PA-X Decreases the Pathogenicity of Highly Pathogenic H5N1 Influenza A Virus in Avian Species by Inhibiting Virus Replication and Host Response

Jiao Hu, Yiqun Mo, Xiaoquan Wang, Min Gu, Zenglei Hu, Lei Zhong, Qiwen Wu, Xiaoli Hao, Shunlin Hu, Wenbo Liu, Huimou Liu, Xiaowen Liu, Xiufan Liu

Animal Infectious Disease Laboratory, School of Veterinary Medicine, and Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonosis, Yangzhou University, Yangzhou, China

ABSTRACT

PA-X is a newly discovered protein that decreases the virulence of the 1918 H1N1 virus in a mouse model. However, the role of PA-X in the pathogenesis of highly pathogenic avian influenza viruses (HPAIV) of the H5N1 subtype in avian species is totally unknown. By generating two PA-X-deficient viruses and evaluating their virulence in different animal models, we show here that PA-X diminishes the virulence of the HPAIV H5N1 strain A/Chicken/Jiangsu/k0402/2010 (CK10) in mice, chickens, and ducks. Expression of PA-X dampens polymerase activity and virus replication both *in vitro* and *in vivo*. Using microarray analysis, we found that PA-X blunts the global host response in chicken lungs, markedly downregulating genes associated with the inflammatory and cell death responses. Correspondingly, a decreased cytokine response was recapitulated in multiple organs of chickens and ducks infected with the wild-type virus relative to those infected with the PA-X-deficient virus. In addition, the PA-X protein exhibits antiapoptotic activity in chicken and duck embryo fibroblasts. Thus, our results demonstrated that PA-X acts as a negative virulence regulator and decreases virulence by inhibiting viral replication and the host innate immune response. Therefore, we here define the role of PA-X in the pathogenicity of H5N1 HPAIV, furthering our understanding of the intricate pathogenesis of influenza A virus.

IMPORTANCE

Influenza A virus (IAV) continues to pose a huge threat to global public health. Eight gene segments of the IAV genome encode as many as 17 proteins, including 8 main viral proteins and 9 accessory proteins. The presence of these accessory proteins may further complicate the pathogenesis of IAV. PA-X is a newly identified protein in segment 3 that acts to decrease the virulence of the 1918 H1N1 virus in mice by modulating host gene expression. Our study extends these functions of PA-X to H5N1 HPAIV. We demonstrated that loss of PA-X expression increases the virulence and replication of an H5N1 virus in mice and avian species and alters the host innate immune and cell death responses. Our report is the first to delineate the role of the novel PA-X protein in the pathogenesis of H5N1 viruses in avian species and promotes our understanding of H5N1 HPAIV.

Influenza A virus (IAV) can infect diverse host species, from wild and domestic birds to mammalian species, and the pathogenesis of IAV is complex due to its remarkable genetic variability. The genome of IAV contains eight RNA segments that encode at least 17 viral proteins, including 8 initially identified proteins (PB2, PB1, PA, HA, NP, NA, M1, and NS1), 2 splicing variants of the M and NS genes (M2 and NS2) (1–3), and the recently identified proteins PB1-N40 (4), PB1-F2 (5), PA-X (6), M42 (7), NS3 (8), PA-N155, and PA-N182 (9). PB1-N40 is an N-terminally truncated version of the PB1 protein that lacks the transcriptase function but can still interact with other polymerase complex subunits and regulate virus replication in a specific genetic background (4). PB1-F2, encoded by an alternative open reading frame (ORF) of PB1, has multiple functions, including the induction of apoptosis (10), aggravation of inflammation (11, 12), and secondary bacterial infection (13). PA-X is a frameshift product of the ribosome and acts to decrease the virulence of the 1918 H1N1 virus in mice (6). PA-N155 and PA-N182 are N-terminally truncated forms of PA, and the mutant viruses lacking these two proteins exhibit attenuated *in vitro* replication and pathogenicity in mice relative to the wild-type (wt) virus (9). M42 is the M2 isoform with an alternative ectodomain that can functionally replace M2 and support efficient viral replication (7). Selman et al. have identified NS3 as the isoform of NS1 and speculated that the codon providing NS3 expression could be associated with host adaptation and the overcoming of

the species barrier (8). However, this is only an arguable hypothesis. Thus, there arises a concern that the presence of these novel proteins may further complicate the pathogenesis of IAV.

The PA-X protein is a fusion protein incorporating the N-terminal 191 amino acids of the PA protein with a short C-terminal sequence (either 61 or 41 codons) encoded by an overlapping ORF (“X-ORF”) in segment 3 that is accessed by +1 ribosomal frame-shifting. Jagger et al. have shown that PA-X decreases viral pathogenicity in mice by comparing the virulence of the wt 1918 H1N1 pandemic virus (1918-wt) and the PA-X-deficient virus (1918-FS)

Received 21 July 2014 Accepted 17 January 2015

Accepted manuscript posted online 28 January 2015

Citation Hu J, Mo Y, Wang X, Gu M, Hu Z, Zhong L, Wu Q, Hao X, Hu S, Liu W, Liu H, Liu X, Liu X. 2015. PA-X decreases the pathogenicity of highly pathogenic H5N1 influenza A virus in avian species by inhibiting virus replication and host response. *J Virol* 89:4126–4142. doi:10.1128/JVI.02132-14.

Editor: A. García-Sastre

Address correspondence to Xiufan Liu, xfliu@yzu.edu.cn.

J.H. and Y.M. contributed equally to this article.

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

doi:10.1128/JVI.02132-14

in a mouse model (6). Moreover, the increased virulence of the PA-X-null mutant is associated with increases in the expression levels of genes of the inflammatory, apoptotic, and T lymphocyte signaling pathways. Recently, Desmet et al. have revealed that the PA-X protein of the 2009 H1N1 pandemic virus is involved in host protein synthesis shutoff, which may contribute to viral evasion of host antiviral activity (14). However, nothing is known about the potential role of PA-X in the pathogenesis of highly pathogenic avian influenza viruses (HPAIV) of the H5N1 subtype.

In the present study, we first demonstrated that PA-X attenuated the pathogenicity of an HPAIV H5N1 strain, A/Chicken/Jiangsu/k0402/2010 (CK10), in a mouse model, confirming the findings of Jagger et al. (6). More importantly, we further determined that loss of PA-X expression increased the virulence of CK10 both in chickens and in mallard ducks and enhanced polymerase activity and virus replication both *in vitro* and *in vivo*. Using a microarray, we also found that loss of PA-X expression enhanced the host response in chicken lungs, represented by high activation of numerous genes associated with the inflammatory response pathway and the interferon (IFN), immune cell, and cell death signaling pathways. Furthermore, stronger apoptosis in chicken and duck embryo fibroblasts and stronger cytokine responses in birds were observed for PA-X-null recombinant viruses than for the wt strain. Overall, our findings reveal the important role of PA-X in decreasing the virulence of H5N1 IAV in both mammalian and avian species.

MATERIALS AND METHODS

Ethics statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The protocols for animal experiments complied with the laboratory animal welfare and ethics guidelines of the Jiangsu Administrative Committee of Laboratory Animals and were approved by that committee (approval number SYXK-SU-2007-0005). All experiments involving live viruses and animals were performed in negative-pressure isolators with HEPA filters in biosafety level 3 (BSL3) animal facilities in accordance with the institutional biosafety manual.

Viruses and cells. CK10 was isolated from a dead chicken. As reported previously, this virus belongs to clade 2.3.2.1, which is dominant in China and is highly pathogenic in multiple animal models, including mice, guinea pigs, chickens, and mallard ducks (15). Reverse genetics-derived CK10 (r-CK10) was generated previously (16). Recombinant viruses harboring mutations at the ribosomal frameshifting sites of the CK10 PA gene that reduce the expression of PA-X were generated based on a strategy established previously (6). The CK-PAX5 virus carries five nucleotide mutations (T568A, C569G, T573C, C574A, and T576A) in the PA gene, and the CK-PAX3 virus harbors three mutations (T573C, C574A, and T576A) (numbering from the A of the AUG codon) (see Fig. 1A). PA-derived plasmids were sequenced to verify the presence of the mutations introduced and the absence of additional unwanted mutations.

Duck embryo fibroblasts (DEF), chicken embryo fibroblasts (CEF), a chicken fibroblast cell line (DF-1), and MDCK, A549, Vero, and HEK 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) (Life Technologies) and antibiotics and were cultured at 37°C under 5% CO₂.

Virus rescue. Virus was rescued as described elsewhere (17). Briefly, the mixture of HEK 293T and MDCK cells (1:1) was transfected with plasmids encoding all eight influenza proteins by using PolyFect transfection reagent (Qiagen, GmbH, Germany). After 48 h, the supernatant was harvested and was used to inoculate 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs in order to obtain virus stocks. To confirm the identities of the

mutant viruses, RNA was extracted, and each gene segment was sequenced to ensure the absence of unwanted mutations.

Cell fractionation and Western blotting. To compare the PA-X expression of the recombinant viruses, MDCK cells were infected with r-CK10, CK-PAX5, or CK-PAX3 at a multiplicity of infection (MOI) of 10. At 36 h postinfection (p.i.), cells were washed twice with phosphate-buffered saline (PBS) and were fractionated for the preparation of whole-cell extracts using the protocol recommended by the manufacturer (Beyotime Institute of Biotechnology, Nantong, China). The proteins were then subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Roche Diagnostics Corporation, Indianapolis, IN) using an electrophoresis system (Bio-Rad, Hercules, CA) and a Mini Trans-Blot electrophoretic transfer system (Bio-Rad). The membranes were blocked for 1 h at room temperature with Tris-buffered saline-Tween (TBST) (20 mM Tris-HCl [pH 7.4], 137 mM NaCl, and 0.1% Tween 20) buffer containing 5% skim milk and were then incubated with a primary monoclonal antibody against β -actin (Abcam, Cambridge, United Kingdom), an anti-PA-X antibody (a polyclonal rabbit antiserum against an H5N1 X-ORF-derived peptide [CAGLPKTVSH RTSPA] [GenScript, Nanjing, China]), or a polyclonal rabbit anti-PA antibody (kindly provided by Hualan Chen) overnight at 4°C. The blots were washed three times for 10 min each time in TBST buffer and were incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Sigma, St. Louis, MO). The expression levels of the PA-X protein relative to those of the control, β -actin, were determined by densitometry using BandScan software, version 5.0. The data presented are representative of the results of three independent experiments.

Animal experiments. To evaluate the role of PA-X in H5N1 virus pathogenicity in mice, groups of five 6-week-old female BALB/c mice were inoculated intranasally (i.n.) with serial dilutions of viruses ($10^{1.0}$ to $10^{3.0}$ 50% embryo infectious doses [EID₅₀]) to determine the median lethal dose in mice (MLD₅₀). Animals were monitored daily for mortality over a period of 14 days as described previously (15). A similar procedure was used to calculate the median lethal doses in chickens and ducks (CLD₅₀ and DLD₅₀, respectively); inoculation doses were $10^{1.0}$ to $10^{5.0}$ EID₅₀ for chickens and $10^{2.0}$ to $10^{5.0}$ EID₅₀ for mallard ducks. Animals showing severe disease signs were euthanized and were recorded as having died on the following day. The viral lethal doses in different animal models were calculated using the Reed and Muench method (18). The mean death time (MDT) of the animals was also calculated.

To compare virus replication and characterize the innate immune response, groups of nine 4-week-old SPF chickens were infected i.n. with $10^{3.0}$ EID₅₀ of each virus, and at 12, 24, and 36 h p.i., three birds per group were euthanized, and organs (hearts, spleens, brains, and lungs) were collected for virus titration in eggs and cytokine gene profiling. For duck experiments, groups of nine 6-week-old mallard ducks were infected i.n. with $10^{5.0}$ EID₅₀ of each virus, and on days 1, 3, and 5 p.i., three ducks per group were euthanized, and organs (hearts, spleens, brains, and lungs) were collected for virus titration in eggs and cytokine gene profiling. To investigate virus replication in mice, groups of nine 6-week-old female BALB/c mice were infected i.n. with $10^{5.0}$ EID₅₀ of each recombinant virus, and on days 1, 2, and 3 p.i., three mice of each group were euthanized, and the whole lungs were collected for virus titration in eggs.

Growth curves. Cells of different types, including A549, Vero, MDCK, CEF, DF-1, and DEF cells, were inoculated at an MOI of 0.01. Supernatants were collected at 12, 24, 48, 72, and 96 h p.i., and virus titers in the culture supernatants were determined in MDCK cells. The virus titer was calculated as the 50% tissue culture infectious dose (TCID₅₀) per ml using the Reed and Muench method (18). The data presented are means $\pm$ standard deviations (SD) for three independent infections.

Luciferase assays. To determine the impact of PA on the synthesis of coexpressed proteins, 293T cells were transfected with either an empty pcDNA3.1+ vector or the pcDNA3.1+ vector expressing a wt or mutated

TABLE 1 Primers for amplification of cytokine genes

Host and primer name	Sequence (5'–3')	GenBank accession no. of target
Duck		
GAPDHF	ATGTTCGTGATGGGTGTGAA	AY436595
GAPDHR	CTGTCTTCGTGTGTGGCTGT	
MXF	TCACACGAAGGCCTATTTTACTGG	NM_204609
MXR	GTCGCCGAAGTCATGAAGGA	
IL-8F	AGGACAACAGAGAGGTGTGCTTG	NM_205498
IL-8R	GCCTTTACGATCCGCTGTACC	
IL-1 β F	GAGATTTTCAACCCGTCACC	DQ393268
IL-1 β R	AGGACTGGGAGCGGGTGTGA	
IL-18F	AGGTGAAATCTGGCAGTGGAAAT	NM_204608.1
IL-18R	ACCTGGACGCTGAATGCAA	
IFN- α -F	TTGTCCTTCCCGGACA	EF053034
IFN- α -R	GCTGAGGGTGTCTGAAGAGGT	
IFN- β -F	CCTCAACCAGATCCAGCATT	AY831397
IFN- β -R	GGATGAGGCTGTGAGAGGAG	
Chicken		
β -actinF	ATTGTCCACCGCAAATGCTTC	NM_205518.1
β -actinR	AAATAAAGCCATGCCAATCTCGTC	
IFN- β F	TGCACAGCATCTACTGCTCTTG	NM_001024836.1
IFN- β R	GTTGGCATCCTGGTGACGAA	
IFN-rF	CTCCCGATGAACGACTTGAG	NM_205149.1
IFN-rR	CTGAGACTGGCTCCTTTTCC	
MX-1F	ATCCATGGTCCAACTTCAGC	NM_204609.1
MX-1R	GCCTCTTGGACACTTTCTGC	
IL-6F	TTCGCCTTTTCAGACCTACCT	EU170468
IL-6R	TGGTGATTTTCTCTATCCAGTCC	
IL-18F	AGGTGAAATCTGGCAGTGGAAAT	NM_204608.1
IL-18R	ACCTGGACGCTGAATGCAA	
CCL-19F	CCAGGAAGGTCCCAATCAA	XM_424980.4
CCL-19R	ACCACAGCAGGACATAGAAGCA	

PA gene together with a *Renilla* plasmid (pTK-RL; kindly provided by Jinhua Liu) using PolyFect transfection reagent (Qiagen). After 24 to 36 h, luciferase production was measured using reagents in the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA). All results are means $\pm$ SD for three independent experiments.

To determine the effect of PA-X expression on polymerase activity, 293T cells were transfected with 200 ng of a luciferase reporter plasmid (p-Luc; kindly provided by Jinhua Liu), a mixture of pcDNA3.1+ plasmid constructs expressing the PB2, PB1, and PA polymerase subunit genes and the NP gene (200 ng each), and 20 ng of a *Renilla* internal control plasmid by using PolyFect transfection reagent (Qiagen). After 24 h, cell lysates were prepared using the Dual-Luciferase reporter assay system (Promega). CEF and DEF were transfected with 400 ng of a luciferase reporter plasmid (paviPolIT-Luc; kindly provided by Hualan Chen), the mixture of pcDNA3.1+ plasmid constructs expressing the polymerase subunits (400 ng each), and 200 ng *Renilla* plasmid. Cell lysates were prepared at 36 h p.i. Luciferase activity was measured using a GloMax 96 microplate luminometer (Promega). All results are means and SD from three independent experiments.

Independent experiments were also carried out to collect whole-cell extracts for Western blotting of the ribonucleoprotein (RNP) components. The primary monoclonal antibody against β -actin (Abcam), a polyclonal rabbit anti-PB2 antibody (kindly provided by Hualan Chen), a polyclonal goat anti-PB1 antibody (kindly provided by Jinhua Liu), a polyclonal rabbit anti-PA antibody (kindly provided by Hualan Chen), or a monoclonal antibody against NP (Santa Cruz, CA, USA) was used. The blots were washed three times for 10 min each time in TBST buffer and were incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse, anti-rabbit, or anti-goat IgG (Sigma). The ex-

pression level of each RNP component relative to that of the control, β -actin, was determined by densitometry using BandScan software, version 5.0. The data shown are representative of the results of three independent experiments.

Microarray analysis. Groups of six chickens were inoculated with $10^{3.0}$ EID₅₀ of r-CK10 or CK-PAX3. An additional six chickens were inoculated with PBS as a mock-infected control. At 12 and 24 h p.i., lungs were collected from three individual birds per group for a microarray assay. Total RNA was extracted from lung tissues using TRIzol reagent (Life Technologies) and was purified using an RNeasy minikit (Qiagen). The RNA was then amplified, labeled, and purified using a GeneChip 3' IVT Express kit (Affymetrix) to obtain aRNA (amplified and biotinylated complementary RNA). The labeled aRNA was purified, fragmented, and hybridized in the Affymetrix working station. Chips were then washed and stained for scanning by a GeneChip Scanner 3000 system.

Raw data were normalized by the MAS 5.0 algorithm in GeneSpring software, version 11.0 (Agilent Technologies). A Student one-sample *t* test and significance analysis of microarrays (SAM) were performed to identify the genes that were significantly differently expressed (SDE) (≥ 2 fold change with a *P* value of >0.05). Functional and canonical pathway analysis of SDE genes was conducted using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood, CA). Fisher's exact test was used to determine the probability that each biological function assigned to the genes within each data set was due to chance alone.

Expression profiles of cytokine genes. Quantitative real-time PCR (qRT-PCR) was used to analyze the expression of cytokine genes in chickens and ducks (IFN- β , IFN- γ , MX-1, interleukin-6 [IL-6], IL-18, and CCL19 for chickens; IFN- α , IFN- β , MX-1, IL-1 β , IL-8, and IL-18 for mallard ducks). Total RNA was isolated from tissues using TRIzol reagent

FIG 1 Evaluation of the biological characteristics of the rescued PA-X-deficient viruses. (A) Mutation sites in the PA genes of the PA-X deficient viruses. The red letters represent nucleotides at the mutation sites. (B) Western blotting for PA, PA-X, and β -actin expression. MDCK cells were infected with the indicated virus at an MOI of 10. After about 36 h, whole-cell lysates were subjected to Western blotting. The levels of PA-X expression relative to the expression of the control, β -actin, were determined by densitometry using BandScan software, version 5.0. The data shown are representative of results from three independent experiments and were standardized to the level of r-CK10 (taken as 100%). (C) *Renilla* luciferase expression in 293T cells cotransfected with the indicated PA or PA-derived mutant plasmid. Cells were transfected with either an empty pcDNA3.1 + vector or a pcDNA3.1 + vector expressing the indicated PA or PA-derived mutant gene together with a *Renilla* plasmid (200 ng of each plasmid) for 24 to 36 h. The results are means and SD for three independent experiments. **, $P < 0.01$. (D and E) Mice were infected with $10^{4.0}$ EID₅₀ of r-CK10, CK-PAX5, or CK-PAX3 ($n = 5$). Mice were humanely killed when they lost $\geq 25\%$ of their initial body weight. Shown are the mean weight loss (D) and survival rates (E) of mice infected with the indicated viruses or mock infected with PBS. (F) Determination of the LD₅₀s of the recombinants in mice, chickens, and ducks. Groups of five animals were inoculated intranasally with 100- μ l-vial dilutions containing different doses of virus ($10^{1.0}$ to $10^{3.0}$ EID₅₀ for mice, $10^{1.0}$ to $10^{5.0}$ EID₅₀ for chickens, and $10^{2.0}$ to $10^{5.0}$ EID₅₀ for mallard ducks). Values are presented as the LD₅₀ (expressed in log₁₀ EID₅₀)/MDT (in days). (G and H) Chickens were infected with $10^{5.0}$ EID₅₀ of r-CK10, CK-PAX5, or CK-PAX3. Survival rates (G) and MDTs (H) are shown. Data are means and SD. *, $P < 0.05$.

(Life Technologies) and was treated with DNase I (Fermentas, Glen Burnie, MD, USA). One microgram of total RNA per sample was reverse transcribed into cDNA using 400 U RevertAid Premium reverse transcriptase (Fermentas) and 100 μ M random primers in the presence of an RNase inhibitor (Fermentas) at 50°C for 30 min. The PCR mixture contained cDNA, 200 nM each primer, and 10 μ l of 2 $\times$ SYBR green PCR master mix (TaKaRa, Shiga, Japan). PCRs were performed in triplicate using the ABI Prism 7300 system (Applied Biosystems, Foster City, CA, USA) with the following cycle profile: 1 cycle at 50°C for 2 min and 1 cycle at 95°C for 5 s, followed by 40 cycles at 95°C for 5 s and 60°C for 31 s. One cycle for the generation of a melting curve was added for all reactions to verify product specificity. The expression value of each gene, relative to that for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β -actin, was calculated using the $2^{-\Delta\Delta CT}$ method. The primers for the target genes are listed in Table 1.

Apoptosis analysis. CEF and DEF were infected with r-CK10, CK-PAX3, or CK-PAX5 at an MOI of 1. At 12 and 24 h p.i., both infected and

noninfected cells were treated with trypsin and were washed three times with PBS. An aliquot of cells (10^6) was stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) (Roche) for 15 min at room temperature in the dark. The stained cells were then analyzed by a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Annexin V has a high affinity for phosphatidylserine (PS), which is translocated from the inner to the outer leaflet of the plasma membrane in the process of apoptosis. PI cannot permeate live cells and apoptotic cells but stains dead cells, binding tightly to the nucleic acids in the cell. Thus, apoptotic cells are those with high annexin V and low PI staining, whereas necrotic cells are highly stained with both annexin V and PI. The proportions of total cells that are apoptotic or necrotic, shown in Fig. 7 and 8, are means $\pm$ SD for at least three independent experiments.

Immunofluorescence. To determine whether apoptotic and necrotic cells were infected with virus, CEF and DEF were costained with annexin V, PI, and an antiserum against viral PA protein. Briefly, cells were first stained with annexin V and PI; then they were fixed with 4% paraformal-

FIG 2 Effect of PA-X on viral replication *in vitro*. A549, Vero, MDCK, DF-1, CEF, and DEF cells were inoculated at an MOI of 0.01. Virus titers were determined in MDCK cells at the indicated time points. Data are means $\pm$ SD for three independent infections. Asterisks indicate significant differences between CK-PAX3 and r-CK10 (*, $P < 0.05$; **, $P < 0.01$).

dehydrate for 15 min, saturated with 0.5% Triton X-100 for 10 min, and blocked with 10% bovine serum albumin for 20 min. Cells were incubated with a rabbit antiserum against PA at 37°C for 30 min. After washing with PBS, cells were incubated with DyLight 405-labeled goat anti-rabbit IgG (H+L) (Beyotime Institute of Biotechnology) at 37°C for 30 min and were observed under a fluorescence microscope.

Statistical analysis. Statistical analyses were performed using SPSS Statistics software. The independent-samples *t* test was used for data analysis. A *P* value of <0.05 was considered significant.

Accession numbers. All primary expression microarray data have been deposited in the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/info/linking.html>) under accession number GSE53932. The genomic sequences of CK10 are available in GenBank under accession numbers JQ638673 to JQ638688.

RESULTS

Loss of PA-X expression increases viral virulence in mice, chickens, and mallard ducks. It has been shown that PA-X decreases the virulence of the 1918 H1N1 pandemic virus in mice (6). To test whether PA-X can exert a similar effect in the H5N1 AIV genetic background, we generated two PA-X-deficient viruses based on the CK10 virus, which is highly lethal to mice without prior adaptation, and evaluated their virulence in mice. We first used Western blotting to investigate whether PA-X could be detected in the lysates of cells infected with r-CK10 or mutant CK10 viruses. As shown in Fig. 1A and B, the mutant CK10 virus carrying five nucleotide mutations in the PA gene showed 15% lower PA-X expression than the r-CK10 virus. At the same time, the level of PA-X expression for the CK-PAX3 virus, harboring three nucleotide mutations in the PA gene, was 47% of that for the r-CK10 virus.

Two independent studies have demonstrated the inhibitory activity of the PA-X protein on plasmid-mediated gene expression (6, 14). In this study, to verify this finding, HEK 293T cells were cotransfected with a plasmid expressing *Renilla* luciferase together with the empty expression vector or a plasmid expressing a wt or mutant PA gene. As shown in Fig. 1C, the level of *Renilla* luciferase

expression in the presence of the CK10 PA gene was greatly reduced (11-fold) from that with the empty vector, whereas the reduction in luciferase activity in the presence of the CK-PAX5 or CK-PAX3 PA gene was much lower (2.77- or 1.6-fold, respectively). These results suggest that PA-X has a suppressive effect on coexpressed proteins.

We next investigated the impact of altered PA-X expression on pathogenicity in mice. As shown in Fig. 1D, PA-X-deficient virus-infected mice displayed more weight loss than mice infected with the wt virus. Although no difference in mortality was observed between mice infected with the wt and mutant viruses at $10^{4.0}$ EID₅₀, the MDT of mutant virus-infected mice was shorter than that of wt virus-infected mice (Fig. 1E). We then determined the MLD₅₀s of the three viruses (Fig. 1F). The MLD₅₀ values of CK-PAX3 and CK-PAX5 were 7.5- and 3.5-fold lower than that of r-CK10, respectively. Overall, these results suggest that loss of PA-X expression increases the virulence of H5N1 IAV in mice, which is in agreement with the data for the 1918 H1N1 virus. We further performed a pathogenicity test on chickens and ducks. We found greater increases in the virulence of CK-PAX3 than in the virulence of CK-PAX5 over that of r-CK10 in both avian models (Fig. 1F). The CLD₅₀ and DLD₅₀ of CK-PAX3 were 6.24- and 58.9-fold lower than those of r-CK10, whereas CK-PAX5 had a CLD₅₀ comparable to that of r-CK10 and a 7.04-fold decrease in the DLD₅₀. Moreover, greater differences in the MDTs for chickens and ducks were observed between CK-PAX3 and r-CK10 than between CK-PAX5 and the wt strain (Fig. 1F). When $10^{5.0}$ EID₅₀ were used, CK-PAX3 took a significantly shorter time to kill chickens than did r-CK10 (Fig. 1G and H). Therefore, these results suggest that the PA-X protein decreases the pathogenicity of H5N1 AIV both in mammalian and in avian species.

PA-X dampens virus replication both *in vitro* and *in vivo*. To test the effect of the reduced PA-X expression on virus replication, we first compared the replication kinetics of r-CK10, CK-PAX3,

FIG 3 Effect of PA-X on viral replication *in vivo*. (A) Viral replication in chickens. Groups of nine 4-week-old SPF chickens were infected intranasally with $10^{3.0}$ EID₅₀ of r-CK10, CK-PAX5 (PAX5), or CK-PAX3 (PAX3). At 12, 24, and 36 h p.i., three birds of each group were euthanized in order to collect organs for virus titration in eggs. (B) Viral replication in ducks. Groups of nine 6-week-old mallard ducks were infected intranasally with $10^{5.0}$ EID₅₀ of the indicated viruses. On days 1, 3, and 5 p.i., three birds of each group were euthanized in order to collect organs for virus titration in eggs. (C) Viral replication in mice. Groups of nine 6-week-old female BALB/c mice were infected intranasally with $10^{5.0}$ EID₅₀ of the indicated viruses. On days 1, 2, and 3 p.i., three mice of each group were euthanized in order to collect organs for virus titration in eggs. Means $\pm$ SD are shown. Asterisks indicate significant differences ($P < 0.05$) from results for r-CK10. The detection limit for virus titers in chickens and ducks is $10^{1.48}$ EID₅₀/g of tissue, and the detection limit in mice is $10^{1.0}$ EID₅₀/ml of the cleared tissue homogenate.

and CK-PAX5 in A549, Vero, MDCK, CEF, DF-1, and DEF cells. We found that CK-PAX3 had higher replication efficiency than r-CK10 in various cell types (Fig. 2). In A549 cells, a significantly higher titer of CK-PAX3 than of r-CK10 was detected at 96 h p.i. In Vero cells, CK-PAX3 replicated to significantly higher titers than r-CK10 at several time points (48, 72, and 96 h). However, no

significant difference in viral replication was observed between PA-X-deficient viruses and the wt virus in MDCK cells. In avian cells, overall, higher virus titers were detected in CK-PAX3-infected cells than in r-CK10 virus-infected cells. Moreover, a significantly higher titer of CK-PAX3 than of r-CK10 was detected in DEF at 72 h p.i.

FIG 4 Effects of PA-X on the activity and expression of the RNP complex. (A) Polymerase activity of the RNP in 293T, CEF, and DEF cells. Cells were transfected in triplicate with a luciferase reporter plasmid (p-Luci for 293T cells; paviPolIT-Luc for CEF and DEF) and a *Renilla* internal control plasmid, together with plasmids expressing CK10 PB2, PB1, and NP and a plasmid expressing either CK10 PA or a CK10 PA-derived mutant. After 24 to 36 h, luciferase activities in cell lysates were measured. The polymerase activity values were normalized to the *Renilla* luciferase activity. Values shown are means $\pm$ SD for three independent experiments and were standardized to those for CK10 PA (taken as 100%). (B) Expression of RNP components from cells cotransfected with plasmids expressing CK10 PB2, PB1, and NP and a plasmid expressing either CK10 PA or a CK10 PA-derived mutant. Levels of viral protein expression were determined by Western blotting. The level of expression of each RNP component relative to the expression of the control, β -actin, was determined by densitometry using BandScan software, version 5.0. Data are representative of the results of three independent experiments and were standardized to those for r-CK10 (taken as 100%).

Next, we investigated virus replication *in vivo*. At an early time point (12 h p.i.), CK-PAX3 could be detected in the lungs, spleens, hearts, and brains of two out of three chickens, whereas r-CK10 was recovered from only one chicken (Fig. 3A). In addition, virus yields for CK-PAX3-infected chickens were higher than those for r-CK10-infected chickens. At 24 h p.i., CK-PAX3 disseminated to the spleens and brains in more chickens than r-CK10 and replicated to a significantly higher titer in the lungs. At 36 h p.i., there was no significant difference in viral loads in the lungs, spleens, and hearts between CK-PAX3 and r-CK10. However, the CK-PAX3 virus was recovered from the brains of more chickens than the r-CK10 virus at this time point. In ducks, CK-PAX3 displayed a replication advantage on day 5 p.i., as evidenced by significantly higher virus titers in the lungs, spleens, and brains of CK-PAX3-infected birds than in those of r-CK10-infected birds (Fig. 3B). At this time point, a significantly higher virus yield in the hearts of

CK-PAX5-infected ducks than in those of r-CK10-infected ducks was also observed. For mice, both PA-X-deficient viruses replicated to significantly higher titers in the lungs than r-CK10 on day 1 p.i. (Fig. 3C). Moreover, virus titers in the spleens of CK-PAX3-infected birds were significantly higher than those for r-CK10-infected birds on day 3 p.i. Collectively, these results demonstrate that PA-X dampens virus replication both *in vitro* and *in vivo*.

Downregulated PA-X expression enhances viral polymerase activity. To investigate whether changes in PA-X expression could influence viral polymerase activity, the activity of the reconstituted RNP was measured in CEF, DEF, and HEK 293T cells by using the luciferase minigenome assay. To determine the inhibitory effect of PA-X on the expression of the cotransfected plasmids, we also analyzed the expression of the RNP components by Western blotting of lysates prepared from cells transfected with plasmids expressing PB2, PB1, and NP and with a plasmid ex-

FIG 5 Downregulation of PA-X expression increases the host global transcriptional response. (A) Number of genes SDE (with ≥ 2 -fold change at a P value of < 0.05) during infection with $10^{3.0}$ EID₅₀ of r-CK10 or CK-PAX3 relative to mock infection at 12 and 24 h p.i. (B) Venn diagram showing the distribution of SDE genes. The Venn diagrams were generated using Venny (www.pangloss.com/seidel/Protocols/venn.cgi). (C and D) Gene Ontology analysis of the SDE genes using IPA at 24 h p.i. (C) Top biofunctions of the SDE genes. (D) Top canonical pathways of the SDE genes. (E and F) Expression patterns of inflammatory response- and cell death-related genes in chicken lungs. Heat maps show the hierarchical clustering of the expression profiles of genes related to the inflammatory response (E) and cell death (F) during infection with r-CK10 or CK-PAX3 at 24 h p.i. Clustering was performed by the hierarchical unweighted-pair group method using average linkages with a Euclidean distance measure. The expression of the genes for which data are displayed was changed ≥ 2 -fold ($P < 0.05$) from that in mock-infected controls.

pressing either wt or mutant PA. As shown in Fig. 4A, the activities of the RNP complex carrying the CK-PAX3 PA gene were 6.18- and 6.04-fold higher than those for r-CK10 in CEF and DEF, respectively. In addition, the activities of the CK-PAX5 RNP complex were 5.24- and 5.12-fold higher than those for r-CK10 in CEF and DEF, respectively. In HEK 293T cells, the activities of the RNP complex in PA-X-deficient mutants were also significantly higher than that of r-CK10. The RNP complex harboring the CK-PAX3 PA gene showed a polymerase activity 3.68-fold higher than that of r-CK10, while the polymerase activity of the CK-PAX5 RNP complex was 3.47-fold higher. When the expression of the RNP components was analyzed, overall, higher expression of these proteins was detected in cells cotransfected with PA-derived mutated plasmids, especially in DEF (Fig. 4B). These findings suggest that loss of PA-X expression markedly enhances viral polymerase activity in different cell types.

The CK-PAX3 virus augments inflammatory and cell death responses in chicken lungs. The host response to IAV has a prominent role in viral pathogenicity, and an excessive immune response may be deleterious to the host (19, 20). To understand the mechanism of the increased virulence of H5N1 AIV caused by the loss of PA-X expression in avian species, we used microarray analysis to compare the global transcriptional responses in the lungs of chickens infected with r-CK10 or CK-PAX3. By screening the genes SDE during virus infection and mock infection, we found a remarkable difference between the transcriptional host responses to these two viruses at 24 h p.i. As shown in Fig. 5A, a total of 1,112 SDE genes were stimulated by CK-PAX3, whereas the corresponding number for r-CK10 is 417. In addition, a Venn diagram summarizing the distribution of SDE genes showed that only 175 genes were shared by CK-PAX3 and r-CK10 (Fig. 5B).

The genes SDE at 24 h p.i. were then uploaded into the IPA

FIG 6 Loss of PA-X expression augments the innate immune response in chicken lungs. (A and B) Hierarchical clustering of expression profiles of innate immune response-related genes during infection with r-CK10 or CK-PAX3 at 12 or 24 h p.i. (A) Genes involved in the activation or trafficking of immune cells (including NK cells, macrophages, dendritic cells [DC], antigen-presenting cells [APC], and T lymphocytes) and cytokine responses. (B) Interferon (IFN) and IFN-related genes. Clustering was performed by the hierarchical unweighted-pair group method using average linkages with a Euclidean distance measure. The expression of the genes for which data are displayed was changed ≥ 2 -fold ($P < 0.05$) from that in mock-infected controls at least at one time point of infection. (C) Modeling of the IFN response pathway in the lungs of chickens infected with CK-PAX3 at 24 h p.i. (red, upregulated; green, downregulated).

FIG 8 Loss of PA-X expression increases cell death in DEF. (A) Flow cytometry analysis of cells infected with CK10 or PA-X-deficient viruses and of mock-infected control cells at 12 and 24 h p.i. Cells were stained with FITC-conjugated annexin V and propidium iodide (PI). Apoptotic cells are those with high annexin V and low PI staining, whereas necrotic cells are highly stained with both annexin V and PI. The percentages of positive cells among the total numbers of cells analyzed are shown. (B and C) Quantitative analyses of wt or PA-X-deficient virus-induced apoptosis (B) and necrosis (C) by flow cytometry. Values are proportions of the total number of cells that are apoptotic or necrotic and are means $\pm$ SD of the results from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

necrosis, since the outcome of this type of cell death for the host is different from that of apoptosis. As with apoptosis, all viruses tested induced significantly more necrotic cells at 12 and 24 h p.i. than the mock infection control, and there was a significant difference in necrosis between CK-PAX3 and r-CK10 at 24 h p.i. (Fig. 7D). In DEF, a similar pattern of apoptosis was observed (Fig. 8B). However, only CK-PAX3 induced significantly more necrotic cells than the mock infection control and the r-CK10 virus at 24 h p.i., a result quite different from those in CEF (Fig. 8C).

To determine whether apoptotic and necrotic cells are infected with virus, CEF and DEF were costained with annexin V, PI, and an antiserum against viral PA protein. We found that viral PA protein can be detected in the majority of apoptotic and necrotic cells (data not shown). However, a few apoptotic or necrotic cells were identified as virus antigen negative, suggesting that these cells underwent cell death during the sample preparation process. These results suggest that, in accord with the results presented in Fig. 7 and 8, virus infection is directly related to apoptosis and necrosis in CEF and DEF. It should be noted that the results of the apoptosis assay *in vitro* are consistent with the *in vivo* data showing high activation of multiple genes involved in the tumor necrosis factor (TNF)/FasL-mediated apoptosis pathway during CK-PAX3 infection (Fig. 7A and B). Taken together, these results indicate that the PA-X protein of H5N1 virus may act as an antiapoptosis factor during virus infection.

Reduced PA-X expression exacerbates cytokine responses in birds. Based on the results of microarray analysis, we further tested the impact of PA-X on the innate immune response by profiling the expression of a set of representative cytokine genes in the lungs, spleens, hearts, and brains of virus-infected chickens and ducks. Overall, in chickens, CK-PAX3 elicited a stronger cytokine response than r-CK10 (Fig. 9 and 10). In the lungs, the viruses tested induced comparable levels of cytokine gene expression at an early time point, 12 h p.i., whereas at 24 and 36 h p.i., the expression of IFN- γ , MX1, IL-6, IL-18, and CCL19 was more highly activated by CK-PAX3 than by r-CK10 (Fig. 9). In agreement with this observation, cytokine responses in the spleens of CK-PAX3-infected birds were exacerbated relative to those for r-CK10 (Fig. 9). However, in the hearts and brains, remarkable differences in cytokine gene expression between CK-PAX3 and r-CK10 were observed mainly at 24 h p.i. (Fig. 10). In addition, in the hearts, there was no significant difference in cytokine gene induction between CK-PAX5 and r-CK10 except for MX-1 and IL-18 expression (Fig. 10).

In ducks, generally, both PA-X-deficient viruses induced more robust cytokine responses than their parental virus (Fig. 11 and 12). As shown in Fig. 11, in the hearts, CK-PAX3 infection significantly increased the expression levels of IFN- α , IFN- β , MX1, and IL-1 β over those with r-CK10 at an early time point, day 1 p.i. Moreover, both PA-X-deficient viruses upregulated the cy-

FIG 9 qRT-PCR analysis of cytokine gene expression in the lungs and spleens of chickens infected with wt or PA-X-deficient virus. Groups of nine 4-week-old SPF chickens were infected intranasally with $10^{3.0}$ EID₅₀ of the indicated viruses. Total RNA was extracted from tissues collected at 12, 24, and 36 h p.i. from three birds of each group. Equal amounts of RNA (1 μ g) from each sample were used for qRT-PCR. The expression level of each cytokine gene was normalized to that of the β -actin gene and is presented as the fold change from the expression level in mock-treated birds. Values are means $\pm$ SD. *, $P < 0.05$.

tokine genes tested to higher levels (except for IL-18) than did r-CK10 on day 3 p.i. On day 5 p.i., the innate immune response was markedly augmented by CK-PAX3, as evidenced by the fact that the expression levels of all the cytokine genes tested were significantly higher with CK-PAX3 than with r-CK10. In the brains, on day 1 p.i., significantly higher expression of the IL-1 β and IL-18 genes was induced by CK-PAX3 than by r-CK10, whereas comparable induction of the cytokine genes tested was observed with all three viruses on day 3 p.i. (Fig. 11). The cytokine response status in the brains on day 5 p.i. resembled that in the hearts. Of note, in the hearts and brains, some cytokine genes, including the IFN- β and MX1 genes, were extremely upregulated (>200 -fold for MX1). In contrast, the cytokine responses in the lungs and spleens were milder than those in the hearts and brains,

but CK-PAX3 still induced markedly higher expression of several cytokines in these organs at several time points (Fig. 12).

Taken together, these findings suggest that the PA-X-null mutants, especially CK-PAX3, manifest a high-cytokine phenotype both in chickens and in ducks, which may partly account for their enhanced virulence in these two animal models.

DISCUSSION

Our study is the first report defining the important role of the PA-X protein in the pathogenicity of H5N1 HPAIV in different host species. We generated two CK10-derived mutants (CK-PAX5 and CK-PAX3) whose PA-X expression was decreased to different levels by reducing the ribosomal frameshifting efficiency of the PA “X-ORF” (Fig. 1A and B). Virulence tests showed that these two

FIG 10 qRT-PCR analysis of cytokine gene expression in the hearts and brains of chickens infected with wt or PA-X-deficient virus. Groups of nine 4-week-old SPF chickens were infected intranasally with $10^{3.0}$ EID₅₀ of the indicated viruses. Total RNA was extracted from tissues collected at 12, 24, and 36 h p.i. from three birds of each group. Equal amounts of RNA (1 μ g) from each sample were used for qRT-PCR. The expression level of each cytokine gene was normalized to that of the β -actin gene and is presented as the fold change from the expression level in mock-treated birds. Values are means $\pm$ SD. *, $P < 0.05$.

mutants had higher virulence in mice, chickens, and mallard ducks than their parental virus, r-CK10 (Fig. 1D to H). Loss of PA-X expression increased polymerase activity and virus replication both *in vitro* and *in vivo* (Fig. 2 to 4). Microarray analysis of lungs from chickens infected with CK-PAX3 or r-CK10 showed that the decrease in PA-X expression resulted in changes in the magnitude of the host response, including marked increases in the inflammatory response pathway and the IFN, immune cell, and cell death signaling pathways (Fig. 5 to 7). Moreover, CK-PAX3 also induced enhanced cell death in avian cells and a more potent innate immune response in birds (Fig. 7 to 12). Taken together, our findings suggest that the PA-X protein acts to attenuate the

pathogenicity of H5N1 AIV in avian species by inhibiting virus replication and the host response.

Jagger et al. discovered the PA-X protein and identified PA-X as a negative regulator of virus pathogenicity in mice in the context of the 1918 H1N1 pandemic virus (6). Therefore, we first revealed the decreased PA-X expression in the H5N1 genetic background by generating two PA-X-deficient viruses based on an H5N1 strain, CK10, and comparing their PA-X expression levels with that of the parental virus (Fig. 1A and B). We then verified the role of PA-X in the pathogenicity of H5N1 viruses in a mouse model and obtained similar results showing that the PA-X protein decreases the pathogenicity of an H5N1 HPAIV in mice (Fig. 1D

FIG 11 qRT-PCR analysis of cytokine gene expression in the hearts and brains of ducks infected with wt or PA-X-deficient virus. Groups of nine 6-week-old mallard ducks were infected intranasally with $10^{5.0}$ EID₅₀ of a recombinant virus or the parental strain. Total RNA was extracted from tissues collected on days 1, 3, and 5 p.i. from three birds of each group. Equal amounts of RNA (1 μ g) from each sample were used for qRT-PCR. The expression level of each cytokine gene was normalized to that of the GAPDH gene and is presented as the fold change from the expression level in mock-infected ducks. Values are means $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$.

through F). In the study on the 1918 H1N1 virus, no significant difference in replication was detected between the wt virus and PA-X-deficient mutants. However, in this study, we found that the PA-X protein affects virus replication both in mammalian cells (A549 and Vero cells) and in mice (Fig. 2 and 3C). The underlying mechanism for the different observations from these two studies is currently unknown. One possible explanation is that PA-X may affect virus replication in a virus subtype-dependent manner. Another possibility is that changes in the host response, rather than virus replication, may be the main driving force for the alteration

in the pathogenicity of the 1918 virus in mice, while for H5N1 viruses, virus replication may also be actively involved in determining virulence.

It is notable that we focus on the role of PA-X in the pathogenesis of H5N1 AIV in its natural host avian species. The pathogenicity of the parental r-CK10 strain in chickens and ducks was increased when the PA-X expression level was decreased, and higher pathogenicity was seen for CK-PAX3 (Fig. 1F). This finding is consistent with the results in mice (Fig. 1D through F), indicating that attenuation of H5N1 viruses caused by PA-X is a common

FIG 12 qRT-PCR analysis of cytokine gene expression in the lungs and spleens of ducks infected with wt or PA-X-deficient virus. Groups of nine 6-week-old mallard ducks were infected intranasally with $10^{5.0}$ EID $_{50}$ of a recombinant virus or the parental strain. Total RNA was extracted from tissues collected on days 1, 3, and 5 p.i. from three birds of each group. Equal amounts of RNA (1 μ g) from each sample were used for qRT-PCR. The expression level of each cytokine gene was normalized to that of the GAPDH gene and is presented as the fold change from the expression level in mock-infected ducks. Values are means $\pm$ SD. *, $P < 0.05$.

feature in mammalian and avian hosts. In parallel, loss of PA-X expression enhanced virus replication in CEF, DEF, chickens, and ducks, further highlighting the possibility that the effect of PA-X on virus replication contributes to the change in virulence (Fig. 2 and 3). Moreover, the minigenome assay showed that the activity of the PA-X-deficient RNP complex was significantly higher than that of the wt RNP complex (Fig. 4A). It has been shown that IAV PA-X can intensively repress the production of the coexpressed protein in the plasmid cotransfection system (6, 14). Here we showed that decreased PA-X expression caused significantly higher polymerase activity and slightly increased expression levels of RNP components under the plasmid cotransfection condition (Fig. 4A and B). Thus, this finding suggests that the low polymer-

ase activity of r-CK10 may be partially associated with the suppressive effect of PA-X on the expression of the RNP components. Interestingly, the significantly increased polymerase activity of the CK-PAX3 mutant RNP complex in avian cells does not translate into significantly enhanced replication in the same cells (except for DEF at 72 h p.i.). We surmised that the actual biological function of the viral RNP complex during infection may be different from the polymerase activity of the reconstituted RNP complex measured in the plasmid transfection system. Moreover, previous studies have also provided evidence that the level of polymerase activity does not always correlate with the efficiency of viral replication *in vitro* (21, 22).

One of the most important findings of our study is that the

PA-X protein can suppress virus-induced apoptosis (Fig. 7C and 8B). Influenza virus can induce apoptosis in multiple cell types, including lymphocytes (23, 24), epithelial cells (25, 26), and immortalized cell lines (10, 27–30); apoptosis then causes cellular and organ damage, contributing to virus pathogenicity (31–33). Thus, the accelerated cell death evoked by CK-PAX3 may be associated with the increased virulence in chickens and ducks. In addition, several viral proteins, including NA, M1, NS1, and PB1-F2, are related to influenza-induced apoptosis (5, 27, 34, 35). NS1 has both proapoptotic (25, 28, 34) and antiapoptotic (36) functions. PB1-F2 is a proapoptotic protein that induces apoptosis via the mitochondrial pathway (10). Recently, Desmet et al. have shown that influenza virus A/WSN/33 (WSN) harboring the PA gene from influenza virus A/California/04/2009 (Cal) induces more apoptotic cells than the wt WSN strain, suggesting that the PA gene is also associated with virus-induced apoptosis (14). Here we identified PA-X as a novel antiapoptosis protein of influenza virus. However, further studies are needed to elucidate the underlying mechanism of PA-X-associated antiapoptosis activity.

Innate immunity is the first line of defense against virus infection, but overactivation of the innate immune response is harmful to the host (16, 20, 37–39). In the present study, a microarray assay showed that CK-PAX3 induced a more potent innate immune response in chickens than its parental counterpart (Fig. 6). We surmise that the enhanced host immune response probably reflects the alleviation of host protein synthesis shutoff when the PA-X expression level is decreased, which may result in the increased virulence of CK-PAX3. In addition, cytokine gene profiling using qRT-PCR confirmed the hyperinduction of the cytokine response by the PA-X-deficient mutants in chickens and ducks (Fig. 9 through 12). However, different patterns of cytokine response were observed in these two avian species. More-intense cytokine responses in the hearts and brains than in the lungs and spleens were observed in ducks, whereas the magnitudes of the cytokine responses in these four organs were similar in chickens. This finding suggests that the extent of the cytokine response in specific organs, such as hearts and brains, may be associated with disease severity in ducks, a possibility consistent with the previous findings highlighting the importance of these organs to the pathogenicity of AIV in ducks (15, 40–43).

To date, several accessory proteins, including M2, NS2, PB1-N40, PB1-F2, PA-X, M42, NS3, PA-N155, and PA-N182, have been identified in IAV (2–9). Among these, PB1-F2 is well studied and plays a critical role in the pathogenicity of influenza virus by regulating apoptosis, inflammation, and secondary bacterial pneumonia (5, 11–13). Of note, Schmolke et al. have shown that PB1-F2 increases the pathogenicity of the H5N1 VN1203 strain in ducks independently of sequence variation at position 66 but affects virulence in mice only in the presence of the N66S polymorphism (44). However, in contrast to the species-specific function of PB1-F2, PA-X manifests a virulence-reducing role both in mice and in ducks. On the other hand, PB1-F2 promotes the systematic dissemination of the virus in experimental ducks and thus contributes to pathogenicity, whereas PA-X acts to decrease virulence by suppressing virus replication in chickens and ducks. In addition, by using high-throughput gene expression assays, Le Goffic et al. found that PB1-F2 increases the virulence of the WSN strain in mice by enhancing immune cell death and the inflammatory response (11). However, our study demonstrated that PA-X attenuates the virulence of r-CK10 in chickens and ducks by inhibiting

the host immune and cell death responses. Therefore, based on these contrasting properties of PB1-F2 and PA-X, we hypothesize that these two additional proteins may have a mutually suppressive relationship that maintains the stable virulence of IAV.

In summary, the present study demonstrates that PA-X diminishes the virulence of H5N1 HPAIV both in mammalian and in avian species. In addition, the exacerbated innate immune response, aggravated cell death, and enhanced viral replication may together contribute to the higher pathogenicity of PA-X-deficient mutants in birds. Thus, our study defines the role of the novel PA-X protein in the pathogenesis of H5N1 AIV and has important implications for understanding the variable pathogenesis of IAV. Additional studies are required to understand whether these findings can be generalized to other IAV subtypes or whether they are unique to H5N1 HPAIV.

ACKNOWLEDGMENTS

We are grateful to Hualan Chen for kindly providing the luciferase reporter plasmid paviPolIT-Luc and viral antibodies (against PB2 and PA); to Jinhua Liu for generously providing the luciferase reporter plasmid p-Luc, the *Renilla* internal control plasmid, and a viral antibody (against PB1); and to Maozhi Hu and Qiuxiang Yan for technical support of flow cytometry. We appreciate Sandie Munier for sharing primers (IL-6 and IFN- γ for chickens).

This work was supported by the Chinese National High-Tech R&D Program (863 Program; grant 2011AA10A200), the earmarked fund for the Modern Agro-Industry Technology Research System (nycytx-41-G07), the National Natural Science Foundation of China (grant 31101827), and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

REFERENCES

1. Palese P. 1977. The genes of influenza virus. *Cell* 10:1–10. [http://dx.doi.org/10.1016/0092-8674\(77\)90133-7](http://dx.doi.org/10.1016/0092-8674(77)90133-7).
2. Lamb RA, Lai CJ. 1980. Sequence of interrupted and uninterrupted mRNAs and cloned DNA coding for the two overlapping nonstructural proteins of influenza virus. *Cell* 21:475–485. [http://dx.doi.org/10.1016/0092-8674\(80\)90484-5](http://dx.doi.org/10.1016/0092-8674(80)90484-5).
3. Lamb RA, Lai CJ, Choppin PW. 1981. Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins. *Proc Natl Acad Sci U S A* 78:4170–4174. <http://dx.doi.org/10.1073/pnas.78.7.4170>.
4. Wise HM, Foeglein A, Sun J, Dalton RM, Patel S, Howard W, Anderson EC, Barclay WS, Digard P. 2009. A complicated message: identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. *J Virol* 83:8021–8031. <http://dx.doi.org/10.1128/JVI.00826-09>.
5. Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, Basta S, O'Neill R, Schickli J, Palese P, Henklein P, Bennink JR, Yewdell JW. 2001. A novel influenza A virus mitochondrial protein that induces cell death. *Nat Med* 7:1306–1312. <http://dx.doi.org/10.1038/nm1201-1306>.
6. 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>.
7. Wise HM, Hutchinson EC, Jagger BW, Stuart AD, Kang ZH, Robb N, Schwartzman LM, Kash JC, Fodor E, Firth AE, Gog JR, Taubenberger JK, Digard P. 2012. Identification of a novel splice variant form of the influenza A virus M2 ion channel with an antigenically distinct ectodomain. *PLoS Pathog* 8:e1002998. <http://dx.doi.org/10.1371/journal.ppat.1002998>.
8. Selman M, Dankar SK, Forbes NE, Jia J-J, Brown EG. 2012. Adaptive mutation in influenza A virus non-structural gene is linked to host switching and induces a novel protein by alternative splicing. *Emerg Microbes Infect* 1:e42. <http://dx.doi.org/10.1038/emi.2012.38>.
9. Muramoto Y, Noda T, Kawakami E, Akkina R, Kawaoka Y. 2013.

- Identification of novel influenza A virus proteins translated from PA mRNA. *J Virol* 87:2455–2462. <http://dx.doi.org/10.1128/JVI.02656-12>.
10. Zamarin D, Garcia-Sastre A, Xiao X, Wang R, Palese P. 2005. Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1. *PLoS Pathog* 1:e4. <http://dx.doi.org/10.1371/journal.ppat.0010004>.
 11. Le Goffic R, Leymarie O, Chevalier C, Rebours E, Da Costa B, Vidic J, Descamps D, Sallenave JM, Rauch M, Samson M, Delmas B. 2011. Transcriptomic analysis of host immune and cell death responses associated with the influenza A virus PB1-F2 protein. *PLoS Pathog* 7:e1002202. <http://dx.doi.org/10.1371/journal.ppat.1002202>.
 12. McAuley JL, Chipuk JE, Boyd KL, Van De Velde N, Green DR, McCullers JA. 2010. PB1-F2 proteins from H5N1 and 20th century pandemic influenza viruses cause immunopathology. *PLoS Pathog* 6:e1001014. <http://dx.doi.org/10.1371/journal.ppat.1001014>.
 13. McAuley JL, Hornung F, Boyd KL, Smith AM, McKeon R, Bennink J, Yewdell JW, McCullers JA. 2007. Expression of the 1918 influenza A virus PB1-F2 enhances the pathogenesis of viral and secondary bacterial pneumonia. *Cell Host Microbe* 2:240–249. <http://dx.doi.org/10.1016/j.chom.2007.09.001>.
 14. 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>.
 15. Hu J, Zhao K, Liu X, Wang X, Chen Z. 2013. Two highly pathogenic avian influenza H5N1 viruses of clade 2.3.2.1 with similar genetic background but with different pathogenicity in mice and ducks. *Transbound Emerg Dis* 60: 127–139. <http://dx.doi.org/10.1111/j.1865-1682.2012.01325.x>.
 16. Hu J, Hu Z, Song Q, Gu M, Liu X, Wang X, Hu S, Chen C, Liu H, Liu W, Chen S, Peng D. 2013. The PA-gene-mediated lethal dissemination and excessive innate immune response contribute to the high virulence of H5N1 avian influenza virus in mice. *J Virol* 87:2660–2672. <http://dx.doi.org/10.1128/JVI.02891-12>.
 17. Hulse-Post DJ, Franks J, Boyd K, Salomon R, Hoffmann E, Yen HL, Webby RJ, Walker D, Nguyen TD, Webster RG. 2007. Molecular changes in the polymerase genes (PA and PB1) associated with high pathogenicity of H5N1 influenza virus in mallard ducks. *J Virol* 81:8515–8524. <http://dx.doi.org/10.1128/JVI.00435-07>.
 18. Reed LJ, Muench H. 1938. A simple method of estimating fifty percent endpoints. *Am J Hyg (Lond.)* 27:493–497.
 19. 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>.
 20. Oldstone MB. 2013. Lessons learned and concepts formed from study of the pathogenesis of the two negative-strand viruses lymphocytic choriomeningitis and influenza. *Proc Natl Acad Sci U S A* 110:4180–4183. <http://dx.doi.org/10.1073/pnas.1222025110>.
 21. Herfst S, Chutinimitkul S, Ye J, de Wit E, Munster VJ, Schrauwen EJ, Bestebroer TM, Jonges M, Meijer A, Koopmans M, Rimmelzwaan GF, Osterhaus AD, Perez DR, Fouchier RA. 2010. Introduction of virulence markers in PB2 of pandemic swine-origin influenza virus does not result in enhanced virulence or transmission. *J Virol* 84:3752–3758. <http://dx.doi.org/10.1128/JVI.02634-09>.
 22. Zhou B, Li Y, Halpin R, Hine E, Spiro DJ, Wentworth DE. 2011. PB2 residue 158 is a pathogenic determinant of pandemic H1N1 and H5 influenza A viruses in mice. *J Virol* 85:357–365. <http://dx.doi.org/10.1128/JVI.01694-10>.
 23. Hinshaw VS, Olsen CW, Dybdahl-Sissoko N, Evans D. 1994. Apoptosis: a mechanism of cell killing by influenza A and B viruses. *J Virol* 68:3667–3673.
 24. Nichols JE, Niles JA, Roberts NJ, Jr. 2001. Human lymphocyte apoptosis after exposure to influenza A virus. *J Virol* 75:5921–5929. <http://dx.doi.org/10.1128/JVI.73.13.5921-5929.2001>.
 25. Lam WY, Tang JW, Yeung AC, Chiu LC, Sung JJ, Chan PK. 2008. Avian influenza virus A/HK/483/97(H5N1) NS1 protein induces apoptosis in human airway epithelial cells. *J Virol* 82:2741–2751. <http://dx.doi.org/10.1128/JVI.01712-07>.
 26. Brydon EW, Smith H, Sweet C. 2003. Influenza A virus-induced apoptosis in bronchiolar epithelial (NCI-H292) cells limits pro-inflammatory cytokine release. *J Gen Virol* 84:2389–2400. <http://dx.doi.org/10.1099/vir.0.18913-0>.
 27. Morris SJ, Price GE, Barnett JM, Hiscox SA, Smith H, Sweet C. 1999. Role of neuraminidase in influenza virus-induced apoptosis. *J Gen Virol* 80(Part 1):137–146.
 28. Price GE, Smith H, Sweet C. 1997. Differential induction of cytotoxicity and apoptosis by influenza virus strains of differing virulence. *J Gen Virol* 78(Part 11):2821–2829.
 29. Schultz-Cherry S, Dybdahl-Sissoko N, Neumann G, Kawaoka Y, Hinshaw VS. 2001. Influenza virus NS1 protein induces apoptosis in cultured cells. *J Virol* 75:7875–7881. <http://dx.doi.org/10.1128/JVI.75.17.7875-7881.2001>.
 30. Takizawa T, Matsukawa S, Higuchi Y, Nakamura S, Nakanishi Y, Fukuda R. 1993. Induction of programmed cell death (apoptosis) by influenza virus infection in tissue culture cells. *J Gen Virol* 74(Part 11): 2347–2355. <http://dx.doi.org/10.1099/0022-1317-74-11-2347>.
 31. Brydon EW, Morris SJ, Sweet C. 2005. Role of apoptosis and cytokines in influenza virus morbidity. *FEMS Microbiol Rev* 29:837–850. <http://dx.doi.org/10.1016/j.femsre.2004.12.003>.
 32. McLean JE, Ruck A, Shirazian A, Pooyaei-Mehr F, Zakeri ZF. 2008. Viral manipulation of cell death. *Curr Pharm Des* 14:198–220. <http://dx.doi.org/10.2174/138161208783413329>.
 33. Tumpey TM, Lu X, Morken T, Zaki SR, Katz JM. 2000. Depletion of lymphocytes and diminished cytokine production in mice infected with a highly virulent influenza A (H5N1) virus isolated from humans. *J Virol* 74:6105–6116. <http://dx.doi.org/10.1128/JVI.74.13.6105-6116.2000>.
 34. Stasakova J, Ferko B, Kittel C, Sereinig S, Romanova J, Katinger H, Egorov A. 2005. Influenza A mutant viruses with altered NS1 protein function provoke caspase-1 activation in primary human macrophages, resulting in fast apoptosis and release of high levels of interleukins 1 β and 18. *J Gen Virol* 86:185–195. <http://dx.doi.org/10.1099/vir.0.80422-0>.
 35. Zhirnov OP, Ksenofontov AL, Kuzmina SG, Klenk HD. 2002. Interaction of influenza A virus M1 matrix protein with caspases. *Biochemistry (Mosc.)* 67:534–539. <http://dx.doi.org/10.1023/A:1015542110798>.
 36. Zhirnov OP, Konakova TE, Wolff T, Klenk HD. 2002. NS1 protein of influenza A virus down-regulates apoptosis. *J Virol* 76:1617–1625. <http://dx.doi.org/10.1128/JVI.76.4.1617-1625.2002>.
 37. Kobasa D, Jones SM, Shinya K, Kash JC, Copps J, Ebihara H, Hatta Y, Kim JH, Halfmann P, Hatta M, Feldmann H, Alimonti JB, Fernando L, Li Y, Katze MG, Feldmann H, Kawaoka Y. 2007. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. *Nature* 445:319–323. <http://dx.doi.org/10.1038/nature05495>.
 38. Cilloniz C, Pantin-Jackwood MJ, Ni C, Goodman AG, Peng X, Proll SC, Carter VS, Rosenzweig ER, Szretter KJ, Katz JM, Korth MJ, Swayne DE, Tumpey TM, Katze MG. 2010. Lethal dissemination of H5N1 influenza virus is associated with dysregulation of inflammation and lipoxin signaling in a mouse model of infection. *J Virol* 84:7613–7624. <http://dx.doi.org/10.1128/JVI.00553-10>.
 39. Suzuki K, Okada H, Itoh T, Tada T, Mase M, Nakamura K, Kubo M, Tsukamoto K. 2009. Association of increased pathogenicity of Asian H5N1 highly pathogenic avian influenza viruses in chickens with highly efficient viral replication accompanied by early destruction of innate immune responses. *J Virol* 83:7475–7486. <http://dx.doi.org/10.1128/JVI.01434-08>.
 40. Kajihara M, Sakoda Y, Soda K, Minari K, Okamatsu M, Takada A, Kida H. 2013. The PB2, PA, HA, NP, and NS genes of a highly pathogenic avian influenza virus A/whooper swan/Mongolia/3/2005 (H5N1) are responsible for pathogenicity in ducks. *Virol J* 10:45. <http://dx.doi.org/10.1186/1743-422X-10-45>.
 41. Bingham J, Green DJ, Lowther S, Klippel J, Burggraaf S, Anderson DE, Wibawa H, Hoa DM, Long NT, Vu PP, Middleton DJ, Daniels PW. 2009. Infection studies with two highly pathogenic avian influenza strains (Vietnamese and Indonesian) in Pekin ducks (*Anas platyrhynchos*), with particular reference to clinical disease, tissue tropism and viral shedding. *Avian Pathol* 38:267–278. <http://dx.doi.org/10.1080/03079450903055371>.
 42. Löndt BZ, Nunez A, Banks J, Nili H, Johnson LK, Alexander DJ. 2008. Pathogenesis of highly pathogenic avian influenza A/turkey/Turkey/1/2005 H5N1 in Pekin ducks (*Anas platyrhynchos*) infected experimentally. *Avian Pathol* 37:619–627. <http://dx.doi.org/10.1080/03079450802499126>.
 43. Kishida N, Sakoda Y, Isoda N, Matsuda K, Eto M, Sunaga Y, Umemura T, Kida H. 2005. Pathogenicity of H5 influenza viruses for ducks. *Arch Virol* 150:1383–1392. <http://dx.doi.org/10.1007/s00705-004-0473-x>.
 44. Schmolke M, Manicassamy B, Pena L, Sutton T, Hai R, Varga ZT, Hale BG, Steel J, Perez DR, Garcia-Sastre A. 2011. Differential contribution of PB1-F2 to the virulence of highly pathogenic H5N1 influenza A virus in mammalian and avian species. *PLoS Pathog* 7:e1002186. <http://dx.doi.org/10.1371/journal.ppat.1002186>.