Interferon-induced cell membrane proteins, IFITM3 and tetherin, inhibit vesicular stomatitis virus infection via distinct mechanisms

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Tetherin and IFITM3 are recently identified interferon-induced cellular proteins that restrict the infection of retroviruses and filoviruses as well as influenza virus and flaviviruses, respectively. In our efforts to further explore their antiviral activity against other viruses and determine their antiviral mechanisms, we found that the two antiviral proteins potently inhibit the infection of vesicular stomatitis virus (VSV), a prototype member of Rhabdoviridae family. Taking advantage of this well-studied virus infection system, we show that although both tetherin and IFITM3 are plasma membrane proteins, tetherin inhibits virion particle release from infected cells, while IFITM3 disrupts an early event after endocytosis of virion particles, but before primary transcription of incoming viral genomes. Furthermore, we demonstrate that both the N-terminal 21 amino acid residues and C-terminal transmembrane region of IFITM3 are required for its antiviral activity. Collectively, our work sheds light on the mechanism by which tetherin and IFITM3 restrict the infection with rhabdoviruses and possible other pathogenic viruses.
The interferon (IFN) system is the first line defense against virus infection in the vertebrates. Infection of cells by viruses can be detected by host cellular pattern recognition receptors (PRRs), such as toll-like receptors and RIG-I-like receptors (1, 33, 42). Engagement of the PRRs with the virus-associated molecular patterns, such as 5′-triphosphate and/or double stranded viral RNA, triggers signaling cascades leading to the synthesis and secretion of type I IFNs, represented by IFN-α and IFN-β (25). Type I IFNs bind to their cognate receptors on the cell surface and activate the receptor-associated janus kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2) through tyrosine phosphorylation, which in turn stimulates the tyrosine phosphorylation of STAT1 and STAT2. The phosphorylated STAT1 and STAT2, in combination with IRF9, form a trimeric ISGF3 transcription factor that translocates into the nucleus and activates the expression of IFN-stimulated genes (ISGs), whose products limit viral replication, regulate cell proliferation and/or modulate host innate and adaptive immune responses (34, 36, 39).

Although IFN treatment of cells induces the expression of hundreds of ISGs, only a handful of ISGs have thus far been demonstrated to instigate antiviral state in cultured cells and only four of them, including ISG15 (IFN-stimulated protein of 15 kDa), myxovirus resistance 1 (Mx1), ribonuclease L (RNase L) and double-stranded RNA activated protein kinase (PKR), have been shown to play a role in mediating the IFN’s antiviral response in vivo with gene knockout mice models (36). While it is most likely that inhibition of the infection of any given virus by IFNs is through induction of multiple ISGs that work cooperatively to disrupt multiple steps of viral replication, identification of individual antiviral ISGs and subsequent elucidation of their mode of action are essential to uncover the antiviral mechanism of IFNs and viral pathogenesis (20, 49, 50).

Recently, several laboratories, including our own, have identified two new families of
IFN-induced cellular proteins, tetherin (or BST-2) and three members of IFN-induced transmembrane family (IFITM) proteins, as novel antiviral ISGs (4, 21, 31). While tetherin inhibits the release of retroviruses and filoviruses by tethering newly budding viral particles on cell surface, which leads to their subsequent endocytosis and degradation in the lysosome (23, 31, 37), IFITMs disrupt an unidentified early event of influenza virus and flavivirus infection (4, 21). Our studies reported herein for the first time demonstrate that expression of either tetherin or IFITMs in human embryonic kidney (HEK) 293-derived cell lines potently inhibits the infection of vesicular stomatitis virus (VSV), a well-studied member of Rhabdoviridae family. As observed for lentiviruses and filoviruses, tetherin does not inhibit VSV RNA replication and protein expression, but rather inhibits the release of virions from infected cells. Conversely, IFITM3, the most potent antiviral IFITM, inhibits the infection of VSV glycoprotein (G) pseudotyped lentiviruses, but does not disrupt the attachment and endocytosis of VSV. However, expression of the ISG significantly reduces the viral primary transcription. Hence, it appears that IFITM3 inhibits an early event after endocytosis of virion particles, but at or before primary transcription of incoming viral genomes. Moreover, we provide strong evidence suggesting that both the N-terminal 21 amino acid residues and C-terminal transmembrane region of IFITM3 are required for its antiviral function. Our work thus reveals the distinct antiviral property of the two newly identified antiviral ISGs, and provides basis for further analyses of the role and mechanism of tetherin and IFITMs in controlling the infection of rhabdoviruses and other pathogenic viruses.

MATERIALS AND METHODS
Cell culture and virus. The parental FLP-IN T Rex cells (Invitrogen) were maintained in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin G, streptomycin, non-essential amino acids, L-glutamine, 10 µg/ml blasticidin and 100 µg/ml Zeocin. FLP-IN T Rex-derived cell lines expressing control protein chloramphenicol acetyl transferase (CAT), tetherin, IFITM1, IFITM2 or IFITM3 were established and maintained as described previously (20). Vero cells were grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 50 U/ml of penicillin and 50 µg/ml of streptomycin. BHK-21 cells were grown in minimal essential medium (MEM, Gibco-BRL) supplemented with 5% FBS, 2 g/l sodium bicarbonate, 50 U/ml of penicillin and 50 µg/ml of streptomycin. Wild type VSV (Indiana serotype) is described previously (15). Recombinant VSV expressing GFP was kindly provided by Dr. Matthias J. Schnell (Thomas Jefferson University, Philadelphia, PA) and propagated in BHK-21 cells. VSV titers were determined by a plaque assay in Vero cells, essentially as described previously (15).

Immunofluorescence  FLP-IN T Rex-derived cell lines were left untreated or treated with 1 µg/ml of tetracycline for 24 h and followed by fixation with 2% paraformaldehyde and permeabilization of the cell membrane with 0.1% Triton X-100. Cells were then stained with either IFITM3- or tetherin-specific antibody and the bound antibodies were visualized by Alexa Fluor 488 goat anti-mouse IgG. Cells were imaged with a Nikon fluorescent microscope and photographed with a charge-coupled device camera.

Analysis of membrane topology of tetherin and IFITM3  Tetherin and IFITM3-expressing FLP-IN cell lines were seeded into 12-well plates at a density of 5x10^5 cells per well
and cultured in the absence or presence of tetracycline (1µg/ml) for 24h. Cells were then
detached from the wells by treatment with PBS containing 0.5M EDTA at 37°C for 5 min and
followed by suspension in FACS buffer, which is PBS containing 2% bovine serum albumin
(BSA). After fixation with PBS containing 1% paraformaldehyde for 30 min at room
temperature, cells were extensively washed with PBS and divided into two tubes. While one tube
was left untreated, another tube was treated with PBS containing 0.2% Tween 20 at 37°C for 15’
to permeabilize the cells. Both the untreated and permeablized cells were suspended in FACS
buffer containing 1 µg/ml of mouse anti-flag antibody (Sigma) and incubated on ice for 1h.
After extensive washes with PBS to remove unbound antibodies, the cells were incubated with
FACS buffer containing fluorescein-549 labeled rabbit anti-mouse IgG on ice for 30 min. The
bound antibodies were analyzed by Guava EasyCyte plus (Millipore) flow cytometer. Cells not
induced with tetracycline were used as a negative control, as well as an isotype matched primary
antibody.

Quantification of viral RNA by real-time RT-PCR FLP-IN T Rex cell lines expressing
CAT, tetherin or IFITM3 were cultured in the absence or presence of tetracycline (1µg/ml) for
24h and followed by infection with VSV at an MOI of 5. Cells were harvested prior to being
infected or at the indicated time points after infection. Total cellular RNA was extracted with
TRIzol reagent by following the direction of manufacturer (Invitrogen). VSV RNA was
measured by a TaqMan RT PCR assay (40). Briefly, primers and probes for TaqMan RT-PCR
detection of VSV RNA were designed and purchased from Integrated DNA Technologies. The
sequences of the forward and reverse primers are 5’-GATAGTACC GGAGGATTGACGACTA-
3’ and 5’-TCAAACCATCCGAGCCATTC-3’, respectively. The dual-labeled probe is 5’–
carboxyfluorescein-TGCACCGCCACAAGGCAGAGA-6-carboxytetramethylrhodamine-3’.
RT-PCR reaction was performed with a TaqMan One-Step RT-PCR Master Mix Reagents kit (Applied Biosystems), as described by the manufacturer, using a 20 µl sample volume with 5µM each of the primers, 25 µM probe, and 100ng of total cellular RNA. TaqMan PCR assays were performed using an ABI 7500 Fast-Real Time PCR System (Applied Biosystems), using the following amplification profile: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, and 44 cycles at 95°C for 15 s and 60°C for 1 min. All samples were tested in triplicate.

**Western blot assay** Cell monolayers were washed once with PBS buffer and lysed with 1x Laemmli buffer. A fraction of cell lysate was separated on sodium dodecyl sulfate-12% polyacrylamide gels and electrophoretically transferred onto PVDF membrane (Bio-Rad). Membranes were blocked with PBS containing 5% nonfat dry milk and probed with antibodies against FLAG tag (Sigma), tetherin (Proteintech Group, Inc.), IFITM3 (Proteintech Group, Inc.), VSV G protein (Rockland, Inc) or β-actin (Chemicon International). Bound antibodies were revealed by HPR-labeled secondary antibodies and visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) according to the protocol of the manufacturer.

**FACS Analysis of GFP-expressing VSV-infected cells** FLP-IN T Rex-derived stable cell lines expressing CAT, tetherin or IFITM3 were seeded into 12-well plates at a density of 5x10^5 cells per well and cultured in the absence or presence of tetracycline (1 µg/ml) for 24 h and followed by infection with VSV-GFP at an MOI of 5. Four hours after infection, cells were detached from the wells by treatment with PBS containing 0.5M EDTA for 5 min at 37°C. Cells were resuspended in FACS buffer (PBS+2%BSA) and gently pelleted by centrifugation at 2,000 rpm for 5 min. Cells were resuspended in fixative solution (PBS containing 1% paraformaldehyde) and incubated at room temperature for 30 min, followed by two times of
washing with PBS and resuspended in FACS buffer. GFP expression was analyzed by using Guava EasyCyte plus (Millipore) flow cytometer.

**VSV-G pseudotyped lentivirus infection**  FLP-IN cells expressing CAT, tetherin or IFITMs were seeded at a density of 5x10^4 cells per well in a 96-well plate. At 24 h post seeding, the cells were cultured in the absence or presence of 1 µg/ml tetracycline for 24 h and followed by infection with the VSV-G protein pseudotyped lentivirus (SABioscience) that express firefly luciferase at a MOI of 2 for 1 h and continued to be cultured with medium without or with tetracycline for additional 48 h. Cells were then lysed and luciferase activity in the cell lysates was quantified by using a Steady-Glo firefly luciferase assay kit (Promega).

**Virus binding and entry assays**  FLP-IN T Rex cells inducible expressing CAT, IFITM3 or tetherin were seeded in 12-well plates at a density of 5x10^5 cells per well and cultured in the absence or presence of tetracycline for 36 h. Cells were infected with VSV at an MOI of 5 for 1 h on ice to allow attachment but impede virus entry. After three washes with PBS, RNA was extracted to measure the amount of cell-bound virus. To assay virus entry into cells by endocytosis, virus inocula were removed after 1 h of binding on ice, cells were washed, pre-warmed medium was added, and the cells were incubated for another 10 min at 37°C. Cells were then washed once with PBS, followed by treatment with 0.25% trypsin for 10 min and again washed three times with PBS to remove any cell-associated virus which had not entered the cytoplasm. Total cellular RNA was extracted to measure the amount of viral genomes that have entered cells by real-time RT-PCR assay.

**Northern Blot hybridization**  Total cellular RNA was extracted with TRIzol reagent (Invitrogen) by following the manufacturer's direction. Five micrograms of total RNA were
fractionated on 1.5% agarose gel containing 2.2M formaldehyde and transferred onto Nylon membranes. Membranes were hybridized with riboprobes specific for VSV N mRNA in the conditions described previously (14).

Establishment of cell lines expressing mutant IFITMs cDNA constructs expressing FLAG-tagged IFITM mutant proteins harboring point mutations in the conserved region and chimeric IFITM proteins consisting of structural domains originated from both IFITM1 and IFITM3 were constructed by overlap extension PCR. The sequences of the primers are available upon request. Briefly, two separate PCRs were performed to amplify two overlapping fragments of the coding region of targeting molecule using four primers. The point mutations were introduced by the two middle primers. The final PCR products were cloned into vector pcDNA5/FRT/ΔCAT. cDNA construct expressing a N-terminally truncated IFITM3 protein was constructed by cloning the PCR products, amplified with a pair of primers bracketing the desired regions of IFITM3 cDNA, into pcDNA5/FRT/ΔCAT vector (20). All the resulting DNA clones were sequenced to verify the desired mutation(s). Stable cell lines inducibly expressing the mutant IFITMs were established by co-transfection of FLP-IN T Rex cells with pOG44 and above made IFITM mutant protein-expressing plasmids and followed by selection and pool of hygromycin and blasticidin resistant cell colonies, essentially as previously described (20).

RESULTS

Expression of IFITMs and tetherin elicits antiviral activity against VSV in HEK293 cells In our recent efforts to systematically identify antiviral ISGs that inhibit hepatitis C virus
(HCV) and dengue virus (DENV) infection, we have established 36 FLP-IN T Rex-derived stable cell lines expressing individual ISGs in a tetracycline-inducible manner (20, 21). Taking advantage of this unique cell culture system, we tested the antiviral activity of the 36 ISGs against infection with VSV. Aside from PKR and ISG20, that have been reported previously to inhibit VSV infection in cultured cells and mice \textit{in vivo} (3, 11, 12, 41), we found that expression of tetherin and two members of IFITM family proteins, IFITM2 and IFITM3, reduced VSV yields by over 1000, 5 and 15-fold, respectively (Fig. 1B and D). Inducible expression of each of the three members of IFITMs and tetherin in FLP-IN T Rex derived cell lines, upon addition of tetracycline in culture media, is demonstrated by Western blot analyses (Fig. 1A and C). The observed antiviral profile of the three IFITMs against VSV is consistent with our previous observation that IFITM3 and to a lesser extent, IFITM2, but not IFITM1, elicit an antiviral activity against both WNV and DENV infection in these cell lines (21).

**Both IFITM and tetherin restrict VSV infection** To further investigate the physiological role of the IFITMs and tetherin in controlling VSV infection, we intended to determine the effects of depletion of basal levels and IFN-induced expression of IFITM and tetherin on VSV infection. Compared with Hela cells transfected with nontargeting siRNA (control), transfection of SMARTpool siRNA targeting IFITM2 and 3 (Fig. 2A) or tetherin (Fig. 2C) not only efficiently reduced basal, but also IFN-α-induced levels of IFITM and tetherin expression, respectively. Interestingly, knocking-down IFITM2/3 expression results in an approximately two to three-fold increase of VSV yields in cells mock-treated or treated with IFN-α (Fig. 2B). Intriguingly, transfection of siRNA targeting tetherin (Fig. 2D) into Hela cells drastically increases permissiveness of the cells to VSV infection, as indicated by approximately
100-fold increase of progeny virus production (Fig. 2D). Moreover, reduction of IFN-induced tetherin expression also significantly attenuated the ability of IFN-α to inhibit VSV infection at lower concentrations (1 - 10 IU/ml). However, in the cells treated with a higher concentration of IFN-α (100 IU/ml), the attenuation effect is decreased, presumably due to the compensation of other IFN-activated antiviral pathways under this condition (Fig. 2D). Hence, our results imply that both IFITM2/3 and tetherin not only restrict VSV infection at their basal levels of expression, but also are physiological mediators of IFN-induced antiviral response against VSV.

Characterization of tetherin and IFITM expression and membrane topology in FLP-IN T Rex cells Tetherin and IFITMs are type I IFN-inducible cell membrane proteins (18). Immunofluorescent staining with tetherin- and IFITM3-specific antibodies demonstrates that upon induction by the addition of tetracycline into culture media, both IFITM3 and tetherin can be detected in the cytoplasm of FLP-IN cells (Fig. 3A and B). The localization of tetherin is enriched on the peripheral zone of cells, most likely on the plasma membrane. Recently, Bruss and colleagues showed that IFITM3 resided in ER membranes (4). However, it was also reported previously that IFITMs localized in the plasma membrane of several types of cells (10). Protein sequence analysis with TMpred program (www.ch.embnet.org) predicts that IFITMs contain two transmembrane regions and a conserved intracellular loop. Such a membrane topology (depicted in Fig. 3C, top diagram) suggests that both the N- and C-terminus of IFITM protein localizes extracellularly. In contrast, tetherin is a type II transmembrane protein with a GPI-anchor at its C-terminus, therefore, its N-terminus localizes intracellularly (depicted in Fig. 3D, top diagram) (31). Because both tetherin and IFITMs engineered into FLP-IN T Rex cells is N-terminally flag-tagged (20), we performed FACS analysis to detect the flag epitope expression in intact and
permeablized FLP-IN T Rex cells expressing IFITM3 or tetherin. As shown in Fig 3 C and D, while the flag epitope can be detected in both permeablized IFITM3 and tetherin-expressing cells, it can only be detected on intact cells expressing IFITM3, but not tetherin. These results thus convincingly demonstrate that IFITM3 is expressed on the plasma membrane of FLP-IN cells with the predicted membrane topology.

**Tetherin and IFITM3 inhibit distinct steps of VSV replication** In order to map the viral replication steps targeted by tetherin and IFITM3, a synchronized VSV infection assay was performed. Briefly, the indicated cell lines were cultured in the absence or presence of tetracycline for 24 h and followed by infection with VSV at an MOI of 5 on ice for 1 h. Such a multiplicity of infection ensures that at least 99% cells are infected with minimally one infectious viral particle. The virus inoculums were then removed and infected cells were cultured with pre-warmed fresh media at 37°C. At the indicated times after infection, culture media and cells were harvested to determine the virus yields and amount of intracellular viral RNA and G protein accumulation. As shown in Fig 4, expression of control protein, CAT, does not affect viral RNA replication, G protein accumulation or virion production. IFITM3 expression, however, significantly reduces the levels of intracellular viral RNA as early as 1 h post infection, and similarly, VSV-G protein accumulation was dramatically reduced at the 6 h post infection. As a consequence, virion production was also inhibited by IFITM3. In stark contrast, expression of tetherin does not affect intracellular viral RNA replication and G protein accumulation, but drastically reduces virion production. Moreover, microscopic inspection indicates that VSV infection causes apparent cytopathic effects (CPE) as demonstrated by cell round-up and
detachment from plates at 6 h post infection. Interestingly, expression of IFITM3, but not tetherin, efficiently inhibited the VSV-induced CPE (Fig. 4D).

To further confirm the distinct antiviral property of tetherin and IFITM3, a synchronized infection assay was performed with a recombinant VSV that expresses green fluorescent protein (GFP). As for all viral proteins, GFP is expressed by the recombinant VSV as an independent transcript unit. Therefore, the level of intracellular GFP can serve as a surrogate marker for viral RNA replication/transcription and protein expression. As shown in Fig. 5, flow cytometry analysis clearly indicates that expression of CAT and tetherin does not affect GFP expression, whereas IFITM3 significantly reduces the percentage of GFP positive cells as well as the mean fluorescent intensity (MFI) of infected cells.

Taken together, the results obtained from these experiments suggest that tetherin most likely inhibits VSV release from infected cells, which is in agreement with the mechanism by which the ISG restricts HIV and Ebola virus infection (23, 31). IFITM3, however, most possibly inhibits either VSV entry into host cells or viral RNA/protein biosynthesis.

**IFITM3 inhibits VSV G protein mediated virus entry** Considering the facts that IFITM3 is a plasma membrane protein and inhibits an unidentified early event of influenza virus and flavivirus infection (4), it is reasonable to envisage that IFITM3 may also inhibit an early event of VSV infection. To test this hypothesis, we first determined the effects of the three IFITMs and tetherin on the infection of a VSV G protein pseudotyped lentivirus that expresses firefly luciferase upon integration of lentiviral vector DNA into host cellular chromosome. As shown in Fig. 6A, expression of CAT and tetherin does not affect the pseudovirus infection.
However, consistent with their antiviral profile observed in Fig. 1, expression of IFITM2 and 3, but not IFITM1, significantly reduced the level of the pseudotyped lentivirus conferred luciferase expression. Because it has been demonstrated independently in several reports that the IFITMs do not inhibit lentiviral infection (4, 31), our result thus most likely indicates that IFITM3 inhibits VSV-G protein mediated pseudovirus entry into cells, but does not disrupt a post entry event of lentiviral replication, such as lentiviral cDNA synthesis, integration and gene expression.

To further validate this observation and determine the precise VSV entry step that is inhibited by the antiviral ISG, a classical virus binding and entry assay was performed (16). Briefly, FLP-IN T Rex cells expressing CAT, tetherin or IFITM3 were cultured in the absence or presence of tetracycline for 24 h. For the attachment assay, these cells were infected with VSV at an MOI of 5 for 1 h on ice, followed by extensive washing steps with cold PBS. Cellular associated viral RNA was measured to quantify virus attachment onto the cells. To determine virus entry, cells were infected on ice as described above, and after extensive washing with cold PBS to remove unattached virions, pre-warmed medium was added and cells were incubated at 37°C for another 10 min. The time interval chosen to monitor VSV entry is based on previous studies showing that the endocytosis of VSV virion particles is extremely efficient and takes less than 5 min (9, 22). In fact, similar results were obtained by harvesting cells between 10 and 30 min of incubation (data not shown). After incubation at 37°C, cells were treated with trypsin and washed three times with complete DMEM medium to remove virions that had not entered into cells. As shown in Fig. 6B, this procedure efficiently removes approximately 99% of bound virions (comparing viral RNA copies obtained from attachment to cells immediately treated with trypsin without temperature shift). Total cellular RNA was extracted and viral RNA was
quantified with real-time RT-PCR assay. The results show that expression of neither control proteins (CAT and tetherin) nor IFITM3 affected VSV attachment and entry into cells (Fig. 6B).

Expression of IFITM3 reduces primary transcription of incoming VSV genomes

While the pseudovirus infection assay suggests that IFITM3 inhibits VSV G protein-mediated virus entry (Fig. 6A), it appears that the ISG does not inhibit the attachment and endocytosis of VSV (Fig. 6B). It is therefore possible that IFITM3 disrupts the intracellular trafficking of the endosome and/or fusion of virion and endosomal membranes. However, due to technical limitations, we were unable to follow endosome trafficking and membrane fusion in VSV infected cells. Instead, in order to further confirm the notion that IFITM3 inhibited an early event of VSV infection, but not viral RNA replication or protein translation, we sought to determine the effect of IFITM3 on the primary transcription of incoming viral genomes. As a negative-stranded RNA virus, upon release of viral nucleocapsids into the cytoplasm through membrane fusion, the first biosynthetic step in VSV replication cycle is primary transcription, which is mediated by virion-associated RNA-dependent RNA polymerase. Because all the essential proteins for the primary transcription of viral mRNA are packaged within the nucleocapsid, primary transcription does not require synthesis of viral or additional host cellular proteins (7, 35).

FLP-IN/IFITM3 cells were left untreated or treated with tetracycline for 24 h, followed by infection of VSV at an MOI of 1, on ice, for 1 h. Cells were then washed twice with PBS to remove unattached viruses and re-fed with pre-warmed complete medium without or with cycloheximide, to inhibit protein translation. Cells were harvested immediately after infection (0 h) or at 1 and 2 h post infection. The levels of viral nucleocapsid protein (N) mRNA were
measured by Northern blot hybridization. As shown in Fig. 7A, treatment of cells with cycloheximide does not affect the accumulation of VSV N mRNA at 1 h after infection (comparing lanes 3 and 4), but significantly reduces viral mRNA accumulation at 2 h after infection (comparing lanes 7 and 8), suggesting that within the first hour post infection, viral mRNA is exclusively derived from primary transcription, which does not require new protein synthesis. Interestingly, expression of IFITM3 significantly reduced the amount of VSV N mRNA from primary transcription (comparing lanes 3 and 5 or lanes 4 and 6). Moreover, as shown in an additional time course study (Figs. 7B and C), VSV N mRNA was readily detectable at 30 min post infection and expression of IFITM3 reduced the levels of the viral mRNA at all the time points tested. Taken together, our results imply that IFITM3 either directly inhibited VSV primary transcription or more likely, an early event after endocytosis, but before primary transcription.

Identification of the structural domains essential for the antiviral function of IFITM3: The polypeptide sequence alignment shows that IFITM2 and IFITM3 share 91% amino acid identity and differ at only 12 amino acid residues that scatter along the 123 amino acids polypeptides (Fig. 8A). In contrast, IFITM1 shares only 70% amino acid identity to IFITM3, with the major differences lying at the N and C termini. The IFITM1 sequence lacks the first 21 amino acids of IFITM3 and differs markedly over the carboxy-terminal 27 amino acids, being both different in sequence and 13 amino acids longer in this region (27) (Fig. 7A). While Brass and colleagues reported that all three IFITM proteins, upon overexpression in A549 cells, inhibit the infection of influenza virus, WNV and dengue virus (4), our results reported herein, and published previously (21), demonstrate that expression of only IFITM3 and IFITM2, but not
IFITM1, in FLP-IN T Rex cells inhibits VSV, WNV and dengue virus infection. Taking advantage of the differential antiviral activity and distinct structural features of IFITM1 and IFITM3, a panel of mutant IFITM proteins, including point mutations in transmembrane region 1, N-terminal truncation of IFITM3 and chimeric IFITM1 and IFITM3 proteins (as illustrated in Fig. 8B) were engineered. FLP-IN T Rex cell lines expressing each of the mutant IFITMs were established. The expression of the desired mutant proteins was verified by Western blot assay (Fig. 8C). The ability of the mutant IFITMs, in comparison with the wild-type IFITM3 and IFITM1, to reduce VSV yield was determined by a plaque assay.

As shown in Fig. 8D, the results reveal the following: First, substitution of the two aminio acid residues in the transmembrane region 1 of IFITM3 with the amino acid residues at the same positions from IFITM1 (IFITM3M68L/P70W) does not affect the antiviral activity of IFITM3. Second, deletion of the N-terminal 21 amino acid residues from IFITM3 (IFITM3TN21) completely abolishes its antiviral activity. Third, replacement of N-terminal variable region of IFITM3 with the corresponding region of IFITM1 (IFITM3EX1) does not impair the antiviral activity of IFITM3, whereas replacement of C-terminal variable region of IFITM3 with the corresponding region of IFITM1 (IFITM3EX2) significantly compromises the antiviral activity of IFITM3. These results thus suggest that both the N-terminal 21 amino acid extension and C-terminal (but not N-terminal) variable region of IFITM3 are important for its antiviral activity. In agreement with this conclusion, neither addition of IFITM3 N-terminal 21 amino acid extension to IFITM1 (IFITM1EXN) nor replacement of C-terminal variable region of IFITM1 with the corresponding region of IFITM3 (IFITM1EX2) was able to confer antiviral activity. Interestingly, because the only difference between the central conserved regions of IFITM1 and IFITM3 is the two amino acid residues (L and W, as indicated in Figs. 8A and B)
which have been shown not to be responsible for the differential antiviral activity of the two proteins, the chimeric protein IFITM3EX1 is structurally equivalent with that simultaneously adding N-terminal extension of IFITM3 to the N-terminus of IFITM1 and replacing C-terminal variable region of IFITM1 with the corresponding C-terminal region of IFITM3. As shown in Fig. 8D, such a chimeric protein is fully functional in inhibition of VSV infection.

C-terminal variable regions of IFITMs determine the steady-state protein levels

While we consistently observed that IFITM3 and IFITM2, but not IFITM1, were able to inhibit virus infection in FLP-IN T Rex cells, we also noticed that upon induction, both IFITM2 and IFITM3 express to a higher level than that of IFITM1 (Fig. 1). It is, therefore, possible that inability of IFITM1 to inhibit virus infection could be due to its relatively lower level of expression in this cell line. Interestingly, in our mutagenesis studies presented in the previous section, we noticed that exchange of C-terminal variable region between IFITM1 and IFITM3 significantly decreased the steady-state level of IFITM3 (comparing mutant IFITM3EX2 with other IFITM3 mutants in Fig. 8C), but increased the steady-state level of IFITM1 (comparing mutant IFITM3EX2 with other IFITM3 mutants in Fig. 8C). To further validate this observation, FLP-IN T Rex cells expressing wild-type IFITM3, IFITM1 and the two C-terminal region exchanged mutants were left untreated or treated with tetracycline. Cells were harvested at 1, 2 and 3 days post induction. Levels of IFITM protein expression were determined by Western blot assay. As shown in Fig. 9, such a side-by-side comparison convincingly demonstrates that the C-terminal regions of IFITMs determine their steady-state protein levels. Because the ISG cDNA is integrated into host chromosome via the single FRT site in FLP-IN T Rex cells and transcribed under the same tet-inducible promoter (20), it is unlikely that the observed difference in the
levels of IFITM protein expression is due to a difference in transcription. Instead, the most possible explanation is that the C-terminal transmembrane regions of IFITMs determine the stability of the proteins. Nevertheless, although expressed in a much higher level in comparison with IFITM1, IFITM1EX2 does not gain any detectable antiviral activity (Fig. 8C). These results thus indicate that failure to inhibit virus infection by IFITM1 is more likely due to its lack of essential functional domains, but not its relatively lower level of expression.

DISCUSSION

Our work identified that IFITM2/3 and tetherin, two families of structurally distinct IFN inducible cell membrane proteins, inhibited VSV infection upon over-expression in HEK293 cells. Based upon the data presented above, we conclude that IFITM proteins inhibit a post-endocytosis event of virus entry, whereas tetherin impairs a late step in VSV replication cycle, most likely, viral particle (virion) release. Our findings not only expand the antiviral spectrum of the two families of ISGs, but also demonstrate that the two ISG restrict VSV infection and are physiological mediators of IFN-induced antiviral response against the virus. Hence, our work provides an explanation for previous observations indicating that IFN inhibited VSV entry and release from infected cells (7, 44, 45).

The IFITM proteins were identified more than two decades ago based on their inducible expression by type I and to a lesser extent, type II IFNs in neuroblastoma cells (46) and originally termed the 1-8 gene family (27). The three human IFITM genes lie adjacent to on chromosome 11 and share very similar gene structures consisting of an interferon-stimulated response element (ISRE) in the immediate 5’ flanking sequence and two exons (27). This family
of proteins has been implicated to play a role in cell adhesion, oncogenesis, germ cell homing and maturation, as well as regulation of endocytosis (10, 26, 43, 46, 47). In addition to being induced by IFNs, IFITM3 expression has been reportedly up-regulated in certain types of cancer cells (2, 48). Furthermore, it was also reported that IFITM3 was responsible for IFN-induced cell growth inhibition (5, 47). However, in agreement with Brass et al (4), even a prolonged (more than three weeks) expression of the IFITMs in FLP-IN T Rex cells did not disturb proliferation of the cells (data not shown).

Our previous studies discovered that while expression of IFITM3 in FLP-IN T Rex cells efficiently inhibited the infection of both West Nile and dengue virus-like particles (VLP), the antiviral protein was not able to inhibit the viral replicon replication when the replicon RNA was directly delivered into cells via transfection. We thus concluded that IFITM3 inhibited a replication step of the flavivirus life cycle prior to translation and replication of incoming viral RNA genomes, i.e., the virus entry and/or uncoating (21). This notion is consistent with a recent report elegantly showing that overexpression of IFITM proteins in several types of cells inhibited the infection of influenza virus envelope protein pseudotyped retroviral particles (4). In this report, we used VSV infection system to further dissect the antiviral mechanism of IFITM3 and provide evidence suggesting that IFITM3 inhibited a VSV entry step after endocytosis, but before primary transcription, with the most likely targets being endosome trafficking and/or fusion of virion and endosomal membranes. In fact, this proposed function of IFITM3 is consistent with the observed subcellular localization of the protein. As demonstrated herein and reported recently by Brass and colleagues, in addition to its plasma membrane localization, IFITM3 also associates with ER membrane and multivesicular compartments that contain endosome-like organelles (4, 5, 47). To further determine the relationship between the
intracellular localization and antiviral function of IFITM3, we added a classical ER retrieval
signal KDEL sequence at the C-terminus of IFITM3 as well as introduced two point mutations
(T118Y and P125S) in the C-terminal transmembrane region in attempt to withhold the protein
within the ER compartment and prevent its endosomal and plasma membrane localization (28,
38). However, FACS analysis suggested that both the mutant proteins were still able to express
on plasma membrane at a similar level of that observed in wild-type IFITM3-expressing cells.
Not surprisingly, both the mutant proteins elicited antiviral response against VSV (data not
shown). In the future, it will be interesting to further explore the relationship between the
subcellular localization and antiviral activity of IFITM3 with both genetic and cell imaging
technologies. In addition, a recent report showed that IFITM1 localized in caveolae of the plasma
membrane and could interact with CAV-1. Deletion mutagenesis clearly revealed that the
hydrophobic transmembrane domains were responsible for the interaction between IFITM1 and
CAV-1 (46). It will be of great interest to test the hypothesis that failure of IFITM1 to inhibit
both VSV and flavivirus infection in HEK293 cell could be due its distinct intracellular
localization.

Tetherin is an interferon-induced, transmembrane and GPI-anchored protein that restricts
the release of numerous enveloped viruses including all retroviruses tested as well as members of
the arenavirus (Lassa) and filovirus (Ebola and Marburg) families (23, 31, 37). In this report, we
further extended its antiviral activity to VSV, a member of Rhabdoviridae family. However, we
found that tetherin was not able to inhibit the release of DENV, a member of Flaviviridae family
(data not shown). Although we could not rule out the possibility that like many other viruses
(24, 29), DENV may encode proteins that antagonize the function of tetherin, these observations
are in agreement with the notion that tetherin is localized in cholesterol enriched microdomains
of plasma membrane and thus inhibits the release of virions that bud from such a membrane microdomains (13, 17, 32). In contrast, the flaviviruses presumably bud from ER membrane and thus, could potentially elude tetherin’s antiviral activity (6, 8, 19, 30). Moreover, consistent with that observed in HIV infection, elimination of GPI-anchor of tetherin by addition of a CV5 tag sequence at its C-terminus severely impaired its antiviral activity against VSV (data not shown), suggesting that its GPI anchor is necessary to inhibit the release of VSV and thus, via a similar mechanism as suggested for tethering HIV and other viruses (31).

In conclusion, our work presented herein advances our understanding of the two newly identified antiviral proteins. Further studies to decipher the structural and cell biological basis of the antiviral activities of IFITM and tetherin may ultimately lead to the development of a broad-spectrum antiviral therapeutic approach against enveloped virus infection by enhancing their antiviral function or disarm the antagonists of viruses against the IFN-induced antiviral proteins.
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FIGURE LEGENDS

FIG. 1. Antiviral activity of IFITMs and tetherin against VSV FLP-IN T Rex cells expressing CAT or the desired ISGs were left untreated or treated with 1 µg/ml of tetracycline for 24 h. (A and C) At this time point, cells were harvested and induction of IFITMs and tetherin expression were confirmed by Western blot analyses with antibody against flag-tag or tetherin protein. (B and D) Alternatively, cells were infected with VSV at an MOI of 0.001 and continued to be cultured in media without or with tetracycline for additional 16 h. VSV titers in the supernatants of the infected cells were determined by a plaque assay with Vero cells. Error bars indicate standard derivations of the means from three experiments. Statistics were performed using the Student’s t-test.

FIG. 2. IFITM and tetherin restrict VSV infection (A) Hela cells were transfected with SMARTpool siRNA targeting IFITM2 and IFITM3 or nontargeting siRNA (control) by following the direction of manufacturer (Dharmacon). Forty-eight hours post transfection, cells were left untreated or treated with indicated concentrations of IFN-α for 24 h. Cells were harvested at this time point for Western blot analysis of IFITM protein in the cell lysates with an antibody recognizing IFITM2 and 3 (Proteintech). β-actin served as loading controls. (B) Following IFN-α treatment, Hela cells were infected with VSV at an MOI of 0.01. Virus yields in culture media harvested at 24 h post infection were determined with a plaque assay and expressed as mean +/- standard error (n=3). * p<0.001. (C) Hela cells were transfected with SMARTpool siRNA targeting tetherin or nontargeting siRNA (control) by following the direction of manufacturer (Dharmacon). Six hours post transfection, cells were left untreated or
treated with indicated concentrations of IFN-α for 24 h. Cells were harvested for Western blot analysis of tetherin expression in the cell lysates or alternatively, infected with VSV at an MOI of 0.01. (D) Virus yields in culture media harvested at 24 h post infection were determined with a plaque assay and expressed as mean+/− standard error (n=3). Statistics were performed using the Student’s t-test. **, p<0.0001; #, p<0.05.

FIG. 3. Expression and membrane topology of IFITM3 and tetherin in HEK293 cells. FLP-IN T Rex cell lines expressing either IFITM3 or tetherin were left untreated or treated with 1 µg/ml of tetracycline for 24h. Expression of IFITM3 (A) and tetherin (B) was visualized by immunofluorescent staining. Cell nuclei were visualized by DAPI staining. (C and D) For FACS analysis, after induction of the ISG expression, cells were either left untreated (intact) or permabilized by incubation with buffer containing 0.1% Triton X-100. Expression of the Flag tag on the surface of intact cells (blue) or in the permeabilized cells (green) was revealed by flow cytometry. Histograms were gated to analyze the population expressing the Flag tag. Diagrams to depict the putative membrane topologies of IFITM3 and tetherin are also presented.

FIG. 4. Identification of the VSV replication step(s) targeted by IFITM3 and tetherin. FLP-IN T Rex cell lines expressing control protein CAT or the antiviral proteins IFITM3 or tetherin were left untreated or treated with 1 µg/ml of tetracycline for 24h prior to be infected with VSV at an MOI of 5. At the indicated time points after infection, culture supernatants and cells were harvested. (A) The levels of cell-associated viral RNA were determined by a real-time RT-PCR assay and the results were expressed as copies per 100ng of total cellular RNA. (B) Virus yields were measured by a plaque assay. Error bars indicate standard derivations of the means (n=3).
(C) Accumulation of viral G protein was determined by Western blot assay. (D) Cells were fixed with 2% paraformaldehyde and photographed with a Nikon microscope.

**FIG 5. IFITM3, but not tetherin, inhibits the synchronized infection of a recombinant VSV that expresses GFP.** FLP-IN T Rex cell lines expressing control protein CAT (A) or the antiviral proteins IFITM3 (B) or tetherin (C) were left untreated (red) or treated with 1 µg/ml of tetracycline (blue) for 24h prior to be infected with VSV-GFP at an MOI of 5. At 4 h post infection, cells were fixed and the levels of GFP expression were analyzed by flow cytometry. (D) Ratio of the mean fluorescence intensity (MFI) in cells treated with tetracycline over the cells that left untreated was plotted as relative MFI. Error bars indicate standard derivation of the means (n=3).

**FIG.6. IFITM3, but not tetherin, inhibits VSV-G protein mediated virus entry.** (A) FLP-IN T Rex cells expressing control protein CAT or the indicated ISGs were left untreated or treated with 1 ug/ml of tetracycline for 24 h. Cells were then infected with VSV-G pseudotyped lentiviral particles at a MOI of 1. Forty-eight hours post infection, intracellular levels of firefly luciferase expressed by the recombinant lentiviral vector were determined. Results represent the means +/- standard derivations (n = 6) of the ratios of light units obtained from wells cultured in the presence of tetracycline over that obtained from wells that were cultured in the absence of tetracycline. (B) FLP-IN T Rex cells inducible expressing CAT, IFITM3 or tetherin were cultured in the absence or presence of tetracycline for 36 h. Cells were infected with VSV at an MOI of 5 for 1 h on ice to allow attachment. After three washes with PBS, RNA was extracted to measure the amount of cell-bound virus. To quantify virus entry, virus inocula were removed
after 1 h of binding on ice, cells were either directly subject to trypsin treatment or incubated for another 10 min at 37°C and followed by trypsin treatment to remove any cell-associated virus which had not entered the cytoplasm. Intracellular viral RNA was measured with a real-time RT-PCR assay and the results were expressed as copies per 100ng of total cellular RNA. Error bars indicate standard derivations of the means (n=3).

**FIG 7.** IFITM3 reduces primary transcription of viral mRNA. (A) FLP-IN/IFITM3 cells were cultured in the absence or presence of tetracycline for 24 h. Cells were infected with VSV at an MOI of 1 for 1 h on ice, followed by cultured at 37°C in the absence or presence of cycloheximide for 1 or 2 h. The levels of VSV nucleocapsid protein (N) mRNA were determined by Northern blot hybridization. 18S rRNA served as loading controls. (B) FLP-IN/IFITM3 cells were cultured in the absence or presence of tetracycline for 24 h and followed by infection with VSV at an MOI of 1 for 1 h on ice. The infected cells were either immediately harvested or harvested at the indicated time points after being cultured at 37°C. The levels of VSV N mRNA were determined Northern blot hybridization and quantified with Quantity One phosphoimaging system (C).

**FIG 8.** Structural function analysis of IFITM3. (A) Amino acid sequence lineup of the three members of the IFITM family proteins. Differences are indicated by amino acid code. Transmembrane domains are indicated as “TM I” and “TM II”. (B) Schematic representation of the structures of IFITM and their mutants. The top panel of diagram shows the overall structural organization of IFITM3 and IFITM1. The lower panels highlight the nature of the mutations in 7 mutant IFITMs (see text for details). (C) Inducible expression of mutant IFITMs in stable FLP-IN T Rex cell lines. Cells were cultured in the absence or presence of tetracycline for 24 h and
the levels of FLAG-tagged IFITM1, IFITM3 and their mutants in cell lysates were determined by Western blot analysis with a monoclonal antibody against FLAG tag. β-actin served as loading control and was detected by using a monoclonal antibody against human β-actin. (D) Stable cell lines that inducibly express CAT, wild-type and mutant IFITMs were left untreated or treated with 1 µg/ml tetracycline for 24 h prior to VSV infection at MOI of 0.001. Sixteen hours later, culture media were harvested. VSV titers in the media of the infected cells were determined by a plaque assay with Vero cells. Error bars indicate standard derivations of the means from three experiments. Statistics were performed using the Student’s t-test.

**FIG 9. The C-terminal regions of IFITMs are responsible for their intracellular steady-state levels.** The indicated stable cell lines were cultured in the absence of tetracycline for 3 days or in the presence of tetracycline for 1, 2 or 3 days. The levels of FLAG-tagged IFITM1, IFITM3 and their mutants in cell lysates were determined by Western blot analysis with a monoclonal antibody against FLAG tag. β-actin served as a loading control and was detected by using a monoclonal antibody against human β-actin.
FIG. 1
FIG. 2
FIG. 3

A  IFITM3                     Merged
Tet +
Tet -

B  Tetherin                   Merged
Tet +
Tet -

C  D

FIG. 3
FIG. 4
FIG. 5
FIG. 8AB

A

B