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PA-X protein contributes to virulence of triple-reassortant H1N2 influenza virus by suppressing early immune responses in swine

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ABSTRACT

Previous studies have identified a functional role of PA-X for influenza viruses in mice and avian species; however, its role in swine remains unknown. Toward this, we constructed PA-X deficient virus (Sw-FS) in the background of a Triple-reassortment (TR) H1N2 swine influenza virus (SIV) to assess the impact of PA-X in viral virulence in pigs. Expression of PA-X in TR H1N2 SIV enhanced viral replication and host protein synthesis shutoff, and inhibited the mRNA levels of type I IFNs and proinflammatory cytokines in porcine cells. A delay of proinflammatory responses was observed in lungs of pigs infected by wild type SIV (Sw-WT) compared to Sw-FS. Furthermore, Sw-WT virus replicated and transmitted more efficiently than Sw-FS in pigs. These results highlight the importance of PA-X in the moderation of virulence and immune responses of TR SIV in swine, which indicated that PA-X is a pro-virulence factor in TR SIV in pigs.

1. Introduction

Influenza A virus continues to result in high morbidity and mortality in both humans and animals despite numerous efforts to produce anti-influenza drugs and vaccines. The high intrinsic mutation rate and the specific structure of influenza A virus' genome is likely a key factor to its capacity to infect multiple mammalian and avian species. Initially, it was deemed that 8 genome segments of influenza virus encoded 10 proteins (Lamb and Lai, 1980; Lamb et al., 1981; Palese, 1977). However, 7 more proteins, PB1-N40, PB1-F2, PA-X, M42, NS3, PA-N155, and PA-N182, have been recently discovered (Chen et al., 2001; Jagger et al., 2012; Muramoto et al., 2013; Selman et al., 2012; Wise et al., 2009, 2012). It is important to pay close attention to the functions of novel proteins as their presence may further complicate the pathogenesis of influenza virus.

PA-X was recently identified as a fusion protein containing a common N-terminal endonuclease domain of 191 amino acids derived from the segment 3 open reading frames fused to a unique C-terminal region of 41 or 61 amino acids, derived from the +1 frameshift open reading frame (X-ORF) (Jagger et al., 2012; Yewdell and Ince, 2012). Comprehensive evolution analysis has shown that the PA-X gene is conserved in influenza viruses, which suggests that PA-X may have functional importance for influenza viruses (Shi et al., 2012). It has been shown that the absence of PA-X expression increased the pathogenicity of the 1918 pandemic H1N1 in mice and highly pathogenic avian H5N1 influenza viruses in mice and multiple avian species (Gao et al., 2015b; Hu et al., 2015; Jagger et al., 2012). However, loss of PA-X attenuated the replication and pathogenicity of avian H9N2 influenza virus in mice (Gao et al., 2015c). Besides, the contrary effects of PA-X on the virulence of 2009 pandemic H1N1 influenza virus have been shown by different studies (Gao et al., 2015b; Hayashi et al., 2015). Phylogenetic analysis suggested that the truncation of PA-X particularly occurred in pigs and dogs (Shi et al., 2012). These results revealed that the function of PA-X may possess strain- or host-specificity. Therefore, the role of PA-X in the virulence of influenza virus in other influenza viruses and hosts remains unclear.

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Pigs have been postulated to play an important role in interspecies transmission by acting as the "mixing vessel" for reassortment between viruses specific to different host species (Scholtissek, 1994). The "triple-reassortment" (TR) swine influenza viruses (SIVs) continued circulating in swine, which has in turn generated a range of additional reassortants. For example, the 2009 pandemic H1N1 influenza virus was proposed to have generated in pigs through reassortment between well-established swine influenza lineages, the "Eurasian avian-like" (EA) lineage, and the North American TR lineage (Garten et al., 2009; Smith et al., 2009; Trifonov et al., 2009). Furthermore, the North American TR lineage SIVs have been detected in Asian swine populations, and showed increased virulence and efficient transmissibility in ferrets, which have caused sporadic human infections (Gray et al., 2007; Pascua et al., 2012; Shinde et al., 2009). However, the function of PA-X protein in SIV in pigs remains unclear. To determine the function of PA-X in SIV, we constructed a PA-X-deficient TR H1N2 SIVs, and compared its biological characteristics *in vitro* and *in vivo* with the wild type strain.

2. Results

2.1. PA-X enhances viral growth in porcine cells and respiratory explants

A reverse genetics system of wild-type H1N2 TR SIV A/swine/Guangdong/1222/2006 (Sw-WT) was established as previously described (Bi et al., 2010; Xu et al., 2016). To evaluate the effect of PA-X on viral function, we generated a PA-X-deficient virus, Sw-FS, by changing the frameshifting (FS) motif from UCC UUU CGU to AGC UUC AGA in segment 3, preventing the formation of PA-X (Fig. 1A). The abolition of the ribosomal frameshift site in Sw-FS did not alter the PA ORF. To test the expression levels of PA-X protein, porcine kidney (PK15) cells were infected with indicated viruses (Sw-WT and Sw-FS) at an multiplicity of infection (MOI) of 5.0. The result showed that PA-X could be detected in PK15 cells infected in Sw-WT but not Sw-FS virus by Western blot (Fig. 1B).

To evaluate the effect of PA-X on the replicative abilities of H1N2 recombinant viruses *in vitro*, the multicycle replication kinetics of PA-X Sw-WT and Sw-FS viruses were compared in PK15 or porcine alveolar macrophages (PAMs) cells infected with each virus at an MOI of 0.01 or 0.1, respectively. In PK15 cells, the virus titers of Sw-FS were significantly lower than Sw-WT from 24 to 48 h post-inoculation (hpi) ($P < 0.05$); similarly, PA-X also significantly increased H1N2 virus replication in PAMs from 12 to 36 hpi ($P < 0.05$) (Fig. 2). An *ex vivo* organ-culture model of the pig respiratory tract maintained at an air-liquid interface as a biologically relevant *in vitro* system was used to study the replication of H1N2 SIVs (Pena et al., 2012b). Briefly, porcine nasal turbinate, trachea, and lung explants cultured in 12-well plates were infected with 10^6 $TCID_{50}$ of each virus, and virus titers were subsequently tested at 24, 48 and 72 hpi. The Sw-FS virus showed weaker replicability than Sw-WT virus in explants of nasal turbinate and trachea at all 3 time-points, and in lung explants at 24 and 48 hpi ($P < 0.05$) (Fig. 3). Collectively, these data indicated that PA-X contributed to the growth ability of H1N2 SIV both *in vitro* and *ex vivo*.

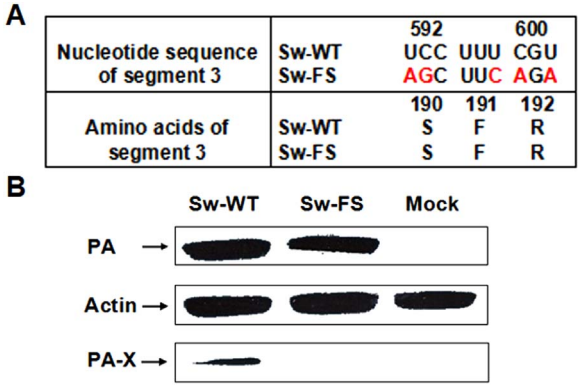

Fig. 1. Generation of PA-X deficient PA-X in H1N2 swine influenza virus. (A) Mutation sites in the segment 3 of the PA-X-deficient H1N2 viruses. The red letters represent mutation sites. (B) Western blotting for the detection of PA, PA-X, and β-actin expression in PK15 cells infected with indicated viruses (Sw-WT and Sw-FS) at an MOI of 5.0.

2.2. PA-X contributes to the suppression of non-viral protein expression and host inflammatory responses in vitro

PA-X has been demonstrated to have a role in host-cell shutoff, which results in a rapid decline of cellular protein synthesis (Gao et al., 2015a, 2015b; Hayashi et al., 2015; Jagger et al., 2012). To assess the contribution of PA-X from SIV in this function, we compared the ability of PA gene of Sw-WT and Sw-FS viruses to suppress non-viral synthesis by co-transfection for 24 h with pEGFP and individual segment 3 plasmids in Human embryonic kidney (293 T) cells. The results showed that the levels of eGFP expression were significantly higher by more than 20% after loss of PA-X ($P < 0.05$) (Fig. 4A). Newly synthesized eGFP and PA proteins were also analyzed by Western blot. As shown in Fig. 4B, eGFP synthesis was strongly suppressed in cells expressing Sw-WT PA at 24hpt. While all of the PA proteins were expressed at similar levels.

Next, to determine the impact of PA-X on host-cell protein synthesis, PAM cells were either uninfected or infected with Sw-WT or Sw-FS at MOI of 5.0, and at 12 h after infection, cells were lysed to determine the β-actin synthesis in infected cells. As shown in Fig. 4C, Sw-WT, but not Sw-FS, strongly reduced β-actin protein expression in PAM cells.

Host shutoff induced by PA-X could also limit host innate immune responses (Gao et al., 2015b, 2015c; Hayashi et al., 2015; Hu et al., 2015; Jagger et al., 2012). To determine whether PA-X altered the induction or magnitude of type I IFNs (IFN-I) and proinflammatory cytokines, we compared cytokine mRNA profiles in PAM cells infected with Sw-WT and Sw-FS viruses at 1.0 MOI at which infection dose showed no statistical differences in viral titers during 6–24 hpi (data not shown). As shown in Fig. 5A, a comprehensive upregulation of IFN-α, IFN-β, TNF-α, IL-1β, IL-6, and IL-12 mRNAs was observed after infecting cells with Sw-WT or Sw-FS viruses. Meanwhile, it is noteworthy that SIV with PA-X showed significantly stronger inhibition from type I IFNs and proinflammatory cytokine mRNAs expression than PA-X deficient virus at 6 and 12 hpi ($P < 0.05$) (Fig. 5A).

IFN-I establishes an antiviral state in infected cells that functions to inhibit viral replication and restrict viral spread. To further confirm the direct effect of PA-X protein on IFN-I production, we expressed the PA-X proteins ectopically in 293 T cells and then infected them with Sendai virus to stimulate the innate immune response. The IFN-β mRNA profiles in 293 T cells were tested at 24hpt. As shown in Fig. 5B, IFN-β mRNA levels in cells expressing full-length PA-X was 3-fold lower than cells expressing PA-X protein from Sw-FS, which only expressed a common N-terminal endonuclease domain of 191 amino acids by changing the frameshifting motif from UCC UUU CGU to AGC UUC AGA in PA segment. These results indicated that PA-X protein could efficiently suppress IFN-I mRNA expression *in vitro*. Taken together, these findings indicated that PA-X, especially its unique C-terminal sequence, contributed to the suppression of non-viral protein synthesis and host innate immune responses.

2.3. PA-X expression is required for efficient virus replication and transmissibility in pigs

To evaluate the effects of PA-X on the virulence of SIV in pigs, 9 pigs from each group were inoculated intranasally with 10^6 $TCID_{50}$ of the Sw-WT or Sw-FS virus, and 3 infected pigs from each group were moved to a separate room with 3 naïve pigs at 24 hpi. Five naïve pigs