PA-X is a virulence factor in avian H9N2 influenza virus

Huijie Gao,1 Guanlong Xu,1 Yipeng Sun,1 Lu Qi,1 Jinliang Wang,1 Weili Kong,1 Honglei Sun,1 Juan Pu,1 Kin-Chow Chang2 and Jinhua Liu1

Correspondence
Jinhua Liu
ljh@cau.edu.cn

1Key Laboratory of Animal Epidemiology and Zoonosis, Ministry of Agriculture, College of Veterinary Medicine and State Key Laboratory of Agrobiotechnology, China Agricultural University, Beijing, PR China

2School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington Campus, Sutton Bonington, UK

H9N2 influenza viruses have been circulating worldwide in multiple avian species, and regularly infect pigs and humans. Recently, a novel protein, PA-X, produced from the PA gene by ribosomal frameshifting, was demonstrated to be an antivirulence factor in pandemic 2009 H1N1, highly pathogenic avian H5N1 and 1918 H1N1 viruses. However, a similar role of PA-X in the prevalent H9N2 avian influenza viruses has not been established. In this study, we compared the virulence and cytopathogenicity of H9N2 WT virus and H9N2 PA-X-deficient virus. Loss of PA-X in H9N2 virus reduced apoptosis and had a marginal effect on progeny virus output in human pulmonary adenocarcinoma (A549) cells. Without PA-X, PA was less able to suppress co-expressed GFP in human embryonic kidney 293T cells. Furthermore, absence of PA-X in H9N2 virus attenuated viral pathogenicity in mice, which showed no mortality, reduced progeny virus production, mild-to-normal lung histopathology, and dampened proinflammatory cytokine and chemokine response. Therefore, unlike previously reported H1N1 and H5N1 viruses, we show that PA-X protein in H9N2 virus is a pro-virulence factor in facilitating viral pathogenicity and that the pro- or antivirulence role of PA-X in influenza viruses is virus strain-dependent.

INTRODUCTION

H9N2 influenza viruses have been circulating worldwide in poultry, resulting in severe economic losses due to reduced egg production or increased mortality associated with co-infection with secondary pathogens (Banks et al., 2000; Bano et al., 2003; Capua & Alexander, 2006). H9N2 influenza viruses have been widely reported to infect mammals, including pigs and humans (Abolnik et al., 2010; Butt et al., 2010; Cong et al., 2007; Sun et al., 2010; Xu et al., 2007); there is evidence that a large number of people have been infected with H9N2 viruses, in particular poultry workers (Coman et al., 2013; Jia et al., 2009; Wang et al., 2009). H9N2 virus infections in humans showed typical human flu-like symptoms, which can easily go undetected or unreported (Butt et al., 2005; Lin et al., 2000). Recent studies showed that H9N2 viruses contributed the six internal genes to the novel H7N9 and H10N8 viruses that are causing severe human infections in China (Chen et al., 2014; Gao et al., 2013; Zhang et al., 2013). H9N2 viruses can be regarded as precursors to emerging subtypes of influenza viruses that are highly infectious to humans. Therefore, it is important to ascertain virulence factors of H9N2 viruses.

Recently, PA-X, arising from ribosomal frameshift in a +1 OTF (X-ORF) extension of a growing PA polypeptide, was identified as a protein (Jagger et al., 2012). It was demonstrated that PA-X plays an important role in inhibiting cellular protein synthesis, suggesting that PA-X contributes to host cell shut-off induced by influenza virus (Desmet et al., 2013; Jagger et al., 2012; Katze et al., 1986a, b). Jagger et al. (2012) also showed that PA-X decreased the virulence of the 1918 H1N1 virus in a mouse model, through modulating host inflammatory response, apoptosis, cell differentiation and tissue remodelling. We recently reported that loss of PA-X expression in 2009 pandemic H1N1 (pH1N1) and highly pathogenic H5N1 viruses increases viral replication and apoptosis in A549 cells, and increases virulence and host inflammatory response in mice (Gao et al., 2015). Loss of PA-X expression also increases the virulence and virus replication of H5N1 virus in avian species, and blunts the host innate immune and cell death response (Hu et al., 2015).
Here, we report that the absence of PA-X in H9N2 virus, contrary to previous findings on pH1N1, highly pathogenic H5N1 and 1918 H1N1 viruses, decreased viral replication and pro-inflammatory response in mice. The absence of PA-X in H9N2 virus also reduced virus-induced suppression of cellular protein synthesis.

RESULTS

Generation of PA-X-deficient H9N2 virus

In the present study, the use of reverse genetics was based on the A/chicken/Hebei/LC/2008 (H9N2 WT) virus (Sun et al., 2011). To evaluate the effect of loss of PA-X expression on viral function, we generated PA-X-deficient virus H9N2-FS by altering the frameshifting motif from UCC UUU CGU to AGC UUC AGA in the PA segment to prevent the formation of PA-X (Fig. 1a) (Jagger et al., 2012). The mutations did not alter the PA ORF. To show that PA-X expression from H9N2-FS was abolished, Madin-Darby canine kidney (MDCK) cells were infected with H9N2 PA-X mutant and WT viruses at m.o.i. 1, and cell lysates were harvested at 12 h post-infection (p.i.). We found that PA-X could be detected in H9N2 WT-infected cells but not in H9N2-FS-infected cells (Fig. 1b).

![Fig. 1. Generation of H9N2 PA-X-deficient viruses. (a) The frameshifting motif of UCC UUU CGU was mutated to AGC UUC AGA (in red) in the PA gene, which did not alter the PA ORF but abrogated the expression of PA-X. (b) PA-X protein expression was abolished in H9N2-FS virus-infected cells. MDCK cells were infected with H9N2-FS and H9N2 WT virus for 12 h. Western blotting was performed on cell lysates with antibodies against PA-X, PB1 or β-actin, as indicated, followed by alkaline phosphatase-conjugated secondary antibodies.](image)

Decreased apoptosis in A549 cells infected with PA-X-deficient H9N2 virus

H9N2 WT and H9N2-FS were used to infect MDCK and human pulmonary adenocarcinoma (A549) cells at m.o.i. 0.01, and the supernatants were collected and titrated at 6, 12, 24, 36, 48, 60, 72, and 84 h p.i. There was no significant difference in the virus output from MDCK cells between H9N2 WT and H9N2-FS viruses (Fig. 2a). In A549 cells, H9N2-FS and H9N2 WT viruses reached maximum virus output at around the same time (48 h p.i.) with comparable peak virus titres; viral titres at indicated time points showed no significant difference between H9N2-FS and H9N2 WT virus (Fig. 2b).

Apoptosis is a contributor to virulence (Roberts & Nichols, 1989; Tumpey et al., 2000). Some viral proteins are able to induce apoptosis, such as NS1 and PB1-F2 (Chanturiya et al., 2004; Chen et al., 2001; Zhirnov et al., 2002). A549 cells were infected with H9N2-FS and H9N2 WT viruses at m.o.i. 1 for 6 and 12 h, and assessed for apoptosis. H9N2-FS virus infection produced less apoptotic cells (1.40 % Annexin-V+ only at 6 h p.i. and 6.59 % at 12 h p.i.) than H9N2 WT virus (3.60 % at 6 h p.i. and 10.73 % at 12 h p.i.) (P<0.05) (Fig. 2c). Cells that were propidium iodide (PI)+ only and Annexin-V+PI+ showed no significant difference between H9N2-FS and H9N2 WT viruses. Overall, the data showed that loss of PA-X in H9N2 virus had little effect on viral replication and produced less apoptosis in A549 cells.

PA-X-deficient H9N2 virus is less pathogenic and causes mild inflammatory response in mice relative to H9N2 WT virus

To assess the effect of PA-X on pathogenicity, mice (15 per group) were intranasally inoculated at 10⁶ TCID₅₀ with each virus. Clinical signs, mortality and weight loss were monitored over 14 days. Three virus-infected mice per day were humanely killed at 3, 5 and 7 days p.i., and lungs were collected for virus titration. H9N2-FS virus infection resulted in no death, whilst H9N2 WT infection caused 33.3 % mortality (Fig. 3a). No significant weight loss was observed in the H9N2-FS virus-infected group, in contrast to the 15 % weight loss in H9N2 WT virus-infected mice (Fig. 3b).

Histopathologically, H9N2-FS virus-infected lung appeared nearly normal. However, in the H9N2 WT virus group, there was extensive vascular congestion and cellular exudate (Fig. 3c). Viral titres of H9N2-FS virus-infected lungs were 8- to 20-fold lower than those of H9N2 WT virus-infected lungs at 3, 5 and 7 days p.i. (P<0.05) (Fig. 3d), consistent with the observed pathology.

Increased pulmonary cytokine/chemokine expression contributes to the severity of influenza virus infection in humans and animal models (Bermejo-Martin et al.,...
Contribution of PA-X to the virulence of the H9N2 virus

Inhibition of host protein synthesis by influenza virus can hinder host antiviral response and promote virus replication (Katze et al., 1986a, b). The PA gene plays a major role in the suppression of host protein synthesis, which is partly mediated by PA-X (Desmet et al., 2013; Jagger et al., 2012). We compared the ability of PA of H9N2 WT and H9N2-FS viruses to suppress non-viral protein synthesis by co-transfections of human embryonic kidney 293T cells for 24 h with H9N2 WT PA or H9N2-FS PA, and pEGFP expression plasmids. EGFP expression was significantly higher by > 20 % when co-transfected with

PA protein is less effective at suppressing protein expression without PA-X

2010; Hagau et al., 2010; Lam et al., 2010; Perrone et al., 2008). We determined the protein levels of seven cytokines and chemokines in the lungs of H9N2 WT and H9N2-FS virus-infected mice at 3 and 5 days p.i. IL-1β, IL-6, the mouse equivalent of human IL-8 (KC), monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α (CCL3), TNF-α and IFN-γ levels from H9N2-FS virus-infected mice were consistently lower than those of H9N2 WT virus-infected mice at both time points (P<0.05) (Fig. 4). Collectively, these results demonstrated that PA-X in H9N2 virus facilitated pathogenicity and upregulated inflammatory response in mice.
H9N2-FS PA compared with H9N2 WT PA co-transfection (Fig. 5a, b). Although the expression level of PA protein of H9N2 was higher than that of H9N2 WT, H9N2-FS PA plasmid was less effective in suppressing EGFP expression than H9N2 WT PA (Fig. 5b). These results suggested that loss of PA-X in H9N2 virus reduced the host shut-off ability of the virus in 293T cells.

**DISCUSSION**

In the present study, we assessed the pathogenic capability of PA-X in avian H9N2 virus. PA-X-deficient H9N2 (H9N2-FS) virus was less virulent than its H9N2 WT counterpart. Absence of PA-X attenuated the H9N2 virus as manifested by decreased viral replication, reduced apoptosis and dampened pro-inflammatory response. Furthermore, PA without PA-X was less able to suppress host protein synthesis. Therefore, we propose that PA-X is a virulence factor in H9N2 virus.

H9N2-FS WT virus-infected mice showed significant weight loss, unlike H9N2-FS virus- and mock-infected mice. Any mouse that lost >30% of its body weight was euthanized. Histopathology in lung of H9N2-FS virus-infected mice was mild-to-normal compared with corresponding H9N2 WT virus infection, which showed vascular congestion and cellular infiltration of bronchioles and alveoli. Bar, 100 μm. (d) Viral lung load based on log_{10}TCID_{50} determination in MDCK cells. *Significant difference between H9N2 WT and H9N2-FS virus (P<0.05). Data represent mean ± SD of three mice per group.

Previous studies, however, have shown that PA-X-deficient H1N1 and H5N1 viruses are more pathogenic than their corresponding WT counterparts (Gao et al., 2015; Hu et al., 2015; Jagger et al., 2012). Enhanced virulence of the 1918 H1N1 virus deficient in PA-X might be due to alterations in the kinetics of host response (Jagger et al., 2012). Jagger et al. (2012) found that loss of PA-X expression in the 1918 pandemic virus upregulated inflammatory, apoptotic and T-lymphocyte signalling pathways.
Elevated virulence, manifested as increased PA expression, ribonucleoprotein polymerase activity and inflammatory response, was seen in PA-X-deficient in pH1N1 and highly pathogenic H5N1 avian influenza viruses (Gao et al., 2015). PA-X is also an antivirulence factor of avian H5N1 virus in avian species as well as in mice (Hu et al., 2015). H5N1 PA-X blunted the global host response in chicken lungs, which included markedly downregulated genes associated with inflammation and cell death, and promoted anti-apoptotic activity in chicken and duck fibroblasts (Hu et al., 2015). In the present study, we found that PA-X played an opposite role in H9N2 virus. Apoptotic and inflammatory responses were decreased with H9N2 PA-X-deficient virus. These observations indicate that the pro- or antivirulence role of PA-X in influenza viruses is virus strain-specific.

Loss of PA-X expression decreased viral replication of H9N2 virus in vivo. H9N2 PA-X-deficient virus showed lower replication levels in mice than H9N2 WT at 3, 5 and 7 days p.i. The poor replication in murine lungs was directly related to the lower pathogenicity and reduced expression of inflammatory cytokines from H9N2 PA-X-deficient virus infection. Hu et al. (2015) showed that PA-X decreases the virulence of H5N1 virus through inhibiting viral replication and the host innate immune response. Jagger et al. (2012) showed that loss of PA-X in 1918 H1N1 virus did not affect viral replication in mice.

**Fig. 4.** Detection of cytokine/chemokine proteins in lungs of mice infected with H9N2 WT and H9N2-FS viruses. Mean ± SD cytokine/chemokine levels are shown (n=3). *Significant difference between H9N2-FS and H9N2 WT (P<0.05).
but increased pathogenicity through enhanced host immune response.

Influenza virus infection can induce host shut-off with a rapid decline of host protein synthesis (Katze et al., 1986a, b) to divert host resources towards viral replication. Inhibition of host protein synthesis also aids in dampening the antiviral response. Therefore, virus-induced host shut-off is closely related to viral replication and pathogenicity.

Recently, the roles of PA and PA-X in the inhibition of cellular protein synthesis were demonstrated (Desmet et al., 2013; Jagger et al., 2012). The N-terminal domain of PA, which includes the endonuclease active site, is sufficient to suppress protein expression and PA-X showed a stronger effect than the corresponding N-terminal domain of PA. We previously showed that the absence of PA-X resulted in PA being less able to suppress co-transfected gene expression for pH1N1 and H5N1.
viruses (Gao et al., 2015). Conceivably, loss of PA-X in these viruses could inhibit host protein synthesis less effectively, which would result in reduced viral replication and virulence. However, loss of PA-X does enhance the virulence of 1918 H1N1, pH1N1 and H5N1 viruses (Gao et al., 2015; Hu et al., 2015; Jagger et al., 2012). We speculate that the decrease in suppression of host protein synthesis exacerbates the host inflammatory response and enhances apoptosis. As 1918 H1N1, pH1N1 and highly pathogenic H5N1 viruses could elicit significantly high levels of pro-inflammatory cytokines, loss of PA-X in such viruses could lead to more severe lung injury and contribute to the enhanced virulence (Kang et al., 2011; Ma et al., 2011; Perrone et al., 2008). In the present study, H9N2 virus is largely a low pathogenicity virus and does not typically induce high levels of cytokines. PA protein in H9N2 virus was less able to suppress GFP expression in the absence of PA-X, suggesting that PA-X also plays a role in the inhibition of host protein synthesis. The level of host shut-off by H9N2-FS could be less effective in promoting viral replication, but more effective in eliciting an antiviral response. In summary, our results show that PA-X of H9N2 virus, unlike the more virulent H5N1, pH1N1 and 1918 H1N1 viruses, is a pro-virulence factor in the facilitation of viral replication and pathogenicity, and that the function of PA-X is virus strain-specific. Therefore, the role of PA-X in other influenza viruses needs to be investigated.

METHODS

Viruses and cells. A/chicken/Hebei/LC/2008 (HB/08, H9N2) virus was isolated from a diseased chicken in Hebei Province, China, in January 2008 and propagated in 10-day-old specific pathogen-free embryonated chicken eggs (Sun et al., 2011). 293T, MDCK and A549 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 10 % FBS (Life Technologies), 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹.

Generation of recombinant viruses by reverse genetics. All eight gene segments were previously amplified by reverse transcription (RT)-PCR from HB/08 virus and cloned into the dual-promoter plasmid pHW2000 (Sun et al., 2011). PA-X-deficient virus H9N2-FS was created by site-directed mutagenesis (QuikChange mutagenesis kit; Agilent) on the corresponding PA gene of H9N2 WT virus, which converted the frameshifting motif from UCU UUU CGU to AGC UUC AGA (U592A, C593G, U597C, C598A and U600A) to prevent the formation of PA-X (Jagger et al., 2012). PCR primer sequences used are available upon request. The PA ORF was unaltered in H9N2-FS. Rescued viruses were detected using haemagglutination assays. The viruses were purified by sucrose density gradient centrifugation; viral RNA was extracted and analysed by RT-PCR, and each viral segment was sequenced to confirm sequence identity.

Viral titration and replication kinetics. TCID₅₀ was determined in MDCK cells using 10-fold serially diluted virus inoculated at 37 °C and cultured for 72 h. The TCID₅₀ values were calculated by the method of Reed & Muench (1938). MDCK and A549 cells were infected with viruses at m.o.i. 0.01, overlaid with serum-free DMEM containing TPCK-trypsin (2 µg ml⁻¹, Sigma-Aldrich) and incubated at 37 °C. Supernatants of infected MDCK and A549 cells were harvested at 6, 12, 24, 36, 48, 60, 72 and 84 h p.i. Virus titres were determined by TCID₅₀ in MDCK cells. Three independent experiments were performed.

Mouse infections. Fifteen mice (6-week-old female BALB/c; Vital River Laboratory) per group were anesthetized with Zoletil (tiletamine-zolazepam; Virbac; 20 µg g⁻¹) and inoculated intranasally with 50 µl 10⁶ TCID₅₀ H9N2 diluted in PBS. All mice were monitored daily for 14 days and mice losing 30 % of their original body weight were humanely euthanized. Three mice were euthanized on 3, 5 and 7 days p.i. for the determination of lung virus titres, histopathology and cytokine levels. Lungs were collected and homogenized in cold PBS. Virus titres were determined by TCID₅₀. All animal research was approved by the Beijing Association for Science and Technology and complied with Beijing Laboratory Animal Welfare and Ethical Guidelines as issued by the Beijing Administration Committee of Laboratory Animals.

Histopathology. A portion of the lung from each euthanized mouse at 5 days p.i. was fixed in 10 % phosphate-buffered formalin and processed for paraffin embedding. Each 5 µm section was stained with haematoxylin and eosin, and examined for histopathological changes. Images were captured with a Zeiss AxioPlan 2IE epifluorescence microscope.

Quantification of cytokine/chemokine protein levels in mouse lungs. Levels of cytokines/chemokines, including IFN-γ, IL-1β, IL-6, KC, TNF-α, MIP-1α (CCL3) and MCP-1, in lungs were determined by cytometric bead array assays [BD Cytometric Bead Array (CBA) Mouse Inflammation kit; BD Bioscience]. Briefly, 50 µl mouse inflammation capture bead suspension and 50 µl detection reagent were added to an equal amount of sample and incubated in the dark for 2 h at room temperature. Subsequently, each sample was washed with 1 ml wash buffer and then centrifuged at 200 g at room temperature for 5 min. Supernatants were discarded and a further 300 µl wash buffer was added. Samples were analysed on a FACSArray bioanalyser (BD Bioscience). Data were analysed using BD CBA software (BD Bioscience). Each chemokine or cytokine was computed as pg (ml homogenate)⁻¹.

Cell death assays. Virus infection assays were conducted in six-well plates. Cells were seeded at a density of 1 × 10⁶ cells per well for overnight incubation in infection media (cell growth media with 1 % BSA was used in place of FBS). Cells were then infected with virus at m.o.i. 1 for 12 h. Cells pooled from the supernatant and monolayer were then harvested, washed and stained with FITC-labelled Annexin-V and PI (Becton Dickinson) for 20 min. After a final wash, cells were resuspended in 100 µl FACS wash buffer (PBS containing 3 % BSA and 0.01 % sodium azide) and analysed on a FACSCalibur (BD Biosciences) with FlowJo software (version 7.6.1). Cell death (apoptosis and necrosis) was defined as Annexin-V- and PI⁺, whilst apoptotic cells were Annexin-V⁺ only. Viable cells were considered as neither Annexin-V⁻ nor PI⁺.

Western blotting. Total cell protein lysates were extracted from transfected 293T cells or infected MDCK cells with CA630 lysis buffer (150 mM NaCl, 1 % CA630 detergent, 50 mM Tris base, pH 8.0). Cellular proteins were separated by 12 % SDS-PAGE and transferred to a PVDF membrane (Amersham Biosciences). Each PVDF membrane was blocked with 0.1 % Tween 20 and 5 % non-fat dry milk in TBS, and subsequently incubated with a primary antibody. Primary antibodies were specific for influenza A virus PA (1 : 3000; GeneTex), influenza A virus PA-X (1 : 2000), polyclonal rabbit antiserum against a H5N1 X-ORF-derived peptide (CAGLPTKVSHRTSPA; Genscript), influenza A virus PB1 (1 : 3000; Thermo Fisher Scientific), GFP (1 : 1000; Abcam) and β-actin (1 : 1000; Santa Cruz). The secondary antibody used was either HRP-conjugated anti-mouse antibody or HRP-conjugated anti-rabbit antibody (1 : 10 000; Jackson Immuno-Research) as appropriate. HRP presence was detected using a Western.
Lightning chemiluminescence kit (Amersham Pharmacia), following the manufacturer’s protocol.

**Statistics.** All statistical analyses were performed using Prism version 5.0 (GraphPad). The two treatment methods were compared by two-tailed Student’s t-test, and multiple comparisons were carried out by two-way ANOVA considering time and virus as factors. Differences were considered statistically significant at \( P < 0.05 \).

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