Influenza A Virus Protein PA-X Contributes to Viral Growth and Suppression of the Host Antiviral and Immune Responses

Tsuyoshi Hayashi, Leslie A. MacDonald, Toru Takimoto

Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, New York, USA

ABSTRACT

Influenza virus infection causes global inhibition of host protein synthesis in infected cells. This host shutoff is thought to allow viruses to escape from the host antiviral response, which restricts virus replication and spread. Although the mechanism of host shutoff is unclear, a novel viral protein expressed by ribosomal frameshifting, PA-X, was found to play a major role in influenza virus-induced host shutoff. However, little is known about the impact of PA-X expression on currently circulating influenza A virus pathogenicity and the host antiviral response. In this study, we rescued a recombinant influenza A virus, A/California/04/09 (H1N1, Cal), containing mutations at the frameshift motif in the polymerase PA gene (Cal PA-XFS). Cal PA-XFS expressed significantly less PA-X than Cal wild type (WT). Cal WT, but not Cal PA-XFS, induced degradation of host β -actin mRNA and suppressed host protein synthesis, supporting the idea that PA-X induces host shutoff via mRNA decay. Moreover, Cal WT inhibited beta interferon (IFN- β) expression and replicated more rapidly than Cal PA-XFS in human respiratory cells. Mice infected with Cal PA-XFS had significantly lower levels of viral growth and greater expression of IFN- β mRNA in their lungs than mice infected with Cal WT. Importantly, more antihemagglutinin and neutralizing antibodies were produced in Cal PA-XFS-infected mice than in Cal WT-infected mice, despite the lower level of virus replication in the lungs. Our data indicate that PA-X of the pandemic H1N1 virus has a strong impact on viral growth and the host innate and acquired immune responses to influenza virus.

IMPORTANCE

Virus-induced host protein shutoff is considered to be a major factor allowing viruses to evade innate and acquired immune recognition. We provide evidence that the 2009 H1N1 influenza A virus protein PA-X plays a role in virus replication and inhibition of host antiviral response by means of its host protein synthesis shutoff activity both *in vitro* and *in vivo*. We also demonstrated that, while the growth of Cal PA-XFS was attenuated in the lungs of infected animals, this mutant induced a stronger humoral response than Cal WT. Our findings clearly highlight the importance of PA-X in counteracting the host innate and acquired immune responses to influenza virus, an important global pathogen. This work demonstrates that inhibition of PA-X expression in influenza virus vaccine strains may provide a novel way of safely attenuating viral growth while inducing a more robust immune response.

Viral infection induces a wide range of host defense mechanisms, such as innate immunity and inflammation triggered by pattern recognition receptors that recognize specific pathogen-associated molecular pattern motifs (1). Type I interferons (IFNs), together with IFN-stimulated genes (ISGs) and inflammatory cytokines, establish a systematic antiviral state that restricts viral replication and spread (1). To counteract the host defense, viruses such as coronaviruses, herpesviruses, and orthomyxoviruses suppress general host protein synthesis, so-called “host shutoff,” through mRNA decay. This shutoff serves to dampen host antiviral factor expression and redirect the host translation apparatus to favor viral protein production (2, 3). Both herpes simplex virus (HSV) and severe acute respiratory syndrome-related coronavirus (SARS-CoV) encode a similar host shutoff protein. HSV virion host shutoff (vhs) and SARS-CoV nsp1 suppress host protein synthesis through endonuclease activity, and this suppression has been shown to play a significant role in viral pathogenesis (2). It has been known that HSV lacking vhs is highly attenuated in a mouse model (4, 5). Several studies also demonstrate that HSV lacking vhs induces more proinflammatory cytokines, such as interleukin 1 β (IL-1 β) and IL-8 in macrophages, is more sensitive to type I IFNs, fails to downregulate major histocompatibility complex I (MHC-I) expression from the infected cell surface, and

allows the activation of dendritic cells (6–8). These findings highlight the importance of vhs in controlling host immune responses to HSV. Likewise, Narayanan et al. showed that SARS-CoV nsp1 suppresses host gene expression in infected cells, including the expression of the type I IFN gene and ISGs like those encoding beta interferon (IFN- β), ISG15, and ISG56 (9). It has also been reported that SARS-CoV nsp1 plays a role in the interruption of the IFN signaling pathway by decreasing the phosphorylation of STAT1 and is necessary for efficient viral growth in the human lung epithelial cell line Calu-3 (10). Mouse hepatitis virus (MHV) nsp1, a homolog of SARS-CoV nsp1, also inhibits cellular gene

Received 5 February 2015 Accepted 3 April 2015

Accepted manuscript posted online 8 April 2015

Citation Hayashi T, MacDonald LA, Takimoto T. 2015. Influenza A virus protein PA-X contributes to viral growth and suppression of the host antiviral and immune responses. *J Virol* 89:6442–6452. doi:10.1128/JVI.00319-15.

Editor: A. García-Sastre

Address correspondence to Toru Takimoto, toru_takimoto@urmc.rochester.edu.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.00319-15

expression, and the viral replication of MHV devoid of nsp1 was severely attenuated in the spleen and liver of infected wild-type (WT) mice. However, replication of the nsp1 depletion mutant was restored in IFN receptor-deficient mice, demonstrating the significant role this protein plays in controlling the immune response (11). Taken together, these previous reports clearly show that virus-induced host shutoff plays a significant role in the modulation of host innate and acquired immune responses to virus infections.

In the case of influenza virus, a novel viral gene product encoded by viral segment 3, named PA-X, was recently identified and found to act as a host shutoff protein (12). PA-X is a fusion protein containing a common N-terminal endonuclease domain of 191 amino acids (aa) with the polymerase PA open reading frame (ORF) fused to a unique C-terminal region of 41 or 61 aa (depending on the strain) produced by ribosomal frameshifting of the +1 reading frame of PA (Fig. 1A) (12–14). Although most influenza virus isolates have 61 C-terminal aa residues, some strains of influenza virus, such as 2009 pandemic H1N1 (pH1N1) viruses, possess a stop codon at position 42 of the C terminus that results in a truncated 41-aa C terminus (12–14). We and Jagger et al. demonstrated that transfection of a PA-X expression plasmid inhibited reporter gene expression to a greater extent than the full PA ORF, suggesting that PA-X could be the major determinant of host shutoff induced by influenza virus (12, 15). Also, PA-X with a mutation at an endonuclease active site (a change of D to A at position 108 [D108A] or K134A) failed to induce host shutoff activity, which implies that PA-X suppresses host protein synthesis through degradation of host mRNA, similar to HSV vhs and SARS-CoV nsp1 (12, 15). Using a recombinant 1918 H1N1 highly pathogenic (HP) influenza virus, Jagger et al. reported that 1918 H1N1 HP WT virus induced the suppression of host protein synthesis to a greater extent than virus expressing lower levels of PA-X through mutation of the frameshift motif (PA-XFS). Despite the difference in host shutoff, both viruses grew to similar titers (12). Global gene expression analysis using lungs of the infected mice revealed that the 1918 PA-XFS virus induced host genes related to immune response or inflammation, apoptosis-related genes, and MHC class I-associated genes in the lungs more strongly than the 1918 WT virus. Interestingly, although the viral growth of both viruses in lungs of the infected mice was almost identical, mice infected with 1918 PA-X FS virus exhibited more severe weight loss than those infected with WT virus, likely caused by the enhanced immune response induced by the failure of PA-X-induced host shutoff. It is, therefore suggested that PA-X may be an accessory protein that modulates the host immune response rather than directly affecting virus proliferation. So far, the effect of PA-X on viral pathogenicity was only tested with 1918 HP virus (12). Unlike HP influenza viruses, seasonal human influenza viruses, including pH1N1 virus, do not induce an excessive host cytokine response, a so-called “cytokine storm” (16–18). Therefore, it is possible that in the case of seasonal influenza A viruses, lack of PA-X expression allows more appropriate innate and acquired immune responses that are able to effectively attenuate virus replication and pathogenicity.

In this study, we evaluated the role of PA-X in viral growth and pathogenicity and host immune response using a currently circulating pH1N1 virus strain (A/California/04/2009 [Cal]). In contrast to the results reported for the 1918 HP virus, we found that Cal PA-XFS virus was attenuated both *in vitro* and *in vivo*. We also

found that Cal PA-XFS induced IFN- β to a greater extent than Cal WT in infected cultured cells and in lungs of infected mice. Importantly, sera collected from surviving mice infected with Cal PA-XFS had higher antihemagglutinin and neutralizing antibody titers than the sera of mice infected with Cal WT. These data show that Cal PA-X contributes to efficient viral growth, possibly by interfering with the host innate response, and prevents the development of the acquired immune response via its host shutoff function.

MATERIALS AND METHODS

Virus and cells. Influenza A virus A/California/04/2009 (H1N1) was provided by R. Webster and R. Webby (St. Jude Children’s Research Hospital, Memphis, TN). MDCK, 293T, and A549 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Corning) supplemented with 8% fetal bovine serum (FBS; Life Technologies) and 1 $\times$ GlutaMAX (Life Technologies). Calu-3 cells, kindly provided by D. Topham (University of Rochester), were maintained in DMEM supplemented with 10% FBS.

Plasmids. pPolCalPA-XFS, containing mutations in the frameshift motif in Cal PA (mutation of nucleotides [nt] 568 to 576 from UCCUU UCGU to AGCUUCAGA), was constructed by site-directed mutagenesis as reported previously (15). For construction of pPolCalHA-E3, the Cal hemagglutinin-E3 (HA-E3) gene was amplified by reverse transcription-PCR using total RNA extracted from A/California/4_NYICE_E3/09 (19) and was subcloned to plasmid pPol-I. For construction of pCAGGSCalPA-X134 Δ Cflag, which does not contain the unique C-terminal regions of PA-X (aa residues 192 to 232), the truncated Cal PA-X 134A Δ C flag gene was amplified by PCR using pCAGGSCalPA-X134Aflag as a template and subcloned to plasmid pCAGGS. Primer sequences are available upon request. Other pPol-I and pCAGGS vectors for virus rescue of Cal viruses, pCAGGSCalPAflag, and pCAGGSCalPA-X134Aflag were constructed previously (15, 20).

Virus rescue. Recombinant Cal viruses were rescued by a 12-plasmid rescue system (21). Briefly, 293T and MDCK cells were cocultured in 6-well plates and transfected, using Lipofectamine 2000 (Invitrogen), with 0.1 μ g each of pPol-I plasmids encoding the 8 segments for synthesis of viral RNAs together with 0.4 μ g each of pCAGGS plasmids carrying the Cal polymerase protein PA, PB1, or PB2 or nucleoprotein (NP) gene. We used pPolCalHA-E3 from A/California/4_NYICE_E3/09 (19), which possesses three amino acid substitutions (K136N, S200P, and D239G) in the HA protein, because stock recombinant Cal virus possessing Cal HA was poorly adapted to the growth conditions in culture cells and in mice as described previously (19). For rescue of Cal PA-XFS, we replaced pPolCalPA with pPolCalPA-XFS. Rescued viruses were plaque purified, and stock virus was propagated in 10-day-old embryonated chicken eggs. Mutations in HA and PA sequences were confirmed by sequence analysis. The viruses were titrated in MDCK cells by plaque assay or in A549 or Calu-3 cells by immunofluorescence analysis of the nucleoprotein.

Production of anti-Cal PA-X MAb. To produce anti-Cal PA-X monoclonal antibody (MAb), we used Cal PA-X 134A protein as the immunogen because the mutation (K134A) in one of the endonuclease active sites allows efficient protein expression, in contrast to that of wild-type PA-X (22). First, the Cal PA-X 134A flag gene was cloned into pVL1392 (Invitrogen). Cal PA-X 134A flag protein was expressed using a baculovirus expression system and purified by tandem affinity purification (TAP), as described previously (23). Inoculation of the purified protein into BALB/c mice and production of hybridoma cell lines were performed as described previously (23).

Western blot analysis. 293T cells were transfected with pCAGGSCalPAflag, pCAGGSCalPA-X134Aflag, or pCAGGSCalPA-X134A Δ Cflag using Lipofectamine 2000. After 24 h, cells were lysed with Triton X lysis buffer (20 mM HEPES [pH 7.5], 1.5 mM MgCl₂, 500 mM NaCl, 0.2 mM EDTA, 1% Triton X-100, and 20% glycerol). The cell lysates were separated by SDS-PAGE (4 to 12% gel; Life Technologies) and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore).

FIG 1 Rescue of Cal PA-XFS possessing mutations in the frameshift motif. (A) Schematic representation of Cal PA and PA-X proteins. Cal PA-X encodes a common N-terminal endonuclease domain (amino acid residues 1 to 191) of PA fused to a unique C-terminal region (41 amino acids) produced by a +1 reading frame of PA mRNA via ribosomal frameshifting. The frameshift motif is shown within a rectangle. (B) Reactivities of MAbs as determined by Western blotting. 293T cells were transfected with the indicated plasmids for 24 h. The cell lysates were subjected to Western blotting using the indicated MAb. pPA, pCAGGSCalPAflag; p134A, pCAGGSCalPA-X134Aflag; p134AΔC, pCAGGSCalPA-X134AΔCflag. (C) A549 cells infected with Cal WT or Cal PA-XFS or 293T cells transfected with pCAGGSCalPA-X134Aflag were labeled with 35 S-labeled methionine and cysteine. The labeled cell lysates were used for immunoprecipitation (IP) by anti-PA-X or anti-PA MAb or for Western blotting (WB) using the indicated MAbs. The data shown are representative of two independent experiments.

The blot was blocked in 2% dry milk and then incubated with primary antibodies as follows: F17-112 (concentrated hybridoma supernatant, 1:1,000), F17-169 (culture hybridoma supernatant, 1:5), F17-278 (culture hybridoma supernatant, 1:5), F17-300 (culture hybridoma supernatant, 1:2), F17-419 (concentrated hybridoma supernatant, 1:1,000), and mouse anti-FLAG MAb (1:1,000; Sigma). The membrane was then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:10,000; Bio-Rad). Similarly, PA, PB1, and PB2 proteins in A549 cells infected with Cal WT or Cal PA-XFS were detected using anti-PA MAb F17-112 (1:1,000), anti-PB1 MAb F7-87 (1:1,000, NR-31693; BEI Resources), or anti-PB2 MAb (1:1,000, NR-4541; BEI Resources). Target proteins were visualized using SuperSignal West Femto maximum sensitivity substrate (Thermo Scientific). Images were captured using the ChemDoc XRS system (Bio-Rad) and analyzed using Quantity One 1-D analysis software (Bio-Rad).

Metabolic labeling. A549 cells were either left uninfected or infected with Cal WT or Cal PA-XFS at a multiplicity of infection (MOI) of 1. After 8 or 12 h postinfection (hpi), cells were labeled with ^{35}S -labeled methionine and cysteine (PerkinElmer) at 50 $\mu\text{Ci}/\text{ml}$ for 30 min in medium lacking methionine and cysteine (Corning). The cells were then lysed with NP-40 lysis buffer (25 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 5% glycerol), and the radiolabeled lysates were analyzed in 4 to 12% SDS-polyacrylamide gels. The dried gels were exposed onto a phosphor screen and visualized using Personal Molecular Imager (Bio-Rad) as described previously (15).

Radioimmunoprecipitation. A549 cells were either left uninfected or infected with Cal WT or Cal PA-XFS at an MOI of 3. At 4 hpi, the cells were radiolabeled for 20 h as described above. Also, 293T cells were transfected with pCAGGSPA-X134Aflag as described above for 8 h, followed by metabolic labeling for 16 h. The labeled cell lysates were used for immunoprecipitation by anti-PA-X MAb F17-419 or anti-PA MAb IF6 (NR19225; BEI Resources). The immunoprecipitated proteins were visualized as described above.

Viral growth kinetics. Confluent Calu-3 cells in a 12-well plate were infected with either Cal WT or Cal PA-XFS at an MOI of 0.025 for 1 h and incubated in DMEM containing 0.15% bovine serum albumin (BSA). At 2, 12, 24, 36, and 48 hpi, 10% of the culture supernatants were collected and titrated on MDCK cells. Viral titers were calculated by the method of Reed and Muench (24) and expressed as 50% tissue culture infective dose ($\text{TCID}_{50}/\text{ml}$).

Quantification of IFN- β protein. Culture supernatants collected from A549 cells either left infected or infected with Cal viruses at 12 or 24 hpi as described above were used for quantification of human IFN- β protein using a Verikine human IFN- β enzyme-linked immunosorbent assay (ELISA) kit (PBL).

qRT-PCR. Total RNAs in culture cells or lung homogenates were extracted using an Illustra RNAspin mini RNA isolation kit (GE Healthcare), following the manufacturer's instructions. The cDNA was synthesized from mRNA by reverse transcription using an oligo(dT)₂₀ primer with the SuperScript III first-strand synthesis system (Life Technologies). The synthesized cDNA was diluted 1:10 and used as the template. PCRs were performed with equal volumes of cDNA, primers specific for target genes, and Power SYBR green PCR master mix (Applied Biosystems). Amplification was done using the 7300 real-time PCR system (Applied Biosystems) with the following program: 1 cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. To create a standard curve with genomic DNA to determine the absolute copy number for each sample, human IFN- β (nt 76 to 753) or mouse IFN- β (nt 21 to 569) was amplified using cDNA synthesized from total RNA of either Calu-3 cells or C57BL/6 mouse lung, respectively. The number of copies of each PCR product was calculated, and 10-fold dilutions of each DNA ranging from 5×10^0 to 5×10^7 copies were used for creating the standard curve. Primers for amplification of human or mouse IFN- β and for quantitative real-time PCR (qRT-PCR) analysis are available upon request.

Northern blot analysis. Northern blot analysis was performed using digoxigenin (DIG)-labeled antisense RNA probes as described previously (25, 26). Briefly, the extracted RNA was separated on a 1% agarose gel prepared by using a formaldehyde-free RNA gel kit (Amresco) and then was transferred onto a positively charged nylon membrane (Hybond-N+; GE Healthcare). Hybridization of the probe and detection of mRNA were performed using a DIG Northern starter kit (Roche) according to the manufacturer's protocol. The DIG-labeled antisense human β -actin RNA probe, corresponding to nt 85 to 880 amplified from A549 cells, was created by *in vitro* transcription with T7 RNA polymerase and DIG-11-UTP. 18S and 28S rRNAs were used as a loading control, assuming that PA-X does not affect the rRNA level as reported for SARS-CoV nsp1 (25, 26).

In vivo experiments. A virus challenge experiment was performed as described previously (19, 27, 28). Six-week-old female specific-pathogen-free C57BL/6 mice were purchased from Taconic Farms, Inc. (Germantown, NY). To determine the 50% mouse lethal doses (MLD_{50}) of the Cal viruses, groups of 5 mice were anesthetized with Avertin (240 mg/kg of body weight) and then infected intranasally with either PBS as a control or Cal WT or Cal PA-XFS at 10-fold serial dilutions containing 10^1 to 10^4 PFU in a 30- μl volume. The MLD_{50} of each virus was calculated by the method of Reed and Muench (24). To monitor morbidity after viral infection, the body weight of each individual mouse was measured daily until 16 days postinfection (dpi). Thirty percent weight loss was considered fatal, and mice reaching this limit were humanely sacrificed.

To determine the virus titers, pathology, and host gene response in lungs of the infected mice, mice were infected intranasally with either PBS or a Cal virus at a dose of 10^2 PFU. At 2, 5, and 8 dpi, groups of 4 mice were humanely sacrificed. Whole lungs were collected and then homogenized in 1 ml of PBS containing gentamicin (50 $\mu\text{g}/\text{ml}$). The clarified supernatants were stored at -80°C until analysis. Infectious virus titers in lung homogenates were determined by plaque assay in MDCK cells and calculated by the method of Reed and Muench (24). For pathological analysis, lungs collected from 1 or 2 mice in each group at 8 dpi were fixed with 10% neutral phosphate-buffered formalin. Fixed specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE). Images of HE-stained tissue sections were taken using an Olympus inverted microscope. All animal experiments were approved by the University of Rochester Committee on Animal Resources.

Plaque neutralization assay. A plaque neutralization assay was performed using sera collected at 16 dpi from surviving mice either left uninfected or infected with Cal WT or Cal PA-XFS virus at a dose of 10^1 , 10^2 , or 10^3 PFU. Each serum sample was serially diluted from 1:250 to 1:8,000 in PBS-gentamicin (100 μl), mixed with 150 PFU/100 μl of Cal WT, and then incubated in MDCK cells to determine plaque numbers.

ELISA. The antibody titer against HA was determined by ELISA as described previously (23). Briefly, purified Cal HA protein (NR-15749; BEI Resources) was used to coat a 96-well plate overnight at 4°C (0.1 $\mu\text{g}/\text{well}$). The plate was blocked with 1% BSA-PBS for 1 h and then was incubated with 3-fold serial dilutions of sera collected from surviving mice for 1 h at 37°C . The plate was then incubated with HRP-conjugated goat anti-mouse IgG (1:5,000 dilution; Bio-Rad) for 1 h at 37°C . Reactions were developed by treatment with the ABTS [2,2'-azinobis(3-ethylbenzothiazolinesulfonic acid)] chromophore (Calbiochem) for 15 min, quenched with 1% SDS, and measured at 405 nm using a SpectraMax plus (Molecular Devices).

RESULTS

Reduced expression of PA-X in Cal PA-XFS-infected cells. To determine if PA-X plays a role in influenza virus growth or pathogenicity or the host immune response to infection by currently circulating pH1N1 influenza virus, we rescued a recombinant A/California/04/09 (Cal) virus possessing mutations in the frameshift motif (UCCUUUCGU to AGCUUCAGA, nt 568 to 576) in the PA gene (Cal PA-XFS) (Fig. 1A). Jagger et al. demonstrated that these mutations reduce frameshift efficiency from 1.3% to

FIG 2 Effects of PA-X expression on host protein synthesis and actin mRNA levels in human cells infected with Cal viruses. (A) A549 cells were either left uninfected or infected with Cal WT or Cal PA-XFS at an MOI of 1. At the indicated time points, cells were labeled with ^{35}S -labeled methionine and cysteine for 30 min, and total lysates were resolved by SDS-PAGE. (B) Densitometric traces of labeled proteins in cells that were either left uninfected or infected with the viruses shown in panel A. (C and D) A549 or Calu-3 cells were either left uninfected or infected with Cal WT or Cal PA-XFS at an MOI of 3. At 4 or 12 hpi, total RNA was extracted from cell lysates and subjected to Northern blot analysis using a DIG-labeled antisense human β -actin probe. 18S and 28S rRNAs were stained with ethidium bromide and are shown as a loading control. The data shown are representative of two (A) or three (C and D) independent experiments.

0.2% without amino acid substitution in PA (12). To test the expression levels of PA-X protein in the infected cells, we first produced hybridoma cell lines which secrete MAbs specific for PA-X. We successfully established 5 hybridoma cell lines. To determine whether these MAbs react with the common N-terminal region between PA and PA-X or the unique C-terminal region of PA-X, we tested the reactivity of MAbs against PA, PA-X mutated at the endonuclease active site (PA-X134A), or PA-X134A Δ C, which does not contain the unique C-terminal domain. As shown by the results in Fig. 1B, two antibodies (F17-278 and F17-419) reacted with PA-X134A but not PA or PAX134A Δ C, indicating that these antibodies bind the unique C-terminal region of PA-X. Three antibodies (F17-112, F17-169, and F17-300) reacted with all three proteins, indicating that these react with N-terminal common regions (Fig. 1B). Radioimmunoprecipitation was then carried out on lysates from cells infected with Cal WT or Cal PA-XFS or transfected with PA-X134A as a control. An antibody that recognizes the unique C-terminal regions of PA-X (F17-419) effectively immunoprecipitated PA-X from Cal WT-infected cells (Fig. 1C). However, we found that the expression of PA-X in cells infected with Cal PA-XFS was strongly reduced compared with that detected in Cal WT-infected cells (Fig. 1C). To determine whether the level of PA-X affects the expression of other viral proteins, we used an anti-PA MAb (IF6) that recognizes the N-terminal common domain and immunoprecipitates the protein ef-

ficiently. We found that polymerase proteins (PB1, PB2, and PA) were precipitated at almost the same levels from both WT- and PA-XFS virus-infected cells. Western blot analysis using specific MAbs further confirmed that the expression levels of each polymerase protein were identical in WT and PA-XFS virus-infected cells, suggesting that reduced expression of PA-X does not affect the expression levels of other viral proteins in the infected cells (Fig. 1C). These data are consistent with the previous report (12) and confirm that mutations at the frameshift motif in PA result in reduced expression of PA-X in infected cells.

PA-X contributes to the shutdown of host protein synthesis and degradation of actin mRNA. To assess the effect of PA-X expression on cellular protein synthesis in infected cells, we compared the levels of host protein synthesis in the human lung adenocarcinoma epithelial cell line A549 following infection with either Cal WT or Cal PA-XFS. At 4 hpi, neither virus protein synthesis nor suppression of host protein synthesis was detected in Cal WT- and Cal PA-XFS-infected cells (data not shown). At 12 hpi, clear selective shutdown of host protein synthesis was detected in Cal WT-infected cells compared to the level of host protein synthesis in mock-infected cells. In contrast, host shutoff activity was attenuated in Cal PA-XFS-infected cells (Fig. 2A and B). Using a plasmid coexpression system, we and Jagger et al. showed that PA-X possessing mutations at endonuclease active sites (K134A or D108A) failed to suppress reporter protein production

(12, 15), implying that PA-X induces cellular mRNA degradation that results in host protein shutoff. To determine cellular mRNA degradation in infected cells, we isolated total RNAs from mock-, Cal WT-, or Cal PA-XFS-infected A549 cells and compared the actin mRNA levels by Northern blotting. At 12 hpi, the actin mRNA level in Cal WT-infected cells was reduced by $50.6\% \pm 7.9\%$ (mean $\pm$ standard deviation) compared to the level in mock-infected cells, while there was only an $11.2\% \pm 7.8\%$ reduction by Cal PA-XFS (Fig. 2C). Likewise, Cal WT infection reduced the actin mRNA level by $52.2\% \pm 7.3\%$ in human airway epithelial cell line Calu-3 cells at 12 hpi, whereas no reduction was seen in Cal PA-XFS-infected cells (Fig. 2D). These data strongly suggest that PA-X contributes to the shutdown of host protein synthesis through cellular mRNA degradation in the context of viral infection.

PA-X expression is required for efficient virus replication and suppression of the host antiviral response *in vitro*. Next, we compared the viral growth kinetics of Cal WT and Cal PA-XFS *in vitro*. Since A549 cells are sensitive to trypsin, which is needed for the multiple-step growth of Cal viruses, we used Calu-3 cells, which support the multistep growth of low-pathogenicity influenza virus without the addition of exogenous trypsin (29). As shown by the results in Fig. 3A, both viruses replicated well in this cell line, though Cal WT grew to higher titers than Cal PA-XFS at 12 and 24 hpi. The more efficient viral growth of Cal WT compared to that of Cal PA-XFS suggests that PA-X may play a role in suppressing host innate immune responses, such as type I IFNs. The induction of type I IFN establishes an antiviral state which impedes viral replication and spread through the host cells. To assess whether this is true, we compared the IFN- β mRNA levels in infected Calu-3 cells by qRT-PCR. We found that Cal PA-XFS induced IFN- β mRNA expression to a level 7.2-fold higher than that induced by Cal WT at 24 hpi (Fig. 3B). We also used ELISA to quantitate the IFN- β released from infected A549 cells. Consistent with the qRT-PCR results, IFN- β proteins were released from Cal PA-XFS-infected cells to a greater extent than from Cal WT-infected cells at 24 hpi (Fig. 3C). These results clearly indicate that PA-X suppresses IFN- β release and enhances viral growth in cultured cells, suggesting that shutoff activity by PA-X plays a role in efficient virus spread.

PA-X contributes to viral growth and inhibition of IFN- β expression in lungs of the infected mice. The above-described data showing the contribution of PA-X to the inhibition of the host antiviral response and to efficient viral growth *in vitro* led us to assess the impact of PA-X expression on virus pathogenicity in mice. First, we determined the 50% mouse lethal dose (MLD_{50}) of both viruses. The MLD_{50} s of Cal WT and Cal PA-XFS were $10^{3.17}$ and $10^{2.84}$ PFU, respectively, showing that reduced expression of PA-X did not attenuate the virus in mice (Fig. 4A). The body weights of mice infected with 10^2 PFU of Cal WT or Cal PA-XFS were reduced to 77.9% or 79.5%, respectively, by 9 dpi. However, mice infected with Cal PA-XFS virus recovered more rapidly than Cal WT-infected mice (Fig. 4B), suggesting that viral clearance in lungs infected with Cal PA-XFS occurs faster than in those infected with Cal WT. This is possibly because Cal PA-XFS fails to suppress the host antiviral response in the lungs due to reduced expression levels of PA-X. To test this hypothesis, we compared the viral growth and IFN- β mRNA levels in lungs of mice infected with 10^2 PFU. As shown by the results in Fig. 5A, at 2 dpi, the viral titers in lungs infected with Cal WT and Cal PA-XFS were almost

FIG 3 Effects of PA-X expression on viral growth and IFN- β expression *in vitro*. (A) Viral growth kinetics in Calu-3 cells. The cells were infected with either Cal WT or Cal PA-XFS at an MOI of 0.025. At the indicated time points, 10% of the supernatant was collected and titrated on MDCK cells. The dashed line indicates the detection limit of the assay. The data represent averages with standard deviations from two independent experiments with assays performed in triplicate ($n = 6$). (B) Calu-3 cells were either left uninfected or infected with Cal WT or Cal PA-XFS at an MOI of 3. At the indicated time points, total RNA was extracted and subjected to qRT-PCR analysis. The copy number in each sample was calculated using a standard curve of 10-fold serial dilutions of human IFN- β gene product. Fold change is the ratio of the copy number of each sample over that of mock-infected cells at 6 h. The mRNA levels represent averages with standard deviations. (C) A549 cells were either left uninfected or infected with Cal WT or Cal PA-XFS at an MOI of 10. IFN- β protein levels in culture supernatants at 12 or 24 hpi were determined by using a human IFN- β ELISA kit. The data represent averages with standard deviations. Asterisks indicate statistically significant differences by the Student t test (*, $P < 0.05$).

identical, and the mean virus titer was $10^{5.7}$ or $10^{5.5}$ PFU/ml, respectively. At 5 dpi, the titers in the lungs of WT Cal-infected mice had increased 3.6-fold and were significantly higher than the titers in the lungs of Cal PA-XFS-infected mice (Cal WT, $10^{6.2}$ PFU/ml, and Cal PA-XFS, $10^{5.7}$ PFU/ml) (Fig. 5A). Although the titers of both viruses were reduced at 8 dpi, the titers of Cal PA-XFS were

FIG 4 Survival and body weight change of mice infected with Cal viruses. (A and B) C57BL/6 mice were either mock infected or infected with Cal WT or Cal PA-XFS at a dose of 10¹, 10², 10³, or 10⁴ PFU intranasally. The survival (A) and body weight (B) of each mouse were monitored daily until 16 dpi (*n* = 5). Asterisks indicate statistically significant differences by the Student *t* test (*, *P* < 0.05).

significantly lower than those of Cal WT, suggesting that viral clearance occurs more efficiently in Cal PA-XFS-infected mice. Despite the reduced level of viral replication, Cal PA-XFS induced IFN- β mRNA to a greater extent than Cal WT in the lungs of infected mice at 5 dpi (Fig. 5B). These findings suggest that PA-X plays a role in efficient virus replication and/or spread, possibly through inhibition of host antiviral gene expression in the lungs of infected mice.

To gain further insight into the pathogenicity of these viruses, we performed histological analysis of the infected lungs at 8 dpi. The lungs of Cal WT-infected mice showed moderate inflammation, with immune infiltrates in alveolar air spaces and the areas surrounding bronchioles, compared to uninfected lungs (Fig. 5C). The lungs of Cal PA-XFS-infected mice showed greater degrees of inflammatory cell infiltration than those of Cal WT-infected lungs, possibly because this mutant induced stronger host cytokine responses than Cal WT due to the lack of PA-X-mediated shutoff activity. These results show that PA-X plays a role in viral growth and inhibition of the host antiviral response and inflammation in infected lungs, while having minimal effect on lethality in mice.

Cal PA-XFS induces stronger humoral immune responses than Cal WT. Next, to test whether PA-X plays a role in humoral immune responses to influenza virus infection, we analyzed the serum antibodies of mice infected with the viruses. First, we performed a plaque neutralization assay using sera collected from surviving mice at 16 dpi. We tested the ability of the sera to inhibit the plaque formation of Cal WT by incubating serial dilutions of serum with 150 PFU of Cal WT prior to infection to MDCK cells. The plaque numbers were compared to those of virus that was mixed with PBS. Sera from Cal PA-XFS-infected mice inhibited plaque formation to a greater extent than those from Cal WT-

infected mice (Fig. 6A to C). From these data, we calculated the serum dilutions needed for a 50% reduction in plaque numbers. As shown by the results in Fig. 6D, the serum dilutions from Cal PA-XFS-infected mice were 2.67-, 1.55-, or 1.33-fold higher than those from Cal WT-infected mice at a dose of 10¹, 10², or 10³ PFU, respectively. These results indicate that Cal PA-XFS infection produced more neutralizing antibodies than Cal WT infection, even though the virus replication of this mutant was reduced in the lungs (Fig. 5A). We also compared the antibody titers against HA protein by standard ELISA. Consistent with the results shown in Fig. 6, the anti-HA antibody titers in sera from Cal PA-XFS-infected mice were 28 to 69% higher than those in sera from Cal WT-infected mice when compared for each inoculation dose (Fig. 7). Similar patterns were seen when we used purified ribonucleoprotein as an antigen (data not shown). These data demonstrate that shutoff activity by PA-X contributes to inhibition of the host humoral immune response in mice.

DISCUSSION

Virus-induced host shutoff is an important step to dampen the host antiviral defense and support preferential virus protein production in infected cells. Influenza A virus is known to shut off host protein synthesis (30). In influenza virus-infected cells, host protein synthesis is specifically blocked, while virus proteins are efficiently produced. How influenza viruses shut off host protein synthesis while still allowing viral protein production remains unsolved. It was previously believed that cap-snatching activity and cellular RNA polymerase II degradation induced by viral polymerase caused general shutoff of host protein synthesis. However, Jagger et al. identified a novel influenza virus protein, PA-X, expressed by PA mRNA by ribosomal frameshifting, that plays a major role in inducing host shutoff (12). Using 1918 HP virus,

FIG 5 Viral growth, IFN- β mRNA expression, and pathology in lungs of the infected mice. C57BL/6 mice were mock infected or infected with Cal WT or Cal PA-XFS at a dose of 10^2 PFU intranasally. At 2, 5, or 8 dpi, four mice from each group were humanely sacrificed and the extracted whole lungs were homogenized in 1 ml of PBS. (A) Virus present in the homogenates was titrated on MDCK cells by plaque assay. (B) Total RNA was extracted from 100 μ l of the homogenates, and IFN- β mRNA levels were determined by qRT-PCR analysis. Fold change is the ratio of the copy number of each sample over that of the control sample at 2 dpi. The data represent averages with standard deviations. Asterisks indicate statistically significant differences by the Student *t* test (*, $P < 0.05$). (C) Representative hematoxylin-and-eosin-stained images of uninfected or infected lungs at 8 dpi. Original magnification, $\times 10$.

they also reported that this protein modulates the host immune response in infected mice (12). In this study, we further assessed the effects of PA-X in virus replication and pathogenicity and host innate and acquired immune responses in the context of currently circulating pH1N1 infection. Similar to the role reported for PA-X in the 1918 HP virus, we found that the pH1N1 PA-X plays a major role in the suppression of host protein synthesis and host mRNA degradation in infected cells (Fig. 2). However, unlike the case with 1918 HP virus, reduced PA-X expression attenuated viral growth in both cultured cells and mouse lungs (Fig. 3A and 5A). Cal PA-XFS virus was cleared more rapidly than Cal WT virus from the lungs of infected mice, while showing no major difference in the 50% lethal dose (LD_{50}) (Fig. 4). These results clearly indicate that the effects of PA-X on viral growth and pathogenicity vary between the highly pathogenic strains and seasonal influenza viruses.

Our data for viral growth kinetics in cultured human lung cells indicate that Cal WT replicates and spreads faster than Cal PA-XFS in Calu3 cells, concomitant with the stronger inhibition of IFN- β expression (Fig. 3). We also detected reduced IFN- β ex-

pression and enhanced virus replication of WT virus in infected mice (Fig. 5A and B). The induction of type I IFNs (IFN- α and IFN- β) is an important step to establish a host antiviral state that restricts virus replication and spread. In fact, previous studies show that pretreatment with IFN- α significantly reduces virus titers in culture cells and the lungs of infected mice (31, 32). Another study also demonstrates that IFN- β knockout mice infected with influenza virus show reduced survival and increased virus replication in the lungs (33). These previous data highlight the importance of interfering with the type I IFN response for efficient virus replication. To date, it has been known that influenza virus nonstructural NS1, PB1-F2, and PB2 proteins play a role in inhibiting the type I IFN response (34–37). In addition to these proteins, our data suggest that general host shutoff by PA-X also contributes to the reduced expression of host antiviral responses *in vitro* and *in vivo*.

The mechanism of how PA-X induces general host shutoff is unknown. Using a plasmid coexpression system, we and Jagger et al., by characterizing PA-X containing mutations at the endonuclease active sites, have reported that endonuclease activity of

FIG 6 Titration of neutralization antibody in sera of mice infected with Cal viruses. (A to C) Neutralization assay was performed using serum samples collected at 16 dpi from surviving mice infected with Cal viruses at a dose of 10^1 , 10^2 , or 10^3 PFU. Each serum sample was serially diluted and mixed with 150 PFU of Cal WT, and then plaque numbers were counted. Data are presented as the percentages of the plaque number of control virus incubated with PBS. (D) Calculated serum dilutions showing 50% plaque inhibition. The data shown are averages with standard deviations of the results from 4 or 5 (control, 10^1 , or 10^2 PFU virus) or 2 or 3 (10^3 PFU virus) individual mice. Asterisks indicate statistically significant differences by the Student *t* test (*, *P* < 0.05).

PA-X is required for suppression of reporter protein production (12, 15). In this study, we directly showed that infection with Cal WT but not with Cal PA-XFS reduced the actin mRNA level in the infected cells (Fig. 2C and D), which is consistent with the role of the other host shutoff proteins HSV vhs1 and SARS-CoV nsp1 (2). Given the fact that PA-X has functional similarity to HSV vhs and SARS-CoV nsp1, which are known to induce host shutoff via host mRNA decay, it is possible that PA-X may interact with host transcription and/or translation apparatus and interrupt host protein expression. Interestingly, Khapersky et al. recently reported that PA-X contributes to depletion of cytosolic poly(A) mRNA and nuclear accumulation of poly(A)-binding protein (38). Further analysis of interactions between host transcription and translation apparatuses and PA-X are needed to better understand how PA-X induces host shutoff via mRNA degradation.

The mice infected with Cal PA-XFS recovered their body weight more rapidly than those infected with Cal WT (Fig. 4B), suggesting that viral clearance occurs faster in Cal PA-XFS-infected mice than in Cal WT-infected mice. In fact, we found that Cal PA-XFS induced more severe inflammation in infected mouse lungs, while showing lethality almost identical to that in mice with lower viral loads in the lungs (Fig. 4 and 5A and C). Host shutoff by PA-X will also suppress the induction of proinflammatory cytokines or chemokines, which lead to infiltration of inflammatory cells like macrophages and neutrophils. Jagger et al. demonstrated that a 1918 HP PA-XFS mutant caused more severe weight loss of mice, as well as high induction of host genes related to the immune response and inflammation in the infected lungs, compared to these parameters in WT infection (12). These observations suggest that the excessive host immune response in the lungs infected with

FIG 7 Titration of anti-HA antibodies in sera of mice infected with Cal viruses. Antibody titers were determined by ELISA on 96-well plate using Cal HA protein (0.1 μ g/well) as an antigen. Serum samples were collected from surviving mice at 16 dpi. Data shown are the averages with standard deviations of the results from 4 or 5 (control, 10¹, or 10² PFU virus) or 2 or 3 (10³ PFU virus) individual mice. Asterisks indicate statistically significant differences by the Student *t* test (*, *P* < 0.05).

PA-XFS virus enhances viral pathogenicity due to the accelerated and increased cytokine response, even though these host responses restrict virus replication and/or spread. It also implies that the magnitude of the host cytokine response, as well as the virus load, plays a significant role in virus pathogenicity.

Interestingly, we found that Cal PA-XFS induced a greater humoral response than Cal WT in mice, even though virus replication was attenuated in the lungs (Fig. 6 and 7). This result indicates that, in addition to its effects on enhanced viral growth and inhibition of the host innate response, PA-X also contributes to the suppression of the acquired immune response. It is likely that, in mice infected with Cal PA-XFS, elevated type I IFN expression induces a stronger B cell response. Indeed, it has been reported that treatment with IFN- α enhances the antibody response in mice through the stimulation of B and T cells (39). Global transcriptional profiling of infected mouse lung tissue showed differential expression of immune regulatory genes between mice infected with WT and PA-XFS influenza viruses, and several MHC

class I-associated genes were upregulated in mice infected with PA-XFS viruses (12). These data raise the possibility that the lack of PA-X would allow an appropriate host response and induce a strong protective immunity.

In summary, using currently circulating influenza A virus, we found that PA-X affects viral growth and host antiviral responses both *in vitro* and *in vivo*. Strikingly, Cal PA-XFS induced stronger humoral responses in mice than Cal WT, even with lower Cal PA-XFS virus replication in the lungs. Our findings suggest that PA-X contributes to virus replication, possibly through the inhibition of host innate and acquired immune responses, and highlight the importance of PA-X's function in influenza virus pathogenicity and host immune responses.

ACKNOWLEDGMENTS

This work was supported by the New York Influenza Center of Excellence, a member of the NIAID CEIRS network, under NIH contract HHSN266200700008C.

We thank Brian M. Ward, David J. Topham, Luis Martinez-Sobrido, Richard Webby, Robert Webster, and Stephen Dewhurst for reagents. We also thank Chutikarn Chaimayo, Colleen O'Dell, and Kris Lambert for technical assistance, Linda Johnstone (Division of Laboratory Animal Medicine, University of Rochester Medical Center) for histology preparation, and Erin Hodges for editing the manuscript.

REFERENCES

- Kumar H, Kawai T, Akira S. 2011. Pathogen recognition by the innate immune system. *Int Rev Immunol* 30:16–34. <http://dx.doi.org/10.3109/08830185.2010.529976>.
- Narayanan K, Makino S. 2013. Interplay between viruses and host mRNA degradation. *Biochim Biophys Acta* 1829:732–741. <http://dx.doi.org/10.1016/j.bbagg.2012.12.003>.
- Walsh D, Mohr I. 2011. Viral subversion of the host protein synthesis machinery. *Nat Rev Microbiol* 9:860–875. <http://dx.doi.org/10.1038/nrmicro2655>.
- Smith TJ, Morrison LA, Leib DA. 2002. Pathogenesis of herpes simplex virus type 2 virion host shutoff (vhs) mutants. *J Virol* 76:2054–2061. <http://dx.doi.org/10.1128/JVI.76.5.2054-2061.2002>.
- Strelow LI, Leib DA. 1995. Role of the virion host shutoff (vhs) of herpes simplex virus type 1 in latency and pathogenesis. *J Virol* 69:6779–6786.
- Samady L, Costigliola E, MacCormac L, McGrath Y, Cleverley S, Lilley CE, Smith J, Latchman DS, Chain B, Coffin RS. 2003. Deletion of the virion host shutoff protein (vhs) from herpes simplex virus (HSV) relieves the viral block to dendritic cell activation: potential of vhs-HSV vectors for dendritic cell-mediated immunotherapy. *J Virol* 77:3768–3776. <http://dx.doi.org/10.1128/JVI.77.6.3768-3776.2003>.
- Tigges MA, Leng S, Johnson DC, Burke RL. 1996. Human herpes simplex virus (HSV)-specific CD8⁺ CTL clones recognize HSV-2-infected fibroblasts after treatment with IFN- γ or when virion host shutoff functions are disabled. *J Immunol* 156:3901–3910.
- Suzutani T, Nagamine M, Shibaki T, Ogasawara M, Yoshida I, Daikoku T, Nishiyama Y, Azuma M. 2000. The role of the UL41 gene of herpes simplex virus type 1 in evasion of non-specific host defence mechanisms during primary infection. *J Gen Virol* 81:1763–1771.
- Narayanan K, Huang C, Lokugamage K, Kamitani W, Ikegami T, Tseng CT, Makino S. 2008. Severe acute respiratory syndrome coronavirus nsp1 suppresses host gene expression, including that of type I interferon, in infected cells. *J Virol* 82:4471–4479. <http://dx.doi.org/10.1128/JVI.02472-07>.
- Wathelet MG, Orr M, Frieman MB, Baric RS. 2007. Severe acute respiratory syndrome coronavirus evades antiviral signaling: role of nsp1 and rational design of an attenuated strain. *J Virol* 81:11620–11633. <http://dx.doi.org/10.1128/JVI.00702-07>.
- Zust R, Cervantes-Barragan L, Kuri T, Blakqori G, Weber F, Ludewig B, Thiel V. 2007. Coronavirus non-structural protein 1 is a major pathogenicity factor: implications for the rational design of coronavirus vaccines. *PLoS Pathog* 3:e109. <http://dx.doi.org/10.1371/journal.ppat.0030109>.
- Jagger BW, Wise HM, Kash JC, Walters KA, Wills NM, Xiao YL, Dunfee RL, Schwartzman LM, Ozinsky A, Bell GL, Dalton RM, Lo A,

- Efstathiou S, Atkins JF, Firth AE, Taubenberger JK, Digard P. 2012. An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. *Science* 337:199–204. <http://dx.doi.org/10.1126/science.1222213>.
13. Shi M, Jagger BW, Wise HM, Digard P, Holmes EC, Taubenberger JK. 2012. Evolutionary conservation of the PA-X open reading frame in segment 3 of influenza A virus. *J Virol* 86:12411–12413. <http://dx.doi.org/10.1128/JVI.01677-12>.
14. Vasin AV, Temkina OA, Egorov VV, Klotchenko SA, Plotnikova MA, Kiselev OI. 2014. Molecular mechanisms enhancing the proteome of influenza A viruses: an overview of recently discovered proteins. *Virus Res* 185:53–63. <http://dx.doi.org/10.1016/j.virusres.2014.03.015>.
15. Desmet EA, Bussey KA, Stone R, Takimoto T. 2013. Identification of the N-terminal domain of the influenza virus PA responsible for the suppression of host protein synthesis. *J Virol* 87:3108–3118. <http://dx.doi.org/10.1128/JVI.02826-12>.
16. Peiris JS, Hui KP, Yen HL. 2010. Host response to influenza virus: protection versus immunopathology. *Curr Opin Immunol* 22:475–481. <http://dx.doi.org/10.1016/j.coi.2010.06.003>.
17. Belser JA, Wadford DA, Pappas C, Gustin KM, Maines TR, Pearce MB, Zeng H, Swayne DE, Pantin-Jackwood M, Katz JM, Tumpey TM. 2010. Pathogenesis of pandemic influenza A (H1N1) and triple-reassortant swine influenza A (H1) viruses in mice. *J Virol* 84:4194–4203. <http://dx.doi.org/10.1128/JVI.02742-09>.
18. Perrone LA, Plowden JK, Garcia-Sastre A, Katz JM, Tumpey TM. 2008. H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice. *PLoS Pathog* 4:e1000115. <http://dx.doi.org/10.1371/journal.ppat.1000115>.
19. Guo H, Santiago F, Lambert K, Takimoto T, Topham DJ. 2011. T cell-mediated protection against lethal 2009 pandemic H1N1 influenza virus infection in a mouse model. *J Virol* 85:448–455. <http://dx.doi.org/10.1128/JVI.01812-10>.
20. Bialas KM, Desmet EA, Takimoto T. 2012. Specific residues in the 2009 H1N1 swine-origin influenza matrix protein influence virion morphology and efficiency of viral spread in vitro. *PLoS One* 7:e50595. <http://dx.doi.org/10.1371/journal.pone.0050595>.
21. Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez DR, Donis R, Hoffmann E, Hobom G, Kawaoka Y. 1999. Generation of influenza A viruses entirely from cloned cDNAs. *Proc Natl Acad Sci U S A* 96:9345–9350. <http://dx.doi.org/10.1073/pnas.96.16.9345>.
22. Hara K, Schmidt FI, Crow M, Brownlee GG. 2006. Amino acid residues in the N-terminal region of the PA subunit of influenza A virus RNA polymerase play a critical role in protein stability, endonuclease activity, cap binding, and virion RNA promoter binding. *J Virol* 80:7789–7798. <http://dx.doi.org/10.1128/JVI.00600-06>.
23. MacDonald LA, Aggarwal S, Bussey KA, Desmet EA, Kim B, Takimoto T. 2012. Molecular interactions and trafficking of influenza A virus polymerase proteins analyzed by specific monoclonal antibodies. *Virology* 426:51–59. <http://dx.doi.org/10.1016/j.virol.2012.01.015>.
24. Reed LJ, Muench H. 1938. A simple method of estimating fifty per cent endpoints. *Am J Epidemiol* 27:493–497.
25. Kamitani W, Narayanan K, Huang C, Lokugamage K, Ikegami T, Ito N, Kubo H, Makino S. 2006. Severe acute respiratory syndrome coronavirus nsp1 protein suppresses host gene expression by promoting host mRNA degradation. *Proc Natl Acad Sci U S A* 103:12885–12890. <http://dx.doi.org/10.1073/pnas.0603144103>.
26. Tanaka T, Kamitani W, DeDiego ML, Enjuanes L, Matsuura Y. 2012. Severe acute respiratory syndrome coronavirus nsp1 facilitates efficient propagation in cells through a specific translational shutoff of host mRNA. *J Virol* 86:11128–11137. <http://dx.doi.org/10.1128/JVI.01700-12>.
27. Bussey KA, Bousse TL, Desmet EA, Kim B, Takimoto T. 2010. PB2 residue 271 plays a key role in enhanced polymerase activity of influenza A viruses in mammalian host cells. *J Virol* 84:4395–4406. <http://dx.doi.org/10.1128/JVI.02642-09>.
28. Bussey KA, Desmet EA, Mattiacio JL, Hamilton A, Bradel-Tretheway B, Bussey HE, Kim B, Dewhurst S, Takimoto T. 2011. PA residues in the 2009 H1N1 pandemic influenza virus enhance avian influenza virus polymerase activity in mammalian cells. *J Virol* 85:7020–7028. <http://dx.doi.org/10.1128/JVI.00522-11>.
29. Zeng H, Goldsmith C, Thawatsupha P, Chittaganpitch M, Waicharoen S, Zaki S, Tumpey TM, Katz JM. 2007. Highly pathogenic avian influenza H5N1 viruses elicit an attenuated type I interferon response in polarized human bronchial epithelial cells. *J Virol* 81:12439–12449. <http://dx.doi.org/10.1128/JVI.01134-07>.
30. Katze MG, DeCorato D, Krug RM. 1986. Cellular mRNA translation is blocked at both initiation and elongation after infection by influenza virus or adenovirus. *J Virol* 60:1027–1039.
31. Haasbach E, Droebner K, Vogel AB, Planz O. 2011. Low-dose interferon type I treatment is effective against H5N1 and swine-origin H1N1 influenza A viruses in vitro and in vivo. *J Interferon Cytokine Res* 31:515–525. <http://dx.doi.org/10.1089/jir.2010.0071>.
32. Ngunjiri JM, Mohni KN, Sekellick MJ, Schultz-Cherry S, Webster RG, Marcus PI. 2012. Lethal H5N1 influenza viruses are not resistant to interferon action in human, simian, porcine or chicken cells. *Nat Med* 18:1456–1457. <http://dx.doi.org/10.1038/nm.2879>.
33. Koerner I, Kochs G, Kalinke U, Weiss S, Staeheli P. 2007. Protective role of beta interferon in host defense against influenza A virus. *J Virol* 81:2025–2030. <http://dx.doi.org/10.1128/JVI.01718-06>.
34. Garcia-Sastre A. 2011. Induction and evasion of type I interferon responses by influenza viruses. *Virus Res* 162:12–18. <http://dx.doi.org/10.1016/j.virusres.2011.10.017>.
35. Gack MU, Albrecht RA, Urano T, Inn KS, Huang IC, Carnero E, Farzan M, Inoue S, Jung JU, Garcia-Sastre A. 2009. Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. *Cell Host Microbe* 5:439–449. <http://dx.doi.org/10.1016/j.chom.2009.04.006>.
36. Graef KM, Vreede FT, Lau YF, McCall AW, Carr SM, Subbarao K, Fodor E. 2010. The PB2 subunit of the influenza virus RNA polymerase affects virulence by interacting with the mitochondrial antiviral signaling protein and inhibiting expression of beta interferon. *J Virol* 84:8433–8445. <http://dx.doi.org/10.1128/JVI.00879-10>.
37. Varga ZT, Ramos I, Hai R, Schmolke M, Garcia-Sastre A, Fernandez-Sesma A, Palese P. 2011. The influenza virus protein PB1-F2 inhibits the induction of type I interferon at the level of the MAVS adaptor protein. *PLoS Pathog* 7:e1002067. <http://dx.doi.org/10.1371/journal.ppat.1002067>.
38. Khaperskyy DA, Emara MM, Johnston BP, Anderson P, Hatchette TF, McCormick C. 2014. Influenza A virus host shutoff disables antiviral stress-induced translation arrest. *PLoS Pathog* 10:e1004217. <http://dx.doi.org/10.1371/journal.ppat.1004217>.
39. Le Bon A, Thompson C, Kamphuis E, Durand V, Rossmann C, Kalinke U, Tough DF. 2006. Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. *J Immunol* 176:2074–2078. <http://dx.doi.org/10.4049/jimmunol.176.4.2074>.