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Original article

Characteristics of IFITM, the newly identified IFN-inducible anti-HIV-1 family proteins

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Abstract

IFN-inducible IFITM proteins (IFITM1, 2, and 3) inhibit the replication of various viruses including HIV-1 through poorly understood mechanisms. Here, we further analyzed characteristics of these newly identified HIV-1 restriction factors. Firstly, in contrast to other anti-HIV-1 proteins, such as tetherin and APOBEC3G, IFITMs were resistant to a down-regulation of surface expression or degradation by HIV-1 proteins. Secondly, the enforced expression of IFITMs reduced the production of HIV-1 viruses from cells transfected with proviral plasmids containing whole viral sequences. Although their inhibitory activities were modest when compared to that of tetherin, IFITMs, but not tetherin, directly reduced the expression of HIV-1 proteins including Gag, Vif and Nef. Of importance, however, IFITMs had no inhibitory effect when these viral proteins were expressed by codon-optimized cDNAs that bypassed the viral-specific expression machinery. Indeed, our results supported the idea that IFITMs interfere with viral protein expression mediated by double-stranded viral RNAs, such as RRE and TAR. Finally, the S-palmitoylation of IFITMs, which is crucial for their anti-influenza virus activity, was not required for their anti-HIV-1 activity, indicating that IFITMs restrict these viruses at different steps. These characteristics lead to a better understanding of the mechanism by which IFITMs restrict HIV-1 and other viruses.

Keywords: IFITM; Tetherin; APOBEC3G; HIV-1; IFN; Antiviral activity

1. Introduction

Type I interferons (IFN) are cytokines of the innate immune system that induce the expression of antiviral proteins upon viral infection. Viral recognition induces the activation of cellular signaling pathways that trigger the production of IFN, which leads to the expression of a set of IFN-stimulated genes that inhibit viral replication through diverse mechanisms [1,2]. The IFN-induced transmembrane (IFITM) genes were identified as IFN-stimulated genes [3]. Among this family of proteins, IFITM1, 2, and 3 are ubiquitously expressed. Although it has been reported that IFITM1 and IFITM3 play distinct roles in mouse primordial germ cell homing and repulsion [4], their precise physiological functions remain largely unknown. Intriguingly, recent studies have revealed that IFITM family proteins are potent inhibitors of influenza virus, West Nile virus, dengue virus [5–8], Marburg and Ebola filoviruses, SARS coronavirus [9], vesicular stomatitis virus [10], HCV [11], and HIV-1 [12,13]. However, it remains unclear how these small proteins, which are composed of approximately 130 amino acids, exert antiviral activity against a broad range of viruses.

Recently, Lu et al. showed that the knockdown of all three *ifitm* genes increased the susceptibility of TZM-bl HeLa cells to HIV-1 infection [12]. Consistent with this result, the enforced expression of IFITM1, 2, or 3 markedly suppressed HIV-1 replication in SupT1 T cells without affecting cell proliferation, the cell cycle, or the cell surface expression of

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the HIV-1 entry receptor CD4 [12]. Schoggins et al. also reported that the enforced expression of IFITM2 or 3 had a similar inhibitory effect on HIV-1 replication using another T cell line, MT-4 [13]. Although HIV-1 entry step was clearly inhibited by IFITM2 and 3, the entry step might not be the primary target of IFITM1 during its restriction of HIV-1 replication, since IFITM1 did not affect HIV-1 entry but efficiently suppressed HIV-1 replication in SupT1 cells [12]. Indeed, it was shown that the intracellular region, rather than the N- or C-terminal extracellular domains, of IFITM1 is required for its inhibitory effect on HIV-1 [12]. Importantly, Lu et al. also showed that the enforced expression of IFITMs led to a reduction in the expression of the structural HIV-1 protein Gag, which might be simply due to the reduced viral replication. Nevertheless, it is also possible that IFITMs directly affect Gag expression.

In addition to the mechanism by which they suppress Gag expression, there are several unanswered questions regarding IFITMs. HIV-1 encodes proteins that antagonize the activities of IFN-inducible antiviral proteins. For instance, tetherin (also known as BST-2 or CD317) blocks the release of nascent virions from infected cells, but the HIV-1 accessory protein Vpu acts as a viral antagonist by inducing the down-regulation of tetherin expression at the cell surface [14–19]. Similarly, the cytidine deaminase APOBEC3G, whose expression is elevated by IFN [20], causes the hyper-mutation of HIV-1 cDNA, but another HIV-1 accessory protein, Vif, antagonizes its anti-HIV-1 activity by inducing the degradation of APOBEC3G [21–27]. However, it has not been examined whether HIV-1 antagonizes the anti-HIV-1 activity of IFITMs. Curiously, in contrast to the findings of two independent studies [12,13], Neil et al. failed to observe an inhibitory effect of the enforced expression of IFITMs on HIV-1 production in a study in which they identified tetherin as an HIV-1 restriction factor [15]. In this study, we therefore attempted to investigate whether HIV-1 proteins affect the expression and localization of IFITMs, how anti-HIV-1 activity of IFITMs and tetherin are different, and how IFITMs affect the expression of HIV-1 proteins in order to further understand the characteristics of these newly identified HIV-1 restriction factors.

2. Materials and methods

2.1. IFITM and tetherin plasmids

The N-terminal Flag-tagged human IFITM1, 2, and 3 cDNAs were provided by Liang [12], and subcloned into the pcDNA3.1 vector (Invitrogen). It was shown that IFITM3 was S-palmitoylated at three membrane-proximal cysteine residues (C71, C72, and C105) [7]. We therefore prepared three different mutants of Flag-tagged IFITM3, in which these cysteines had been mutated (singly or in combination) to alanine (C1/2A, C3A, and C1/2/3A; see Fig. 6A for details). As these cysteines are conserved in IFITM2 (C70, C71, and C104), similar mutants of IFITM2 were also prepared. The mutants were prepared using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) and appropriate mutagenic primers and cloned into the pcDNA3.1 vector. The nucleotide sequences of the mutants were verified using the BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Extracellular Flag-tagged human tetherin (BST-2-exFlag) cloned into the pCAGGS vector was prepared as described previously [28].

2.2. HIV-1 plasmids

A proviral NL43 plasmid and a *vpu*-deleted mutant version of the plasmid (pNL-uE65) were provided by Adachi [29]. A proviral JRFL plasmid in which the *nef* gene had been replaced with that of the SF2 strain was prepared as described previously [30]. A codon-optimized Gag–GFP fusion expression plasmid (synGag–GFP) cloned into the pcDNA3.1 vector (Ohmine et al., manuscript under review) was kindly provided by Y. Ikeda (Mayo Clinic, Rochester, MN). Codon-optimized Vpu (Vphu) and Vif (HVif) expression plasmids cloned into the pcDNA3.1 vector were obtained through the NIH AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD), both of which were derived from the NL43 strain [31]. Two different Nef (the SF2 strain) expression plasmids were prepared as described previously [32,33]: Nef fused to the C-terminus of the extracellular/transmembrane regions of CD8 (CD8-Nef) was cloned into the pRc/CMV vector and Nef fused to the N-terminus of GFP (Nef–GFP) was cloned into the pAcGFP-N1 vector (Clontech). The NL43 strain Rev expression plasmid cloned into the pCAGGS vector was prepared as described previously [28]. In this study, we also created Gag and Gag/Pol expression plasmids (pCA-Gag-RRE and pCA-GagPol-RRE, respectively) by inserting PCR-amplified NL43-derived *gag* and *gag/pol* genes (nucleotides 790–2292 and 790–5096, respectively) together with a Rev-response element (RRE; nucleotides 7759–7992) into the pCAGGS vector.

2.3. Transfection

HEK293 cells (Invitrogen) were maintained in DME medium (Wako, Osaka, Japan) supplemented with 10% FCS (Nichirei Biosciences, Tokyo, Japan). The cells were seeded onto 12-well tissue culture plates at a density of 1.8×10^5 cells/well and transfected with various plasmids using 4 μl/well Lipofectamine 2000 reagent (Invitrogen), as described previously [30,32]. The total amount (1.6 μg/well) of the plasmid was normalized using appropriate control (empty) vectors. After 6 h of transfection, the culture medium was replaced with fresh medium, and the cells were cultured for an additional 42 h and then subjected to p24 Gag protein ELISA, Western blotting, flow cytometric analysis, or immunofluorescence analysis. In another experiment (see Fig. 5), the double-stranded RNA-dependent protein kinase (PKR) inhibitor C16 (imidazolo-oxindole; Sigma) was added to the culture (0.1% v/v) during the changing of the medium. The inhibitor was dissolved in DMSO (Wako), and the same volume of DMSO was used as a vehicle control.

2.4. p24 Gag ELISA

Viral production was assessed by measuring the concentration of p24 Gag protein in the culture supernatant [30]. The supernatants of the transfected 293 cells were clarified by brief centrifugation, and their p24 concentrations were analyzed by ELISA (ZeptoMetrix, Buffalo, NY). The absorbance of each well was measured at 450 nm with a microplate reader (Bio-Rad Laboratories).

2.5. Western blotting

The preparation of the total cell lysates and Western blotting was performed essentially as described previously [34]. Briefly, the cells were lysed on ice for 30 min with Nonidet P-40 lysis buffer (1% Nonidet P-40, 50 mM Tris, and 150 mM NaCl) containing protease inhibitors (1 mM EDTA, 1 μM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). The cell lysates were centrifuged and the resultant supernatants were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The proteins were transferred to a nylon membrane (Hybond-P; GE Healthcare). The antibodies used were as follows: anti-Gag (#65-004; Bio-Academia, Osaka, Japan), anti-Nef (#2949; NIH AIDS Research and Reference Reagent Program), anti-Vif (#319; NIH AIDS Research and Reference Reagent Program), anti-GFP (#FL; Santa Cruz Biotechnology), anti-Flag (clone M2; Sigma), and anti-actin (#C-2; Santa Cruz Biotechnology). The detection was performed with HRP-labeled secondary antibodies (anti-rabbit or anti-mouse IgG; GE Healthcare), the Immunostar LD Western blotting detection reagent (Wako), and an image analyzer (ImageQuant LAS 4000; GE Healthcare).

2.6. Flow cytometry

The cell surface expression of the Flag-tagged IFITM or Flag-tagged tetherin was assessed by the flow cytometric analysis, essentially as described previously [35]. The transfected 293 cells were detached using the enzyme-free cell dissociation buffer (Gibco), stained on ice for 30 min with PE-labeled anti-Flag antibody (60 μg/ml; Columbia Biosciences, Columbia, MD), and analyzed using a FACSCalibur (Becton Dickinson) and Cell Quest Software (Becton Dickinson). In a selected experiment (see Fig. 2C), 293 cells stably expressing human CD4 [32] were transfected and analyzed for the cell surface expression of CD4 using PE-labeled anti-CD4 antibody (clone RPA-T4, eBioscience).

2.7. Immunofluorescence

For immunostaining, the transfected 293 cells were directly fixed in 2% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained with the primary antibodies for 12 h followed by labeled secondary antibodies [32,33]. The following primary antibodies were used: anti-Flag (clone M2; Sigma, to detect Flag-tagged IFITM proteins) and anti-Gag (#65-004; BioAcademia). Anti-mouse IgG-AlexaFluor488 and anti-rabbit IgG-AlexaFluor568 (both from Molecular Probes) were used as the labeled secondary antibodies. Nuclei were stained with DAPI (Molecular Probes), and fluorescent signals were visualized with a BZ-8000 fluorescent microscope (Keyence, Osaka, Japan) equipped with Plan-Fluor ELWD 20×/0.45 objective lenses (Nikon). Image processing was performed using a BZ-analyzer (Keyence) and the Adobe Photoshop software (Adobe Systems).

2.8. Statistical analysis

The statistical significance of differences between samples was determined using the Student's *t*-test. p Values less than 0.05 were considered significant.

3. Results and discussion

3.1. IFITM3 and tetherin restrict HIV-1 at different steps

It has been shown that the knockdown of IFITMs increases the susceptibility of TZM-bl HeLa cells to HIV-1 infection and that their enforced expression suppresses HIV-1 replication and Gag expression in SupT1 cells [12]. However, in a study in which the enforced expression of tetherin was found to strongly inhibit viral release from 293 cells, IFITMs failed to display a similar inhibitory effect [15]. Therefore, we initially compared their anti-HIV-1 activities in the same system, i.e., the co-transfection into 293 cells. Viral production was monitored by assessing the concentration of p24 Gag protein in the culture supernatants. In this study, we found that IFITM3 significantly reduced viral production when it was co-transfected with the proviral NL43 plasmid containing the whole viral sequence (Fig. 1A, left graph). A similar inhibitory effect was also observed when IFITM3 was co-transfected with the *vpu*-deleted NL43 mutant (NL43-ΔVpu; right graph). When analyzed using the TZM-bl reporter cells, the infectivity of the WT viruses produced in the presence of IFITM3 was comparable to that of the control viruses (data not shown). Tetherin also reduced viral production, but its inhibitory effect was more marked when it was co-transfected with NL43-ΔVpu (Fig. 1A). This was due to the ability of Vpu to down-regulate the cell surface expression of tetherin [14–19]. In the experiment shown in Fig. 1A, we used 0.6 μg IFITM3 expression plasmid. When the same amount of plasmid was used, tetherin displayed a lower expression level (Fig. 1B) but stronger inhibitory activity (Fig. 1A) than IFITM3. These results indicated that the inhibitory effect of IFITM3 on viral production is modest when compared with that of tetherin, which explains why Neil et al. [15] failed to observe an inhibitory effect of IFITMs in their study. The importance was that IFITM3 and tetherin restricted HIV-1 at different steps because IFITM3 but not tetherin significantly reduced the expression levels of the p55 and p24 Gag proteins in the cells (Fig. 1C). It therefore appears that tetherin inhibits the release of viruses without affecting intracellular Gag expression whereas IFITM3 directly reduces intracellular Gag expression, and thereby suppresses viral production.

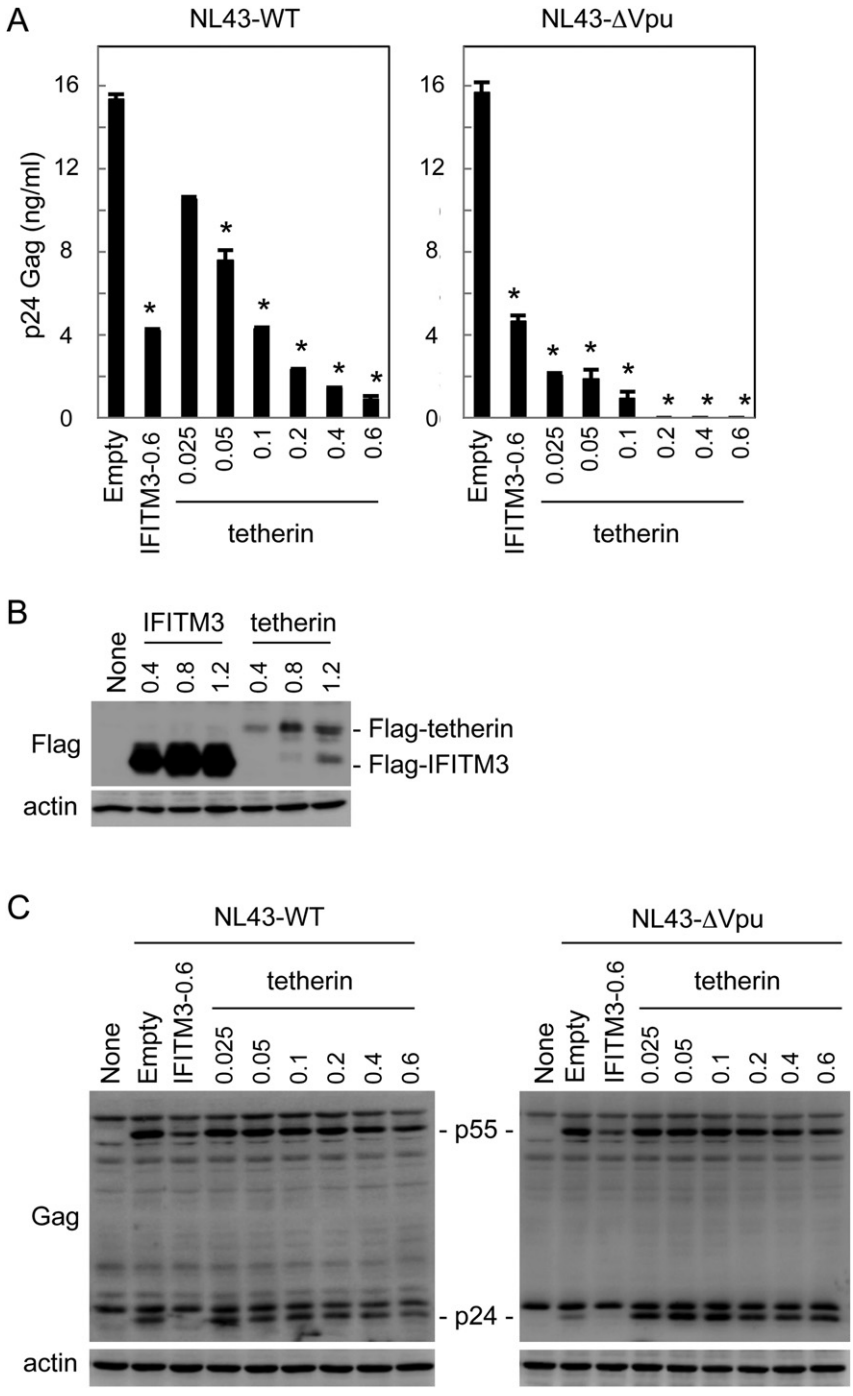

Fig. 1. The effects of IFITM3 and tetherin on viral production and Gag expression. (A) The 293 cells were transfected with the wild-type (NL43-WT, 0.6 μg) or *vpu*-deleted NL43 proviral plasmid (NL43-ΔVpu, 0.6 μg), or co-transfected with the empty vector (Empty, 0.6 μg), the IFITM3 expression plasmid (0.6 μg), or the indicated amount (0.025–0.6 μg) of the tetherin expression plasmid. The cells were cultured for 2 days, and the p24 Gag concentrations in the culture supernatants were determined by ELISA. Data are shown as the mean ± SD of triplicate assays and are representative of two independent experiments with similar results. $^{*}p < 0.05$. (B) The 293 cells were transfected with the empty vector (None), or the indicated amount (0.4, 0.8, or 1.2 μg) of the IFITM3 or tetherin expression plasmid, lysed after being cultured for 2 days, and analyzed for the expression of Flag-tagged IFITM3 or tetherin by Western blotting using anti-Flag antibody. The actin blot is a loading control. (C) The 293 cells were transfected as described in panel A, lysed after being cultured for 2 days, and analyzed for the expression of p55 and p24 Gag by Western blotting using anti-Gag antibody. The actin blot is a loading control. Data shown are representative of two independent experiments with similar results.

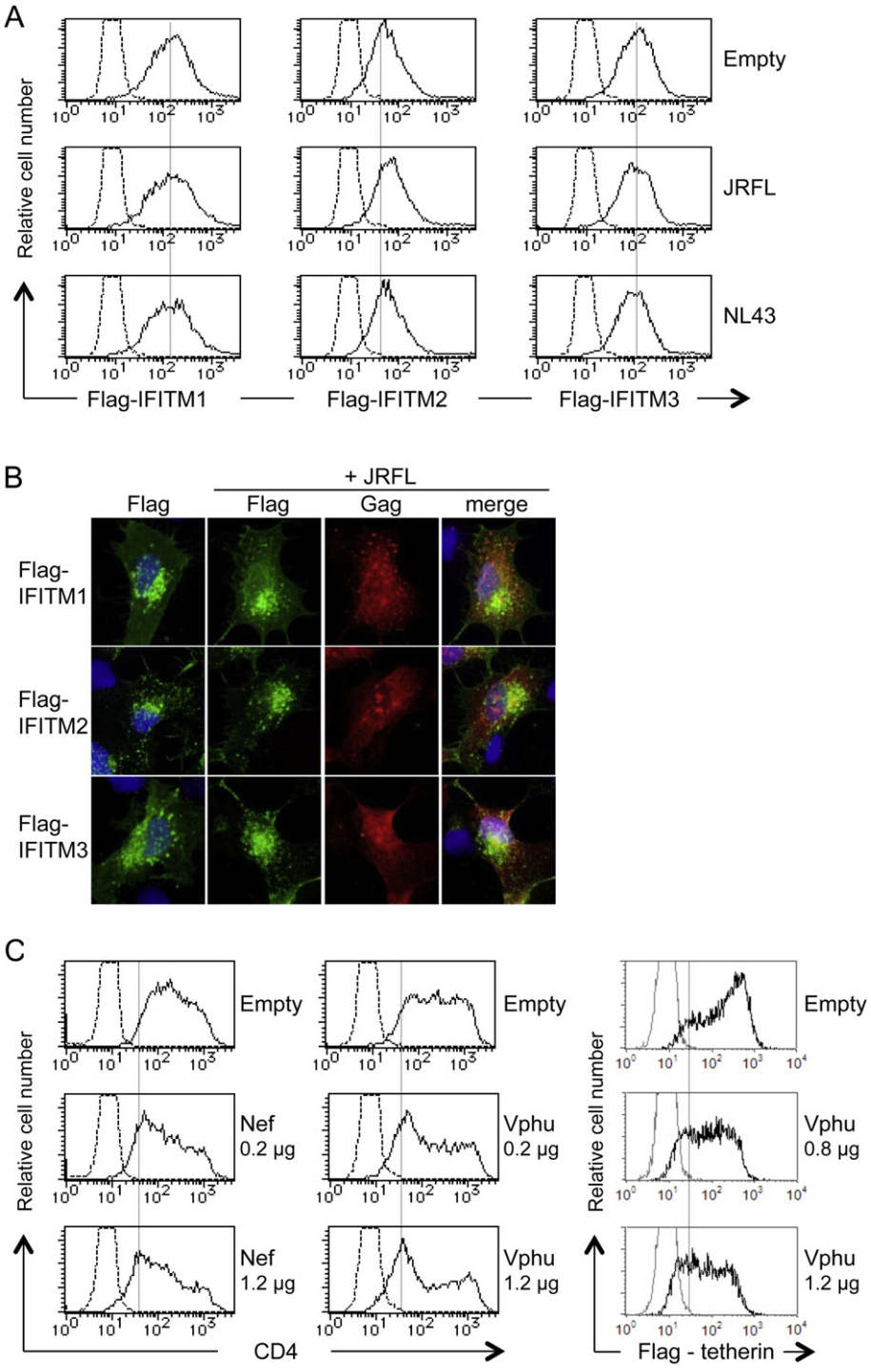

Fig. 2. The effects of HIV-1 proteins on the cell surface expression and intracellular distribution of IFITMs. (A) The 293 cells were co-transfected with the Flag-tagged IFITM1, -2, or -3 expression plasmid (0.4 μg) and the following plasmids (1.2 μg): the empty vector (Empty), the proviral NL43 plasmid, or the JRFL plasmid. The cells were cultured for 2 days, detached from the wells using the enzyme-free cell dissociation buffer, and analyzed for the cell surface expression of Flag-tagged IFITMs by flow cytometry using PE-labeled anti-Flag antibody. Data shown are representative of two independent experiments with similar results. (B) The 293 cells were transfected with the Flag-tagged IFITM1, 2, or 3 expression plasmid alone (0.4 μg) or co-transfected with the proviral JRFL plasmid (1.2 μg), cultured for 2 days, and co-stained with anti-Flag antibody (green), anti-Gag antibody (red), and DAPI (blue). (C) The 293 cells stably expressing human CD4 were transfected with the empty vector (Empty), the CD8-Nef fusion expression plasmid (0.2 or 1.2 μg), or the codon-optimized Vpu expression plasmid (Vphu, 0.2 or 1.2 μg). The cells were analyzed as in panel (A) using PE-labeled anti-CD4 antibody. Alternatively, 293 cells were co-transfected with Flag-tagged tetherin expression plasmid (0.4 μg) and the following plasmids: the empty vector (Empty, 1.2 μg) or the codon-optimized Vpu expression plasmid (Vphu, 0.8 or 1.2 μg). The cells were cultured for 2 days and analyzed as in panel (A). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

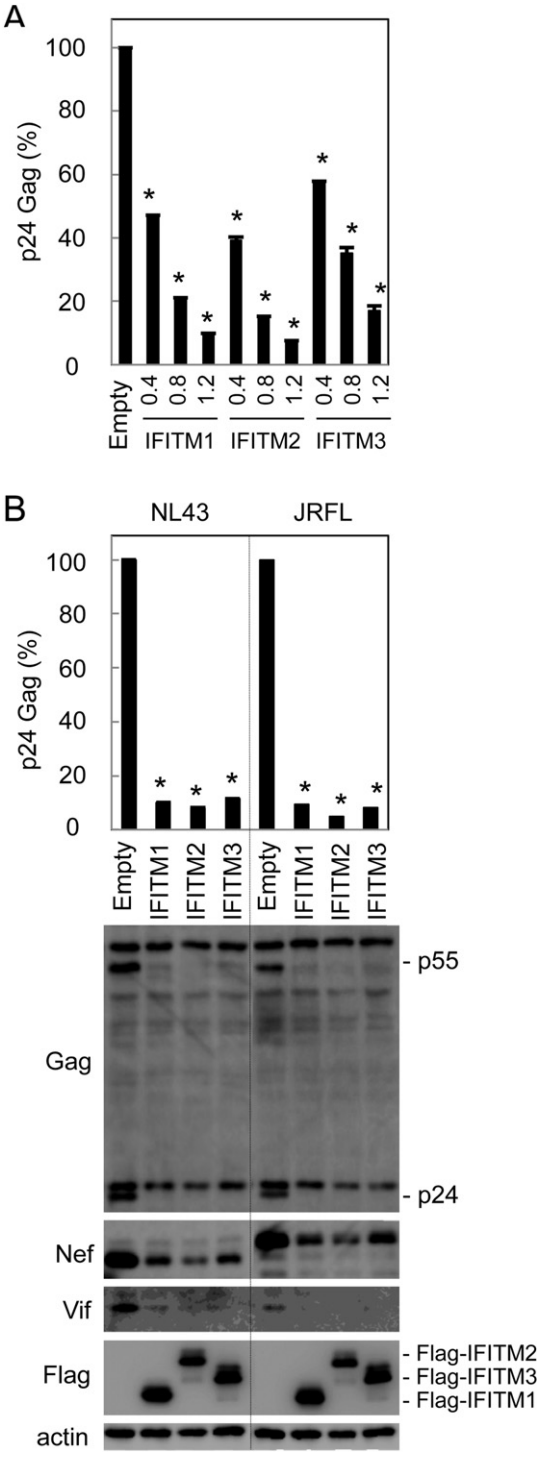

Fig. 3. The effects of IFITMs on viral production and the expression of Gag, Nef, and Vif. (A) The 293 cells were co-transfected with the proviral NL43 plasmid (0.4 μg) and the indicated amount (0.4, 0.8, or 1.2 μg) of the IFITM expression plasmids. The cells were cultured for 2 days, and the concentration of p24 Gag in the culture supernatants was determined by ELISA. The results are expressed as a percentage of the value for the sample on the far left. Data are shown as the mean ± SD of triplicate assays. (B) The 293 cells were transfected with the proviral NL43 or JRFL plasmid (0.4 μg), or co-transfected with the empty vector (Empty, 1.2 μg) or IFITM expression plasmid (1.2 μg), as indicated. The cells were cultured for 2 days, and the concentration of p24 Gag in the culture supernatants was determined by ELISA and analyzed as described in panel A. Alternatively, the cells were lysed and analyzed for the expression of Gag, Nef, Vif, or Flag-tagged IFITMs by Western blotting (lower blots). The actin blot is a loading control. (A and B) Data shown are representative of two independent experiments with similar results. *$p < 0.05$.

3.2. The cell surface expression, total expression level, and intracellular localization of IFITMs are unaffected by HIV-1 proteins

Tetherin and APOBEC3G are well-characterized HIV-1 restriction factors, but it is also known that HIV-1 proteins counteract their activities. Vpu and Vif induce the down-regulation of the cell surface expression of tetherin [14–19] and the degradation of APOBEC3G [21–27], respectively. Indeed, tetherin inhibited the release of the Vpu (−) viruses more strongly than it inhibited the release of the wild-type viruses (Fig. 1A). On the other hand, IFITM3 exhibited comparable inhibitory activity to these two viruses (Fig. 1A). Therefore, we next examined whether HIV-1 proteins affect the localization or expression of IFITM family proteins. The Flag-tagged IFITM1, 2, or 3 expression plasmid was co-transfected with the proviral plasmid (JRFL or NL43), but we did not detect any obvious changes in the cell surface expression of IFITMs (Fig. 2A). Although IFITM3 has been found to localize not only to the plasma membrane but also to the perinuclear region [7], we did not detect any obvious changes in the intracellular distribution of IFITMs after their co-transfection with the proviral JRFL plasmid (Fig. 2B). We also tested the effect of the overexpression of individual HIV-1 proteins. The Flag-tagged IFITM1, 2, or 3 expression plasmid was co-transfected with the codon-optimized version of the Vpu (Vphu) or Vif (HVif) plasmid, which bypassed the complicated viral-specific expression machinery and allowed efficient expression [31], and the cell surface expression of Flag-IFITMs was analyzed by flow cytometry using anti-Flag antibody. Nef was also added to the analysis, as it down-regulates the expression of multiple cell surface proteins including CD4 [36]. However, none of the HIV-1 proteins down-regulated the cell surface expression of IFITMs (data not shown). The expression levels of Vpu and Nef under our experimental conditions were sufficient to down-regulate tetherin and CD4, respectively (Fig. 2C). The expression level of Vif under our experimental conditions was sufficient to induce the degradation of APOBEC3G (Western blotting, data not shown). These results indicate that IFITMs are resistant to the down-regulation of cell surface expression or the degradation by HIV-1 proteins. These findings are important because they imply that any HIV-1 proteins, unlike those of tetherin and APOBEC3G, do not counteract the anti-HIV-1 activities of IFITMs.

3.3. IFITMs reduce the expression of several HIV-1 proteins when they were expressed by viral-specific machinery

We next attempted to understand how IFITMs suppress the production of HIV-1 viruses. There was no obvious difference in the inhibitory effect on viral production among IFITMs (Fig. 3A). All the IFITMs reduced the production of both the

NL43 and JRFL strain viruses (Fig. 3B, bar graph), and this effect was strongly associated with reduced Gag expression (Fig. 3B, Gag blot). Importantly, we found that IFITMs also reduced the expression of Nef and Vif (Nef and Vif blots). However, more important finding was that IFITM proteins did not induce any reduction in the expression of these viral proteins when they were co-transfected with the codon-optimized Gag expression plasmid (Fig. 4A, synGag–GFP blot), the Nef expression plasmid (Fig. 4A, Nef–GFP blot), or the codon-optimized Vif expression plasmid (Fig. 4B, HVif blot). It is well characterized that Gag and Vif RNAs contain a double-stranded region (Rev response element; RRE), and the incompletely spliced RNAs encoding these viral proteins require the binding of HIV-1 Rev protein to the RRE sequences for their nuclear export and subsequent expression [37,38]. Codon-optimization bypasses this complicated mechanism and allows these proteins to be expressed in a Rev-independent manner [31,37,38]. Thus, our results raised the possibility that IFITMs selectively interfere with the Rev/RRE-mediated expression of Gag and Vif. Indeed, IFITMs displayed an inhibitory effect on Gag expression in an artificial Rev-dependent expression system (Fig. 4C). In this system, the codon-unoptimized and RRE sequence-containing Gag–Pol and Gag expression plasmids (Gag–Pol–RRE and Gag-RRE) were expressed at normal levels only in the presence of the Rev plasmid (Fig. 5B, first 2 lanes). As a result, we

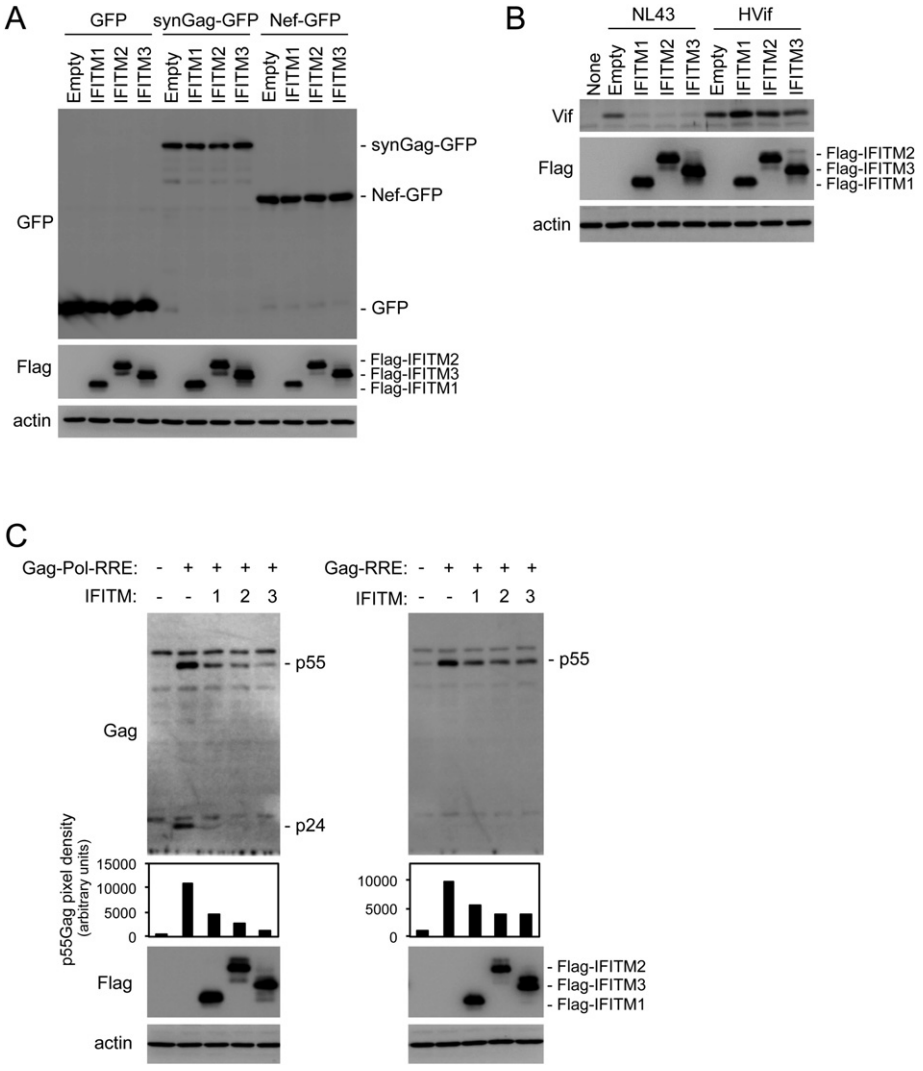

Fig. 4. The effects of IFITMs on the expression of the codon-optimized Gag, Nef and Vif, and the Rev/RRE-mediated expression of Gag. (A) The 293 cells were transfected with the GFP expression plasmid (0.1 μg), the codon-optimized Gag–GFP fusion expression plasmid (synGag–GFP, 0.2 μg), or the Nef–GFP expression plasmid, or co-transfected with the indicated IFITM expression plasmid (1.2 μg). (B) The 293 cells were transfected with the empty vector (None), the proviral NL43 plasmid (0.4 μg), or the codon-optimized Vif expression plasmid (HVif, 0.4 μg), or co-transfected with the indicated IFITM expression plasmid (1.2 μg). (A and B) The transfected cells were cultured for 2 days, lysed, and analyzed for the expression of GFP or GFP fusion proteins, Vif, and the Flag-tagged IFITM proteins by Western blotting. (C) The 293 cells were transfected with the Gag–Pol–RRE (0.3 μg) or Gag-RRE (0.3 μg) expression plasmid in combination with the Rev (0.1 μg) and/or IFITM (1.2 μg) expression plasmids, as indicated. The cells were cultured for 2 days, lysed, and analyzed for the expression of Gag and the Flag-tagged IFITM proteins by Western blotting. The profile created by quantifying the band pixel densities of p55Gag is also shown. (A–C) The actin blot is a loading control. Data shown are representative of two independent experiments with similar results.

found that IFITM1, 2, and 3 significantly reduced Rev/RRE-mediated Gag expression (lanes 3–5), as they did in the proviral plasmid-mediated expression system (see Fig. 3B).

However, the finding that IFITMs interfere with Rev/RRE-mediated expression does not explain the fact that they also reduced the proviral plasmid-mediated expression of Nef (see Fig. 3B) because Nef RNA does not contain RRE, and therefore its expression is independent of the function of Rev [37,38]. Thus, we next examined whether another viral double-stranded RNA, i.e., the *trans*-activation response (TAR) element, was involved in the inhibitory activity of IFITMs. All of the HIV-1 RNAs including that encoding Nef contain the TAR element at their 5′ end, which binds and activates the double-stranded RNA-dependent protein kinase (PKR) [39]. Once activated, PKR phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2α), which reduces the efficiency and rate of the translation initiation of proteins including viral proteins [39]. On the other hand, the HIV-1 Tat protein counteracts PKR activation via various complicated mechanisms, and the combination of the inhibitory pathways that prevent PKR activation determines the level of viral expression [39]. In this study, we found that the small molecule PKR inhibitor C16 restored the expression of p55Gag, Vif, and Nef, when it was added at effective but non-cytotoxic concentrations (0.2–0.8 μM) [40,41] to the cells that had been co-transfected with the proviral NL43 plasmid and IFITM3 (Fig. 5A). Meanwhile, C16 did not restore Gag expression in the Tat/TAR-independent but Rev/RRE-dependent expression system (Fig. 5B), which was consistent with the fact that TAR, but not RRE, induces strong PKR activation [39]. Therefore, our results (Figs. 3–5) suggested that IFITMs reduced the expression of Gag, and possibly Vif, by interfering with both Rev/RRE- and Tat/TAR-mediated expression, and reduced the expression of Nef by interfering with Tat/TAR-mediated expression. Again, the inhibitory effect was not non-specific because IFITMs did not affect the levels of these viral proteins when they were expressed via the system that bypassed the viral-specific machinery (see Fig. 4A and B).

3.4. The S-palmitoylation of IFITMs is not required for their anti-HIV-1 activity

A previous study demonstrated that IFITM3 was post-translationally modified by S-palmitoylation, which is crucial for its activity against influenza virus infection [7]. Therefore, we finally examined whether the S-palmitoylation of IFITMs is important for their anti-HIV-1 activity. To this end, we prepared three different mutants of Flag-tagged IFITM3 and IFITM2, in which the S-palmitoylated cysteine residues were mutated singly or in combination to alanine (Fig. 6A). Consistent with the finding that the first two

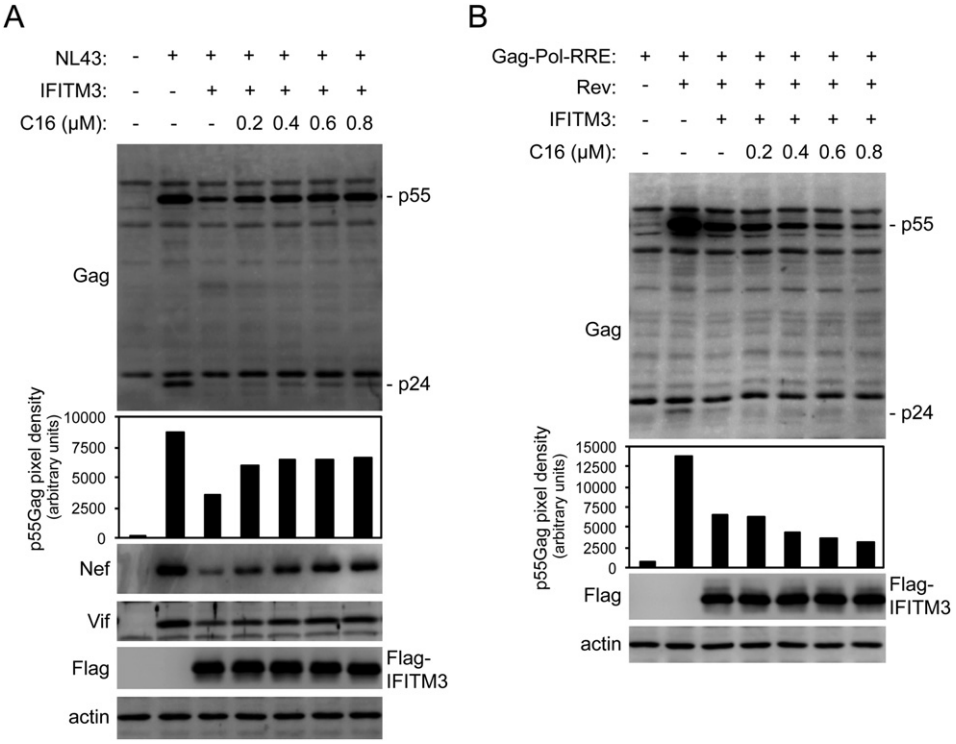

Fig. 5. The effect of the PKR inhibitor C16 on the suppression of Gag, Nef, and Vif expression by IFITM. (A) The 293 cells were transfected with the proviral NL43 plasmid (0.6 μg) and IFITM3 expression plasmid (1.0 μg), as indicated. (B) The 293 cells were transfected with the expression plasmids for Gag–Pol–RRE (0.3 μg), Rev (0.1 μg) and IFITM3 (1.0 μg), in the indicated combinations. (A and B) After 6 h transfection, the PKR inhibitor C16 was added to the culture at the indicated concentration (0.2–0.8 μM), and the cells were cultured for an additional 42 h. The cells were lysed and analyzed for the expression of Gag, Nef, Vif, and the Flag-tagged IFITM3 proteins. The actin blot is a loading control. The profile created by quantifying the band pixel densities of p55Gag is also shown. Data shown are representative of two independent experiments with similar results.

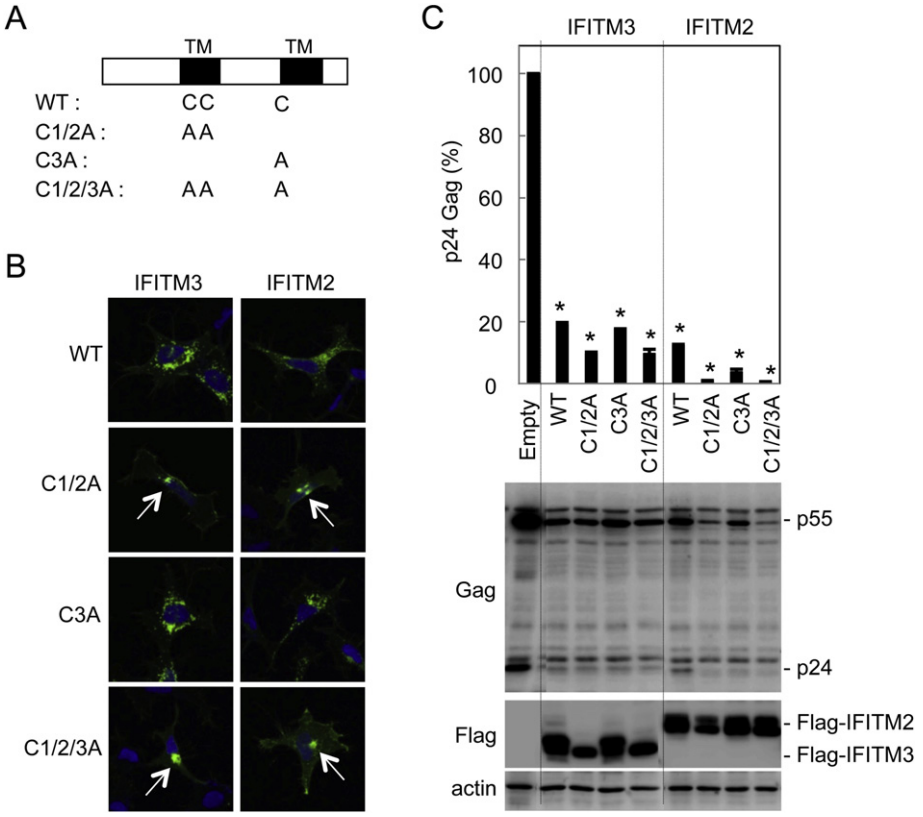

Fig. 6. The effects of S-palmitoylation-deficient IFITM mutants on viral production and Gag expression. (A) The IFITM mutants used are shown schematically. C, cysteine; A, alanine; TM, transmembrane. (B) The 293 cells were transfected with the expression plasmids (1.2 μg) for the wild-type (WT) or three mutants (C1/2A, C3A, and C1/2/3A) of IFITM3 or IFITM2. After 2 days transfection, the cells were fixed and co-stained with DAPI (blue) and anti-Flag antibody to detect Flag-tagged IFITM proteins (green). The C1/2A and C1/2/3A mutants displayed a distinct intracellular distribution (arrows) compared with the WT and C3A mutant. (C) The 293 cells were transfected with the proviral NL43 plasmid (1.0 μg), or co-transfected with the indicated IFITM expression plasmid (0.6 μg). The cells were cultured for 2 days, and the concentration of p24 Gag in the culture supernatants was determined by ELISA (bar graph). The results are expressed as percentages of the value for the sample on the far left. Data are shown as the mean ± SD of triplicate assays. Alternatively, the cells were lysed, and analyzed for the expression of Gag and the Flag-tagged IFITMs by Western blotting (lower blots). The actin blot is a loading control. (B and C) Data shown are representative of two independent experiments with similar results. $*p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cysteines were more heavily S-palmitoylated than the third cysteine [7], the mutants in which the first two cysteines were substituted (C1/2A and C1/2/3A) displayed more marked changes in their intracellular localization (Fig. 6B, arrows). However, unlike in the case of the anti-influenza virus activity of IFITM3, it was found that the S-palmitoylation was not necessary for the anti-HIV-1 activity of IFITMs because all of the mutants of IFITM3 and IFITM2 reduced the concentrations of p24 Gag in the supernatants (Fig. 6C, bar graph) and the expression levels of p55 and p24 Gag proteins in the cells (Gag blot). These results clearly indicated that IFITMs restrict HIV-1 and influenza viruses at distinct steps.

3.5. Conclusion

Both knockdown and enforced expression experiments demonstrated that IFITMs restrict HIV-1 replication [12,13]. In this study, we extended the findings of Lu et al. [12] and revealed that the enforced expression of IFITMs interfered with the production of HIV-1 proteins such as Gag, Vif, and Nef only when viral double-stranded RNAs (RRE and/or TAR) mediated their expression. These findings suggested that IFITM bind directly to viral double-stranded RNA. Indeed, a previous report raised the possibility that IFITM1 is an RRE-binding protein [P. Constantoulakis et al., Inhibition of Rev-mediated HIV-1 expression by an RNA binding protein encoded by the interferon-inducible 9–27 gene, Science 259 (1993) 1314–1318.]. However, as the report was retracted [M. Campbell et al., Science 264 (1994) 492.], careful studies will be necessary in order to clarify the exact mechanisms by which IFITMs interfere with the viral protein expression mediated by the double-stranded viral RNAs such as RRE and TAR. Studies will be also necessary to explain why there was no obvious difference in the anti-HIV-1 activity among three IFITM proteins in our transfection assay, in contrast to the study by Lu et al. [12]. Despite these unanswered questions, the present study demonstrated that IFITMs possess different characteristics from other anti-HIV-1 proteins such as tetherin and APOBEC3G and supported the idea that IFITMs restrict HIV-1 and influenza viruses at distinct steps.

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