Type I interferon protects cells from virus infection through the induction of a group of genes collectively named interferon-stimulated genes (ISGs). In this study, we utilized short hairpin RNA (shRNA) to deplete ISGs in SupT1 cells in order to identify ISGs that suppress the production of human immunodeficiency virus type 1 (HIV-1). Among the ISG candidates thus identified were interferon-induced transmembrane (IFITM) proteins, including IFITM1, IFITM2, and IFITM3, that potently inhibit HIV-1 replication at least partially through interfering with virus entry. Further mutagenesis analysis shows that the intracellular region, rather than the N- and C-terminal extracellular domains, is essential for the antiviral activity of IFITM1. Altogether, these data suggest that the IFITM proteins serve as important components of the innate immune system to restrict HIV-1 infection.

Mammalian cells produce type I interferon in response to virus infection (reviewed in references 8 and 25). Viral proteins and viral nucleic acids are detected by pathogen recognition receptors (PRRs) as pathogen-associated molecular patterns (PAMPs). These PRRs are either membrane associated, such as Toll-like receptor 3 (TLR3) and TLR7 on endosomes, or cytosolic, such as retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), nucleotide-binding oligomerization domain-containing 2 (NOD2), and absent in melanoma 2 (AIM2). These recognition events activate signaling transduction pathways that trigger production of type I interferon and other cytokines. Type I interferon then augments the expression of hundreds of genes, named interferon-stimulated genes (ISGs), that subvert virus replication by a variety of mechanisms (reviewed in references 50 and 53).

Human immunodeficiency virus type 1 (HIV-1) infection also triggers type I interferon production (17). Plasmacytoid dendritic cells, the major interferon producers, release large quantities of interferon, partially through recognition of HIV-1 RNA by TLR7 (23). Although the clinical benefit of engaging interferon in treating HIV-1 infection is uncertain due to the controversial results of clinical trials (16, 31, 51, 60), HIV-1 replication in cultured cells is clearly inhibited by interferon (4, 19, 22, 24, 48, 49, 68). This inhibition must have exerted selection pressure on HIV-1 in vivo in light of the multiple measures that the virus has evolved to evade interferon restriction. For example, HIV-1 infection causes a gradual decrease in the number of plasmacytoid dendritic cells, as well as a reduced capacity of these cells to produce interferon (13, 35, 62). In addition, HIV-1 infection causes depletion of interferon regulatory factor 3 (IRF-3), which plays a key role in Toll-like receptor- and RIG-I-mediated innate immune signaling (12, 45). Equally important, HIV-1 encodes proteins that antagonize individual ISGs. Examples are Vpu (viral protein u), which counteracts BST-2 (bone marrow stromal cell antigen 2, also known as tetherin/CD317/HM1.24) that otherwise blocks virus release from the cell surface (44, 64), and Vif (viral infectivity factor), which antagonizes APOBEC3G (apolipoprotein B mRNA editing enzyme 3G), a cytidine deaminase that causes hypermutation of HIV-1 cDNA (57).

Interferon impedes multiple steps of HIV-1 replication in both the early and late phases (1, 11, 18–22, 24, 28–30, 40, 49, 58, 59, 67, 68). These inhibition activities are the functions of distinct ISGs. For example, PKR (protein kinase R) inhibits HIV-1 production by suppressing protein translation (43). RNase L degrades HIV-1 RNA, thus restricting HIV-1 replication (36). TRIM22 (tripartite motif protein 22) disrupts Gag assembly (3), BST-2 blocks the release of HIV-1 particles from the cell surface (44, 64). ISG15 impedes HIV-1 virus production by causing ISGylation of viral Gag protein and cellular factors, such as CHMP5 (chromatin-modifying protein 5) (46, 47). APOBEC3G restricts HIV-1 replication by causing hypermutation of viral cDNA during reverse transcription (37, 57). TRIM5α from Old World monkeys targets viral core following HIV-1 entry and destroys the viral reverse transcription complex (37, 63). Although APOBEC3G and TRIM5α are constitutively expressed, their levels are elevated by interferon, which potentiates their antiviral function (10, 54). Recent studies by Bass et al. identified the interferon-induced transmembrane (IFITM) proteins as potent inhibitors of influenza A H1N1 virus, West Nile virus, and dengue virus in their genome-wide RNA interference (RNAi) screen (6). In this study, we further show that IFITM proteins also inhibit HIV-1 replication.

**MATERIALS AND METHODS**

Plasmid DNA, viruses, and antibodies. The cDNA clones of IFITM1, IFITM2, and IFITM3 were kindly provided by Ju-Tao Guo (26). The cDNA sequences of...
these (iso) genes were amplified by PCR using primer pairs IFITM1-1/IFITM1-2, IFITM2-3/IFITM2-4, and IFITM3-3/IFITM3-4 (the primer sequences are provided in Table S1 in the supplemental material). The PCR products were digested with restriction enzymes BamHI and EcoRI and inserted into the pRetroX-Tight-Pur retrovector (Clontech) to create DNA constructs Tet-IFITM1, Tet-IFITM2, and Tet-IFITM3. A Flag tag was attached to the N terminus of each IFITM. IFITM1 mutants were generated by PCR using primers that are listed in Table S1 in the supplemental material. The infectious HIV-1 proviral DNA clone BH10 was obtained from the NIH AIDS Research and Reference Reagent Program. The NLEY1-IRES (internal ribosome entry site) and the NLEY1-ES-IRES DNA constructs were kindly provided by David Levy (33) and the pCMV-BlasM-Vpr plasmid DNA by Warner Greene (9). HIV-1 stocks were generated by transfecting 293T cells with the proviral DNA clone BH10, NLEY1-IRES, or NLEY1-ES-IRES. Note that both the BH10 and NLEY1-IRES viruses are infectious, whereas the NLEY1-ES-IRES virus lacks HIV-1 envelope protein and needs to be pseudotyped for entry. When necessary, the vesicular stomatitis virus (VSV) glycoprotein (G) was used to pseudotype HIV-1 particles. The amounts of viruses in the stock were determined by measuring the activity of viral reverse transcriptase or the level of viral CA(p24) antigen.

Creating doxycycline-inducible IFITM cell lines. Retroviral particles were prepared by transfecting 1 µg of each of the Tet-IFITM1, Tet-IFITM2, and Tet-IFITM3 plasmid DNAs into the packaging cells GP2-293 (Clontech), together with 0.1 µg of the VSV G DNA. These viruses were used to infect SupT1 cells, together with viruses expressing the rTA activator (Clontech). Stably transduced cell lines were selected in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% glucose (Invitrogen) supplemented with 0.4 µg/ml puromycin (Sigma). The cells were incubated for 24 h before being infected with wild-type HIV-1 (BH10) (20 ng CA(p24)). The levels of viral proteins were assessed by Western blotting using anti-IFITM1, anti-IFITM2, and anti-IFITM3 antibodies, respectively.

Virus infection assays. IFITM SupT1 cells (10 6 cells for each line) were treated with doxycycline (500 ng/ml) for 16 h before being infected with HIV-1 named NLEY1-IRES equivalent to 100 ng CA(p24). The NLEY1-IRES virus bears HIV-1 envelope protein, is infectious, and expresses the yellow fluorescent protein (YFP). Cells were stained with YFP-positive cells were scored by flow cytometry. The amounts of viruses in the supernatants were determined by infecting TZM-bl indicator cells.

For spreading infection, 2 × 10 6 IFITM SupT1 cells were induced with doxycycline (500 ng/ml) for 16 h before being infected with wild-type HIV-1 (BH10) (20 ng CA(p24)). Viral replication was monitored by measuring the levels of viral reverse transcriptase activity in supernatants over different time intervals.

Measuring HIV-1 reverse transcription products. SupT1 cells (2 × 10 6) were exposed to the wild-type BH10 viruses (equivalent to 100 ng CA(p24)) for 1 h at 37°C before the virus inocula were washed off. A control, nevirapine (1 µM) treatment, was also performed. Cells were harvested 16 h after incubation. The levels of infectious viral DNA and total DNA was extracted with the DNeasy tissue kit (Qiagen). Real-time PCR was performed with equal amounts of DNA from different samples using the following primer pairs: 5'-TTAGACCACTGTCGAGCCTGGGAG-3' / 5'-GGTGTCGAGGATCTCTAGTTACC-3' to amplify the early viral DNA and 5'-TTGTTGGCGCTCTGGTG-3' / 5'-GACTGTTCTGGCTGGAAG-3' to amplify the late viral DNA (34). The reactions were performed with the LightCycler FastStart DNA Master SYBR green 1 system (Roche) in accordance with the manufacturer’s instructions. The PCR conditions were 95°C for 10 s, 62°C for 5 s, and 72°C for 7 s. In order to measure the viral DNA that is integrated into cellular chromosomal DNA, the first round of PCR was performed with primers 5'-GGCTCCCCAACAGGGCTGAGGACTTACAG-3' / 5'-GTTCCTGACTCAGTCTTCCC-3' to amplify the viral DNA and 5'-TTGTTGGCGCTCTGGTG-3' / 5'-GACTGTTCTGGCTGGAAG-3' to amplify the late viral DNA (35). The reactions were performed at 94°C for 1 min to denature DNA templates, followed by 12 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 3.3 min. The amplified DNA products were quantified in real-time PCR as described above with primers 5'-TTAGACCACTGTCGAGCCTGGGAG-3' / 5'-GTTCCTGACAGCGCCCA CGTCTGAG-3', which amplify the HIV-1 long terminal repeat (LTR) region (73).

Immunofluorescence microscopy. 293 cells were seeded into slide chambers prior to transfection with vector DNA expressing Flag-tagged IFITM1, IFITM2, and IFITM3. Forty hours after transfection, the cells were fixed with 4% paraformaldehyde, permeabilized with 1% phosphate-buffered saline for 10 min at room temperature. After permeabilization with 0.1% Triton X-100 for 10 min at room temperature and晒情隐著?
incubation in a blocking solution (2% FBS) for 2 h, the cells were stained with rabbit anti-Flag primary antibody (1:500 dilution; 2 h at room temperature) and Alexa Fluor 488-conjugated secondary anti-rabbit antibody (1:2,000 dilution; 40 min at room temperature). Images were taken using the Zeiss Pascal laser scanning confocal microscope.

RESULTS

Knockdown of IFITM1 increases HIV-1 production in the presence of IFN-α2b. With an aim to identify ISGs that have anti-HIV-1 activity, we first tested the effect of IFN-α2b on HIV-1 production in several human cell lines, as well as in cord blood mononuclear cells (CBMCs), in order to find a cell line that was suitable for the screening assay. The results showed a greater than 100-fold decrease in HIV-1 production in both SupT1 cells and CBMCs following IFN-α2b treatment, as opposed to a less pronounced HIV-1 reduction in Jurkat, U937, and 293T cells (see Fig. S1 in the supplemental material). We then proceeded with SupT1 cells and performed microarray analysis to determine the genes that were upregulated by IFN-α2b. A total of 94 genes showed a more than 2-fold increase in their mRNA expression (see Table S2 in the supplemental material). In order to determine which of these ISGs inhibit HIV-1 production, we used the Mission shRNA products from Sigma to knock down each individual ISG. Sixty-seven out of these 94 ISGs were covered by shRNA clones that were available at Sigma (see Table S2 in the supplemental material). We first created stably transduced shRNA SupT1 cell lines, including a scrambled shRNA cell line as the control, and then treated these cells with IFN-α2b (500 U/ml) for 20 h prior to Western blotting. The fold change in HIV-1 production for each shIFITM1 clone is shown. The results shown are the averages of two independent infection experiments.

FIG. 1. (A to C) Effect of ISG knockdown on HIV-1 production in the presence of IFN-α2b. Each ISG was targeted with 3 to 5 shRNA clones. The shRNA-transduced SupT1 cells were treated with IFN-α2b for 16 h before being infected with a subtype B HIV-1 (BH10). Production of infectious HIV-1 particles was determined by infecting TZM-bl indicator cells. The fold change for each shRNA was calculated by dividing the amount of infectious HIV-1 made from the shRNA SupT1 cells by that from the control shRNA SupT1 cells. The results are summarized in three graphs. The candidate genes are highlighted by arrows. (D) Western blots of SupT1 cells that were stably transduced with either the control shRNA vector or the shIFITM1 clones. Cells were treated with IFN-α2b (500 U/ml) for 20 h prior to Western blotting. The fold change in HIV-1 production for each shIFITM1 clone is shown. The results shown are the averages of two independent infection experiments.
IFITM1 has three homologs in the human genome, i.e., IFITM2, IFITM3, and IFITM5 (38, 42). Unlike IFITM1, -2, and -3, IFITM5 is solely expressed in osteoblasts and is involved in bone mineralization (42); thus, it was not further investigated in this study. When the effects of IFITM2 and IFITM3 on HIV-1 replication were examined using the Tet-On system, we found that IFITM2 and IFITM3 also markedly suppressed HIV-1 infection, albeit to a lesser extent than IFITM1 (Fig. 2C). Further experiments showed that the induced expression of IFITM1, IFITM2, or IFITM3 did not affect cell proliferation, the cell cycle, or cell surface expression of CD4 (see Fig. S2 in the supplemental material), supporting a likely direct inhibition of HIV-1 replication by IFITM1, IFITM2, and IFITM3.

**FIG. 2.** IFITM proteins inhibit HIV-1 replication in SupT1 cells. (A) Illustration of the domain structures of IFITM proteins. The transmembrane domains are highlighted in boxes. A Flag tag was attached to the N termini of IFITM proteins. The conserved amino acid residues are highlighted in red letters. (B) Doxycycline-induced expression of IFITM proteins in SupT1 cells. Stably transduced SupT1 cells were exposed to different amounts of doxycycline (0, 50, and 500 ng/ml) for 16 h before the cell lysates were harvested and subjected to Western blotting using anti-Flag antibodies to detect the expression of IFITM proteins on infection by HIV-1 NLEY1-ES-IRES that was pseudotyped with VSV G protein. The results revealed that IFITM3 reduced virus infection by approximately 55% whereas IFITM1 and IFITM2 led to 30% reduction (Fig. 3D). The more dramatic impact of IFITM3 on VSV G protein-mediated infection is consistent with a recent report showing that IFITM3 inhibits VSV infection by targeting distinct steps of the virus life cycle (66).

We then performed a HIV-1 virion fusion assay to test whether IFITM proteins affect virus entry. The fusion virus particles were prepared by transfecting 293T cells with an infectious HIV-1 proviral DNA clone, BH10, together with BlaM-Vpr DNA. The HIV-1 entry inhibitor T20 (1 μg/ml) effectively blocked the entry of BH10/BlaM-Vpr viruses but did not affect entry mediated by VSV G protein (Fig. 4A). When the BH10/BlaM-Vpr viruses were used to infect the IFITM cell lines, IFITM2 and IFITM3 diminished HIV-1 entry by 2- to 3-fold, whereas IFITM1 did not exert any such effect (Fig. 4B and C). Since IFITM1 differs from IFITM2 and IFITM3 mainly by the lack of an amino acid stretch in the N-terminal region, we speculated that the elongated N-terminal region may allow IFITM2 and IFITM3 to affect virus entry.

Indeed, we tested whether IFITM proteins affect virus entry. The fusion virus particles were prepared by transfecting 293T cells with an infectious HIV-1 proviral DNA clone, BH10, together with BlaM-Vpr DNA. The HIV-1 entry inhibitor T20 (1 μg/ml) effectively blocked the entry of BH10/BlaM-Vpr viruses but did not affect entry mediated by VSV G protein (Fig. 4A). When the BH10/BlaM-Vpr viruses were used to infect the IFITM cell lines, IFITM2 and IFITM3 diminished HIV-1 entry by 2- to 3-fold, whereas IFITM1 did not exert any such effect (Fig. 4B and C). Since IFITM1 differs from IFITM2 and IFITM3 mainly by the lack of an amino acid stretch in the N-terminal region, we speculated that the elongated N-terminal region may allow IFITM2 and IFITM3 to affect virus entry.
FIG. 3. IFITM proteins inhibit an early step of HIV-1 replication. (A) IFITM2 and IFITM3 diminished the number of HIV-1-infected cells. Following doxycycline treatment (0 and 500 ng/ml) for 16 h, SupT1 cells were infected with the NLEY1-IRES virus, which expresses YFP. Forty hours after infection, the number of YFP-positive cells was scored by flow cytometry. (B) IFITM proteins suppressed production of infectious HIV-1 particles. The amounts of infectious virus particles were determined by infecting TZM-bl indicator cells. The fold change was calculated by dividing the values from doxycycline-treated cells (+Dox) by the values from untreated cells (−Dox). The results shown are the averages of three independent infection experiments shown in panel A. The error bars indicate standard deviations. (C) SupT1 cells were infected with the NLEY1-IRES virus for 2 h before exposure to doxycycline (500 ng/ml) treatment. The effects of IFITM proteins on the number of infected cells and the amounts of infectious HIV-1 are summarized in the bar graph. (D) The IFITM SupT1 cell lines were first treated with Dox (500 ng/ml) for 16 h and then infected with NLEY1-ES-IRES viruses that were pseudotyped with VSV G protein. The number of YFP-positive cells was scored 40 h after infection. The results of three independent infections are summarized in the bar graph.
To test this, we created three IFITM3 mutants named Δ(1-6), Δ(1-11), and Δ(1-16) that lacked various lengths of the N-terminal sequences (Fig. 4D). The results of HIV-1 virion fusion assays showed that the Δ(1-6) mutant and the wild-type IFITM3 diminished virus entry by about 40% as opposed to a 20% reduction caused by the Δ(1-11) and the Δ(1-16) mutants (Fig. 4E), suggesting a role of the 7-FFSPV-11 peptide in restricting virus entry. Taken together, these data suggest that IFITM2 and IFITM3 interfere with HIV-1 entry, which represents one mechanism underlying their anti-HIV-1 activities.

**IFITM2 and IFITM3 associate with endocytosis.** Virus entry involves binding to the receptor molecules on cell surfaces and subsequent endocytosis of virus particles (39). Recent studies suggest that HIV-1 entry also involves the endocytosis process (41). Different viruses escape the endosome pathway at different stages. Some viruses, such as influenza virus, travel to the late endosomes to accomplish membrane fusion. We envision that in order to elicit a direct effect on virus entry, IFITM proteins may need to associate with the endocytosis pathway. To test this, we fed cells with Alexa Fluor 555-conjugated transferrin and determined whether IFITM proteins colocalize with transferrin.

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**FIG. 4.** Effect of IFITM proteins on HIV-1 entry. (A) T20 blocks the infection of SupT1 cells by the BH10/BlaM-Vpr viruses. SupT1 cells were infected with the BH10/BlaM-Vpr or the NLEY1-ES-ires/VSV G/BlaM-Vpr viruses in the absence or presence of the entry inhibitor T20 (1 μg/ml) for 2 h before cells were harvested to detect BlaM activity. The numbers of cells with successful HIV-1 entries are scored in windows Q2. (B and C) IFITM SupT1 cell lines were treated with Dox (500 ng/ml) for 16 h before being infected with BH10/BlaM-Vpr viruses. The results of three independent HIV-1/BlaM-Vpr fusion experiments are summarized in the bar graph. The error bars indicate standard deviations. (D) Illustration of IFITM3 mutations. The N-terminal sequences of IFITM3 were deleted to create mutants Δ(1-6), Δ(1-11), and Δ(1-16). Their expression in the Dox-inducible SupT1 cell lines was examined in Western blots. (E) The Δ(1-11) and Δ(1-16) mutants are less effective in inhibiting HIV-1 entry than the wild-type IFITM3. The results of three independent HIV-1 fusion experiments are shown in the bar graph.
ized with the endocytosed transferrin. Although IFITM1 exhibited barely any colocalization with transferrin, a strong association was observed between the endocytosed transferrin and IFITM2 or IFITM3 (Fig. 5). This result suggests that IFITM2 and IFITM3, but not IFITM1, associate with the endocytosis pathway and may exert an effect on the fate of the endocytosed cargos, such as virus particles.

**IFITM1 suppresses HIV-1 Gag production.** IFITM1 does not affect HIV-1 entry yet efficiently inhibits virus replication in SupT1 cells. In order to gain insights into this restriction activity of IFITM1, we first measured the effect of IFITM1 on HIV-1 reverse transcription by real-time PCR. Viral DNA was not detected by PCR in SupT1 cells that were infected by HIV-1 in the presence of a reverse transcriptase inhibitor named nevirapine (1 μM) (data not shown), which validates the idea that the amplified DNA products from HIV-1-infected cells in the absence of nevirapine treatment are reverse transcribed viral DNA. Consistent with the 40% to 50% decrease in virus entry caused by IFITM2 and IFITM3, the early and the late HIV-1 cDNA products diminished by similar magnitudes (Fig. 6A). No effect of IFITM1 was observed on the production of HIV-1 cDNA (Fig. 6A). We next performed Alu-gag PCR to assess the level of HIV-1 DNA that was integrated into cellular chromosomal DNA (73). The results did not reveal any effect of IFITM1 on HIV-1 DNA integration (Fig. 6A). Interestingly, when HIV-1 Gag protein and its processing products, including p40 and p24, were examined in Western blots, a significant reduction was detected in SupT1 cells that expressed any of the three IFITM proteins (Fig. 6B), suggesting that IFITM1, as well as IFITM2 and IFITM3, adversely affect viral Gag expression, which must have led to diminished virus production.

The **C-terminal sequence of IFITM1 regulates its antiviral activity.** It is interesting that IFITM1 does not affect HIV-1 entry but inhibits HIV-1 replication in SupT1 cells more severely than IFITM2 and IFITM3. In order to understand the antiviral activity of IFITM1, we performed mutagenesis studies to determine which domains of IFITM1 are critical for inhibiting HIV-1 replication. IFITM1 can be divided into five domains interspersed with its two transmembrane domains (Fig. 7A). We first deleted the C-terminal sequence of IFITM1 and generated three mutants named Δ(108-125), Δ(112-125), and Δ(117-125) (Fig. 7A and B). The results of spreading-infection assays showed that these three mutants inhibited HIV-1 replication in SupT1 cells even more effectively than did the wild-type IFITM1 (Fig. 7C). Consistent with this, these mutants reduced the number of infected cells and the amounts of infectious virus particles to a greater degree than the wild-type IFITM1 (Fig. 7D). In order to exclude the possibility that the increased antiviral activities of these IFITM1 mutants were a result of their artificially high expression, we compared the doxycycline-induced expression of one mutant, Δ(108-125), to that of the IFN-α/β-induced endogenous IFITM1. Comparable levels in Δ(108-125) and endogenous IFITM1 were shown by the results of Western blotting (Fig. 7E). These data suggest not only that the C-terminal sequence of IFITM1 is dispens-
able for the antiviral activity of IFITM1, but that its presence may impair the antiviral function of the protein.

We next assessed the role of the N-terminal sequence of IFITM1 in inhibiting HIV-1 replication. Two mutants were accordingly created by deleting either 21 or 29 amino acids from the N terminus in the context of the Δ(108-125) deletion that not only inhibited HIV-1 replication more strongly than wild-type IFITM1, but also severely diminished the number of infected cells in the one-cycle infection assay. Despite its low expression level, the Δ(1-21)/Δ(108-125) mutant inhibited HIV-1 replication as efficiently as the wild-type IFITM1 and the Δ(108-125) mutant (Fig. 8). HIV-1 replication was not affected by the Δ(1-29)/Δ(108-125) mutant due to the loss of its expression (Fig. 8). These results suggest that at least the first 21 amino acids of the N-terminal region are not required for IFITM1 to inhibit HIV-1 replication.

The intracellular region of IFITM1 was also subjected to mutagenesis analysis by changing every 4 amino acids to alanines. Eight mutants were created in this way (Fig. 8A). The results of Western blots showed that these mutants were not equally well expressed (Fig. 8B). Nevertheless, the VTG and QY mutants suppressed HIV-1 replication as effectively as the wild-type IFITM1 and the Δ(108-125) mutant (Fig. 8C and D). MVGK and ST partially repressed HIV-1 replication, whereas the SVKS, RDRK, KC, and LNI mutants did not exert an evident inhibitory effect (Fig. 8C and D). However, given the low expression levels of these six mutants, the data are insufficient to conclude that there is a direct involvement of the mutated amino acids in inhibiting HIV-1 infection. Nonetheless, the results do suggest that the amino acids altered in the VTG and QY mutants do not have a role in the IFITM1 anti-HIV-1 function.

FIG. 7. The anti-HIV-1 activity of IFITM1 does not depend on its C-terminal sequence. (A) Illustration of IFITM1 deletion mutants. Sequences from the IFITM1 C terminus were deleted. (B) Doxycycline-induced expression of IFITM1 mutants in SupT1 cells. (C) HIV-1 replication in SupT1 cells that express IFITM1 mutants. SupT1 cells were cultured in media with or without doxycycline (500 ng/ml). The results shown represent three independent spreading-infection experiments. (D) Infection of SupT1 cells with the NLEY1-IRES virus. For details, refer to the legend to Fig. 3B. (E) Comparison of doxycycline-induced expression of IFITM1 and Δ(108-125) to that of IFN-α2b-induced endogenous IFITM1. Cell lines were exposed to doxycycline (500 ng/ml), IFN-α2b (500 U/ml), or both for 16 h before the levels of IFITM1 and Δ(108-125) were assessed in Western blots using anti-IFITM1 antibody. Tubulin was probed as the internal control.
Knockdown of IFITM proteins increases susceptibility of TZM-bl cells to HIV-1 infection. IFITM proteins were recently shown to inhibit infection by influenza A virus, West Nile virus, and dengue virus (6, 27). However, an inhibition of HIV-1 infection was not measured in the HeLa-CD4⁺ cells when IFITM3 was knocked down with siRNA oligonucleotides (6). Consistent with this, we also failed to observe an increase in HIV-1 infection when depleting each of the three IFITM pro-
FIG. 9. Knockdown of IFITM1, IFITM2, and IFITM3 increases HIV-1 infection of TZM-bl cells. (A) Expression of endogenous IFITM1, IFITM2, and IFITM3 in different cell lines. Western blots were probed with anti-IFITM1, -IFITM2, or -IFITM3 antibody. (B) Western blots to assess the knockdown of IFITM1, IFITM2, and IFITM3 in TZM-bl cells that were transfected with mixtures of siRNA oligonucleotides. (C) Following transfection with siRNA oligonucleotides, TZM-bl cells were exposed to HIV-1 infection. The levels of luciferase activity in the TZM-bl cell lysates were measured 40 h after infection. The values (relative luciferase units [RLU]) represent the susceptibility of TZM-bl cells to HIV-1 infection under different siRNA treatments. The results shown are the averages of three independent experiments.

DISCUSSION

There are four human ifitm genes, i.e., ifitm1, ifitm2, ifitm3, and ifitm5 (38, 42). IFITM5 is expressed in osteoblasts and plays a role in bone mineralization (42). IFITM1, IFITM2, and IFITM3 are ubiquitously expressed, with IFITM1 and IFITM3 responding to interferon stimulation due to the presence of a functional interferon stimulation response element (ISRE) in their promoters (38). IFITM proteins have been reported to play a role in a number of cell functions, such as oncogenesis (7, 15), cell adhesion (14), and immune cell signaling (5). High levels of IFITM proteins have also been observed in certain types of tumors, such as colorectal tumors and astrocytoma (2, 56). Nonetheless, these proteins do not seem to play a vital role in embryogenesis, since mice that are knocked out for all ifitm genes appear to develop normally (32). Encoded by interferon-stimulated genes, the antiviral functions of IFITM proteins have not been well appreciated until the recent discovery of their strong inhibition activities against influenza A virus, West Nile virus, dengue virus, and vesicular stomatitis virus (6, 27, 66). Studies also show that palmitoylation of the cysteine residues is required for the antiviral function of IFITM3 (72). Our current study demonstrates that IFITM proteins also inhibit HIV-1 infection, thus expanding the spectrum of viruses under the control of this small ISG family.

One mechanism behind the restriction activity of IFITM proteins involves impeding virus entry. Studies by Brass and colleagues have shown that murine leukemia virus (MLV) particles that were pseudotyped with envelope proteins from influenza A virus, West Nile virus, and dengue virus, but not from alphaviruses, were sensitive to restriction by IFITM proteins (6). One implication of this observation is that IFITM proteins inhibit the entry step that is mediated by the envelope proteins of certain viruses. This function of IFITM is now supported by the results of an assay that directly measures HIV-1 entry (Fig. 4).

Brass and colleagues depleted the endogenous IFITM3 protein in a CD4+ HeLa cell line and did not observe an effect on infection by an HIV-1 IIb strain (6). This is most likely because HeLa cells express all three IFITM proteins, and each IFITM protein is a potent inhibitor of HIV-1 infection. Indeed, when we performed similar experiments in TZM-bl cells, which are HeLa-derived CD4-expressing cells, an increase in HIV-1 infection was observed only when all three IFITM proteins were simultaneously depleted by siRNA oligonucleotides (Fig. 9).

IFITM proteins are membrane associated, a feature that is considered necessary for directly interfering with virus entry (6). The IFITM protein family is characterized by two transmembrane domains and a highly conserved intracellular region. The N and C termini project away from the plasma membrane toward the extracellular environment (6, 61, 66). Interestingly, this topological presentation inversely mirrors that of BST-2, which inhibits the release of a variety of enveloped viruses from the cell surface (reviewed in reference 55). Although the molecular mechanism behind the IFITM restriction action remains to be elucidated, it seems unlikely that it directly targets viral envelope proteins or viral receptors, given the different viruses that IFITM proteins inhibit. Since virus entry often proceeds through endocytosis (reviewed in reference 39), it is possible that IFITM proteins are involved in endocytosis, such as by detecting and eliminating virus invaders before infection is established. In support of this speculation, Brass and colleagues have shown that murine leukemia virus (MLV) particles that were pseudotyped with envelope proteins from influenza A virus, West Nile virus, and dengue virus, but not from alphaviruses, were sensitive to restriction by IFITM proteins (6).

Among the viruses tested thus far, arenaviruses are resistant to restriction by IFITM proteins (6). This suggests that arenavirus envelope proteins may have a mechanism to evade...
IFITM restriction. Entry of arenaviruses, such as Lassa virus (LASV), lymphocytic choriomeningitis virus (LCMV), and machupo virus (MACH), begins with endocytosis at the plasma membrane and ends at the late endosome with low-pH-triggered membrane fusion (reviewed in reference 32). It is noted that LASF and LCMV exploit alpha-dystroglycan (alpha-DG) as the receptor and enter cells via an endocytosis process that is independent of clathrin, dynamin, and caveolin, whereas MACH uses transferrin receptor 1 to enter cells via clathrin-mediated endocytosis. In addition, the glycoproteins (GPs) of arenaviruses contain a stable signal peptide (SSP) of unusual length that becomes an integral component of the mature GP complex (including GP1, GP2, and SSP) (69–71). This SSP component may have a role in communication between the cytoplasmic domain (CTD) and the fusion-active ectodomain, which represents a unique feature of arenavirus GP-mediated entry. Further studies should determine which of these features or other unknown properties of arenavirus GPs allow these viruses to be resistant to IFITM proteins.

Targeting virus entry may not be the only mechanism by which IFITM proteins inhibit HIV-1 replication. For example, the 2- to 3-fold decrease in entry as a result of IFITM2 or IFITM3 expression does not seem to fully account for the over 5-fold decrease in HIV-1 production as measured in the one-cycle virus infection assay (Fig. 3B). It is possible that the results of the virion fusion assay may have underestimated the impact of IFITM proteins on virus entry in the context of a natural HIV-1 infection, during which cells are exposed to a dose of viruses much lower than the large quantity of concentrated HIV-1 particles that were used for the virion fusion assay. It is also notable that IFITM1 does not affect HIV-1 entry yet strongly inhibits HIV-1 replication. The latter observation adds to the argument that IFITM proteins may affect more than one step of HIV-1 replication.

The effect of interferon on HIV-1 entry was recently assessed by Goujon and Malim in a number of human immune cell lines (20). Their results revealed a moderate inhibitory effect in CD4+ T cells, which is, to some extent, consistent with the 2- to 3-fold reduction in HIV-1 entry seen with IFITM2 or IFITM3 in our study (Fig. 4). Given the constitutive expression of IFITM proteins in different cell lines (Fig. 9A), the exact impact of IFITM proteins on HIV-1 entry awaits further experimentation using IFITM knockout cells.

In summary, our results demonstrate that IFITM proteins restrict HIV-1 infection. Given the evidence that other viruses, including influenza A virus, West Nile virus, and dengue virus, are inhibited by IFITM proteins, this small ISG family emerges as an integral component of the interferon-mediated innate immune system that helps to defend against important human viral pathogens.

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AUTHOR’S CORRECTION

The IFITM Proteins Inhibit HIV-1 Infection

Jennifer Lu,1,2‡ Qinghua Pan,1‡ Liwei Rong,1 Wei He,1,2 Shan-Lu Liu,3,4 and Chen Liang1,2,3,4

McGill AIDS Centre, Lady Davis Institute-Jewish General Hospital, Montreal, Quebec, Canada H3T 1E21; and Department of Medicine2 and Department of Microbiology and Immunology,3 McGill University, Montreal, Quebec, Canada H3A 2B4; and Department of Molecular Microbiology and Immunology, Bond Life Sciences Center, University of Missouri, Columbia, Missouri 65211-73104

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