Interferon induction of IFITM proteins promotes infection by human coronavirus OC43

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IFNs are a family of cytokines that are essential for the antiviral response in vertebrates. Not surprisingly, viruses have adapted to encode virulence factors to cope with the IFN response. Intriguingly, we show here that all three types of interferons, IFN-α, IFN-γ, and IFN-λ, efficiently promote infection by a human coronavirus, HCoV-OC43, one of the major etiological agents of common cold, through the induction of IFN-inducible transmembrane (IFITM) proteins. IFITMs typically exert their antiviral function by inhibiting the entry of a broad spectrum of viruses into their host cells, presumably by trapping and degrading invading virions within the endocytic compartments. In contrast, HCoV-OC43 uses IFN-induced human IFITM2 or IFITM3 as an entry factor to facilitate its infection of host cells. Reverse genetics analyses suggest that the structural motifs critical for the IFITM proteins' enhancement of HCoV-OC43 infection are distinct from those required for inhibiting infection by other viruses. We also present evidence showing that IFITM family members work as homo- and hetero-oligomers to modulate virus entry. The observed enhancement of HCoV-OC43 infection by IFNs may underlie the propensity of the virus to invade the lower respiratory tract under inflammatory conditions.

evasion of IFN response | viral pathogenesis | proviral function of IFITM

Coronaviruses (CoVs) are a large family of enveloped, positive-stranded RNA viruses with a broad host range. Six CoVs have been identified thus far as human pathogens (HCoVs) causing either mild upper respiratory tract infections (HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1) or severe acute respiratory syndrome [SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV)] (1, 2). Although regarded as one of the major etiological agents of the common cold, HCoV-OC43 occasionally causes severe infection of upper and lower respiratory tracts in infants and elderly people (3, 4). In our efforts to evaluate the role of inflammatory cytokines in HCoV-OC43 infection and pathogenesis, we found that, unlike other human CoVs that are sensitive to IFN-induced antiviral response (5), HCoV-OC43 infection of cell lines derived from human respiratory tract epithelia and hepatocytes was enhanced drastically not only by IFN-α but also by type II and type III IFNs, i.e., IFN-γ and IFN-λ. Furthermore, we found that this enhancement occurs because HCoV-OC43 uses the IFN-inducible transmembrane (IFITM) proteins to facilitate its entry into host cells.

IFITMs are widely expressed IFN-inducible proteins that localize in the membranes of cell plasma and endocytic vesicles and restrict infection by many pathogenic viruses (6–11). Studies by us and by others have revealed that IFITMs do not inhibit viruses from binding to and entering their host cells by endocytosis but instead trap the incoming virions in endocytic compartments, resulting in their degradation (12, 13). Although there is evidence suggesting that IFITMs may inhibit membrane fusion between virions and endosomal membranes, additional mechanisms, such as the disruption of signaling pathways regulating vesicle trafficking and alteration of endosome environments, cannot be excluded (14, 15). In this study, we obtained evidence showing that although the distant N-terminal region of IFITMs is critical for inhibiting influenza A virus (IAV) entry, the sequence-divergent C-terminal region is the critical determinant for IFITM enhancement of HCoV-OC43 infection. Although the detailed molecular mechanisms by which IFITM proteins modulate the entry of the variety of viruses remain to be explored further, the extraordinary ability of HCoV-OC43 to hijack IFITM proteins as its entry factor and to evade other IFN-induced antiviral pathways may play a role in its pathogenesis in humans.

Results

IFNs Promote HCoV-OC43 Infection. To determine the role of inflammatory cytokines in HCoV-OC43 infection and pathogenesis, we initially tested the effects of IFNs on the viral infection of five cell lines derived from human respiratory tract epithelia and hepatocytes. We found that all three types of IFNs (IFN-α, IFN-γ, and IFN-λ) enhanced HCoV-OC43 infection of the human lung cancer cell line NCI-H520 (Fig. 1) and the hepatoma cell line Huh7.5 (*SI Appendix*, Fig. S1) in a dose-dependent manner, as judged by significant increases in the percentages of infected cells (Fig. 1*A*), amounts of intracellular viral nucleocapsid protein (N) (Fig. 1*B*), viral RNA (Fig. 1*C*), and yields of progeny virions (*SI Appendix*, Fig. S1*D*). However, IFNs enhanced HCoV-OC43 infection of the human hepatoma cell line Huh7 (*SI Appendix*, Fig. S2) and the human lung cancer cell line NCI-H460 (*SI Appendix*, Fig. S3) only modestly at lower concentrations and slightly inhibited the viral infection at higher concentrations. On the other hand, the cytokines modestly inhibited HCoV-OC43 infection of another human lung cancer cell line A549 in a dose-dependent manner (*SI Appendix*, Fig. S4).

IFITM Proteins Are Required for HCoV-OC43 Infection of Host Cells. In deciphering the molecular mechanism of the cell-line–specific IFN enhancement of HCoV-OC43 infection, we noticed that

Significance

Here we report that all three types of IFNs, the primary mediators of host innate and adaptive antiviral responses, promote infection by human coronavirus HCoV-OC43. They do so through the IFN-induced transmembrane proteins that normally restrict a broad spectrum of viruses but serve as entry factors for HCoV-OC43 to infect its host cells. Our finding reveals a unique mechanism by which HCoV-OC43 evades host antiviral immune responses and suggests that the cytokine response to infection or noninfectious stimuli can be co-opted to promote the infection and spreading of opportunistic pathogens that have evolved adaptations similar to that of HCoV-OC43.

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The authors declare no conflict of interest.

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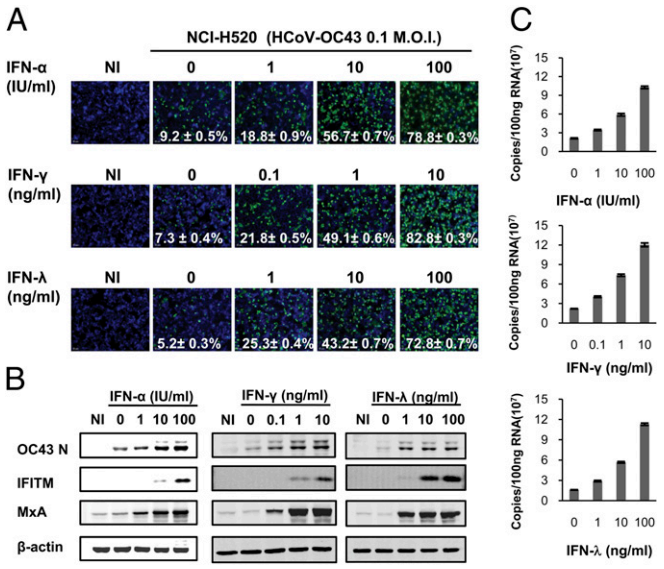

Fig. 1. IFNs enhance HCoV-OC43 infection of NCI-H520 cells. Cells were treated with IFN-α, IFN-γ, or IFN-λ for 12 h and then were infected with HCoV-OC43 at an MOI of 0.1. (*A*) Cells were fixed at 24 h hpi, and virally infected cells were visualized by immunofluorescence (IF) staining of HCoV-OC43 N protein (green). Cell nuclei were visualized by DAPI staining. (*B*) HCoV-OC43 nuvcleoprotein, IFITM, and MxA were determined by Western blot. (*C*) Intracellular viral RNA was quantified by quantitative RT-PCR (qRT-PCR) and is presented as copy number per 100 ng total RNA. Error bars indicate SDs ($n = 4$). Differences in viral RNA between IFN- and mock-treated cells are statistically significant ($P < 0.001$).

although all three types of IFNs efficiently induced the expression of MxA and IFITM proteins in the five cell lines tested, basal expression of IFITMs was not detected in the lines in which IFN treatment significantly enhanced HCoV-OC43 infection (Fig. 1 and *SI Appendix*, Figs. S1–S4). Moreover, the susceptibility of the five cell lines to HCoV-OC43 was positively correlated with their levels of basal IFITM expression (Fig. 2). These observations suggest that IFITM proteins may facilitate HCoV-OC43 infection and mediate the IFN enhancement of the viral infection of cells with low basal IFITM expression.

To investigate this hypothesis, we transduced Huh7.5 cells with a retroviral vector expressing individual human or mouse IFITM proteins or bone marrow stromal cell antigen 2 (BST-2)/tetherin, another IFN-inducible cell membrane protein, as a control. Intriguingly, expression of human IFITM2 or 3 and mouse IFITM1 or 3, but not human IFITM1 or mouse IFITM2, significantly increased the infection by HCoV-OC43 (*SI Appendix*, Fig. S5 *A–C*). We further demonstrated that expression of IFITM3 increased the susceptibility of Huh7.5 cells to HCoV-OC43 infection under a wide range of multiplicity of infection (MOI), as judged by significant increases in the percentages of infected cells (Fig. 3*A*), amounts of intracellular viral N protein (Fig. 3*B*), viral RNA (Fig. 3 *C* and *D*), and yields of progeny virions (Fig. 3 *E* and *F*). Moreover, expression of IFITM3 efficiently promoted not only the initial infection from inocula but also cell-to-cell spreading of HCoV-OC43 (*SI Appendix*, Fig. S5 *D–F*). Interestingly, we also showed that knockdown of the basal expression of IFITM2 and/or IFITM3, but not IFITM1, in Huh7 cells by shRNA significantly inhibited HCoV-OC43 infection, as indicated by the reduced accumulation of intracellular HCoV-OC43 N protein (Fig. 3*G*) and RNA (Fig. 3*H*). Furthermore, knocking down the IFN-α–induced expression of IFITM2 and/or IFITM3, but not IFITM1, in Huh7.5 cells also significantly attenuated the IFN-α enhancement of HCoV-OC43 infection (Fig. 3 *I* and *J*). Similarly, although ectopic expression of human IFITM2 or 3, but not IFITM1, in NCI-H520 cells significantly enhanced HCoV-OC43 infection, lowering the basal expression of IFITM2 and/or IFITM3 in A549 or NCI-H460 cells by shRNA significantly inhibited HCoV-OC43 infection (*SI Appendix*, Fig. S6).

Taken together, our results suggest not only that IFN-induced IFITMs mediate the enhancement of HCoV-OC43 infection by IFNs in cells that express lower basal levels of the proteins but also that IFITM proteins, specifically IFITM2 and IFITM3, most likely are required for the virus to infect efficiently all the host cells tested.

IFITM Proteins Facilitate HCoV-OC43 Entry into Host Cells. Because IFITM proteins localize in the plasma and endolysosomal membranes (6), we hypothesized that these IFN-induced proteins may be host factors that facilitate the entry of HCoV-OC43, as opposed to their more widely recognized role as restriction factors affecting many other viruses. In support of this hypothesis, expression of human IFITM 2 or 3 and mouse IFITM 1 or 3 in Huh7.5 cells increased the infection efficiency of HCoV-OC43 envelope protein pseudotyped lentiviral particles (HCoV-OC43pp) by more than 100-fold (Fig. 4*A*). However, in agreement with the results obtained with live virus infection, expression of human IFITM1 or mouse IFITM2 only modestly enhanced or apparently did not alter the susceptibility of Huh7.5 cells to HCoV-OC43pp infection. In contrast, as reported by others (8), all the human or mouse IFITM proteins significantly inhibited the infection of Huh7.5 by lentiviral particles pseudotyped with envelope protein of IAV (IAVpp) or SARS-CoV (SARSpp), but not Lassa fever virus (LASVpp) (Fig. 4 *B–D*).

The IFITM enhancement of HCoV-OC43 infection was demonstrated further by overexpression of human IFITM3 in 10 different cell lines derived from human hepatocytes and the epithelia of human respiratory tract, kidney, and colon, which significantly enhanced the infection by HCoV-OC43pp but inhibited the infection by IAVpp. The extent to which IFITM3

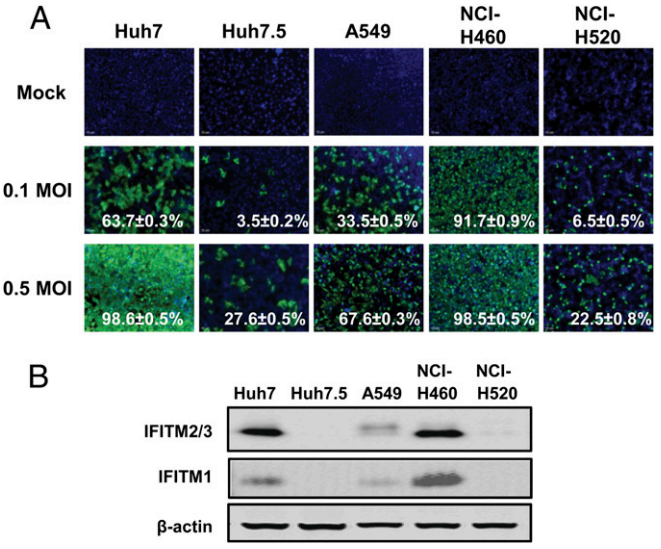

Fig. 2. Susceptibility of human hepatoma and lung cancer cell lines to HCoV-OC43 infection is correlated with their basal levels of IFITM protein expression. (*A*) Huh7m Huh7.5, A549, NCI-H460, and NCI-H520 cells were mock-infected or infected with the indicated MOI of HCoV-OC43. At 24 hpi, cells were fixed, and virally infected cells were visualized by immunofluorescence staining of HCoV-OC43 N protein (green). Cell nuclei were visualized by DAPI staining. (*B*) IFITM protein expression in the five cell lines was determined by a Western blot assay with a polyclonal antibody against IFITM3, which also efficiently recognizes IFITM2 and weakly cross-reacts with IFITM1, and a mAb against IFITM1.

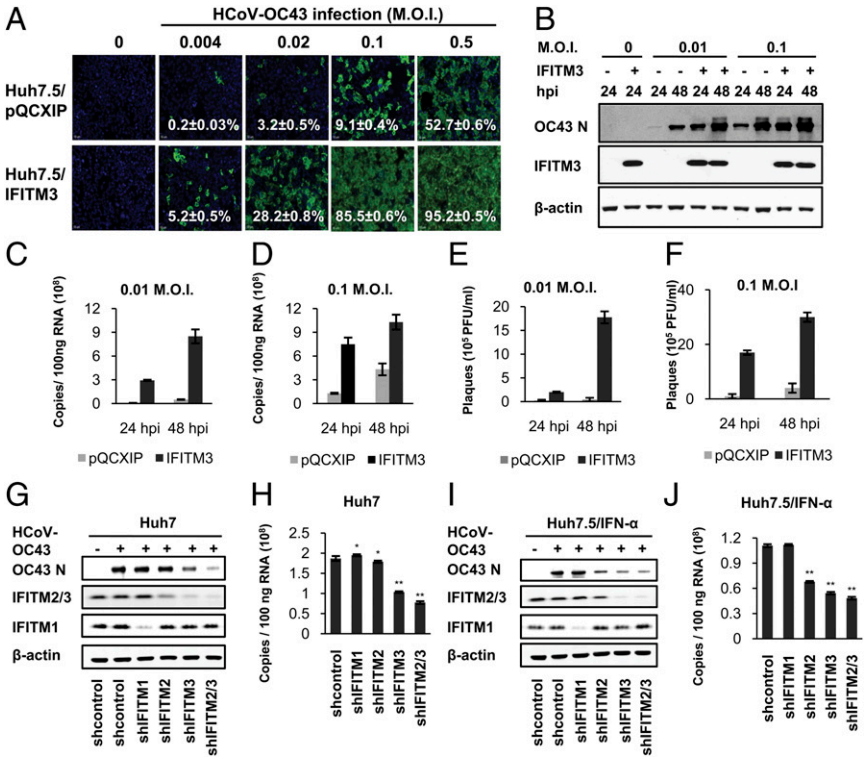

Fig. 3. IFITM3 enhances HCoV-OC43 infection of human hepatoma cells. (*A*) HCoV-OC43–infected Huh7.5 cells stably expressing human IFITM3 or transduced with control vector (pQCXIP) were visualized by IF staining of N protein at 24 hpi. (*B*) Viral N protein and Flag-tagged IFITM3 were determined by Western blot. (*C* and *D*) Viral RNA was quantified by qRT-PCR. (*E* and *F*) Viral yields were determined with a plaque assay. (*G* and *H*) Huh7 cells stably expressing an IFITM-specific or a scrambled shRNA were infected with HCoV-OC43 at an MOI of 0.1. Viral N protein and IFITMs (*G*) and viral RNA (*H*) were determined at 24 hpi. (*I* and *J*) Huh7.5 cells stably expressing a scramble or IFITM-specific shRNA were treated with 100 IU/mL IFN-α for 12 h and were infected with HCoV-OC43 at an MOI of 0.1. Viral nucleoprotein, IFITMs, and viral RNA were determined at 24 hpi. In *G* and *I*, intracellular IFITM protein levels were determined by Western blot using a rabbit polyclonal antibody that efficiently recognizes both IFITM2 and 3 and weakly cross-reacts with IFITM1 or a mAb that specifically recognizes IFITM1. In *E*, *F*, *H*, and *J*, intracellular viral RNA was quantified by qRT-PCR and presented as copy number per 100 ng total RNA. Error bars indicate SDs ($n = 6$). Differences in viral RNA between scramble or IFITM-specific shRNA-expressing cells were analyzed statistically (*$P < 0.05$, **$P < 0.001$; *t* test).

enhances HCoV-OC43pp infection of these cell lines is reversely correlated with their levels of basal IFITM expression (*SI Appendix*, Fig. S7). Moreover, knocking down the expression of IFITM3 in Huh7 cells significantly inhibited infection by HCoV-OC43pp but enhanced infection by IAVpp and did not affect infection by LASVpp (Fig. 4 *E* and *F*).

Mechanism of IFITM Enhancement of HCoV-OC43 Entry. To determine the entry step(s) facilitated by IFITM proteins, we first demonstrated that expression of human IFITM3 in Huh7.5 cells did not increase the attachment of HCoV-OC43 (*SI Appendix*, Fig. S8*A*). In addition, preincubation of Huh7.5 cells stably expressing N-terminal Flag-tagged IFITM3 with antibodies against IFITM3 or the N-terminal Flag tag that is detectable on the cell surface (6, 12) did not affect the attachment of and infection by HCoV-OC43 (*SI Appendix*, Fig. S8*B*). Furthermore, although it has been shown that HCoV-OC43 binds to 9-O-acetylated sialic acid–containing receptors to agglutinate mouse, rat, and avian erythrocytes (16), the specific receptor molecules that mediate the infectious entry of the virus remain to be identified (1). Interestingly, although, as expected, removal of sialic acids on the cell plasma membrane by sialidase treatment significantly inhibited IAVpp infection, it apparently did not affect HCoV-OC43 binding to and infection of Huh7.5 cells, whether or not those cells stably express IFITM3 (*SI Appendix*, Fig. S8 *C* and *D*). The results thus suggest that sialic acid is not the cellular receptor for HCoV-OC43 infection of the hepatoma cells and does not play a role in IFITM-mediated enhancement of virus entry.

To characterize further the impact of IFITM3 on HCoV-OC43 entry, we performed a synchronized infection assay in the absence or presence of NH_4Cl, which neutralizes endosomal pH and consequentially prevents the low-pH–dependent activation of viral envelope protein for triggering the fusion between the viral envelope and endosomal membranes. The study revealed that viral RNA amplification was undetectable within 6 h post-infection (hpi) in both IFITM3-expressing and -nonexpressing Huh7.5 cells (*SI Appendix*, Fig. S9*A*). However, expression of IFITM3 shortened the elapsed time of viral RNA amplification by ∼3 h. Interestingly, NH_4Cl treatment delayed the onset of viral RNA amplification by ∼6 h and reduced viral RNA levels by more than 10-fold at 24 hpi in both IFITM3-expressing and -nonexpressing Huh7.5 cells. In agreement with these results, we further demonstrated that IFITM3 efficiently enhanced the infection by HCoV-OC43pp and inhibited the infection by SARS-CoVpp in the absence or presence of NH_4Cl (*SI Appendix*, Fig. S9 *B* and *C*). Those results clearly indicate that, like SARS-CoV, HCoV-OC43 enters host cells by an acid-dependent endosomal route and that IFITM3 does not affect the low-pH–dependent activation of viral envelope proteins for fusion but most likely alters the fusion activity of host cellular membranes to inhibit or facilitate virus entry. In support of this notion, treatment of cells with amphotericin B, an antibiotic that alters the structure and fluidity of the cell membrane and that recently was reported to prevent IFITM3-mediated restriction of IAV infection (17), also efficiently attenuated IFITM3-mediated enhancement

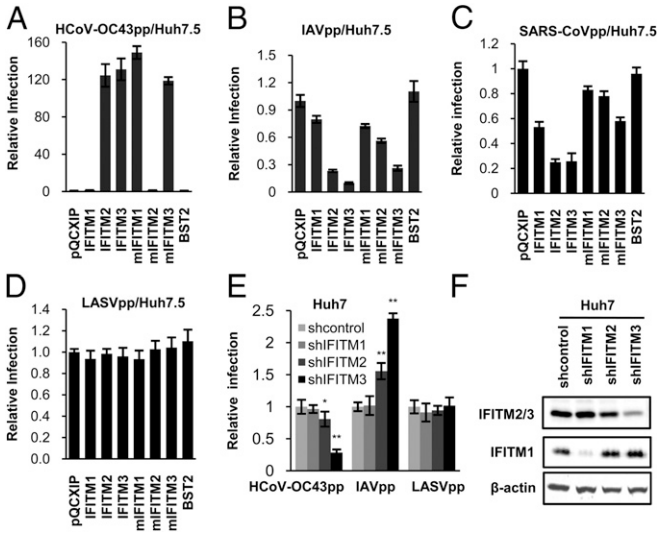

Fig. 4. IFITM3 enhances HCoV-OC43 entry. (*A–D*) Huh7.5 cells stably expressing individual human or mouse IFITM proteins or BST-2 or transduced with empty vector (pQCXIP) were infected with HCoV-OC43pp (*A*), IAVpp (*B*), SARS-CoVpp (*C*), or LASVpp (*D*). Luciferase activities were determined at 48 hpi. Relative infection represents the luciferase activity normalized to that of cells transduced with empty vector. Error bars indicate SDs ($n = 6$). Differences in relative pseudovirus infection between Huh7.5 cells expressing the indicated ISG and those transduced with empty vector (pQCXIP) are statistically significant ($P < 0.001$), except for BST2. (*E*) Huh7 cells stably expressing an IFITM-specific or scramble shRNA were infected with HCoV-OC43pp, IAVpp, or LASVpp. Pseudovirus infection was measured by luciferase assay at 48 hpi; relative infection represents the luciferase activity normalized to that of cells expressing scramble shRNA. Error bars indicate SDs ($n = 6$). Differences in relative pseudovirus infection between scramble and IFITM-specific shRNA-expressing cells were statistically analyzed (*$P < 0.05$, **$P < 0.001$; *t* test). (*F*) Intracellular total IFITM protein and IFITM1 levels were determined by Western blot using a rabbit polyclonal antibody that efficiently recognizes both IFITM2 and 3 and weakly cross-reacts with IFITM1 and a mAb that specifically recognizes IFITM1.

of HCoV-OC43pp infection and inhibition of SARS-CoVpp infection (*SI Appendix*, Fig. S10).

The C-Terminal Domain Is Critical for IFITM to Enhance HCoV-OC43 Infection but Not to Inhibit IAV Entry. Reverse genetics analyses were performed next to identify critical structural motifs essential for IFITMs to enhance HCoV-OC43 entry. IFITM contains a conserved CD225 domain flanked by sequence-divergent N- and C-terminal variable regions (Fig. 5 and *SI Appendix*, Fig. S11*A*). Although deletion of the N-terminal 21-aa residue unique to IFITM2 or 3 (IFITM3TN21) significantly impaired its ability to inhibit IAV entry (12, 18), the deletion apparently did not affect its ability to enhance HCoV-OC43pp infection (Fig. 5). This result is consistent with the observation that mouse IFITM1, which lacks the N-terminal extension, efficiently enhances HCoV-OC43pp infection but only modestly inhibits IAVpp infection.

To determine the role of sequence-divergent C-terminal regions of IFITM1 and IFITM3 in their differential effects on HCoV-OC43 entry, we swapped the corresponding regions of the two proteins. Like IFITM1, the two resulting chimeric proteins, designated IFITM1EX2 and IFITM3EX2, demonstrated similar activity in restricting the entry of IVApp, and IFITM3EX2 significantly inhibited the entry of HCoV-OC43. However, IFITM1EX2 enhanced HCoV-OC43 entry with an efficiency similar to that of IFITM3 (Fig. 5). Hence, the variable C-terminal regions of IFITMs are key determinants for modulating the entry of HCoV-OC43 but not of IAV. Moreover, there is a striking correlation between the enhancement of HCoV-OC43 infection and the length of the C-terminal regions in the human and mouse IFITMs (Fig. 5 and *SI Appendix*, Fig. S11*A*). The amino acid sequence alignment shows that human IFITM1 and mouse IFITM2, the two IFITMs that fail to enhance HCoV-OC43 infection, had a C-terminal extension of 18 and 17 aa, respectively. Intriguingly, sequential deletion of the C-terminal 18-a aresidues from human IFITM1 resulted in mutant IFITM1 proteins with gradually increasing ability to enhance the infection by HCoV-OC43pp, but the mutants' ability to restrict IAVpp infection remained unchanged (Fig. 5).

IFITM Proteins Function as Homo- or Hetero-Oligomers. Although expression of human IFITM1 slightly enhanced HCoV-OC43 and HCoV-OC43pp infection of Huh7.5 cells (Fig. 4*A* and *SI Appendix*, Fig. S5), its ectopic expression in Huh7 and 293 cells that express high basal levels of IFITM proteins significantly inhibited HCoV-OC43pp infection (Figs. 5 and 6*A*). Moreover, the gene-swapping experiments showed that the C-terminal variable region of IFITM1 is the key determinant of the inhibitory effect, as judged by IFITM3EX2 inhibition and IFITM1EX2 enhancement of HCoV-OC43pp infection (Fig. 5). The apparent dominant-negative effect suggests that IFITM1 may form hetero-oligomers with IFITM2 and/or 3 and suppress their functions. In support of this hypothesis, immunoprecipitation assays demonstrated that IFITM1 can associate with itself and other IFITM proteins, such as IFITM3, but not with MS4A4A, a plasma membrane protein serving as a control (19) (Fig. 6*C*). Moreover, compared with its wild type, IFITM1 with F75A and

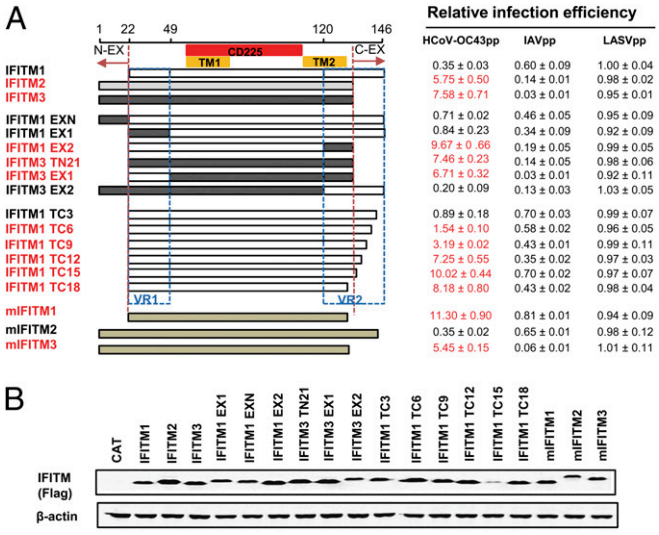

Fig. 5. IFITM structural domains essential for enhancing HCoV-OC43 infection are distinct from those required for inhibiting IAV infection. (*A*) (*Left*) Schematic presentation of the structure domains and construction strategy of mutant IFITMs. FLP-IN T Rex cells that tetracycline-inducibly express the indicated IFITM were left untreated or were treated with 1 μg/mL of tetracycline for 24 h to induce IFITM expression. Cells then were infected with HCoV-OC43pp, IAVpp, or LASVpp. Luciferase activities were determined at 48 hpi. (*Right*) The relative infection efficiency represents the luciferase activity in cells cultured with tetracycline normalized to that in cells cultured without tetracycline. Mean ± standard deviation ($n = 6$) are presented. IFITMs and their mutants significantly ($P < 0.001$) modulate the infection by HCoV-OC43pp (except for IFITM1 TC3) and IAVpp but not infection by LASVpp. Wild-type and mutant IFITMs that enhance HCoV-OC43pp infection are highlighted in red. (*B*) FLIP-IN T Rex 293 cells that tetracycline-inducibly express chloramphenical acetyl transferase (CAT) or an indicated IFITM protein were cultured in the presence of 1 μg/mL of tetracycline for 24 h. Expression of Flag-tag IFITMs was detected by Western blot assay using anti-Flag mAb.

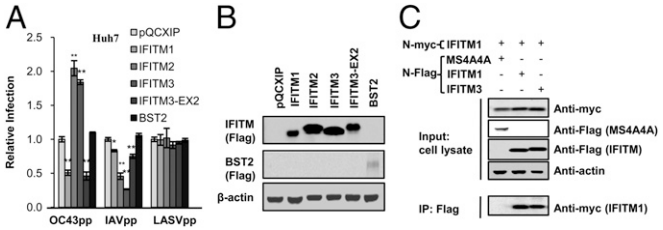

Fig. 6. Evidence suggesting that IFITM proteins function as homo- or hetero-oligomers. (*A*) Huh7-derived cell lines stably expressing individual human IFITM proteins and tetherin/BST-2 or transduced by an empty retroviral vector were infected with HCoV-OC43pp, IAVpp, or LASVpp. Firefly luciferase activities were determined at 48 hpi. Relative infectivity represents luciferase activity normalized to that of cells transduced with empty vector. Error bars indicate SDs ($n = 6$). Differences in pseudovirus infection between cells expressing IFITM and cells transduced by an empty vector were statistically analyzed (*$P < 0.05$, **$P < 0.001$; *t* test). (*B*) Proper expression of the desired human IFITM or BST-2 by the Huh7-derived stable cell lines were confirmed by a Western blot assay using a mAb against Flag tag. (*C*) 293T cells were cotransfected with a plasmid expressing *N*-myc–tagged IFITM1 and a plasmid expressing Flag-tagged MS4A4A, IFITM1, or IFITM3. Cells were lysed at 48 h posttransfection and were immunoprecipitated with a mAb against the FLAG epitope and blotted with a mAb against the myc epitope.

F78A mutations [the two amino acid residues essential for oligomerization of IFITMs (20)] demonstrated a significantly reduced ability to suppress HCoV-OC43pp infection even at low levels of accumulation in 293 cells (*SI Appendix*, Fig. S11). Similarly, expression of IFITM3 with F75A and F78A mutations not only failed to inhibit IAVpp infection but also significantly compromised its ability to enhance HCoV-OC43pp infection (*SI Appendix*, Fig. S11) (20). Hence, like palmitoylation and ubiquitination (20–22), IFITM oligomerization is required for both enhancing and inhibiting virus entry (*SI Appendix*, Fig. S11).

Discussion

IFNs are the primary antiviral cytokines that inhibit virus infection by the induction of hundreds of IFN-stimulated genes (ISGs). Although when individually expressed many ISGs inhibit infection by certain viruses through direct interaction with viral components or induction of cellular antiviral pathways (7, 23, 24), the cytokines usually inhibit viral infection of cells by establishing an antiviral state by modulating the expression and functionality of multiple antiviral proteins in concert (25). Not surprisingly, although many pathogenic viruses evolve strategies to evade IFN antiviral response by inhibiting ISG induction and antiviral function or by hijacking specific IFN-induced antiviral proteins to promote their replication, IFN enhancement of a viral infection rarely occurs. For instance, although human cytomegalovirus hijacks BST-2/tetherin to promote its entry into host cells and co-opts viperin to facilitate its replication, the virus is less sensitive to IFNs (26, 27). In principle, IFN enhancement of a viral infection can occur only when a virus hijacks one or several IFN-induced proteins or pathways to promote its replication and also is refractory to all other IFN-induced antiviral pathways. Indeed, that IFNs do not efficiently inhibit HCoV-OC43 infection of cells expressing high basal levels of IFITMs (*SI Appendix*, Figs. S2–S4) and that IFN-α treatment efficiently inhibited hepatitis C virus (HCV) infection of Huh7 and Huh7.5 cells (28, 29) and vesicular stomatitis virus (VSV) infection of the three lung cancer cell lines used in this study (*SI Appendix*, Fig. S12) strongly suggest that HCoV-OC43 is indeed refractory to the functional antiviral state induced by IFN-α, at least in certain cell lines. Our results thus suggest that HCoV-OC43 not only hijacks IFITMs to facilitate its entry into host cells but also may evade and/or antagonize other IFN-induced antiviral pathways.

Concerning the mechanism of IFITM modulation of virus entry, similar to inhibiting the entry of other viruses, IFITMs do not affect the binding and low-pH dependence of HCoV-OC43 entry but most likely promote the low-pH–activated membrane fusion between the viral envelope and endosomal membranes. Although the detailed molecular mechanism remains to be elucidated further, our work reported here provides mechanistic insights into the IFITM enhancement of HCoV-OC43 infection and also sheds light on the mechanism by which IFITMs inhibit the entry of other viruses.

Three possible membrane topologies had been proposed for IFITM proteins (*SI Appendix*, Fig. S11*B*). However, it was demonstrated recently that IFITM3 is predominantly a type II transmembrane protein (30), suggesting that only its short C terminus is exposed on the cell surface or lumen of endosomes and thus potentially can interact with viral envelopes. Interestingly, the C-terminal domains of IFITM proteins are critical for enhancing HCoV-OC43 infection. However, both wild-type mouse IFITM1 and human IFITM1 with the C-terminal 18-aa residue deleted efficiently promote HCoV-OC43 infection. A plausible explanation is that the C-terminal domains of IFITMs may participate in a critical intramembrane interaction with the HCoV-OC43 receptor and/or other host factors to promote the viral fusion, but the extended C terminus of human IFITM1 may disrupt such an interaction and thus fails to enhance the virus infection.

Second, the N-terminal 21-aa residue extension of IFITM2 and 3 is required for their full antiviral activity against VSV, IAV, and Dengue virus in cultured cells (12, 18, 20, 31) and for efficient restriction of IAV infection in humans (18) but is not essential for their enhancement of HCoV-OC43 entry. That deletion of the N-terminal 21-aa residues or Y20A mutation of IFITM3 results in its predominant cell periphery localization suggests that IFITM promotion of HCoV-OC43 entry may occur in early endosomes (20, 31).

Third, it appears that the proper S-palmitoylation and ubiquitination of IFITM3, which regulate its subcellular localization and stability, are required for both inhibition of IAV infection and enhancement of HCoV-OC43 infection. Moreover, it was reported recently that F75 and F78 in the conserved CD225 domain of IFITM3 are essential for its oligomerization and antiviral activity. We further extended this observation to show that these residues also are required for IFITM3 to enhance HCoV-OC43 infection. We also present evidence showing that different members of IFITM family interact with each other to form hetero-oligomers, which may modify their biological activity. Hence, modulation of virus entry by IFNs is quantitatively determined not only by the abundance of IFITM proteins but also by the proportion of the different IFITM family members within a cell.

Finally, in elucidating the mechanism by which IFITMs restrict virus entry, a "tough membrane" model is proposed, which postulates that intramembranous interactions between adjacent IFITMs alter the fluidity and bending modulus of the host cellular membrane, making it resistant to the viral fusion machinery (20). Alternatively, it has been proposed recently that IFITMs induce the accumulation of cholesterol in the endosomal membrane, making the membrane rigid and hard to fuse with viral envelope (15). Although these hypothetic models explain some aspects of IFITM's inhibition of virus entry, an obvious challenge is how the tough or rigid membrane selectively inhibits the entry of IFITM-sensitive viruses but not the entry of many other viruses. Another concern is that the IFITM-induced tough membrane not only may restrict virus entry but also may alter cell physiology, particularly endocytic vesicle trafficking and fusion. More importantly, our finding that IFITMs enhance HCoV-OC43 infection further challenges this model and suggests that IFITM modulates virus entry through fine-tuned interactions between IFITMs and the entry machinery of specific viruses, not through overt alterations in the

fluidity and structure of cellular membranes at certain subcellular compartments.

Instead, a likely scenario could be that the specificity of IFITM modulation of virus entry is determined by recruitment of IFITMs to specified membrane microdomains, such as lipid rafts, that contain specific viral receptors and/or other host entry factors. This recruitment efficiently homes the IFITMs to a location where they can block the entry of multiple viruses that use lipid rafts for viral fusion without needing to interact with the host receptor of each virus (32). Moreover, IFITM interaction with viral receptors and/or host entry factors and the alterations they induce in membrane curvature and fluidity at the sites of viral fusion may not always result in membrane modifications that inhibit fusion with viral envelopes. Instead, under certain circumstances such as HCoV-OC43 infection, these interactions may facilitate viral fusion. In support of this notion, it has been demonstrated that IFITM1 inhibits HCV infection via interaction with the viral coreceptors CD81 and occludin (33). In addition, the essential role of cellular receptors and their associated endosomal membrane components in IFITM modulation of virus entry, as predicted by our model, also explains the phenomenon that IFITM proteins promote the entry of HCoV-OC43 but inhibit the entry of other human coronaviruses, such as SARS-CoV and HCoV-229E (11, 34). This apparently contradictory activity is possible because—although the spike proteins of human coronaviruses, especially the C-terminal portions encoding the fusion machinery, are well conserved—these viruses infect cells via distinct cellular receptors that interact differentially with IFITMs at different subcellular compartments. Along this line, our model also predicts that the identification of IFITM-associated cellular proteins may reveal cellular receptors and other host entry factors of HCoV-OC43.

Our finding reported here may be relevant to HCoV-OV43 infection and pathogenesis in humans. For instance, induction of IFITMs in the epithelia of the lower respiratory tract or other tissues by inflammatory cytokines may pave the way for HCoV-OC43 invasion of these tissues. However, in humans the efficient evasion and hijacking of the IFN response by HCoV-OC43 generally result in only mild clinical manifestations, not in a life-threatening infection. A likely explanation for this phenomenon is that HCoV-OC43 may not induce a robust IFN response in vivo and normally is controlled by other branches of host innate and adaptive immune responses. Alternatively, it also is possible that, although its entry into host cells is facilitated by IFITM proteins, HCoV-OC43 is, in fact, still sensitive to IFN response in vivo. Hence, it is important to investigate further the pathobiological role of IFN response in HCoV-OC43 infection in cultures of primary human bronchi epithelia and, ideally, in humans or animal models in vivo. Nevertheless, although the pathobiological significance of IFN enhancement of HCoV-OC43 infection remains to be determined, our work has firmly established the critical role of IFITMs in HCoV-OC43 infection of host cells. Detailed analyses of the interaction between IFITMs and viral entry machinery and IFITM-induced membrane alterations at the site of viral fusion ultimately will reveal how the proteins regarded as antivirals restrict or promote virus entry into their host cells.

Materials and Methods

Information on the sources of experimental materials, procedures for establishing Huh7.5- and NCI-H520-derived stable cell lines expressing wild-type and mutant IFITM proteins, methods for detecting and quantifying of viral and pseudoviral particle infection, and protein and RNA analyses is provided in *SI Appendix, Materials and Methods*. Data are statistically analyzed by student *t* tests. FLIP-IN T Rex-derived stable cell lines expressing IFITM proteins in a tetracycline-inducible manner were described previously (7, 13).

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