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

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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 coinfection 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 (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.](insert)

(a) Nucleotide sequence of PA

<table>
<thead>
<tr>
<th>WT</th>
<th>592</th>
<th>UCC</th>
<th>UUU</th>
<th>600</th>
<th>CGU</th>
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<tr>
<td>FS</td>
<td>AGC</td>
<td>UUC</td>
<td>AGA</td>
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(b) Amino acids of PA

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<thead>
<tr>
<th>190</th>
<th>191</th>
<th>192</th>
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<td>S</td>
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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.,

![Fig. 2. Apoptosis was evaluated in A549 cells infected with PA-X-deficient H9N2 virus.](insert)

(a) 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.
PA protein is less effective at suppressing protein expression without PA-X

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

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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 (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.

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 g 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 (QuickChange mutagenesis kit; Agilent) on the corresponding PA gene of H9N2 WT virus, which converted the frameshifting motif from UCC UUU CGU to AGC V and PI (Becton Dickinson) for 20 min. After a final wash, cells were resuspended in 100 fL FACS wash buffer (PBS containing 3 % BSA and 0.01 % sodium azide) and analysed on a FACS Calibur (BD Biosciences). Data were analysed using BD CBA software (BD Bioscience). Samples were analysed on a FACSArray bioanalyser (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 × 10\(^5\) 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 fL 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 (MA 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), GHP (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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