Identification of Interferon-Stimulated Gene Proteins That Inhibit Human Parainfluenza Virus Type 3

M. A. G. Rabbani,^a Michael Ribauda,^a Ju-Tao Guo,^b Sainen Barik^a

Department of Biological, Geological and Environmental Sciences and Center for Gene Regulation in Health and Disease, Cleveland State University, Cleveland, Ohio, USA^a; Baruch S. Blumberg Institute, Doylestown, Pennsylvania, USA^b

ABSTRACT

A major arm of cellular innate immunity is type I interferon (IFN), represented by IFN- α and IFN- β . Type I IFN transcriptionally induces a large number of cellular genes, collectively known as IFN-stimulated gene (ISG) proteins, which act as antivirals. The IFIT (interferon-induced proteins with tetratricopeptide repeats) family proteins constitute a major subclass of ISG proteins and are characterized by multiple tetratricopeptide repeats (TPRs). In this study, we have interrogated IFIT proteins for the ability to inhibit the growth of human parainfluenza virus type 3 (PIV3), a nonsegmented negative-strand RNA virus of the *Paramyxoviridae* family and a major cause of respiratory disease in children. We found that IFIT1 significantly inhibited PIV3, whereas IFIT2, IFIT3, and IFIT5 were less effective or not at all. In further screening a set of ISG proteins we discovered that several other such proteins also inhibited PIV3, including IFITM1, IDO (indoleamine 2,3-dioxygenase), PKR (protein kinase, RNA activated), and viperin (virus inhibitory protein, endoplasmic reticulum associated, interferon inducible)/Cig5. The antiviral effect of IDO, the enzyme that catalyzes the first step of tryptophan degradation, could be counteracted by tryptophan. These results advance our knowledge of diverse ISG proteins functioning as antivirals and may provide novel approaches against PIV3.

IMPORTANCE

The innate immunity of the host, typified by interferon (IFN), is a major antiviral defense. IFN inhibits virus growth by inducing a large number of IFN-stimulated gene (ISG) proteins, several of which have been shown to have specific antiviral functions. Parainfluenza virus type 3 (PIV3) is major pathogen of children, and no reliable vaccine or specific antiviral against it currently exists. In this article, we report several ISG proteins that strongly inhibit PIV3 growth, the use of which may allow a better antiviral regimen targeting PIV3.

Parainfluenza virus type 3 (PIV3) is a nonsegmented, negative-strand RNA virus belonging to the *Paramyxoviridae* family and a major cause of lower respiratory tract infection in humans and cattle (1–3). Human PIV3 is a particularly serious pathogen in young children, second in clinic importance only to respiratory syncytial virus (RSV), another member of the same family (3). There is currently no specific treatment or approved vaccine for PIV3. However, RNA viruses in general, and paramyxoviruses in particular, induce type I interferon (IFN), a major arm of host innate immunity, mainly by activating a cytoplasmic RNA helicase of the RIG-I family, which is followed by a signaling cascade ultimately leading to transcriptional induction of IFN genes (4–6). Early reports showed that PIV3 is highly sensitive to IFN (7), suggesting that this natural antiviral mechanism holds the potential to be harnessed. Type I IFN by itself has no antiviral activity, but upon binding to its cognate receptor on the cell surface, it triggers the so-called IFN response pathway, in which transcription factors STAT1 and STAT2, along with IRF9, forms a tripartite complex (known as ISGF3) (8, 9) that transcriptionally induces several hundred IFN-stimulated genes (ISGs), some of which have products that have been shown to possess antiviral activity (10–13). While several ISG proteins, such as MxA and PKR (protein kinase, RNA activated), are well known for their relatively broad-spectrum antiviral activity, others may be virus specific (11–13). Regardless, the vast majority of ISG proteins have not been functionally or biochemically characterized. In this study, we have used a combination of overexpression and knockdown strategies to identify several ISG proteins that are potent inhibitors of PIV3

replication. We have also demonstrated that the inhibitory properties of some of these are part of an IFN response. These results advance our understanding of the molecular mechanism by which IFNs control PIV3 infection.

MATERIALS AND METHODS

Cells, virus, and IFN. The following cells were used: A549, human alveolar carcinoma type II-like epithelial cells; HEp-2, human laryngeal carcinoma epithelial cells; LLC-MK2, monkey kidney epithelial cells; and the U series cells, which are chemically mutagenized cell lines derived from fTGH fibrosarcoma cells (14). The tetracycline (Tet)-inducible ISG cell lines in a HEK293 background and their culture conditions have been described in detail previously (15, 16). All cells were grown in monolayers in Dulbecco's minimum essential medium (DMEM), complete with L-glutamine, heat-inactivated fetal bovine serum (FBS; 10%), penicillin (100 IU/ml), and streptomycin (100 μ g/ml). L-Tryptophan (Trp) was from Sigma (St. Louis, MO).

RSV strain Long was grown in HEp-2 cells, and human PIV3 strain JS was grown in LLC-MK2 cells. The titers of these viruses, needed for cal-

Received 4 August 2016 Accepted 26 September 2016

Accepted manuscript posted online 5 October 2016

Citation Rabbani MAG, Ribauda M, Guo J-T, Barik S. 2016. Identification of interferon-stimulated gene proteins that inhibit human parainfluenza virus type 3. *J Virol* 90:11145–11156. doi:10.1128/JVI.01551-16.

Editor: R. M. Sandri-Goldin, University of California, Irvine

Address correspondence to Sainen Barik, s.barik@csuohio.edu.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

cultivation of multiplicity of infection (MOI), were also determined by serial dilution and focus/plaque assay on the same cells.

Universal type I IFN (recombinant human IFN- α A/D-Bg1II; PBL Sciences), which signals through both the human and mouse IFN- α receptors (17), was used for the experiment whose results are shown in Fig. 1. However, when our subsequent studies veered away from mouse cells, the universal IFN was phased out and replaced with traditional recombinant human IFN- α (e.g., see Fig. 7B), purchased from Chemicon International (EMD Millipore; catalog number IF007). Both kinds of type I IFN were used at a final concentration of 1,000 U/ml. Where mentioned, recombinant human IFN- γ (BD Biosciences; catalog number 554616) was used at a concentration of 60 ng/ml.

Plasmids. The pcDNA3.1 clone of human viperin (virus inhibitory protein, endoplasmic reticulum associated, interferon inducible) with an N-terminal FLAG tag has been described previously (18). Human IFIT expression plasmids were constructed by cloning in a modified pcDNA3 vector, generated by inserting six 30-nucleotide repeats of the c-Myc peptide into the vector (19). In these plasmids, N-terminally myc-tagged IFIT proteins were expressed from a vector containing the cytomegalovirus (CMV) promoter.

RNA interference (RNAi). A549 cells were seeded in antibiotic-free DMEM supplemented with FBS. At 50 to 60% confluence of the monolayer, IFN- γ was added to a final concentration of 60 ng/ml, and this concentration was maintained in all subsequent steps until lysate collection. Duplex small interfering RNA (siRNA) against human IDO (indoleamine 2,3-dioxygenase) and the complete transfection reagent system were from Santa Cruz Biotechnology (catalog numbers sc-45939 and sc-45064, respectively). The siRNA transfection was performed according to the manufacturer's protocol. At 48 h after transfection, PIV3 was added at an MOI of 2; cell lysates were made after another 24 h and analyzed by immunoblotting. A549 cell clone 2-18, stably expressing lentiviral short hairpin RNA (shRNA) against IDO, and control cell clone NC-3, expressing scrambled shRNA (NC-3), have been described in detail previously (20). These cells were treated with IFN- γ and infected with PIV3 as described above.

Antibodies. Custom rat antibody was made commercially (Biosynthesis, Inc., Lewisville, TX) against the synthetic peptide ¹²KRNQEINQL ISPRSTSLNS of PIV3 nonstructural protein C. The peptide was used as an antigen in three rats, and the antisera were tested against PIV3-infected cell lysate by immunoblotting (IB; also called Western blotting). Commercial primary antibodies used in immunoblotting included PIV3 HN antibody (Abcam; ab49756), IDO antibody (Santa Cruz; sc-25809), mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (G-9; Santa Cruz; sc-365062), anti-FLAG M2 (Sigma; F1804), and rabbit polyclonal anti-myc (Thermo Scientific; PA1-981). The following horseradish peroxidase-conjugated secondary antibodies for immunoblotting were purchased from Santa Cruz Biotechnology: goat anti-mouse IgG (sc-2031) and goat anti-rat IgG (sc-2032). For microscopy, the secondary antibodies were Alexa Fluor 488 goat anti-rat (Thermo Scientific; A11006) and Alexa Fluor 594 anti-rabbit (Invitrogen; 21442).

Immunoblotting and microscopy. Lysis of cells, SDS-PAGE, and immunoblotting were performed as described previously (21). Horseradish peroxidase, conjugated to the secondary antibody, was developed by chemiluminescence in a LI-COR Odyssey Fc imaging system.

For microscopy, 293T cells (1.35×10^6 /well in 6-well plates) were seeded on poly-L-lysine-coated coverslips, prepared by treating the coverslips with a 0.01% solution of poly-L-lysine (Sigma; P4707) for 6 to 7 min. Cells were transfected 8 h later with plasmids expressing myc-tagged IFIT proteins, using Lipofectamine 2000. The same preoptimized amounts of plasmids, tested by immunoblotting, were used in these experiments, and their efficiencies of transfection were found to be approximately equal (data not shown). Cells were infected with PIV3 at an MOI of ~ 1 at 24 h after transfection and processed for immunostaining after another 18 h. Cells were washed twice with phosphate-buffered saline (PBS) and then fixed with cold 4% paraformaldehyde for 20 min. The

fixed cells were washed twice with PBS and incubated with primary antibody (anti-myc, or anti-PIV3-C) in PBS plus 0.5% Triton X-100 for 2 h at room temperature, followed by washing with PBS and incubation with appropriate secondary antibody for 1.5 h in the dark at room temperature. The antibody dilutions were as follows: PIV3 C, 1:100; Myc, 1:200; anti-rat Alexa Fluor 488, 1:400; and anti-rabbit Alexa Fluor 594, 1:200. Slides were mounted in Vectashield with 4',6-diamidino-2-phenylindole (DAPI), sealed with clear nail polish, and visualized on an EVOS FL imaging system (Thermo Fisher Scientific) with a 10 $\times$ objective.

For quantification of single-cell PIV3 growth inhibition by IFIT proteins, we conducted densitometry of the red and green colors of individual stained cells from the respective (unmerged) fields using ImageJ (NIH). The two numbers for each cell were tabulated in Excel. The highest green color (PIV3) for each IFIT was taken as 100, and green colors of all other cells were calculated as its percentage and plotted on the y axis. The amount of red color of the same cells was plotted on the x axis. Nonlinear (logarithmic) regression was then used to generate the line of best fit.

Quantitative reverse transcription-PCR (qRT-PCR). For the analysis of PIV3 mRNA concentrations, infected-cell mRNA was isolated with a magnetic mRNA isolation kit (New England BioLabs), and cDNA was made using a SuperScript III RT-PCR kit (Invitrogen). Primers specific for the six PIV3 mRNAs were used; GAPDH primers were used for normalization of the samples. Quantitative PCR was performed for 40 cycles using SYBR green PCR master mix (Life Technologies) in a StepOne Plus real-time PCR detection system (Applied Biosystems). The melting temperatures of amplified products were analyzed to confirm the specificity of the reaction. All samples were run in triplicate, and fold changes were calculated using the threshold cycle ($\Delta\Delta C_T$) method.

RESULTS

Detection of PIV3 nonstructural protein C. In our studies of the IFN sensitivity of PIV3, we sought a rapid, antibody-based assay of viral growth, useful for immunoblotting as well as immunofluorescence. In our experience, available antibodies against the virion structural proteins (mostly the HN protein) detected the input virus, including dead virions, especially in older viral stocks, often producing a significant background that did not represent replicating virus. In contrast, a viral nonstructural protein, by definition, should indicate actively replicating virus and productively infected cells. However, to our knowledge, no commercial antibody is currently available against PIV3 nonstructural proteins. We therefore raised a peptide antibody against PIV3 C protein, a nonstructural protein. The antibody was highly specific and exhibited all the properties expected of it. Specifically, (i) it detected a single ~ 26 -kDa band, matching the predicted size of the C polypeptide, in the immunoblot analysis of infected-cell lysate, made after at least 15 h of infection; (ii) it did not react with purified PIV3 virions (data not shown); and (iii) it did not react with uninfected cell extracts or extracts made at early stages of infection (Fig. 1), when viral translation is likely below the threshold of detection. Thus, we used this C antibody in all our PIV3 detection studies described here.

Type I IFN sensitivity of PIV3 requires canonical IFN signaling. In order to determine the IFN sensitivity of our PIV3 preparation, we optimized the concentration of IFN at a low MOI. Based on these results, we used an IFN concentration of 1,000 IU/ml in the cell culture studies of PIV3. As shown (Fig. 1), time-dependent growth of PIV3 (at 18 and 24 h postinfection) was detected in an immunoblot with PIV3 C antibody but could not be detected in IFN-treated cells in the same period. Thus, we confirmed the high IFN sensitivity of PIV3 seen previously (7). We then extended these studies to human cell lines genetically devoid

FIG 1 Type I IFN-mediated inhibition of PIV3 by the canonical IFN response pathway. Universal type I IFN (1,000 U/ml) was added to the indicated human cell monolayers (fTGH, U2A, and U6A), and 20 h later, PIV3 was added at an MOI of 1. Cells were harvested at the indicated times post-PIV3 addition, and total extract was processed for immunoblotting using antibodies that are described in Materials and Methods. Note the abrogation of PIV3 growth, measured by C protein, in IFN-treated, parental fTGH cells but not in cell lines deficient in STAT2 and IRF9. GAPDH was the loading control.

of three major members of the IFN response pathway, namely, IRF9^{-/-} U2A cells, STAT1^{-/-} U3A cells, and STAT2^{-/-} U6A cells (14). PIV3 growth in these mutant cells was significantly resistant to IFN (Fig. 1), demonstrating that the anti-PIV3 function of IFN requires the canonical ISGF3 pathway and thus likely draws on the ISG proteins, the end products of this pathway.

IFIT1 is a major anti-PIV3 factor among the IFIT family ISG proteins. The IFIT family of proteins in humans consists of four members: IFIT1 (ISG56), IFIT2 (ISG54), IFIT3 (ISG60), and IFIT5 (ISG58) (22, 23). While virtually absent in unstimulated cells, IFITs are rapidly expressed following IFN treatment, IFIT1 being the most abundant. Emerging studies of various IFIT proteins have begun to reveal their antiviral functions and their mechanisms of action; IFIT1, for example, has been shown to inhibit hepatitis C virus (HCV) (24) and human papillomavirus (HPV) (25). To test if any IFIT has activity against PIV3, we expressed each IFIT by transient transfection and tested PIV3 growth by immunoblotting. The results (Fig. 2) showed that all four recombinant clones produced the corresponding myc-tagged IFIT, although with equal amounts of plasmid transfected, IFIT1 was least expressed in comparison to the other three (Fig. 2, top panel). Nonetheless, when tested against PIV3 (Fig. 2, bottom panel), IFIT1 exhibited the strongest anti-PIV3 activity. This is despite the fact that the recombinant IFIT1 protein was consistently the most weakly expressed among the four IFITs. Comparison of IFIT levels in PIV3-infected and uninfected cells also showed that PIV3 did not reciprocally affect the expression of any IFIT.

Since immunoblot (Western) assays rely on efficient transfection of the whole population of cells, we decided to study the IFIT-PIV3 relationship in individual cells. However, the traditional approach of fluorescence-activated cytometry was not successful in our hands, primarily due to inefficient immunostaining of the infected cells under flow cytometry conditions. We there-

FIG 2 Inhibition of PIV3 by recombinant IFIT proteins. myc-tagged IFIT plasmids were transfected into A549 cell monolayers (1.6 μ g of plasmid/well in a 12-well plate), and PIV3 at an MOI of 1 was added 24 h later (bottom panel). Cells were lysed at 24 h after addition of the virus, and total extract was analyzed by immunoblotting as for Fig. 1. GAPDH was the loading control. Note the strongest inhibition of PIV3 (indicated by asterisk) by the small amount of expressed myc-IFIT1. Essentially similar results were obtained with 293T cells (results not shown).

fore resorted to microscopy of fixed adherent cells, in which the cells were immunostained with both anti-myc (detecting IFIT) and anti-C (detecting PIV3) antibodies. In representative fields, we have marked a few IFIT and PIV3 costained cells with arrows (Fig. 3). The general trend was the least number of costained cells in IFIT1, indicating that it was the most inhibitory, whereas IFIT2 and IFIT5 had the most costained cells and IFIT3 appeared to be somewhere in between. However, we realized that due to stochastic (cell-to-cell) variation of IFIT expression and PIV3 growth, all cells will not be stained uniformly by either color. Moreover, for an inhibitory IFIT, there will be a reciprocal relationship between the two colors, such that in most cells one color will mask the other, thus not generating a composite color. Therefore, we ignored the visual colocalization to avoid any bias and digitally quantified the red and green colors of each cell from the respective color panel as described in Materials and Methods. Multiple cells were thus quantified for each IFIT, and red (IFIT) versus green (PIV3) intensities were plotted for each cell. The results (Fig. 4A) revealed a strong inverse relationship between IFIT1 levels and PIV3 growth, while this relationship was weaker between IFIT3 and PIV3. At smaller amounts, IFIT1 was pronouncedly stronger anti-PIV3 than IFIT3. For example, at arbitrary unit 5 on the x axis (red, for IFIT), IFIT1 caused a 40% reduction of PIV3 signal, whereas at the same amount, IFIT3 reduced PIV3 by only ~20%. With IFIT2 and IFIT5, the response was flat, indicating that PIV3 grew well regardless of the level of expression of these two IFITs. Taken together, the immunoblotting and microscopic results document a strong anti-PIV3 effect of IFIT1.

To further verify the stronger anti-PIV3 effect of IFIT1 over IFIT3, we performed a head-to-head comparison of the two. We transfected cells with increasing amounts of the myc-tagged IFIT plasmids and tested IFIT protein expression and corresponding

FIG 3 Single-cell images of PIV3 inhibition by recombinant IFIT proteins. 293T cells were transfected with myc-IFIT plasmids, infected with PIV3, and then stained for myc-IFIT (red), PIV3 C (green), and DAPI as described in Materials and Methods. Colocalization of red and green indicates replication of PIV3 in IFIT-expressing cells. Due to the difficulty of visualizing the merged color in this reproduced image, we have used arrows to point to a few representative red-green costained cells; they represent resistance of PIV3 to that particular IFIT. Note that this is only a gross indicator of inhibition; a quantitative analysis of these images is presented in Fig. 4A.

PIV3 inhibition. The results (Fig. 4B) clearly revealed the generally poor expression of recombinant IFIT1 compared to that of IFIT3. In fact, the largest amount of IFIT1 plasmid we could test (3.6 µg) without cell loss produced less protein than the smallest amount of IFIT3 plasmid tested (0.1 µg). Regardless of the mechanism of this highly dissimilar expression, even the smallest amount of IFIT1 protein (e.g., at 0.6 µg of plasmid) inhibited PIV3 more strongly than the largest amount of IFIT3 protein (e.g., at 1.2 µg of plasmid). These results confirm the microscopic results (Fig. 4A) showing that IFIT1 is a stronger antiviral against PIV3, particularly discernible at small quantities of both proteins. Quantitative RT-PCR using gene-specific primers revealed that IFIT1 lowers

the steady-state level of all PIV3 gene mRNAs by a factor in the range of 4 to 8 (Fig. 4C), suggesting that IFIT1 does not inhibit PIV3 by affecting the synthesis or stability of a specific viral mRNA but likely has a general effect on PIV3 growth.

Screening of other ISG proteins for anti-PIV3 activity. Having shown that IFIT1 (ISG56) possesses anti-PIV3 activity, we searched for other ISGs that may also inhibit PIV3. To this end, we used a recently engineered panel of HEK293 cell lines in which a selected group of ISGs, commonly induced by IFN, were overexpressed from a Tet-inducible promoter (15, 16). We have successfully used this ISG cell panel in the past to show that three of the ISG proteins (*viz.*, ISG20, PKR, and viperin) inhibit the HCV rep-

FIG 4 (A) Image analyses of single-cell PIV3 growth inhibition by recombinant IFIT proteins. The red and green colors of individual stained cells in the images in Fig. 3 were quantified and graphed as described in Materials and Methods and in Results. Note the highest inhibition of PIV3 by IFIT1 (sharp drop from 100), particularly appreciable at a low IFIT1 signal. (B) Direct comparison between IFIT1 and IFIT3 for anti-PIV3 activity. Increasing amounts of the two myc-tagged recombinant IFIT plasmids were transfected into A549 cells, and the levels of expressed IFIT and PIV3 growth were measured by immunoblotting for myc tag and C protein, respectively (as for Fig. 2). The numbers indicate amounts of plasmid transfected per well in a 12-well plate. The different proteins are marked by arrows, and GAPDH served as the loading control. (C) Quantitative RT-PCR of the indicated PIV3 mRNAs, isolated from IFIT1-transfected and control untransfected A549 cells, was performed, and the fold reduction by IFIT1 plotted, as described in Materials and Methods. Graphs were plotted in Excel, with each column (bar) representing the mean of three values of the fold change and error bars representing SDs.

licon (15) and five ISG proteins (the three above plus IFITM2 [IFN-induced transmembrane protein 2] and IFITM3) inhibit West Nile virus (WNV) and dengue virus (DENV) (16).

We tested a total of 12 ISG cell lines in this panel (15, 16) by measuring PIV3 growth in immunoblots. The results (Fig. 5A) show that four Tet-induced ISG proteins, namely, IDO (indoleamine 2,3-dioxygenase), IFITM1, PKR (protein kinase, RNA activated), and viperin, had the strongest PIV3-inhibitory effects, while DCK (deoxycytidine kinase) was weakly inhibitory and the rest showed little or no inhibition.

To rule out that the effect of these ISG proteins was manifested solely on PIV3 C protein, we used one recent lot of commercial HN antibody, with which we had had only occasional success in immunoblotting and only when using freshly harvested PIV3 virus. We repeated the aforementioned experiment of Fig. 5A but this time performed immunoblotting using both HN and C antibodies on the same extract, so that the results could be directly compared. A total of six representative ISG proteins were tested; the results (Fig. 5B) clearly show that the effects of a given ISG protein on C and HN were generally comparable, although the HN measurement appeared to show a slightly higher background, likely because HN is a structural protein and the antibody could

detect noninfectious virions in the preparation, as mentioned earlier. Nonetheless, in measuring proteins, IDO, IFITM1, and viperin scored as strongly inhibitory, DCK scored as a weak inhibitor, and IFITM2 and UBE2L6 did not inhibit at all. As these results established the equivalence of measuring HN and C, we used the C antibody for all our subsequent experiments for the reasons stated earlier. We then went on to reproduce these results in A549 cells of alveolar epithelial origin, which are relevant for PIV3. We chose viperin as an example and expressed recombinant viperin in A549 cells by transient transfection with the pcDNA3.1-FLAG-viperin plasmid, and we found that PIV3 growth was strongly inhibited in these cells (Fig. 5C). Finally, we quantified the infectious progeny virus liberated in the culture media by plaque assay and found that the Tet-induced IDO cell line generated ~50-fold less virus than the uninduced cell line (Fig. 5D), suggesting that the induction of IDO led to a net decline in overall yield of the virus progeny.

Tryptophan suppresses the inhibitory effect of IDO on PIV3.

It is quite likely that each ISG has its own mechanism of inhibition of PIV3, which we would like to address in detail in future studies. While IFIT1, viperin, and PKR are well-studied antiviral factors, relatively little is known about IDO and IFITM proteins. Here, we

FIG 5 Screening of ISGs for anti-PIV3 activity. The indicated FLAG-tagged ISGs in stably expressing HEK293 cell lines (31) were induced by the addition of tetracycline (Tet; 1 μ g/ml), and PIV3 (MOI, 1) was added 24 h later. At 24 h thereafter, cells were processed for immunoblotting as described in Materials and Methods. Control cells did not receive Tet. GAPDH served as a protein loading control. (A) Initial screening using PIV3 C antibody. (B) Confirmatory testing of a subset of ISGs with PIV3 HN antibody. (C) Parallel confirmation of inhibition by viperin using recombinant viperin plasmid in A549 cells. Transient transfection and PIV3 infection have been described in Materials and Methods. (D) PFU of progeny PIV3 liberated from the Tet-induced (+Tet) and uninduced (–Tet) IDO cell line at different time points postinfection (0, 4, 12, 18, 24, 36, and 42 h) were plotted using Excel. Data are mean $\pm$ SDs of three values.

report our preliminary results on the mechanism of the anti-PIV3 effect of IDO. IDO is known to catalyze the degradation of the essential amino acid L-tryptophan (Trp) to generate N-formylkynurenine. To test whether the anti-PIV3 effect of IDO is due to depletion of Trp or production of N-formylkynurenine, we performed a series of experiments testing whether addition of exogenous Trp can counteract the antiviral effect of IDO. We tested a range of Trp concentrations and found that Trp concentrations of 50 μ g/ml and higher fully restored PIV3 (Fig. 6A) and RSV (Fig. 6B) growth

in Tet-induced IDO-expressing cells. These results indicate that it is the lack of Trp, rather than the production of any downstream product, that is the cause of IDO's inhibitory effect. Also, all four RSV proteins that could be detected by the polyclonal antibody were equally affected and also equally restored by Trp. Together, these results suggest that when IDO is overexpressed, it functions as a relatively broad-spectrum inhibitor of paramyxoviruses. We then replicated these studies with A549 cells (Fig. 7) and first used the type II IFN, IFN- γ , which is a more potent inducer of IDO than type I IFN in most cell types (26, 27). Indeed, we con-

FIG 6 (A) Suppression by the antiviral activity of IDO by Trp. Tet-mediated induction of recombinant FLAG-IDO expression in HEK293 cells and PIV3 infection were carried out as described in the text. Trp at the indicated final concentration was added to the medium at the same time as the virus, and cells were processed for immunoblotting at 24 h thereafter. (B) Experiment similar to that in panel A, with RSV infection instead of PIV3; the four RSV proteins detected by the polyclonal antibody are indicated. Both viruses were used at an approximate MOI of 1.

firmed this and showed that the IFN- γ -induced IDO caused severe inhibition of PIV3 (Fig. 7A). We conclude that regardless of whether it is expressed by recombinant means or by induction with an agonist, IDO can act as a potent inhibitor of PIV3 by depleting the intracellular tryptophan pool. Virus growth could be significantly rescued by exogenous Trp, as low as 10 μ g/ml (Fig. 7A). Next, we used the type I IFN IFN- α for these studies, since it is a well-known antiviral in diverse cell types and induces hundreds of ISGs, whereas the effect of IFN- γ on nonimmune cells is limited and leads to the induction of fewer and largely nonoverlapping ISGs (11). In HT1080 fibrosarcoma cells, for example, >29-fold upregulation of IFIT1 mRNA expression occurs upon IFN- α treatment, but there is no increase with IFN- γ (11). Since we induced endogenous IDO with IFN- γ in order to demonstrate its anti-PIV3 effect in epithelial A549 cells, we wanted to see how these results compare with those obtained with IFN- α . As shown in Fig. 7B, IFN- α induced an extremely small amount of IDO, which we could detect only by highly overexposing the blot. Nonetheless, PIV3 was severely inhibited in these IFN- α -treated cells. As IFN- α induces many antiviral ISG proteins, and we know that several of them in fact inhibit PIV3, we reasoned that the

FIG 7 Antiviral role of endogenous IDO and viral rescue by Trp. Where indicated, A549 IDO gene was induced by the addition of IFN- γ (60 ng/ml) (A) or IFN- α (1,000 U/ml) (B) to the medium. Virus infection was performed as for Fig. 6. Note the grainy appearance of the IDO blot in panel B, which is due to the fact that it had to be overexposed to visualize the small amounts of IDO, characteristic of induction by IFN- α , in contrast to the high induction by IFN- γ (A).

observed inhibition of PIV3 may be more due to the other ISG proteins than the small amount of IDO made. Indeed, this seemed to be the case, since addition of Trp (up to 200 μ g/ml) was unable to rescue PIV3 growth in the IFN- α -treated cells (Fig. 7B), whereas a much lower concentration was able to do so in the IFN- γ -treated cells (Fig. 7A). We conclude that overexpressed IDO can act as a major antiviral factor, but when it is underexpressed and all other antiviral ISG proteins are also induced by a physiological agonist (such as type I IFN), the latter may work together to generate a more dominant antiviral effect.

In a reciprocal strategy, we investigated if knocking down IDO expression by RNAi improves virus growth. The knockdown was performed in two formats: using transiently transfected siRNA (Fig. 8A) and using a cell line in which precursor shRNA was stably expressed (Fig. 8B). In both kinds of cells, IDO was induced by IFN- γ as before, and a modest restoration of PIV3 growth was observed compared to that in control cells in which IDO was not knocked down (e.g., compare the PIV3 C protein levels in lane b with lane a and lane d with lane c).

Anti-PIV3 role and membrane localization of IFITM proteins. Like the IFIT family, the IFITM family also consists of four paralogs in humans, namely, IFITM1, IFITM2, IFITM3, and IFITM5 (28), which are highly similar in sequence. All are inducible by IFN and, like the IFITs, exhibit overlapping as well as unique activities against diverse viruses (16, 28–35). However, unlike IFIT, the IFITM proteins reside primarily in membranes (hence the M designation) and thus specifically block viral replication by abrogating membrane fusion (31–34). Interestingly, IFITM1 is the only IFITM family member that is primarily localized in the cell membrane, and it inhibits PIV3, whereas

FIG 8 Knockdown of IDO enhances virus growth. Silencing of IDO expression by transient transfection of IDO siRNA (A) or stable expression of shRNA in A549 cells and PIV3 infection (B) was conducted as described in Materials and Methods. Induction of the IDO gene by IFN- γ and the immunoblotting were done essentially as for Fig. 7A. Lanes marked a to d are described in Results.

IFITM2, IFITM3, and IFITM5 reside in various cytoplasmic organellar membranes, such as in endosomes or lysosomes (28, 35). IFITM3, in particular, normally resides in the endocytic compartment through the use of a classic tyrosine-based sorting signal (YEML) (36) and did not inhibit PIV3. Since fusion of paramyxoviruses, including PIV3, occurs at the cell surface (37–42), we hypothesized that IFITM3 could inhibit PIV infection if it was localized to the cell membrane. To test this, we used IFITM3 mutants in which Tyr20 was altered to Ala (Y20A) or Asp (Y20D), both of which result in the localization of the mutant proteins to cell membrane (36; unpublished observation of the Ju-Tao Guo laboratory). Interestingly, both mutants were found to be highly inhibitory to PIV3 (Fig. 9). These results suggest that the localization of IFITM proteins to the plasma membrane may be a major determinant of their anti-PIV3 property. It remains to be seen whether IFITM1 in fact inhibits specific steps of PIV3 fusion and entry.

DISCUSSION

Interferon-stimulated genes (ISGs) are a diverse group of about 500 genes that are induced by interferons (IFNs) (43–47). Although the first ISGs were discovered about 25 years ago, insight into their antiviral roles has been limited to a few classical ISGs, such as those encoding MX1, OAS, and PKR. Attempts to understand the antiviral mechanism of several ISG proteins have resulted in fundamental discoveries concerning regulation of translation, posttranslational modification, RNA and protein stability, and membrane transport (12, 13, 28, 29, 43, 48, 49). However, the

FIG 9 Antiviral activity of mutant IFITM3. FLAG-tagged wild-type IFITM3 and Tyr20 mutants were induced by Tet in the HEK293 cell line, the cells were infected with PIV3, and immunoblotting was performed essentially as described for the ISG screening in Fig. 5.

antiviral function of the vast majority of ISGs remains unknown. Recent studies have revealed that some ISG proteins have antiviral activity against multiple viruses, whereas others are relatively virus specific (15, 16, 28, 29, 49, 50). Moreover, multiple ISG proteins have been shown to inhibit the same virus (28, 29, 49, 50). In this study, analysis of a small set of ISG proteins led to the identification of five that when overexpressed strongly inhibited human PIV3; these are the IDO (indolamine 2,3-dioxygenase), IFIT1, IFITM1, PKR, and viperin. In what follows, we discuss the implications of our findings.

IDO is a heme-containing enzyme which, as mentioned before, catalyzes the degradation of the essential amino acid L-tryptophan (Trp) to N-formylkynurenine. Being the first and the rate-limiting enzyme of the tryptophan catabolism pathway, IDO is strategically important for tryptophan homeostasis. Relatively extensive early studies of intracellular pathogens, such as *Toxoplasma gondii* and *Chlamydia* spp., showed a general antimicrobial role of IDO, although the mechanism has remained controversial (51, 52). Similar to our findings with RSV and PIV3, Trp was shown to counteract the inhibitory effect of IDO on measles virus (26), another member of this family, and on herpes simplex virus (HSV) and hepatitis B virus, two DNA viruses (53, 54). These results support the intracellular Trp depletion mechanism, since if the PIV3-inhibitory effect were due to increased concentration of Trp degradation products, then even larger amounts of such products would have been generated upon addition of more Trp. Although IDO may be less important in the context of IFN- α due to the induction of many other antiviral ISGs, it is certainly a major antiviral force in cells that do respond to IFN- γ . Similar to our findings with lung epithelial A549 cells, in neuronal cells IFN- γ strongly induced IDO expression, causing inhibition of HSV replication, which could be rescued by Trp (54). Interaction of IDO with several other viruses, such as HIV, cytomegalovirus, RSV, hepatitis virus, and influenza A virus, has been unraveled recently, but mainly in the context of T cell responses and induced immunity (52, 55–58). The effect of IDO on PIV3, however, was appreciated in nonimmune cells; it is thus representative of a cell-intrinsic mechanism that directly inhibits viral gene expression. How low Trp levels specifically inhibit the virus and not the cell is currently an enigma, especially because these two entities use the same cytoplasmic translational machinery to incorporate Trp into the proteins. We hypothesize that the viral translation occurs in

specialized cytoplasmic compartments and that a higher concentration gradient of cytosolic Trp may be needed for its entry into these compartments or in the molecular proximity. Future experiments will test this, but there is mounting evidence for specialized compartments of viral replication and assembly, often referred to as inclusion bodies, Negri bodies, or viroplasm (59–61). In some instances, such as in rabies virus, a nonsegmented negative-strand RNA virus, the presence of all viral gene mRNA inside Negri bodies was experimentally demonstrated (61). In RSV, the viral inclusion bodies have also been shown to antagonize innate immunity (59).

IFIT1 is a member of the TPR domain IFIT family, the antiviral role of which and the pertinent mechanisms have begun to receive considerable attention. The four members of the human IFIT family regulate translation by differentially recognizing the 5' termini of their target RNA (28, 48). The exact features in the RNA that they recognize is a matter of some debate, but the current consensus is that they bind to 2'-O-unmethylated 5' cap structure (cap 0) and/or 5'-triphosphates (5'-ppp) on the RNA, which are generally absent in cellular mRNA but found in transcripts of intracellular pathogens, such as viruses that lack cap methyltransferase activity and bacteria (62–67). IFIT1, in particular, shows a high preference for mRNA with unmethylated cap (63–67), whereas IFIT5, which had no inhibitory effect on PIV3, prefers capless, 5'-ppp RNA (67). We have ignored the modest inhibition seen with IFIT3, since it was discernible only at much larger amounts; moreover, no inhibition was observed in the Tet-inducible HEK293 cell line expressing IFIT3 (data not shown). Recent studies have shown that IFIT1 is primarily responsible for IFN-induced inhibition of PIV5 (formerly known as SV5), a rubulavirus of the *Paramyxoviridae* family (68). The cap structures of neither PIV5 nor PIV3 mRNAs are known, but those of a few other nonsegmented negative-strand RNA viruses, such as vesicular stomatitis virus (VSV), Newcastle Disease virus (NDV), and RSV, have been reported. The VSV cap is fully 2'-O methylated (69–71), that of NDV is unmethylated (72), and RSV mRNAs contain a mixture of unmethylated and methylated caps, the latter being favored at higher concentrations of the methyl donor (*S*-adenosylmethionine) *in vitro* (73, 74). PIV5 also appears to have a fully methylated cap (68), yet PIV5 is efficiently inhibited by IFIT1. In view of this diversity, it is possible that a fraction of PIV3 mRNAs may be unmethylated and susceptible to IFIT1-mediated translational inhibition. Alternatively, or additionally, IFIT1 may recognize a PIV3-transcribed 5'-ppp RNA, and the major such RNA in nonsegmented negative-strand RNA viruses is the leader RNA, a short promoter-proximal transcript of ~45 nucleotides. The exact physiological role of leader RNA remains a mystery, but it has been shown to bind viral nucleocapsid protein N (75), which regulates the viral transcription-to-replication switch (76), and the host La antigen (58). The relative amounts of leader RNA bound to these proteins and the timing of the binding need to be determined in order to evaluate the amount available to IFIT1, which is itself induced via the IFN pathway. How this binding affects viral gene expression will be an important area of future research.

PKR was initially identified for its ability to inhibit viral protein translation (77). PKR is a Ser/Thr kinase, inducible by IFN, but it is enzymatically activated by binding to double-stranded RNA (dsRNA). The activated PKR phosphorylates the eukaryotic initiation factor 2 α subunit (eIF2 α) (78, 79), thus inhibiting cap-dependent protein translation. This mechanism explains the

broad antiviral activity of PKR, since the vast majority of mRNAs of all viruses are capped. It also agrees with the lack of antiviral effect of the kinase-dead PKR mutant (PKR-Mt in Fig. 5). We believe that the anti-PIV3 activity of PKR is also due to this mechanism.

Viperin is a “radical SAM domain” protein, highly conserved in all vertebrates. It is a relatively broad antiviral, with demonstrated activity against a variety of DNA and RNA viruses (80, 81), such as cytomegalovirus, Chikungunya virus (CHIKV), DENV, hepatitis C virus, Japanese encephalitis virus (JEV), VSV, Sendai virus, WNV, rhinovirus, yellow fever virus (YFV), lymphocytic choriomeningitis virus, Bunyamwera virus, reovirus, and, recently, RSV (82). Unfortunately, in spite of considerable knowledge of the various domains, structure, and cellular location of viperin, the mechanism of how it inhibits such a diverse panel of viruses remains unclear. A recent study suggests that viperin inhibits RSV morphogenesis and egress (83). However, in our assays, PIV3 growth was carried out for a single cycle (24 h post-virus addition) whereby we measured the intracellular, nonstructural C protein, and therefore, virion egress was irrelevant. Thus, viperin appears to inhibit a step(s) of intracellular replication of PIV3, which needs to be unraveled.

Because of its weak PIV3-inhibitory activity, we have not attached much significance to DCK (Fig. 5A and B), an enzyme that catalyzes the phosphorylation of multiple deoxyribonucleosides (84). Increased DCK activity results in increased production of nucleoside triphosphates, which is, therefore, expected to occur in IFN-treated cells. However, it is unclear as to how deoxynucleotides or their phosphates may inhibit an RNA virus such as PIV3.

Recently, in two large-scale studies (29, 50), nearly 400 ISGs were overexpressed by lentiviral expression in human hepatoma Huh7 cells and STAT1^{-/-} fibroblasts, and growth of various representative viruses was quantified by using recombinant virus strains expressing reporter green fluorescent protein (GFP). The viruses included HCV, WNV, CHIKV, Venezuelan equine encephalitis virus (VEEV), YFV, DENV, HIV-1, and several members of the *Paramyxoviridae* family, namely, measles virus, NDV, human metapneumovirus, RSV, and PIV3. These studies revealed multiplicity as well as overlap of the antiviral roles of the ISG proteins. In brief, 47 were found to inhibit one or more viruses; the broadly acting effectors included IRF1, RIGI, MDA5, and IFITM3, while more targeted antiviral specificity was observed with DDX60, IFI44L, IFI6, IFITM2, MAP3K14, MOV10, NAMPT, OASL, RTP4, TREX1, and UNC84B. However, ISG proteins that inhibited PIV3 in our studies did not match; in fact, only two—IRF1 and MAP3K14—were found to inhibit PIV3 by ~65%, while a modest inhibition, ~20 to 30%, was seen with CD9, IRF2, TMEM51, and B4GALT5. With RSV, similar modest inhibition was found with IRF2, IRF7, MAP3K14, HPSE, and THBD, and surprisingly, with cGAS, a DNA sensor. Four ISG proteins, namely, viperin (82), OASL (85), IFITM1 and IFITM3 (86), specifically shown to inhibit RSV in directed studies, were not found to be inhibitory in the screen. The apparent differences could be due to a number of reasons, such as differences in cell lines, expression levels of the ISG proteins, viral load, and assay methods. It is known, for example, that the antiviral effect of IFN and that of ISG proteins can often be concentration dependent; it is quite possible that the vectors used in the specific ISG studies generated more recombinant ISG proteins than the lentiviral vectors used in the large-scale screening. Lastly, joint expression of

two ISG proteins showed additive antiviral effects similar to those of moderate IFN doses (29); this is perhaps expected, as IFN induces a plethora of ISG proteins, many of which inhibit the same virus, thus exerting a firm control on virus growth. We have not coexpressed multiple ISG proteins in our studies, nor did we silence more than one, since our goal was to identify individual ISG proteins in a selected group of relatively well-known ISG proteins. It is also known that regardless of the large number of ISG proteins induced, a single ISG protein may act as the principal inhibitor, as has been shown for IFIT1-mediated inhibition of WNV and PIV5 (63, 68) and IFITM3-mediated inhibition of influenza A virus (87), while a few others may play minor roles. We anticipate that our results will form the foundation of mechanistic studies in the future addressing the relative importance of each ISG protein and how these proteins inhibit PIV3.

ACKNOWLEDGMENTS

This research was supported in part by NIH grant AI109569.

Sincere thanks are due to Ganes C. Sen and George Stark (both at Lerner Research Institute, Cleveland Clinic) for the IFIT knockout mouse embryonic fibroblast cells and the fTGH, U2A, U3A, and U6A cell lines, respectively; Vincent Piguet (Cardiff University, Wales, UK) for the FLAG-tagged viperin plasmid; and James Koropatnick (Western University and Lawson Health Research Institute, London, Ontario, Canada) for the A549 clones stably expressing IDO shRNA and its scrambled control.

FUNDING INFORMATION

This work, including the efforts of Sailen Barik, was funded by HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) (AI109569).

REFERENCES

- Sato M, Wright PF. 2008. Current status of vaccines for parainfluenza virus infections. *Pediatr Infect Dis J* 27:S123–S125. <http://dx.doi.org/10.1097/INF.0b013e318168b76f>.
- Banerjee AK, Barik S, De BP. 1991. Gene expression of nonsegmented negative strand RNA viruses. *Pharmacol Ther* 51:47–70. [http://dx.doi.org/10.1016/0163-7258\(91\)90041-J](http://dx.doi.org/10.1016/0163-7258(91)90041-J).
- Hall CB. 2001. Respiratory syncytial virus and parainfluenza virus. *N Engl J Med* 344:1917–1928. <http://dx.doi.org/10.1056/NEJM200106213442507>.
- Fensterl V, Chattopadhyay S, Sen GC. 2015. No love lost between viruses and interferons. *Annu Rev Virol* 2:549–572. <http://dx.doi.org/10.1146/annurev-virology-100114-055249>.
- Radoshevich L, Dussurget O. 2016. Cytosolic innate immune sensing and signaling upon infection. *Front Microbiol* 7:313.
- Barik S. 21 July 2016. What really rigs up RIG-I? *J Innate Immun* <http://dx.doi.org/10.1159/000447947>.
- Atreya PL, Kulkarni S. 1999. Respiratory syncytial virus strain A2 is resistant to the antiviral effects of type I interferons and human MxA. *Virology* 261:227–241. <http://dx.doi.org/10.1006/viro.1999.9835>.
- Levy DE, Kessler DS, Pine R, Darnell JE, Jr. 1989. Cytoplasmic activation of ISGF3, the positive regulator of interferon- α -stimulated transcription, reconstituted in vitro. *Genes Dev* 3:1362–1371. <http://dx.doi.org/10.1101/gad.3.9.1362>.
- Fu XY, Kessler DS, Veals SA, Levy DE, Darnell JE, Jr. 1990. ISGF3, the transcriptional activator induced by interferon α , consists of multiple interacting polypeptide chains. *Proc Natl Acad Sci U S A* 87:8555–8559. <http://dx.doi.org/10.1073/pnas.87.21.8555>.
- Fensterl V, Sen GC. 2009. Interferons and viral infections. *Biofactors* 35:14–20. <http://dx.doi.org/10.1002/biof.6>.
- Der SD, Zhou A, Williams BR, Silverman RH. 1998. Identification of genes differentially regulated by interferon α , β , or γ using oligonucleotide arrays. *Proc Natl Acad Sci U S A* 95:15623–15628. <http://dx.doi.org/10.1073/pnas.95.26.15623>.
- Schoggins JW. 2014. Interferon-stimulated genes: roles in viral pathogenesis. *Curr Opin Virol* 6:40–46. <http://dx.doi.org/10.1016/j.coviro.2014.03.006>.
- Schneider WM, Chevillotte MD, Rice CM. 2014. Interferon-stimulated genes: a complex web of host defenses. *Annu Rev Immunol* 32:513–545. <http://dx.doi.org/10.1146/annurev-immunol-032713-120231>.
- Li X, Leung S, Qureshi S, Darnell JE, Jr, Stark GR. 1996. Formation of STAT1-STAT2 heterodimers and their role in the activation of IRF-1 gene transcription by interferon- α . *J Biol Chem* 271:5790–5794. <http://dx.doi.org/10.1074/jbc.271.10.5790>.
- Jiang D, Guo H, Xu C, Chang J, Gu B, Wang L, Block TM, Guo JT. 2008. Identification of three interferon-inducible cellular enzymes that inhibit the replication of hepatitis C virus. *J Virol* 82:1665–1678. <http://dx.doi.org/10.1128/JVI.02113-07>.
- Jiang D, Weidner JM, Qing M, Pan XB, Guo H, Xu C, Zhang X, Birk A, Chang J, Shi PY, Block TM, Guo JT. 2010. Identification of five interferon-induced cellular proteins that inhibit West Nile virus and dengue virus infections. *J Virol* 84:8332–8341. <http://dx.doi.org/10.1128/JVI.02199-09>.
- Ashour J, Morrison J, Laurent-Rolle M, Belicha-Villanueva A, Plumlee CR, Bernal-Rubio D, Williams KL, Harris E, Fernandez-Sesma A, Schindler C, García-Sastre A. 2010. Mouse STAT2 restricts early dengue virus replication. *Cell Host Microbe* 8:410–421. <http://dx.doi.org/10.1016/j.chom.2010.10.007>.
- Mangeat B, Cavaghiotti L, Lehmann M, Gers-Huber G, Kaur I, Thomas Y, Kaiser L, Piguet V. 2012. Influenza virus partially counteracts restriction imposed by tetherin/BST-2. *J Biol Chem* 287:22015–22029. <http://dx.doi.org/10.1074/jbc.M111.319996>.
- Hui DJ, Terenzi F, Merrick WC, Sen GC. 2005. Mouse p56 blocks a distinct function of eukaryotic initiation factor 3 in translation initiation. *J Biol Chem* 280:3433–3440. <http://dx.doi.org/10.1074/jbc.M406700200>.
- Maleki Vareki S, Rytelewski M, Figueredo R, Chen D, Ferguson PJ, Vincent M, Min W, Zheng X, Koropatnick J. 2014. Indoleamine 2,3-dioxygenase mediates immune-independent human tumor cell resistance to olaparib, gamma radiation, and cisplatin. *Oncotarget* 5:2778–2791. <http://dx.doi.org/10.18632/oncotarget.1916>.
- Swedan S, Musiyenko A, Barik S. 2009. Respiratory syncytial virus non-structural proteins decrease levels of multiple members of the cellular interferon pathways. *J Virol* 83:9682–9693. <http://dx.doi.org/10.1128/JVI.00715-09>.
- Fensterl V, Sen GC. 2015. Interferon-induced Ifit proteins: their role in viral pathogenesis. *J Virol* 89:2462–2468. <http://dx.doi.org/10.1128/JVI.02744-14>.
- Vladimer GI, Górna MW, Superti-Furga G. 2014. IFITs: emerging roles as key anti-viral proteins. *Front Immunol* 5:94.
- Raychoudhuri A, Shrivastava S, Steele R, Kim H, Ray R, Ray RB. 2011. ISG56 and IFITM1 proteins inhibit hepatitis C virus replication. *J Virol* 85:12881–12889. <http://dx.doi.org/10.1128/JVI.05633-11>.
- Saikia P, Fensterl V, Sen GC. 2010. The inhibitory action of P56 on select functions of E1 mediates interferon's effect on human papillomavirus DNA replication. *J Virol* 84:13036–13039. <http://dx.doi.org/10.1128/JVI.01194-10>.
- Obojes K, Andres O, Kim KS, Däubener W, Schneider-Schaulies J. 2005. Indoleamine 2,3-dioxygenase mediates cell type-specific anti-measles virus activity of gamma interferon. *J Virol* 79:7768–7776. <http://dx.doi.org/10.1128/JVI.79.12.7768-7776.2005>.
- Ozaki Y, Edelstein MP, Duch DS. 1988. Induction of indoleamine 2,3-dioxygenase: a mechanism of the antitumor activity of interferon gamma. *Proc Natl Acad Sci U S A* 85:1242–1246. <http://dx.doi.org/10.1073/pnas.85.4.1242>.
- Diamond MS, Farzan M. 2013. The broad-spectrum antiviral functions of IFIT and IFITM proteins. *Nat Rev Immunol* 13:46–57.
- Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, Bieniasz P, Rice CM. 2011. A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature* 472:481–485. <http://dx.doi.org/10.1038/nature09907>.
- Brass AL, Huang IC, Benita Y, John SP, Krishnan MN, Feeley EM, Ryan BJ, Weyer JL, van der Weyden L, Fikrig E, Adams DJ, Xavier RJ, Farzan M, Elledge SJ. 2009. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. *Cell* 139:1243–1254. <http://dx.doi.org/10.1016/j.cell.2009.12.017>.
- Pereira JM, Chin CR, Feeley EM, Brass AL. 2013. IFITMs restrict the replication of multiple pathogenic viruses. *J Mol Biol* 425:4937–4955. <http://dx.doi.org/10.1016/j.jmb.2013.09.024>.
- Li K, Markosyan RM, Zheng YM, Golfetto O, Bungart B, Li M, Ding S, He Y, Liang C, Lee JC, Gratton E, Cohen FS, Liu SL. 2013. IFITM

- proteins restrict viral membrane hemifusion. *PLoS Pathog* 9:e1003124. <http://dx.doi.org/10.1371/journal.ppat.1003124>.
33. Bailey CC, Zhong G, Huang IC, Farzan M. 2014. IFITM-family proteins: the cell's first line of antiviral defense. *Annu Rev Virol* 1:261–283. <http://dx.doi.org/10.1146/annurev-virology-031413-085537>.
 34. Desai TM, Marin M, Chin CR, Savidis G, Brass AL, Melikyan GB. 2014. IFITM3 restricts influenza A virus entry by blocking the formation of fusion pores following virus-endosome hemifusion. *PLoS Pathog* 10:e1004048. <http://dx.doi.org/10.1371/journal.ppat.1004048>.
 35. Mudhasani R, Tran JP, Retterer C, Radoshitzky SR, Kota KP, Altamura LA, Smith JM, Packard BZ, Kuhn JH, Costantino J, Garrison AR, Schmaljohn CS, Huang IC, Farzan M, Bavari S. 2013. IFITM-2 and IFITM-3 but not IFITM-1 restrict Rift Valley fever virus. *J Virol* 87:8451–8464. <http://dx.doi.org/10.1128/JVI.03382-12>.
 36. Jia R, Xu F, Qian J, Yao Y, Miao C, Zheng YM, Liu SL, Guo F, Geng Y, Qiao W, Liang C. 2014. Identification of an endocytic signal essential for the antiviral action of IFITM3. *Cell Microbiol* 16:1080–1093. <http://dx.doi.org/10.1111/cmi.12262>.
 37. Jardetzky TS, Lamb RA. 2014. Activation of paramyxovirus membrane fusion and virus entry. *Curr Opin Virol* 5:24–33. <http://dx.doi.org/10.1016/j.coviro.2014.01.005>.
 38. Plattet P, Plemper RK. 2013. Envelope protein dynamics in paramyxovirus entry. *mBio* 4:e00413–13. <http://dx.doi.org/10.1128/mBio.00413-13>.
 39. Hemingway BR, Yu Y, Galinski MS. 1994. Paramyxovirus mediated cell fusion requires co-expression of both the fusion and hemagglutinin-neuraminidase glycoproteins. *Virus Res* 31:1–16. [http://dx.doi.org/10.1016/0168-1702\(94\)90066-3](http://dx.doi.org/10.1016/0168-1702(94)90066-3).
 40. Yao Q, Hu X, Compans RW. 1997. Association of the parainfluenza virus fusion and hemagglutinin-neuraminidase glycoproteins on cell surfaces. *J Virol* 71:650–656.
 41. Lamb RA, Paterson RG, Jardetzky TS. 2006. Paramyxovirus membrane fusion: lessons from the F and HN atomic structures. *Virology* 344:30–37. <http://dx.doi.org/10.1016/j.virol.2005.09.007>.
 42. Porotto M, Palmer SG, Palermo LM, Moscona A. 2012. Mechanism of fusion triggering by human parainfluenza virus type III: communication between viral glycoproteins during entry. *J Biol Chem* 287:778–793. <http://dx.doi.org/10.1074/jbc.M111.298059>.
 43. Staeheli P. 1990. Interferon-induced proteins and the antiviral state. *Adv Virus Res* 38:147–200. [http://dx.doi.org/10.1016/S0065-3527\(08\)60862-3](http://dx.doi.org/10.1016/S0065-3527(08)60862-3).
 44. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. 1998. How cells respond to interferons. *Annu Rev Biochem* 67:227–264. <http://dx.doi.org/10.1146/annurev.biochem.67.1.227>.
 45. Leaman DW, Chawla-Sarkar M, Jacobs B, Vyas K, Sun Y, Ozdemir A, Yi T, Williams BR, Borden EC. 2003. Novel growth and death related interferon-stimulated genes (ISGs) in melanoma: greater potency of IFN-beta compared with IFN-alpha2. *J Interferon Cytokine Res* 23:745–756. <http://dx.doi.org/10.1089/107999003772084860>.
 46. Geiss GK, Carter VS, He Y, Kwiciszewski BK, Holzman T, Korth MJ, Lazaro CA, Fausto N, Bumgarner RE, Katze MG. 2003. Gene expression profiling of the cellular transcriptional network regulated by alpha/beta interferon and its partial attenuation by the hepatitis C virus nonstructural 5A protein. *J Virol* 77:6367–6375. <http://dx.doi.org/10.1128/JVI.77.11.6367-6375.2003>.
 47. Borden EC, Sen GC, Uze G, Silverman RH, Ransohoff RM, Foster GR, Stark GR. 2007. Interferons at age 50: past, current and future impact on biomedicine. *Nat Rev Drug Discov* 6:975–990. <http://dx.doi.org/10.1038/nrd2422>.
 48. Li MM, MacDonald MR, Rice CM. 2015. To translate, or not to translate: viral and host mRNA regulation by interferon-stimulated genes. *Trends Cell Biol* 25:320–329. <http://dx.doi.org/10.1016/j.tcb.2015.02.001>.
 49. Schoggins JW, Rice CM. 2011. Interferon-stimulated genes and their antiviral effector functions. *Curr Opin Virol* 1:519–525. <http://dx.doi.org/10.1016/j.coviro.2011.10.008>.
 50. Schoggins JW, MacDuff DA, Imanaka N, Gainey MD, Shrestha B, Eitson JL, Mar KB, Richardson RB, Ratushny AV, Litvak V, Dabelic R, Manicassamy B, Aitchison JD, Aderem A, Elliott RM, García-Sastre A, Racaniello V, Snijder EJ, Yokoyama WM, Diamond MS, Virgin HW, Rice CM. 2014. Pan-viral specificity of IFN-induced genes reveals new roles for cGAS in innate immunity. *Nature* 505:691–695.
 51. Mellor AL, Munn DH. 2003. Tryptophan catabolism and regulation of adaptive immunity. *J Immunol* 170:5809–5813. <http://dx.doi.org/10.4049/jimmunol.170.12.5809>.
 52. Schmidt SV, Schultze JL. 2014. New insights into IDO biology in bacterial and viral infections. *Front Immunol* 5:384.
 53. Mao R, Zhang J, Jiang D, Cai D, Levy JM, Cuconati A, Block TM, Guo JT, Guo H. 2011. Indoleamine 2,3-dioxygenase mediates the antiviral effect of gamma interferon against hepatitis B virus in human hepatocyte-derived cells. *J Virol* 85:1048–1057. <http://dx.doi.org/10.1128/JVI.01998-10>.
 54. Adams O, Besken K, Oberdörfer C, MacKenzie CR, Rüsing D, Däubener W. 2004. Inhibition of human herpes simplex virus type 2 by interferon gamma and tumor necrosis factor alpha is mediated by indoleamine 2,3-dioxygenase. *Microbes Infect* 6:806–812. <http://dx.doi.org/10.1016/j.micinf.2004.04.007>.
 55. Sage LK, Fox JM, Mellor AL, Tompkins SM, Tripp RA. 2014. Indoleamine 2,3-dioxygenase (IDO) activity during the primary immune response to influenza infection modifies the memory T cell response to influenza challenge. *Viral Immunol* 27:112–123. <http://dx.doi.org/10.1089/vim.2013.0105>.
 56. Ajamian F, Wu Y, Ebeling C, Ilarraza R, Odemuyiwa SO, Moqbel R, Adamko DJ. 2015. Respiratory syncytial virus induces indoleamine 2,3-dioxygenase activity: a potential novel role in the development of allergic disease. *Clin Exp Allergy* 45:644–659. <http://dx.doi.org/10.1111/cea.12498>.
 57. Liu P, Jamaluddin M, Li K, Garofalo RP, Casola A, Brasier AR. 2007. Retinoic acid-inducible gene I mediates early antiviral response and Toll-like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. *J Virol* 81:1401–1411. <http://dx.doi.org/10.1128/JVI.01740-06>.
 58. Bitko V, Musiyenko A, Bayfield MA, Maraia RJ, Barik S. 2008. Cellular La protein shields nonsegmented negative-strand RNA viral leader RNA from RIG-I and enhances virus growth by diverse mechanisms. *J Virol* 82:7977–7987. <http://dx.doi.org/10.1128/JVI.02762-07>.
 59. Lifland AW, Jung J, Alonas E, Zurla C, Crowe JE, Jr, Santangelo PJ. 2012. Human respiratory syncytial virus nucleoprotein and inclusion bodies antagonize the innate immune response mediated by MDA5 and MAVS. *J Virol* 86:8245–8258. <http://dx.doi.org/10.1128/JVI.00215-12>.
 60. Novoa RR, Calderita G, Arranz R, Fontana J, Granzow H, Risco C. 2005. Virus factories: associations of cell organelles for viral replication and morphogenesis. *Biol Cell* 97:147–172. <http://dx.doi.org/10.1042/BC20040058>.
 61. Lahaye X, Vidy A, Pomier C, Obiang L, Harper F, Gaudin Y, Blondel D. 2009. Functional characterization of Negri bodies (NBs) in rabies virus-infected cells: evidence that NBs are sites of viral transcription and replication. *J Virol* 83:7948–7958. <http://dx.doi.org/10.1128/JVI.00554-09>.
 62. Terenzi F, Hui DJ, Merrick WC, Sen GC. 2006. Distinct induction patterns and functions of two closely related interferon-inducible human genes, ISG54 and ISG56. *J Biol Chem* 281:34064–34071. <http://dx.doi.org/10.1074/jbc.M605771200>.
 63. Daffis S, Szretter KJ, Schriewer J, Li J, Youn S, Errett J, Lin TY, Schneller S, Zust R, Dong H, Thiel V, Sen GC, Fensterl V, Klimstra WB, Pierson TC, Buller RM, Gale M, Jr, Shi PY, Diamond MS. 2010. 2'-O methylation of the viral mRNA cap evades host restriction by IFIT family members. *Nature* 468:452–456. <http://dx.doi.org/10.1038/nature09489>.
 64. Szretter KJ, Daniels BP, Cho H, Gainey MD, Yokoyama WM, Gale M, Jr, Virgin HW, Klein RS, Sen GC, Diamond MS. 2012. 2'-O methylation of the viral mRNA cap by West Nile virus evades ifit1-dependent and -independent mechanisms of host restriction in vivo. *PLoS Pathog* 8:e1002698. <http://dx.doi.org/10.1371/journal.ppat.1002698>.
 65. Habjan M, Hubel P, Lacerda L, Benda C, Holze C, Eberl CH, Mann A, Kindler E, Gil-Cruz C, Ziebuhr J, Thiel V, Pichlmair A. 2013. Sequestration by IFIT1 impairs translation of 2'-O-unmethylated capped RNA. *PLoS Pathog* 9:e1003663. <http://dx.doi.org/10.1371/journal.ppat.1003663>.
 66. Kimura T, Katoh H, Kayama H, Saiga H, Okuyama M, Okamoto T, Umemoto E, Matsuura Y, Yamamoto M, Takeda K. 2013. Ifit1 inhibits Japanese encephalitis virus replication through binding to 5' capped 2'-O unmethylated RNA. *J Virol* 87:9997–10003. <http://dx.doi.org/10.1128/JVI.00883-13>.
 67. Kumar P, Sweeney TR, Skabkin MA, Skabkina OV, Hellen CU, Pestova TV. 2014. Inhibition of translation by IFIT family members is determined by their ability to interact selectively with the 5'-terminal regions of cap0-, cap1- and 5'ppp- mRNAs. *Nucleic Acids Res* 42:3228–3245. <http://dx.doi.org/10.1093/nar/gkt1321>.

68. Andrejeva J, Norsted H, Habjan M, Thiel V, Goodbourn S, Randall RE. 2013. ISG56/IFIT1 is primarily responsible for interferon-induced changes to patterns of parainfluenza virus type 5 transcription and protein synthesis. *J Gen Virol* 94:59–68. <http://dx.doi.org/10.1099/vir.0.046797-0>.
69. Ogino T, Banerjee AK. 2011. An unconventional pathway of mRNA cap formation by vesiculoviruses. *Virus Res* 162:100–109. <http://dx.doi.org/10.1016/j.virusres.2011.09.012>.
70. Ogino T, Banerjee AK. 2007. Unconventional mechanism of mRNA capping by the RNA-dependent RNA polymerase of vesicular stomatitis virus. *Mol Cell* 25:85–97. <http://dx.doi.org/10.1016/j.molcel.2006.11.013>.
71. Rahmeh AA, Li J, Kranzusch PJ, Whelan SP. 2009. Ribose 2'-O methylation of the vesicular stomatitis virus mRNA cap precedes and facilitates subsequent guanine-N-7 methylation by the large polymerase protein. *J Virol* 83:11043–11050. <http://dx.doi.org/10.1128/JVI.01426-09>.
72. Colonna RJ, Stone HO. 1976. Newcastle disease virus mRNA lacks 2'-O-methylated nucleotides. *Nature* 261:611–614. <http://dx.doi.org/10.1038/261611a0>.
73. Barik S. 1993. The structure of the 5' terminal cap of the respiratory syncytial virus mRNA. *J Gen Virol* 74:485–490. <http://dx.doi.org/10.1099/0022-1317-74-3-485>.
74. Liuzzi M, Mason SW, Cartier M, Lawetz C, McCollum RS, Dansereau N, Bolger G, Lapeyre N, Gaudette Y, Lagacé L, Massariol MJ, Dô F, Whitehead P, Lamarre L, Scouten E, Bordeleau J, Landry S, Rancourt J, Fazal G, Simoneau B. 2005. Inhibitors of respiratory syncytial virus replication target cotranscriptional mRNA guanylation by viral RNA-dependent RNA polymerase. *J Virol* 79:13105–13115. <http://dx.doi.org/10.1128/JVI.79.20.13105-13115.2005>.
75. Blumberg BM, Giorgi C, Kolakofsky D. 1983. N protein of vesicular stomatitis virus selectively encapsidates leader RNA in vitro. *Cell* 32:559–567. [http://dx.doi.org/10.1016/0092-8674\(83\)90475-0](http://dx.doi.org/10.1016/0092-8674(83)90475-0).
76. Blumberg BM, Leppert M, Kolakofsky D. 1981. Interaction of VSV leader RNA and nucleocapsid protein may control VSV genome replication. *Cell* 23:837–845. [http://dx.doi.org/10.1016/0092-8674\(81\)90448-7](http://dx.doi.org/10.1016/0092-8674(81)90448-7).
77. Metz DH, Esteban M. 1972. Interferon inhibits viral protein synthesis in L cells infected with vaccinia virus. *Nature* 238:385–388. <http://dx.doi.org/10.1038/238385a0>.
78. Pindel A, Sadler A. 2011. The role of PKR in the interferon response. *J Interferon Cytokine Res* 31:59–70. <http://dx.doi.org/10.1089/jir.2010.0099>.
79. Yim HC, Williams BR. 2014. Protein kinase R and the inflammasome. *J Interferon Cytokine Res* 34:447–454. <http://dx.doi.org/10.1089/jir.2014.0008>.
80. Helbig KJ, Beard MR. 2014. The role of viperin in the innate antiviral response. *J Mol Biol* 426:1210–1219. <http://dx.doi.org/10.1016/j.jmb.2013.10.019>.
81. Seo JY, Yaneva R, Cresswell P. 2011. Viperin: a multifunctional, interferon-inducible protein that regulates virus replication. *Cell Host Microbe* 10:534–539. <http://dx.doi.org/10.1016/j.chom.2011.11.004>.
82. McGillivray G, Jordan ZB, Peebles ME, Bakaletz LO. 2013. Replication of respiratory syncytial virus is inhibited by the host defense molecule viperin. *J Innate Immun* 5:60–71. <http://dx.doi.org/10.1159/000342473>.
83. Jumat MR, Huong TN, Ravi LI, Stanford R, Tan BH, Sugrue RJ. 2015. Viperin protein expression inhibits the late stage of respiratory syncytial virus morphogenesis. *Antiviral Res* 114:11–20. <http://dx.doi.org/10.1016/j.antiviral.2014.11.007>.
84. Eriksson S. 2013. Is the expression of deoxynucleoside kinases and 5'-nucleotidases in animal tissues related to the biological effects of nucleoside analogs? *Curr Med Chem* 20:4241–4248. <http://dx.doi.org/10.2174/0929867311320340004>.
85. Zhu J, Zhang Y, Ghosh A, Cuevas RA, Forero A, Dhar J, Ibsen MS, Schmid-Burgk JL, Schmidt T, Ganapathiraju MK, Fujita T, Hartmann R, Barik S, Hornung V, Coyne CB, Sarkar SN. 2014. Antiviral activity of human OASL protein is mediated by enhancing signaling of the RIG-I RNA sensor. *Immunity* 40:936–948. <http://dx.doi.org/10.1016/j.immuni.2014.05.007>.
86. Zhang W, Zhang L, Zan Y, Du N, Yang Y, Tien P. 2015. Human respiratory syncytial virus infection is inhibited by IFN-induced transmembrane proteins. *J Gen Virol* 96:170–182. <http://dx.doi.org/10.1099/vir.0.066555-0>.
87. Everitt AR, Clare S, Pertel T, John SP, Wash RS, Smith SE, Chin CR, Feeley EM, Sims JS, Adams DJ, Wise HM, Kane L, Goulding D, Digard P, Anttila V, Baillie JK, Walsh TS, Hume DA, Palotie A, Xue Y, Colonna V, Tyler-Smith C, Dunning J, Gordon SB, the GenISIS Investigators, the MOSAIC Investigators, Smyth RL, Openshaw PJ, Dougan G, Brass AL, Kellam P. 2012. IFITM3 restricts the morbidity and mortality associated with influenza. *Nature* 484:519–523. <http://dx.doi.org/10.1038/nature10921>.