessed by Western blotting. The results of a representative experiment are shown and were confirmed in three separate experiments. Bands corresponding to tetherin are highlighted by an asterisk. The tetherin signal is heterogeneous, due to heterogeneity in tetherin glycosylation. (B) NCI-H358 cells were transfected with scrambled siRNA (control) or tetherin-specific siRNA and subsequently infected with A/PR/8/1934 (H1N1) (abbreviated PR8), A/Hamburg/4/2009 (H1N1) (abbreviated 2009), and a reassortant PR8 virus equipped with HA and NA of 2009 (PR8 × 2009 HA/NA) or A/Aichi/2/1968 (H3N2) (PR8 × 1968 HA/NA) at an MOI of 0.1 (PR8, PR8 × 2009 HA/NA, and PR8 × 1968 HA/NA) or 0.3 (2009). The average from three independent experiments performed with triplicates samples is shown. Error bars indicate standard error of the mean (SEM).

fied with 2,6-linked sialic acid (41). In order to address whether release of NA-based particles is suppressed by tetherin, we performed the VLP production as described above but coexpressed tetherin. Release of both 1918 NA- and WSN NA-based VLPs was markedly reduced upon tetherin expression, and this effect was rescued upon coexpression of HIV-1 Vpu (Fig. 1B and C), which diminished tetherin levels in the transfected cells, as expected (12–14). These results indicate that release of 1918 NA- and WSN NA-based VLPs is readily detectable and sensitive to inhibition by tetherin. The NA-based VLP assay thus provides a suitable tool for the analysis of a potential tetherin-antagonizing activity of HA.

The hemagglutinin of the pandemic 1918 influenza virus antagonizes tetherin. We next analyzed whether the 1918 HA and WSN HA proteins can rescue VLP release driven by their cognate NAs from inhibition by tetherin. The tetherin-mediated release inhibition of 1918 NA-based VLPs was rescued by expression of both Vpu and 1918 HA (Fig. 2A), and tetherin counteraction by Vpu but not 1918 HA was associated with a reduction in tetherin expression (not shown). In contrast, release of WSN NA-VLPs from tetherin-positive cells was facilitated by Vpu but not WSN HA (Fig. 2B and C). Finally, 1918 HA failed to consistently rescue the release of WSN NA-based VLPs from inhibition by tetherin (not shown), suggesting that tetherin antagonism by HA requires the presence of the corresponding NA. Collectively, these results indicate that the HA of the pandemic 1918 influenza virus but not the HA of the laboratory-adapted WSN can antagonize tetherin in the presence of its cognate NA.

The 2009 pandemic influenza virus is resistant to tetherin. Our findings with the VLP system raised the possibility that seasonal and/or laboratory-adapted FLUAV (WSN was obtained upon repeated passage of a seasonal virus in animals) might be more sensitive to inhibition by tetherin than pandemic FLUAV. To investigate this, we infected tetherin-transfected 293T cells, removed input virus by washing, and assessed FLUAV release in culture supernatants. All experiments were conducted in the absence of exogenously added trypsin, in order to ensure tetherin integrity and to prevent multicycle FLUAV spread. First, we expressed in 293T cells increasing amounts of tetherin or an irrelevant membrane protein, the severe acute respiratory syndrome (SARS) coronavirus entry receptor angiotensin-converting enzyme 2 (ACE2), and then infected the cells with either pandemic FLUAV (A/Hamburg/4/2009 [H1N1]) or laboratory-adapted FLUAV (A/PR/8/1934 [H1N1]), followed by quantification of viral release via focus formation assay. Notably, release of the laboratory-adapted virus was inhibited by tetherin but not ACE2 expression, and the inhibition efficiency correlated with the amount of tetherin expression (not shown), while similar effects were not observed for the pandemic virus. To extend these observations, we next compared the impacts of tetherin on release of a seasonal FLUAV (A/Panama/2007/1999 [H3N2]), laboratory-adapted FLUAV (A/PR/8/1934 [H1N1] and A/WSN/1933 [H1N1]), or pandemic FLUAV (A/Hamburg/4/2009 [H1N1]). Release of all seasonal and laboratory-adapted FLUAV was markedly inhibited by tetherin, while release of the pandemic virus was not affected (Fig. 3B), in keeping with our previous finding (Fig. 3A). Finally, analysis of MDCK cells, which are highly permissive to FLUAV replication, revealed that expression of tetherin reduced release of A/PR/8/1934 but not A/Hamburg/4/2009 (H1N1), despite comparable release from control transfected cells (Fig. 3C) (note that the reduced titers of A/PR/8/1934 upon infection at an MOI of 1 compared to MOI of 0.1 were most likely due to cytopathic effects). These results suggest that sensitivity to inhibition by tetherin is strain dependent, with A/Hamburg/4/2009 (H1N1) and potentially other pandemic viruses being resistant to tetherin while several seasonal or laboratory-adapted viruses are tetherin sensitive.

NA and HA confer tetherin resistance to pandemic viruses. We next addressed the question whether the hemagglutinin (HA)- and neuraminidase (NA)-encoding segments account for the relative tetherin insensitivity of the 2009 pandemic FLUAV. For this

endeavor, we employed an A/PR/8/1934 reassortant equipped with the HA and NA segments from pandemic A/Hamburg/4/2009. This virus, termed PR8 × 2009 HA/NA, and the corresponding wild-type (wt) viruses were tested for tetherin sensitivity, employing transfected 293T cells, as described above. Release of wt A/PR/8/1934 was readily inhibited upon tetherin expression, while A/Hamburg/4/2009 release was not affected (Fig. 3D), confirming our previous observations. Importantly, the reassortant virus PR8 × 2009 HA/NA was resistant against tetherin's antiviral activity. Furthermore, a reassortant virus expressing the HA and NA segments of pandemic A/Aichi/2/1968 (H3N2) in the background of A/PR/8/1934 (PR8 × 1968 HA/NA) turned out to be resistant against inhibition by tetherin (Fig. 3E), indicating that the HA and NA proteins from several pandemic FLUAV can protect these viruses from inhibition by tetherin, at least in transfected 293T cells. In order to analyze whether HA and NA can also rescue FLUAV from inhibition by endogenous tetherin, we used siRNA knockdown to reduce tetherin levels in the lung-derived cell line NCI-H358 (Fig. 4A). The knockdown of tetherin expression markedly augmented release of A/PR/8/1934 but not A/Hamburg/4/2009 (Fig. 4B), and this effect was dependent on HA and NA, since release of PR8 reassortants bearing HA and NA of pandemic viruses was not modulated by tetherin knockdown. Thus, the HA and NA proteins of pandemic FLUAV can antagonize exogenous and endogenous tetherin.

Tetherin antagonism by HA and NA depends on the virion context and correlates with the ability to reduce tetherin expression. In order to obtain initial insights into determinants and mechanisms underlying tetherin antagonism by FLUAV, we next asked whether HA/NA are able to counteract inhibition of HIV budding by tetherin. This question was first addressed in a previously described VLP system (42), in which formation and release of VLPs are driven by HIV-1 p55 Gag. Release of Gag-VLPs was inhibited by tetherin, and this blockade was alleviated by expression of Vpu (Fig. 5A), as expected. In contrast, expression of 1918 HA and NA, either alone or in combination, did not rescue VLP release from inhibition by tetherin (Fig. 5A) (note that HA expression reduced VLP release due to binding to sialic acids [41]). Similarly, infection of Gag-expressing 293T cells with FLUAV (A/Hamburg/4/2009, PR8 × 1968 HA/NA) did not abrogate the tetherin-dependent blockade of VLP release (Fig. 5B). Thus, the ability of HA/NA to antagonize tetherin depends on the virion context.

Several tetherin antagonists reduce tetherin expression levels in infected cells. Therefore, we asked whether FLUAV resistant to tetherin interfere with tetherin expression levels. Tetherin expression in transfected 293T cells was not reduced upon infection with A/PR/8/1934, while a modest but statistically significant decrease in tetherin levels was observed in A/Hamburg/4/2009-infected cells (Fig. 6). Moreover, the introduction of the HA and NA segments from pandemic viruses into A/PR/8/1934 endowed the reassortants with the ability to reduce tetherin expression (Fig. 6). The efficiency of tetherin downregulation was dependent on the amount of tetherin plasmid transfected, and downregulation was also observed in NCI-H358 cells (not shown). These observations suggest that HA- and NA-dependent interference with tetherin expression might contribute to tetherin antagonism of FLUAV.

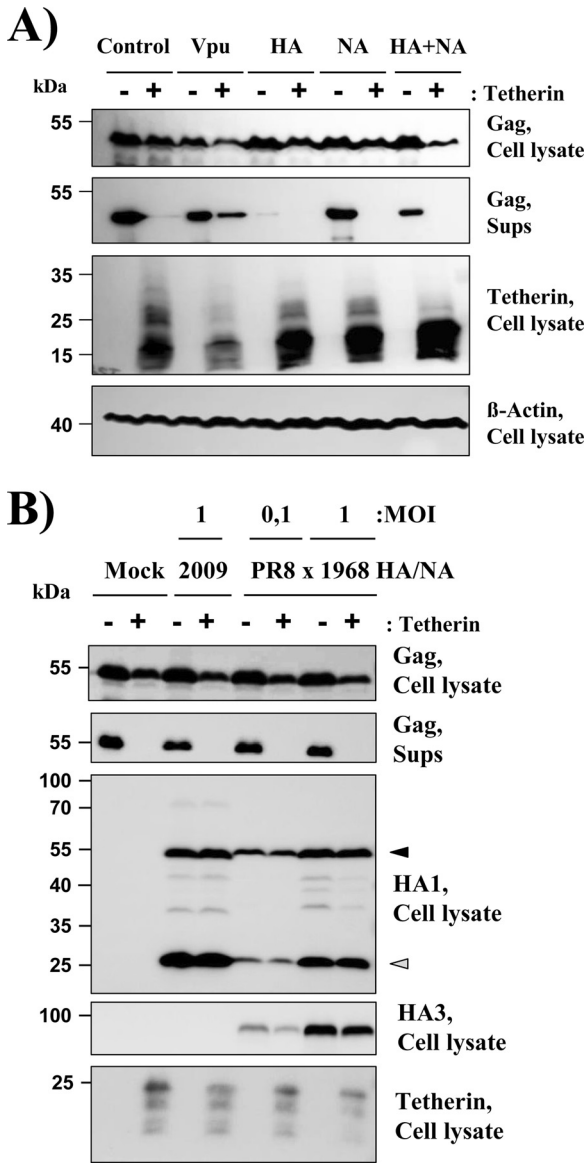

FIG 5 Tetherin antagonism by hemagglutinin and neuraminidase depends on the virion context. (A) 293T cells were cotransfected with a plasmid encoding HIV-1 Gag and an empty plasmid or tetherin-encoding plasmid. In addition, cells were transfected with plasmids encoding Vpu, 1918 HA, or 1918 NA or encoding no protein (control). At 40 h posttransfection, the presence of Gag in culture supernatants and cell lysates was determined by Western blotting. In addition, the expression of tetherin and β-actin in cell lysates was determined. Similar results were obtained in three separate experiments. (B) 293T cells were cotransfected with a plasmid encoding HIV-1 Gag and an empty plasmid or tetherin-encoding plasmid and infected with the indicated FLUAV at the indicated MOIs. As a negative control, the cells were mock infected. At 24 h postinfection, supernatants (sups) were analyzed for expression of Gag, while cell lysates were analyzed for expression of Gag, tetherin, β-actin, and FLUAV proteins (anti-FLUAV H1N1 antibody was used for detection of viral proteins in A/Hamburg/4/2009 [H1N1]-infected cells, while an HA-specific antibody was employed to probe lysates from PR8 × 1968 HA/NA-infected cells). Similar results were obtained in at least three separate experiments. Bands with the molecular masses expected for NP and M1 proteins are marked with black-filled (NP) and gray-filled (M1) triangles. MOI, multiplicity of infection; HA, hemagglutinin; NA, neuraminidase.

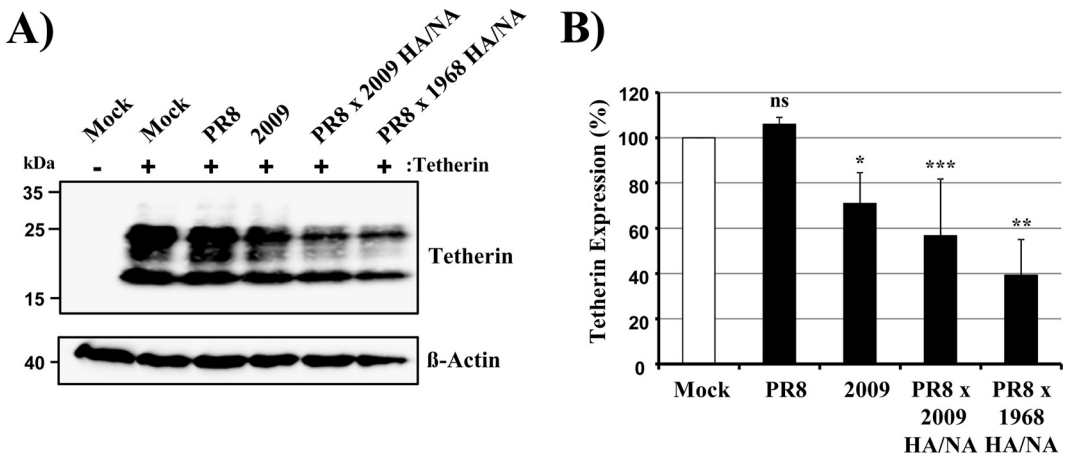

FIG 6 Neuraminidase and hemagglutinin of pandemic FLUAV reduce tetherin expression. (A) 293T cells were transfected with a tetherin-encoding plasmid or empty plasmid and infected with A/PR/8/1934 (H1N1) (abbreviated PR8), A/Hamburg/4/2009 (H1N1) (abbreviated 2009), and a reassortant PR8 virus equipped with HA and NA of 2009 (PR8 × 2009 HA/NA) or A/Aichi/2/1968 (H3N2) (PR8 × 1968 HA/NA) at an MOI of 0.1 (PR8, PR8 × 2009 HA/NA, and PR8 × 1968 HA/NA) or 0.3 (2009). At 1 h postinfection, the virus-containing medium was replaced by fresh culture medium, and cells were cultured for 24 h. Thereafter, cells were lysed and expression of tetherin and β-actin in cell lysates determined by Western blotting. Similar results were obtained in three separate experiments. (B) The tetherin and β-actin signals measured for panel A were quantified using ImageJ software and relative tetherin expression determined. Results represent the average from three independent experiments. Tetherin levels in mock-infected cells were set as 100%. The statistical significance of differences between tetherin expression in mock-infected and FLUAV-infected cells was assessed by Student's *t* test.

DISCUSSION

Tetherin can restrict the release of a variety of enveloped VLPs, including retro-, filo-, and arenavirus-like particles, by forming a clamp between the infected cell and the budding virion (7, 43). However, several but not all of the corresponding authentic viruses encode tetherin antagonists, which allow for efficient viral spread in tetherin-positive target cells (7, 43). Elucidating how these proteins inhibit tetherin may reveal novel targets for antiviral intervention and is thus in the spotlight of current research efforts. The present study shows that several seasonal or laboratory-adapted FLUAV are inhibited by tetherin, while several pandemic viruses antagonize tetherin via their HA and NA proteins. Moreover, our work demonstrates that tetherin antagonism by the HA and NA proteins is dependent on the virion context and might involve interference with tetherin expression.

Studies employing FLUAV VLP systems provided evidence that release of FLUAV-like particles can be restricted by tetherin (18, 23), although some viral NAs counteract tetherin with modest efficiency (20, 21, 23), potentially by altering tetherin glycosylation (21). However, these studies did not examine HA and NA jointly for tetherin antagonism and/or did not analyze HA and NA proteins from pandemic FLUAV. Our results show that both 1918 NA and WSN NA can drive VLP budding, although with differential efficiency, and that VLP release is inhibited by tetherin. Notably, coexpression of 1918 HA rescued release of 1918 NA-based VLPs from inhibition by tetherin, while the same effect was not observed for WSN NA and HA. In contrast, 1918 HA failed to consistently antagonize tetherin in the context of WSN NA-driven VLP release. These results demonstrate that 1918 HA can antagonize tetherin in the presence of its cognate NA and suggest that WSN HA may not be able to do so, although the interpretation of the latter finding is complicated by the less robust expression of WSN HA compared to 1918 HA.

WSN was derived from a seasonal FLUAV, which was passaged in animals, while the FLUAV of the year 1918 is a pandemic virus. The differential ability of the HA proteins of these viruses to rescue NA-driven VLP release from inhibition by tetherin suggested that seasonal or laboratory-adapted and pandemic FLUAV might differ in their susceptibilities to inhibition by tetherin. Indeed, all seasonal and laboratory-adapted FLUAV tested were inhibited by tetherin, while the pandemic virus A/Hamburg/4/2009 was resistant. Furthermore, the analysis of reassortant viruses showed that the introduction of the HA and NA segments of a pandemic virus into the PR8 background was sufficient to confer tetherin resistance to an otherwise susceptible virus. This finding is not in conflict with most previous studies documenting FLUAV inhibition by tetherin, which exclusively examined seasonal or laboratory-adapted FLUAV (17–19, 21), and parallels the documented observation that the surface proteins of Sendai virus, F and HN, are both required for tetherin antagonism (24). Dittmann and colleagues recently showed comparable inhibition of pandemic 2009 virus by tetherin and the antiviral effector IFITM3. However, the inhibition efficiency was low, while blockade of a swine FLUAV assayed in parallel was robust (22). Collectively, the results obtained with VLPs and authentic FLUAV indicate that certain pandemic FLUAV are resistant to tetherin and that tetherin resistance is determined by HA and NA proteins. In this context, it is noteworthy that recent studies documented an HA-dependent resistance of pandemic FLUAV to innate immune proteins of the collectin family, suggesting that the surface proteins of pandemic viruses might be better adapted to resist attack by effectors of the innate immune system than their counterparts in seasonal viruses (44, 45).

The finding that release of seasonal and laboratory-adapted FLUAV can be inhibited about 10-fold in the 293T cell system employed in the present study is of note, given that two studies reported similar effects (20, 46) while three independent ones found that inhibition of FLUAV release by tetherin is minor (2- to 5-fold) or absent (17–19). Differences in the cellular systems, in particular the efficiency of tetherin expression, most likely ac-

count for these discrepant findings. Therefore, future studies should determine which copy numbers of tetherin at the cell surface are required for inhibition of FLUAV release and whether the inhibitory activity of tetherin is to some degree dependent on the cellular context.

Many viral tetherin antagonists remove tetherin from the site of viral budding, the plasma membrane. For instance, the HIV-1 Vpu protein degrades tetherin and additionally interferes with tetherin transport to the cell surface (6, 7, 12–14). In contrast, the Ebola virus glycoprotein counteracts tetherin without interfering with tetherin expression or cellular localization (27, 42, 47–49), and it is currently unclear how this protein interferes with tetherin's antiviral activity. The viruses encoding HA and NA proteins capable of counteracting tetherin also reduced tetherin expression, at least when small amounts of plasmid were transfected, and it is conceivable that the two processes are linked. However, it will be challenging to investigate this possibility, since blockade of proteasomal and endosomal/lysosomal degradation of proteins will also interfere with FLUAV entry into host cells, which is known to depend on endosomal low pH and activity of proteasomes (50, 51). Finally, it is noteworthy that infection with A/Hamburg/4/2009, A/PR/8/1934, and PR8 × 2009 HA/NA did not interfere with tetherin expression at the cell surface, and only a modest reduction was observed upon PR8 × 1968 HA/NA infection in some experiments (data not shown). Lack of interference with tetherin surface expression might thus account for the observation that HA and NA expression or FLUAV infection did not rescue retroviral VLPs from inhibition by tetherin (an additional explanation is discussed below).

An alternative and mutually nonexclusive explanation for the strain dependence of tetherin antagonism might come from differences in the viral envelope: it is conceivable that the dense packaging of HA and NA in the viral membrane sterically impedes the anchoring of tetherin in the lipid bilayer. As a consequence, isolate-specific differences in the efficiency of virion incorporation of HA and NA, which have been documented (52), may result in differential tetherin sensitivity. It may thus be worthwhile to determine whether tetherin-resistant and -susceptible viruses differ in the amounts of HA and NA copies incorporated into viral particles and whether such differences impact susceptibility to inhibition by tetherin. Finally, it is noteworthy that Vpu rescues HIV- and FLUAV-derived VLPs from inhibition by tetherin, while the tetherin-antagonizing activity of HA/NA is FLUAV specific. The dependence of HA/NA-mediated tetherin antagonism on the virion context is not unprecedented, since the Ebola virus glycoprotein rescues release of retro- and filoviral but not arenaviral VLPs from tetherin-positive cells (53). In fact, the context dependence of tetherin antagonism further underlines that efficient virion incorporation of HA and NA might contribute to tetherin inhibition. Thus, HIV-like particles use different compartments of the plasma membrane for budding relative to FLUAV-like particles (54) and might thus incorporate fewer HA/NA copies than their FLUAV counterparts.

Collectively, the present study provides evidence that pandemic FLUAV counteract tetherin via their HA/NA proteins, which might promote viral spread in the host, given that tetherin is constitutively expressed in the human lung (55) and is inducibly expressed upon IFN treatment (7). Future studies need to determine the underlying mechanism and might uncover novel targets for antiviral intervention. Furthermore, it will be interesting to examine if seasonal FLUAV are generally tetherin sensitive, which is at first sight counterintuitive but might be the result of adaptation of HA to immune pressure.

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