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Inhibition of Virus-like Particle Release of Sendai virus and Nipah virus, but not that of Mumps virus, by Tetherin/CD317/BST-2

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ABSTRACT

Tetherin (also known as BST-2 or CD317) has recently been identified as a potent IFN-induced anti-viral protein that inhibits the release of diverse enveloped virus particles from infected cells. The anti-viral activity of tetherin on a number of enveloped viruses, including retroviruses, filoviruses and arenaviruses, has been examined. Here, we show that tetherin is also capable of blocking the release of virus-like particles (VLPs) driven by the matrix protein of Sendai virus. Together with inhibition of Nipah virus VLP release by tetherin, these results indicate that paramyxoviruses are to be added to the list of viruses that are susceptible to tetherin inhibition. Tetherin co-localized with Nipah virus matrix proteins and accumulated in cells, indicating that it is present at, or recruited to, sites of particle assembly. It should be noted, however, that tetherin was not effective against the release of paramyxovirus mumps VLPs, indicating that certain enveloped viruses may not be sensitive to tetherin activity.

Key words: *Sendai virus, Nipah virus, Mumps virus, Paramyxovirus, Tetherin/CD317/BST-2*

The family *Paramyxoviridae* includes numerous human and animal pathogens such as measles virus, mumps virus, respiratory syncytial virus, canine distemper virus, Newcastle disease virus, Nipah virus and Hendra virus. Sendai virus (SeV), which belongs to the genus *Respirovirus* of the family *Paramyxoviridae*, is a respiratory tract pathogen of rodents[19]. Nipah virus (NiV) and mumps virus (MuV) belong to distinct genera, the genus *Henipavirus* and the genus *Rubulavirus*, respectively. Paramyxoviruses form at cellular membranes of infected cells by a budding process. The matrix (M) protein plays a key role in the budding process by binding to the inner surface of the plasma membrane and viral nucleocapsids, forming a virus particle structure[6,10]. Expression of the M protein alone in cultured cells causes membrane budding, and virus-like particles (VLPs) are released into the culture medium. In recent years, the VLP budding system has been well established and proved efficient for study of enveloped virus egress[21,28,33,35,36, reviewed in 10].

Interferons (IFNs) play a crucial role in the innate immune response of mammals against virus infection whereby host cells protect other cells from further viral infection by the expression of numerous anti-viral factors upon recognition of viral nucleic acids[8,17]. Recently, tetherin, also called BST-2 or CD317, was identified as an intrinsic host defense protein that restricts the release of enveloped viruses[24]. Tetherin is constitutively expressed on several cell types, including mature B cells, T cells, monocytes, macrophages, plasma cells and plasmacytoid cells[38], while its expression is enhanced by αIFN[3,31]. The ability of tetherin to inhibit the release of a broad spectrum of enveloped virus particles and its expression in various tissues by

Abbreviations: HIV-1: human immunodeficiency virus-1; MuV: mumps virus; NiV: Nipah virus; ORF: open reading frame; PIV5: parainfluenza virus-5; SeV: Sendai virus; VLP: virus-like particle

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stimulation of IFN suggest that it may function as part of IFN-induced innate immunity against enveloped viruses.

Tetherin is a small type II transmembrane protein consisting of 181 amino acids with a molecular weight of 30 to 36 kDa, depending on its glycosylation state[13,18]. It has an unusual topology with an N-terminal transmembrane domain and a luminal C-terminus modified by a glycosylphosphatidylinositol (GPI) membrane anchor, which enables the tetherin molecule to interact with the cell or virion lipid bilayer at each end. The two membrane anchors are connected by the central extracellular domain of an extended coiled-coil structure with two potential N-linked glycosylation sites. The ectodomain also contains three cysteine residues that mediate homo-dimerization through disulfide linkages[2,29]. Glycosylation contributes to the correct transport and folding of the protein[29]. Both dimerization of tetherin molecules and its GPI anchor are essential for restriction of retroviral particle release[2,24,29].

Following the discovery of tetherin as an inhibitor of HIV-1 infection in the absence of its antagonist, Vpu protein, it was revealed that tetherin is also capable of blocking the release of a variety of prototype retroviruses and subsequently enveloped viruses[23,37]. Since then, the spectrum of inhibition of enveloped virus release by tetherin and its mechanism of action have been intensively investigated. To date, potent anti-viral activity of tetherin has been shown against diverse families of enveloped viruses including retroviruses (all classes; HIV-1, HIV-2, SIV, HTLV-I, MLV, EIAV, Spumaviruses and XMRV)[7,14,20], filoviruses (Ebola virus and Marburg virus)[14,15], arenaviruses (Lassa virus and Machupo virus)[30,31], γ-herpesviruses (Kaposi's sarcoma-associated herpesvirus)[22,27], rhabdoviruses (vesicular stomatitis virus)[39] and paramyxoviruses (Nipah virus)[30].

In the present study, we analyzed the ability of tetherin to restrict VLP release of paramyxoviruses from different genera, including SeV, NiV and MuV. We found that the expression of tetherin significantly inhibited the release of M proteins of SeV as well as NiV but did not have an effect on MuV M protein. The possible mechanism of the difference will be discussed.

MATERIALS AND METHODS

Cells and antibodies

Human renal epithelial cell-derived 293T cells were propagated in Dulbecco's minimum essential medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel) and penicillin-streptomycin (Invitrogen). The following antibodies were used in Western blotting. Rabbit polyclonal antibody (pAb) against synthetic peptides, 48-KIYTPGANERKYNNY-62 of NiV M, was used for detection of NiV M protein. Rabbit anti-serum against purified SeV virions was described by Kiyotani et al[16]. Monoclonal antibodies (mAbs) against HIV-1 Gag p24 (Virogen Corporation, Watertown, MA, U.S.A.), HA-tag (HA.C5; Applied Biological Materials, Richmond, BC, Canada) and FLAG-tag (M2; Sigma-Aldrich Japan, Japan) were also used.

Plasmids

The full-length cDNA clone of Nipah virus M protein (DDBJ/EMBL/GenBank Accession No. NC002728) was synthesized from oligonucleotides. Sixteen 75-nucleotide single-stranded DNA fragments that cover the entire M cDNA with addition of the *Hin*dIII and *Kpn*I sites and the *Xho*I site to the respective N-terminal and C-terminal ends were synthesized. They have 15-nucleotide overlaps at both ends and were connected with neighbor fragments by using PCR. The cDNA fragment of the Gag protein of the HXB2 clone of HIV-1 strain IIIB (DDBJ/EMBL/GenBank Accession No. K03455) was a gift from Yuko Morikawa (Kitasato University, Japan). The cDNA fragment of the M protein of mumps virus (MuV) was generated by RT-PCR using genomic RNA purified from MuV RW strain as a template with simultaneous addition of 5'HA tag. These cDNA fragments were inserted into the multi-cloning site of the pCAGGS.MCS expression vector under the cytomegalovirus immediate early enhancer and the chicken β-actin promoter[25]. These plasmids were named pCAG-NiV M, pCAG-Gag and pCAG-HA-MuV M, respectively. pCAGGS.MCS was provided by Yoshihiro Kawaoka (Tokyo University, Japan). pCAG-SeV M and pCAG-SeV N for expression of SeV M and N proteins, respectively, were described previously[35].

Tetherin/CD317/Bst-2 cDNA was purchased from the Open Biosystems (Huntsville, AL, U.S.A.) and subcloned into pCAGGS.MCS with simultaneous addition of an HA-tag at the N-terminus to generate pCAG-HA-tetherin. The cDNA of HIV-1 Vpu protein was amplified by PCR from pNL4-3, which contains a full-length genomic cDNA of HIV-1 LAV provirus[1] (Accession No. AF324493), and cloned into pCAGGS.MCS with addition of an FLAG epitope tag at the N terminus to generate pCAG-FL-Vpu. pNL4-3 plasmid was provided by Tsutomu Murakami (National Institute of Infectious Diseases, Japan). All of the constructs were confirmed by DNA sequencing.

VLP release assay and Western blotting

293T cells cultured in 6-well plates were transfected with the indicated plasmids using the FuGENE HD reagent (Roche Diagnostics Japan,

Japan) following the manufacturer's instructions. Unless otherwise stated, 1 μg of each plasmid was used per well. An empty pCAGGS vector, pCAGGS.MCS, was used to normalize the total DNA amount for all assays. At 24 hr post-transfection, cell lysates and supernatants were harvested. The supernatant was then centrifuged at 40,000 rpm for 2 hr through a 20%(w/w) sucrose cushion in a Beckman SW55 rotor. The pellet and cells were suspended in 50 μl and 200 μl of SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% sodium dodecyl sulfate [SDS], 2.5% 2-mercaptothanol, 0.002% bromophenol blue, 10% glycerol), respectively. Equal volumes (10 μl) of samples from suspended VLP and cell lysates were analyzed by 12% SDS-PAGE followed by Western blotting using specific antibodies. Subsequently, blots were probed with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG as secondary antibodies. Protein bands were detected by a chemiluminescent method using Luminata Western HRP Substrates (Merck Millipore, Billerica, MA, U.S.A.) and analyzed using an LAS-1000 image analyzer (Fuji Film, Tokyo, Japan). VLP budding rate (% VLP release) was calculated as the ratio of matrix protein present in VLPs to that in cell lysates.

Immunofluorescence microscopy

Immunofluorescent staining was performed basically as described previously[12]. 293T cells cultured in 6-well plates containing glass coverslips were transfected with the indicated plasmids using the FuGENE HD transfection reagent. At 24 hr post-transfection, cells were fixed with 0.5% formaldehyde solution and then treated with 0.1% Triton X-100 in phosphate-buffered saline (PBS). The cells were then stained with anti-NiV pAb and anti-HA mAb as primary antibodies and Alexa Fluor 546-conjugated anti-rabbit IgG goat pAb and Alexa Fluor 488-conjugated anti-mouse IgG goat pAb (Invitrogen) as secondary antibodies. Coverslips were mounted on glass slides and a BZ-9000 microscope (Keyence Japan, Osaka, Japan) was used for deconvoluted fluorescent imaging.

RESULTS

Inhibition of the release of VLPs from NiV and SeV by tetherin

First, we examined the effect of tetherin on the release of VLPs generated by the expression of M proteins from NiV and SeV. 293T cells were transfected with pCAG-NiV M or pCAG-SeV M, and respective protein expression in the cells was

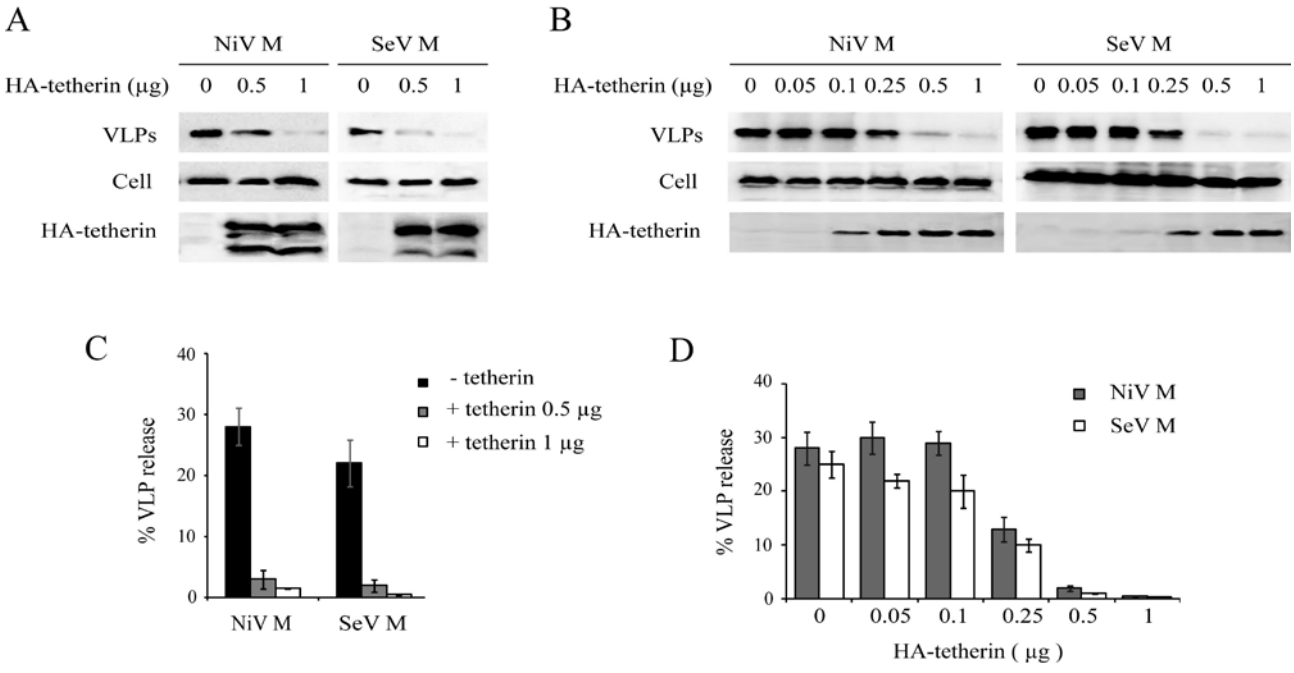

Fig. 1. Tetherin inhibits budding of NiV M- and SeV M-VLPs.

(A) Human 293T cells were transfected with pCAG-NiV M or pCAG-SeV M (1 μg) together with pCAG-HA-tetherin (0.5 or 1 μg) or an empty plasmid. At 24 hr post-transfection, cell lysates and culture medium were collected. VLPs in the medium were pelleted through a 20% sucrose cushion, and M proteins in VLPs were analyzed by Western blotting. Expression of HA-tetherin and M proteins in cell lysates was also determined by Western blotting using corresponding antibodies. (B) Analysis of dose-dependent inhibition of the release of paramyxovirus VLPs by tetherin. 293T cells were transfected with pCAG-NiV M or pCAG-SeV M (1 μg) and increasing amounts of pCAG-HA-tetherin (0 to 1 μg). Expression of M proteins and HA-tetherin was determined by Western blot analysis. (C and D) M proteins in VLPs and cell lysates from experiments A and B were quantitated by an LAS-1000 imaging analyzer. Budding rates (% VLP release) were calculated as described in Materials and Methods and plotted in a graph. Bars represent averages and error bars represent standard deviations from at least three independent experiments.

observed at 24 hr post-transfection by Western blotting. Significant amounts of the M proteins were also detected in the VLP fraction from the culture medium (Fig. 1A). VLP budding rates were calculated and plotted in a graph (Fig. 1C).

Co-expression of tetherin with transfection of 0.5 or 1 μg of pCAG-HA-tetherin per dish inhibited VLP release of these viruses without affecting M protein expression (Fig. 1A, 1C). Furthermore, the efficiency of NiV M- and SeV M-VLP release was reduced in a dose-dependent manner by the increase of tetherin expression (0 to 1 μg) (Fig. 1B, 1D), suggesting that tetherin has an inhibitory effect on the release of NiV M- and SeV M-VLPs. Notably, tetherin appeared as multiple bands on the gel with estimated sizes of 21 and 35 kDa (Fig. 1A), presumably because of heterogeneous glycosylation[13].

Accumulation and colocalization of tetherin and NiV M protein in cells

To confirm that inhibition of paramyxovirus VLP production by tetherin occurs at the step of particle release, as observed for HIV-1[5], we carried out immunofluorescence microscopic analysis of the expression and localization of NiV M and tetherin in cells. 293T cells were transfected with pCAG-NiV M or pCAG-HA-tetherin to examine individual expression, and co-transfection of pCAG-NiV M and pCAG-HA-tetherin was also performed. When NiV M was expressed alone, the M protein was distributed diffusely throughout the cytoplasm with a small amount along the cell membrane. A similar pattern of protein distribution was observed for cells solely expressing tetherin. Accumulation of NiV M-VLPs was observed in 293T cells co-expressing tetherin but not in those without tetherin (Fig. 2), which is in accord with the observation that tetherin restricts the release of NiV M-VLPs from cells. Moreover, possible colocalization of NiV M protein and the tetherin suggests retention of VLPs containing NiV M on the cell membranes by tetherin (Fig. 2).

Confirmation of our VLP production system and tetherin inhibition by HIV-1 Gag protein

We prepared the HA-tagged tetherin cDNA and used it in our VLP production system. To confirm that our VLP production system was reliable, we examined the inhibition of retroviral HIV-1 Gag polyprotein release, which is well known to be highly susceptible to tetherin restriction. Transfection of 293T cells with pCAG-Gag produced HIV-1 Gag protein in the VLP fraction as well as in the cells (Fig. 3A). Co-expression of tetherin with transfection of 0.5 or 1 μg of pCAG-HA-tetherin per dish dramatically inhibited VLP

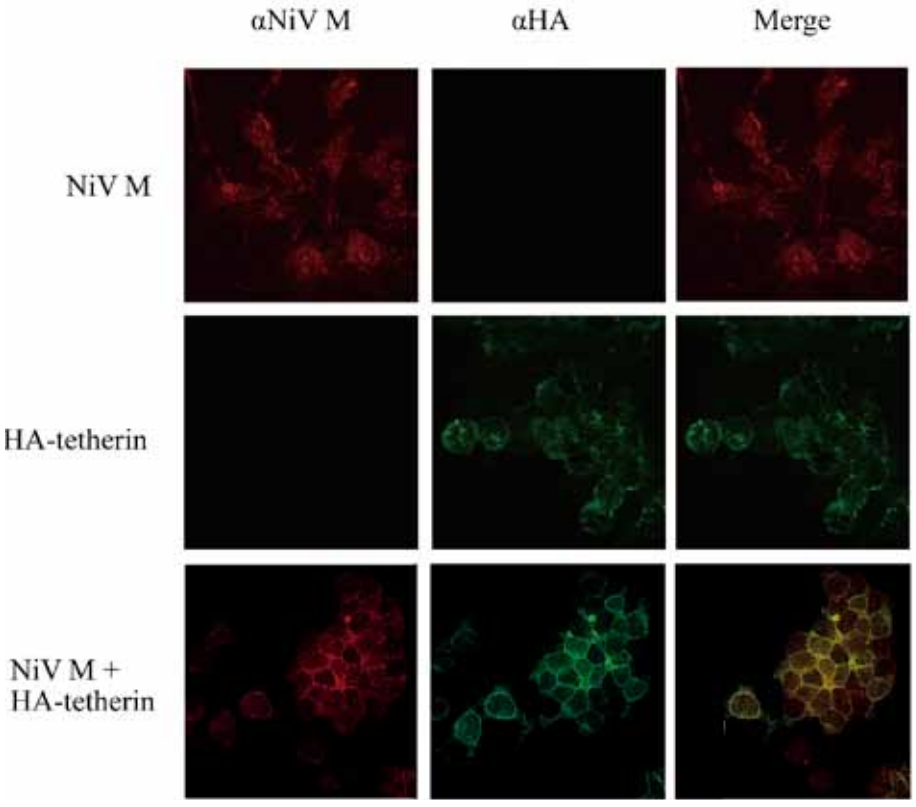

Fig. 2. Immunofluorescence analysis of NiV M and HA-tetherin expression in 293T cells. Cells were transfected individually (top two rows) or co-transfected with pCAG-NiV M and pCAG-HA-tetherin (third row). At 24 hr post-transfection, cells were fixed and stained for immunofluorescence with anti-HA (αHA, green) and anti-NiV M (αNiV M, red) antibodies. Merged images are also shown (Merge).

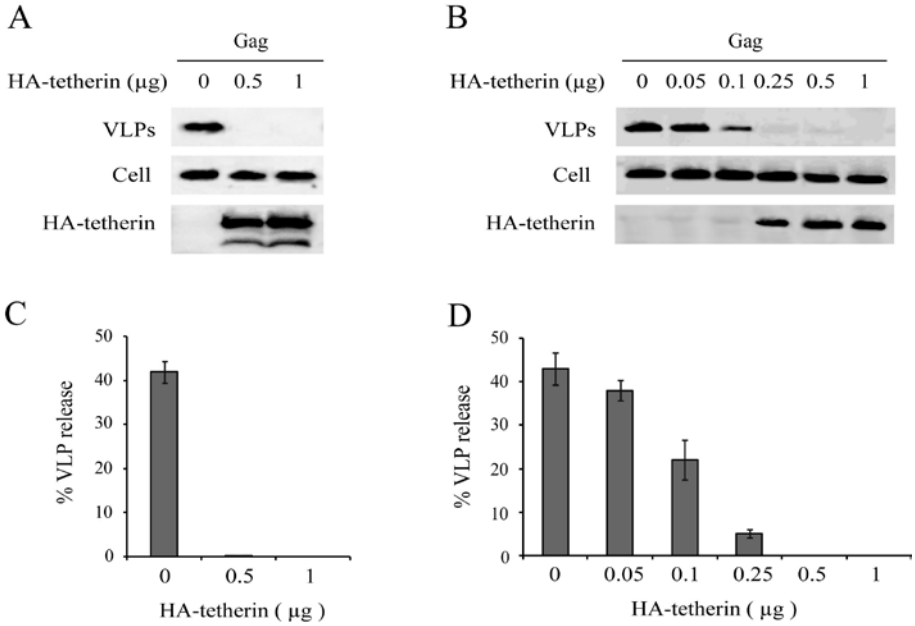

Fig. 3. Tetherin inhibits budding of HIV-1 Gag protein.
(A) 293T cells were transfected with pCAG-Gag (1 μg) together with pCAG-HA-tetherin (0.5 or 1 μg) or an empty plasmid. At 24 hr post-transfection, cell lysates and culture medium were collected and VLPs in the medium were pelleted through a 20% sucrose cushion. Gag proteins in VLPs and cell lysates were analyzed by Western blotting with an antibody against Gag p24 protein. Expression of HA-tetherin in cell lysates was also determined by Western blotting. (B) Analysis of dose-dependent inhibition of HIV-1 Gag release by tetherin. 293T cells were transfected with pCAG-Gag and increasing amounts of pCAG-HA-tetherin (0 to 1 μg). (C and D) HIV-1 Gag proteins present in VLPs and cell lysates from experiments A and B were quantitated by an imaging analyzer and plotted in a graph as described in the legend of Fig. 1.

release of the Gag protein; 0.5 μg of pCAG-HA-tetherin almost completely inhibited VLP release (Fig. 3A, 3C). It appears that the inhibition of the release of Gag VLPs was rather stronger than those of NiV M- and SeV M-VLPs (Fig. 1). The efficiency of Gag release was also reduced in a dose-dependent manner by increase of tetherin expression (0 to 1 μg) (Fig. 2B, 2D).

Tetherin anti-budding activity is antagonized by HIV-1 Vpu.

Viral protein U (Vpu), an 81-amino-acid type I integral membrane phosphoprotein expressed from the HIV-1 genome[4,34], counteracts tetherin activity[23,24]. To investigate whether the expression of Vpu could also counteract the inhibitory effect of tetherin on paramyxovirus M VLP release, cells were transfected with pCAG-Vpu along with pCAG-HA-tetherin. Vpu did not affect the yield of VLPs in the absence of tetherin (data not shown). In the case of Gag, Vpu completely antagonized the activity of tetherin, resulting in absolute recovery of HIV-1 Gag release, as expected (Fig. 4). Moreover, in the case of NiV M and SeV M, the inhibitory effect of tetherin was also reversed by the expression of Vpu, again without having an effect on M protein expression (Fig. 4). The results suggest that tetherin is capable of restricting the release of VLPs of these paramyxoviruses probably by a mechanism similar to that for HIV-1 Gag.

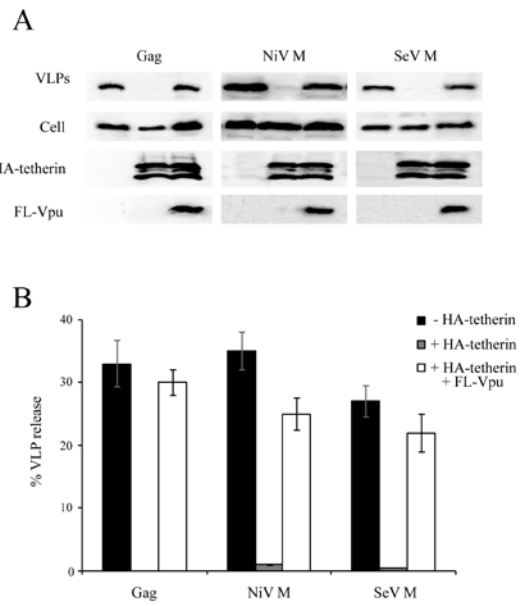

Fig. 4. Inhibitory effects of tetherin on VLPs release were abrogated by Vpu.
(A) 293T cells were transfected with pCAG-Gag, pCAG-NiV M or pCAG-SeV M alone or in combination with pCAG-FL-Vpu and/or pCAG-HA-tetherin as indicated in the figure. VLPs in clarified supernatants were pelleted through a 20% sucrose cushion. Western blotting analysis was performed with antibodies against Gag, NiV M and SeV M proteins, and anti-Flag and anti-HA antibodies. (B) Matrix proteins in VLPs and cell lysates from experiments A and B were quantitated by an imaging analyzer. Budding rates (% VLP release) were calculated and plotted in a graph. Bars represent averages and error bars represent standard deviations from at least three independent experiments.

Tetherin failed to restrict MuV M-VLP release.

Mumps virus (MuV) M protein was expressed, and VLP release and its inhibition by tetherin were investigated. Surprisingly, the release of MuV M-VLPs was unaffected by co-expression of tetherin (Fig. 5A). In this experiment, SeV nucleocapsid (N) protein was also included as a non-budding control, and the N protein was not actually secreted into the culture medium (Fig. 5A).

The unresponsiveness of MuV M VLPs to tetherin was confirmed by using different amounts of pCAG-HA-tetherin (0.5 and 1 μg) (Fig. 5B, 5C). Tetherin expression did not reduce the budding efficiency of MuV M-VLP, unlike in the cases of other paramyxoviruses (NiV M and SeV M).

DISCUSSION

In the present study, we generated VLPs by expressing the NiV M protein. Expressed NiV M protein was efficiently released into the culture medium. The efficiency appeared to be similar to that of the previously reported release of VLPs driven by expression of the SeV M protein[35]. Interestingly, release of the VLPs in both cases was inhibited by simultaneous expression of tetherin. Inhibition of SeV M-VLPs by tetherin is the first report, although that of NiV M-VLPs has recently been reported[30]. NiV M and tetherin molecules appeared to co-localize in transfected cells by immunofluorescent staining, suggesting a direct effect of tetherin on the NiV M-VLP. To confirm the reliability of our VLP production system, we also expressed HIV-1 Gag protein and tetherin. Tetherin inhibited the release of HIV-1 Gag protein as reported previously[24], and the extent of inhibition was almost the same as, or better than, those of NiV M- and SeV M-VLPs. The effects of Vpu, an antagonist of tetherin derived from HIV-1, on NiV M- and SeV M-VLPs, were also investigated. Vpu suppressed the anti-budding activity of tetherin against those paramyxovirus VLPs. To be noted is that the VLP release by the expression of MuV M protein was not inhibited, suggesting the presence of tetherin-non-effective VLPs.

The expression of tetherin is induced by type I IFN, suggesting that tetherin is a part of the general IFN-induced anti-viral response[3,31]. Many viral infections could potentially trigger tetherin expression, which could in turn restrict the spread of the virus. Since the discovery of tetherin, the list of viruses susceptible to it, including retroviruses, filoviruses and arenaviruses, has been growing. We have shown that the release of VLP, driven by paramyxovirus M proteins of SeV, was significantly inhibited. Together with NiV M protein, these findings add paramyxoviruses to the list of tetherin's targets, although the release of MuV M protein was not inhibited by tetherin.

One of the key features of tetherin that enables it to target a diverse group of viruses lies in its unique structure with membrane anchors at both ends. Tetherin binds to the lipid bilayer of virions or cellular membranes via both its N-terminal transmembrane domain and its C-terminal GPI anchor. So far, results of biochemical and structural studies support the simplest mode of restriction, a direct tethering mechanism in which

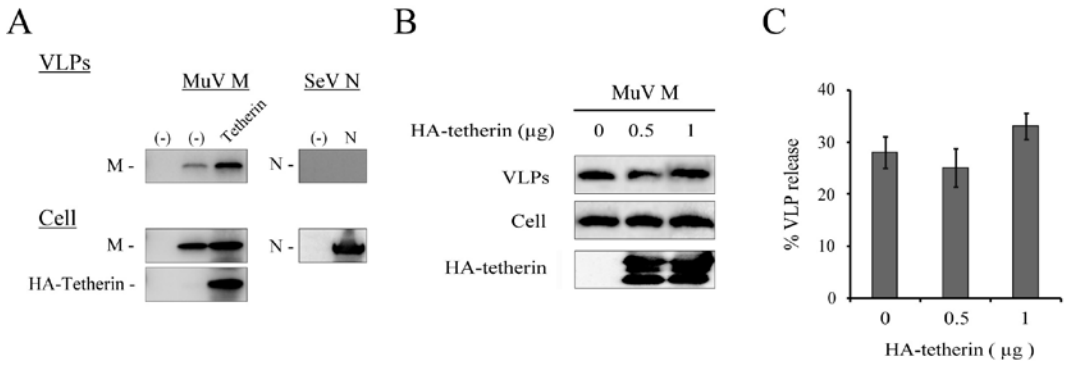

Fig. 5. Tetherin is not effective against MuV M-VLP release.

(A) 293T cells were transfected with pCAG-HA-MuV M (1 μg) together with pCAG-HA-tetherin or an empty plasmid (1 μg). At 24 hr post-transfection, VLPs in the medium were pelleted through a 20% sucrose cushion, and matrix proteins in VLPs and cell lysates were analyzed by Western blotting. Expression of HA-tetherin in cell lysates was also determined by Western blotting. 293T cells were also transfected with pCAG-SeV N and treated similarly as a non-budding control in order to confirm the experimental system. (B) 293T cells were transfected with pCAG-HA-MuV M (1 μg) together with pCAG-HA-tetherin (0.5 or 1 μg) or an empty plasmid. At 24 hr post-transfection, VLPs in the medium were pelleted through a 20% sucrose cushion and M proteins in VLPs were analyzed by Western blotting. Expression of HA-tetherin and M proteins in cell lysates was also determined by Western blotting with appropriate antibodies. (C) MuV M protein in VLPs and cell lysates from experiment B was quantitated by an imaging analyzer, and budding rates (% VLP release) were calculated and plotted in a graph. Bars represent averages and error bars represent standard deviations from at least three independent experiments.

parallel tetherin dimers physically crosslink virions to the cellular membrane or to other virion membranes[2,5,9,11,29,40]. Consistent with these reports, striking co-localization of both proteins along the possible budding site (Fig. 2) indicates that tetherin may act in a direct restriction manner to retain paramyxovirus on host cells.

Some enveloped viruses such as HIV-1, Ebolavirus and γ-herpesvirus are basically insensitive to tetherin inhibition. These viruses encode tetherin-antagonists, Vpu[24], G[15] and K5/MIR2[22,27] proteins, respectively, in their genomes. In the case of resistance of MuV M-VLP release to tetherin, we have to consider a reason other than the presence of an antagonist, since the VLP production system lacks other viral proteins.

Expression of the M protein of parainfluenza virus-5 (PIV5), previously called simian virus 5 (SV5), did not induce formation of VLPs, and efficient VLP formation needs co-expression of one of its glycoproteins, F or HN, and a nucleocapsid protein[33]. Expression of mumps virus M protein induced formation of VLPs with low efficiency, but co-expression of F and N proteins drastically enhanced VLP release[21]. PIV5 and MuV belong to the genus *Rubulavirus* of the family *Paramyxoviridae*. On the other hand, expression of M proteins of viruses of other genera such as SeV (genus *Respirovirus*), NiV (genus *Henipavirus*), Newcastle disease virus (genus *Avulavirus*) and measles virus (genus *Morbillivirus*) cause full release of VLPs, and co-expression of other viral proteins did not enhance VLP release[26,28,32,35] (Kong, W.-S., an unpublished observation). It is tempting to speculate that there is a difference in the virus budding mechanism between Rubulaviruses and other genera, for example, a difference in association of their matrix proteins with host factors such as membrane trafficking proteins or lipid rafts, and that it may cause the different reactivity against tetherin. This question remains to be answered until confirmation of other Rubulaviruses can be made.

In summary, we have demonstrated that a novel host defense factor, tetherin, also blocks the release of paramyxovirus NiV M- and SeV M-VLPs, reinforcing the fact that tetherin can act in a broad way as a characteristic of innate immune effectors. In contrast, release of MuV M-VLPs was insensitive to tetherin. Further analysis of the difference in these VLPs may elucidate the egress mechanism of enveloped viruses and the mechanism of inhibition of VLP release by tetherin.

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