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REVIEW

PA-X: a key regulator of influenza A virus pathogenicity and host immune responses

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Abstract

PA-X, a fusion protein belonging to influenza A viruses (IAVs), integrating the N-terminal 191 amino acids of PA gene and the ribosomal frame-shifting product that lengthens out to 41 or 61 amino acids. Since its discovery in 2012, multiple functions have been attributed to this small protein, including a process, where wide-spread protein synthesis in infected host cells is shut down (called host shutoff), and viral replication, polymerase activity, viral-induced cell apoptosis, PA nuclear localization, and virulence are modulated. However, many of its proposed functions may be specific to strain, subtype, host, or cell line. In this review, we start by describing the well-defined global host-shutoff ability of PA-X and the potential mechanisms underlying it. We move on to the role played by PA-X in modulating innate and acquired immune responses in the host. We then systematically discuss the role played by PA-X in modulating the virulence of influenza viruses of different subtypes and host origins, and finish with a general overview of the research advances made in identifying the host cell partners that interact with PA-X. To uncover possible clues about the differential effects of PA-X in modulating viral virulence, we focus on systemically evaluating polymorphisms in PA-X from various viral subtypes and hosts, including avian and human H5N1, H5N6, H9N2, and H7N9 viruses. Finally, we conclude with a proposition regarding the possible future research directions for this important protein.

Keywords Influenza virus · PA-X · Host shutoff · Immunomodulatory proteins · Pathogenesis · Virus–host interaction

Abbreviations

IAV	Influenza A viruses
PB2	Polymerase basic protein 2
PB1	Polymerase basic protein 1
PA	Polymerase acidic protein
HA	Hemagglutinin
NP	Nucleoprotein
NA	Neuraminidase
M	Matrix protein
NS	Non-structural protein
AIVs	Avian influenza viruses
GISAID	Global initiative on sharing avian influenza data
RdRP	RNA-dependent RNA polymerase RdRp complex
EIV	Equine influenza virus
CIV	Canine influenza virus
TR	Triple-reassortment
SIV	Swine influenza virus
MHC	Major histocompatibility complex
RIG-I	Retinoic acid-induced gene protein I
CPSF30	Cleavage and polyadenylation specificity factor 30
IPS-1	Interferon beta promoter stimulating factor 1
NLRP3	NOD-like receptor family pyrin domain containing 3
AP-MS	Affinity purification and mass spectrometryAP-MS
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HSV-1	Herpes simplex virus 1
RSV	Respiratory syncytial virus

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JEV	Japanese encephalitis virus
CCHFV	Crimean-congo hemorrhagic fever virus
HIV	Human immunodeficiency virus
HPAIV	Highly pathogenic avian influenza virus
LPAIV	Low pathogenic avian influenza virus

Introduction

Influenza A virus (IAV) is the most diverse and epidemiologically significant pathogen associated with severe disease manifestations in humans [1]. Wild aquatic birds are the natural reservoirs for IAV, but it can infect a variety of animals, including poultry, aquatic animals (e.g., seals, dolphins, and whales) and terrestrial mammals (e.g., humans, horses, pigs, mink, cats, dogs, and tigers) [2, 3]. Two common mechanisms are used by IAV to cross the host barrier and acquire high virulence in various animals. One mechanism involves the acquisition of adaptive mutations and/or genetic reassortment, a well-known strategy used by several pandemic viruses, including H1N1 (1918 Spanish flu), H2N2 (1957 Asian flu), H3N2 (1968 Hong Kong flu), H1N1 (2009 pandemic), and H7N9 (2013 Chinese epidemic) [4–8]. Another virulence mechanism is where multiple viral accessory proteins are encoded on a single gene segment. The IAV genome comprises eight negative-sense RNA segments that were initially assumed to encode the following ten proteins: polymerase basic proteins 1 (PB1) and 2 (PB2), polymerase acidic protein (PA), nucleoprotein (NP), hemagglutinin (HA), neuraminidase (NA), matrix proteins 1 (M1) and 2 (M2), and non-structural proteins 1 (NS1) and 2 (NS2). However, over the past 16 years, another seven novel proteins have been gradually discovered, including PB1-F2 [9], PB1-N40 [10], PA-X [11], M42 [12], NS3 [13], PA-N155, and PA-N182 [14]. Among these accessory proteins, PB1-F2 and PA-X, have been extensively studied and found to share some similarities in modulating virulence in IAV.

Here, we review current knowledge on the PA-X protein, including the polymorphism characteristics among the different virus subtypes and host species, host-shutoff activity, the role of PA-X in modulating host innate and adaptive responses, the contribution of PA-X to the pathogenesis of IAV, and provide a summary of known PA-X host partners. Finally, we propose several potential research areas that may accelerate understanding about the role played by this novel protein during influenza virus infections, especially for outbreaks of human infections with emerging IAVs, such as H7N9 virus.

Polymorphisms in the PA-X protein from viruses with different subtypes and host origins

The PA-X protein is a fusion protein with an N-terminal 191 amino acid leader sequence originating from PA and a C-terminal region of 61 or 41 codons encoded by an overlapping open-reading frame (ORF) ("X-ORF"), which is accessed by one ribosomal frame shift in the PA gene (Fig. 1). Based on the lineage-specific differences in the distribution of X-ORF lengths, two major X-ORF groups have been classified [11, 15]. About 75% of the isolates have a 61-codon X-ORF, which basically covers all host species and HA/NA subtypes [15]. The remaining 25% carry the truncated form of PA-X, where a 41-codon X-ORF created by nonsense mutations occurs mainly at codon 42 [15]. These truncated PA-X proteins overwhelmingly come from the 2009 human pandemic H1N1 virus, the triple reassortant swine H1N1 virus (a cluster of classic swine H1N1 viruses), equine H7N7, canine H3N8, canine H3N2, and bat influenza virus [15]. Of note, it has been shown that the truncated form of PA-X evolved convergently in viruses from pigs or dogs (H3N2 and H3N8), suggesting that it is associated with the adaptation and emergence of influenza virus in these host species [15].

Global host-shutoff activity by the PA-X protein

Influenza virus infection results in a rapid decline of global host protein synthesis in infected cells, a process known as host shutoff. This process allows the virus to escape host innate immune recognition and shut down host antigen processing to subvert acquired immunity, enabling it to escape host restriction and promote its multiplication and spread. It has been shown that multiple mechanisms are related to host shutoff in IAV-infected cells. However, recent studies have focused on the following three main mechanisms of global inhibition of host protein expression by IAV infection: (1) blockade of cellular mRNA processing and nuclear export by NS1 [16–19]; (2) degradation of host RNA Pol II by the viral RNA-dependent RNA polymerase RdRp complex (RdRP) [20–22]; and (3) wide-spread host mRNA degradation by the PA-X protein [11, 23–31]. In this review, we focus mainly on the shutoff activity of PA-X protein.

Discovery of host-shutoff ability by the PA-X protein

By co-transfecting reporter plasmids that encode β-galactosidase or the 1918 NP gene together with the wild

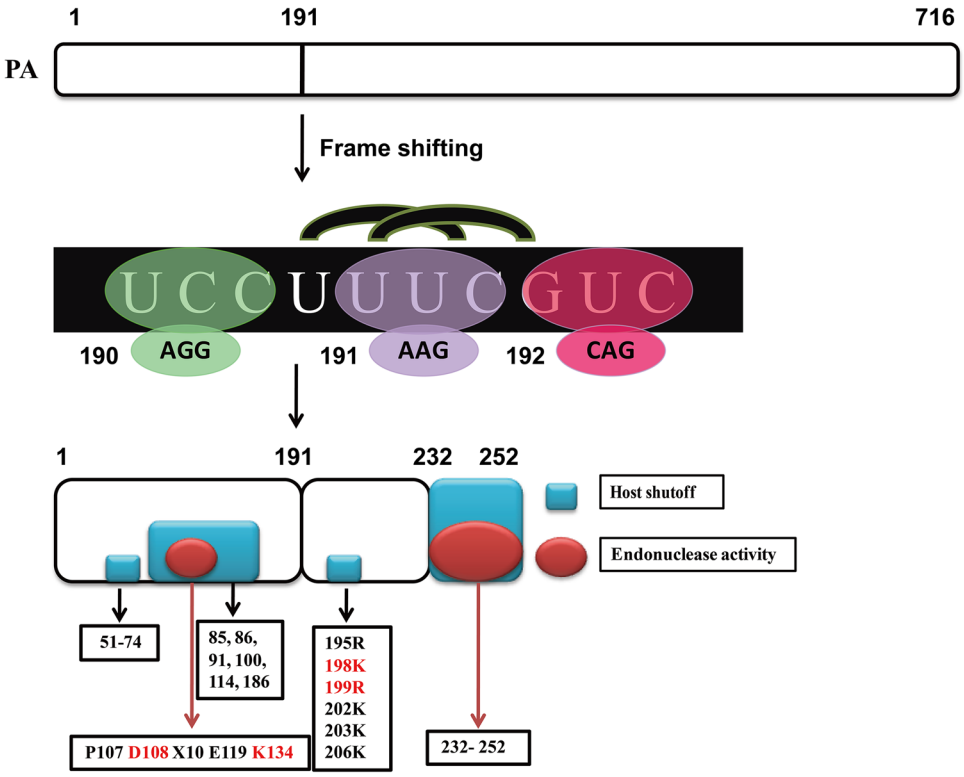

Fig. 1 Proposed mechanism of PA-X protein formation and the functional area in PA-X. The PA-X open-reading frame encodes either 61 or 41 amino acids as indicated. In addition, the X-ORF product lies largely within a linker region between the PA N- and C-terminal domains. The frame-shifting motif locates in the sequence of **UCC UUU CGUC**, after shifting, the sequence changed to **UUC GUC**. Functional domains are distinguished by shape and color: blue bar (areas that important for host-shutoff activity) and red ellipse (areas that important for endonuclease activity)

type or the PA-X-deficient 1918 PA segment, Jagger et al. were the first researchers to discover that PA-X can mediate the suppression of plasmid-driven gene expression [11]. Using global transcriptional profiling, they further demonstrated that down-regulation of PA-X expression leads to an accelerated global host response in virus-infected mouse lungs, notably inflammatory, apoptotic, and T lymphocyte responses [11]. Many later studies have also reported on PA-X-mediated host-shutoff activity in human H1N1 virus [23, 26, 27, 29, 32–34], avian H9N2 virus [35], avian H5N1 virus [24, 36], equine influenza virus (EIV) and canine influenza virus (CIV) of the H3N8 subtype [37], swine H1N2 influenza virus [38], and triple-reassortment (TR) H1N2 swine influenza virus (SIV) [39]. Therefore, the universal expression of PA-X in IAVs and the relatively conservative genetic characteristics of the X-ORF suggest that the shutoff ability of the PA-X protein may also be a common functionality in all influenza virus strains. However, further studies are needed to verify this hypothesis in viruses with different subtypes and in various host cell lines.

Functional domains of the host-shutoff activity in the PA-X protein

The previous studies have shown the involvement of the PA protein in host-shutoff activity; however, the potential function domains could only be mapped after PA-X was discovered [11]. The common mechanism used for host-shutoff activity, as shared by PA and PA-X, has been ascribed to the N-terminal RNA endonuclease domain of these proteins, taking the form of PD(D/E)XK (P107 D108 × 10 E119 K134) in the nuclease family (Fig. 1) [11, 23, 30, 40, 41]. However, over time, other molecular mechanisms also have been shown to be involved in this process. Very early on, Desmet et al. showed that the N-terminal domain of the PA protein is responsible for host shutoff; specifically, helix 4 (amino acids 85, 86, 91, 100, 114, and 186) and the 51–74 amino acid flexible loop (Fig. 1) [23]. These researchers also found that the PA-X C-terminal sequence also plays a role in suppressing expression of the reporter gene [23]. Thereafter, two independent studies demonstrated that the initial 15 amino acids (positions 192–206) in the PA-X C-terminal region are sufficient for the full shutoff activity of PA-X and that six basic amino acids (195R, 198K, 199R, 202K, 203K, and 206K) also play a key role (Fig. 1) [26, 27]. However, differing significantly from these findings, three other studies reported on the contribution made by the last 20 C-terminal residues in PA-X-mediated shutoff activity (Fig. 1). In 2015, Gao et al. reported that amino acids covering 233–252 of the PA-X C-terminus also strongly suppress gene expression and enhance viral replication and virulence in three different strains (human pandemic H1N1 2009, avian H5N1, and avian H9N2) [32]. Simultaneously, Bavagnoli et al. also provided direct evidence that the last 20 amino acids in the PA-X C-terminal region are important for endonuclease activity, and this was assumed to contribute

to host shutoff by the virus (Fig. 1) [42]. Moreover, unlike the specific role played by PA in cutting single-stranded (ss)RNA, PA-X is capable of digesting both ssRNA and double-stranded (ds)RNA, with a preference for ssRNA substrates, such as poly r(A) or poly r(U), which suggests that PA-X has a wide-spread degradation ability for various host RNAs [42]. Consistent with the results from both these studies, by comparing the PA-X proteins from EIV and CIV H3N8 viruses, Feng et al. found that position 231 (Ser) and the C-terminal elongated tail both contribute to the stronger host-shutoff ability of EIV PA-X relative to CIV PA-X [37]. Collectively, these studies reveal that both the N- and C-termini of PA-X contribute to its overall shutoff ability. Quite recently, by expressing PA-X in yeast, Oishi et al. further mapped 22 new amino acid mutations (including sites located in the endonuclease active sites, such as P107S, D108N, and E119N) that contribute to a decrease in shutoff activity [43]. However, further studies are needed to elucidate the potential mechanisms related to host-shutoff regulation by these newly identified amino acids.

Potential regulatory mechanism for host shutoff in the PA-X protein

PA-X selectively targets cellular mRNAs while sparing viral mRNAs, thereby ensuring successful viral replication while defeating an effective anti-viral response in the host. The mechanism involved has been partially elucidated by Khaperskyy et al. who found that PA-X selectively degrades host RNA polymerase II (Pol II)-transcribed mRNA by interacting with the host's 5′->3′ Xrn1 exonuclease activity and that PA-X likely operates in the cell nucleus [29]. Moreover, the shutoff activity is strongly associated with nuclear accumulation of the PA-X protein, with the process majorly mediated by four conserved basic residues (198K, 199R, 202K, and 203K) in X-ORF [29]. In addition, unlike herpes simplex virus 1 (HSV-1) vhs protein [44] and SARS nsp1 protein [45], which all selectively degrade translatable RNA polymerase II (Pol II) transcripts, PA-X also targets Pol II synthesized non-coding RNAs for degradation, highlighting the distinct features of PA-X in mediating host shutoff. Similarly, Hayashi et al. also found that the first nine amino acids are sufficient for nuclear localization and that three basic amino acids (195K, 198K, and 199R) are key residues for host shut off ability and nuclear accumulation in PA-X [27]. Therefore, taking the results from the two studies together, it seems likely that 198K and 199R are the crucial residues controlling the localization of PA-X and both are correlated with host-shutoff function. However, contrasting with the results from the Khaperskyy et al.'s study, Hayashi et al. showed that PA-X can degrade mature mRNAs synthesized in the nucleus and cytoplasm alike and that destruction of mRNA by PA-X in the nucleus was more efficient than in the cytoplasm, thereby revealing the multiple functional sites of action for PA-X-specific mRNA degradation [27].

Concerted effect of PA-X and NS1 in host shutoff and pathogenicity

Another well-characterized host-shutoff mechanism employed by IAV is the NS1-mediated blockade of the cleavage and polyadenylation specificity factor (CPSF) function observed in some strains from humans, which is one of the hallmarks of adaptation to humans by IAV [16–19]. NS1 is involved in the early nuclear phase of the host shutoff, whereas PA-X participates in the late cytoplasmic phase of this process [28]. In the nuclear stages, another mechanism used by IAV is the cap-snatching activity of viral RDRP, which targets host pre-mRNAs to supply capped primers for viral transcription [20–22]. Early on in the viral infection, these three host-shutoff mechanisms act in concert to inhibit the host cell's ability to initiate an effective anti-viral response. In the late period of the infection, IAV induces host cell apoptosis, thereby completely eliminating the ability of the host cell to synthesize new proteins for its own use. When considering the role of NS1 and PA-X in viral pathogenicity and host shutoff, it is very interesting to see the combined effects of them in modulating viral virulence. Therefore, recently, Nogales et al. investigated the interplay of PA-X and NS1-mediated host shutoff in viral replication and pathogenesis using a temperature-sensitive 2009 pandemic H1N1 virus [31]. These researchers found that viruses that simultaneously encode PA-X and NS1, or are deficient in both proteins, are highly attenuated in vivo, suggesting that there is a strict balance between NS1 and PA-X proteins during host gene expression regulation, viral replication, and pathogenesis. Therefore, it seems likely that optimal control of host protein synthesis by IAV PA-X and/or NS1 protein contributes to efficient virus replication and, consequently, to virulence, a hypothesis strengthened by the findings that wide-spread host mRNA degradation by PA-X only occurs during the late infection stage for IAV [28]. However, additional studies examining the concerted effect of NS1 and PA-X in inhibiting host protein expression in different viral strains need to be undertaken to augment our current understanding of IAV-associated host shutoff and provide new clues about the role played by PA-X in viral replication, pathogenesis, and host adaptation.

Role of PA-X in modulating the host's innate and acquired immune responses

PA-Xs role in modulating the host's innate immune response

Jagger et al. were the first researchers to show that down-regulation of PA-X expression markedly elevated the 1918 H1N1-induced immune response in mouse lungs; specifically that IFN-γ, CCR5, CD28, IL-7, and IL-15 signaling pathways, which are associated with lymphocyte activation and/or proliferation or other aspects of cell-mediated immunity, were enhanced [11]. PA-X also markedly suppresses several major histocompatibility complex class I-associated genes and activates many integrins and extracellular matrix components [11]. All of these perturbations in host response pathways may further affect lymphocyte activation and immune cell function, leading to an aberrant immune response and subsequent host immunopathology [46]. Similarly, using microarray analysis, our previous study also found that loss of PA-X expression significantly activates the global host response of the H5N1 influenza virus in chicken lungs, especially the inflammatory and cell death response [24]. Other studies have also shown that decreased PA-X expression clearly elevates the host innate immune response in mouse lungs infected with avian H5N1 virus [36], human 2009 pandemic H1N1 virus [33, 34, 36], and in porcine alveolar macrophages (PAM) cells infected with swine H1N2 virus [39]. In contrast, Gao et al. showed that loss of PA-X expression in H9N2 virus inhibits proinflammatory cytokine and chemokine responses [35]. Elsewhere, by transfecting plasmids expressing only PA-X in cell cultures, Feng et al. found that PA-X markedly elevated a number of genes, notably, innate immune response-related genes, ubiquitin ligases, vesicle transport and budding-related genes, and the genes associated with protein post-translational modifications in the Golgi complex [37]. Therefore, it seems likely that the inhibition effect of PA-X on the innate immune response is strain-specific or subtype-specific.

PA-Xs role in modulating the host's acquired immune response

To investigate the effect of PA-X on the humoral immune response, Hayashi et al. systematically compared serum antibodies from mice infected with wild-type viruses (A/California/04/09, H1N1, or Cal) and the PA-X-deficient Cal PA-XFS virus. They found that the Cal PA-XFS virus stimulated more neutralizing antibodies and higher levels of anti-HA and anti-NP antibodies than the wild-type virus, suggesting that the shutoff activity of the PA-X protein may also be involved in dampening down the host's humoral immune response [33].

Other immunomodulatory proteins in influenza viruses

Considering the universal role for PA-X in modulating the host's innate and adaptive immune responses, undoubtedly, PA-X can be classified as an immunomodulatory protein. As mentioned already, NS1 is another well-characterized immunomodulatory protein [47–50]. NS1 is a relatively small polypeptide with various interesting functions, one of which is its ability to antagonize the innate immune response by inhibiting the type I interferon system at multiple levels. NS1 acts as an antagonist during various stages of the anti-viral response, including (1) pre-transcriptional inhibition of interferon expression by interacting with components of the retinoic acid-induced gene protein I (RIG-I) signaling axis [47, 51–56], (2) co- and post-transcriptional inhibition by limiting host gene expression by blocking the cellular pre-mRNAs 3′ end-processing factor CPSF30 [19, 48, 55, 57–59], and (3) post-translational inhibition of anti-viral genes by antagonizing the PKR Ser/Thr kinase [60–64] and the OAS RNAse L-pathway activator [62].

IAV also encodes several proteins (PB1-F2, PB2, PA, and M2) that have been identified as affecting the host's innate immune response to a certain degree. PB1-F2 blocks the IFN response by interacting directly with the components (RIG-I/MAVs protein complex) of the interferon pathway [65, 66]. PB1-F2 also promotes the inflammatory response and contributes to viral virulence and the acquisition of secondary bacterial infections [67–71]. As for PB2 and PA, these two proteins affect host cell protein synthesis by facilitating cap snatching from host cell's mRNAs while indirectly suppressing the host's anti-viral response [40, 72]. However, PB2 also limits IFN-β expression by interfering with mitochondrial anti-viral signaling [73], or by inhibiting IFN-β promoter activation by regulating RIG-I and interferon beta promoter stimulating factor-1 [74]. Along with its cap-snatching activity [40], PA also plays an important role in inhibiting host type I IFN signaling [75], as well as affecting cytokine production and contributing to virulence in H5N1 viruses [76, 77] and in seasonal H3N2 in mice [78]. As for the M2 ion channel, it has been shown to activate inflammasomes by stimulating the NOD-like receptor family pyrin domain containing 3 protein [79]. Together, these immunomodulatory proteins represent new potential targets for the development of next-generation vaccines, which could exhibit improved duration of protection and protective immunity efficacy; among them, NS1 protein is a well-verified example [80–82].

Role of PA-X in modulating influenza virus virulence in various animal models

Effects of loss of PA-X expression on virulence in influenza virus

After the PA-X protein was discovered in 2012, the role played by it in modulating viral virulence has become gradually clearer. By generating PA-X-deficient viruses, accumulating numbers of studies have defined the important role that PA-X plays in modulating viral replication and virulence in influenza virus. As shown in Table 1, PA-X actively modulates viral pathogenicity in different viral subtypes in various animal models, including mice [11, 24, 32–35, 83], chickens [24], ducks [24], and pigs [38, 39]. In 2012, Jagger et al. were the first group to show that loss of PA-X expression increases host inflammatory and apoptosis responses and enhances virulence in the 1918 pandemic H1N1 virus in mice, but exerts no effect on viral replication in vitro or in vivo [11]. Next, several independent studies revealed that down-regulated PA-X expression enhances viral replication, polymerase activity, apoptosis, inflammatory response, and H5N1 virulence in chickens, ducks, and mice [24, 36, 84]. With the 2009 pandemic H1N1 virus, Gao et al. [36] and Hayashi et al. [33] showed that loss of PA-X expression increases viral virulence in mice, whereas Leea et al. [34] found that down-regulation of PA-X expression attenuates viral replication and virulence in mice. However, although the absence of PA-X in 2009 pandemic H1N1 increased viral pathogenicity in the mice, viral replication became attenuated in both cultured human cells and mice [33].

Interestingly, Gao et al. [35] reported that loss of PA-X expression in the H9N2 virus decreased its virulence in mice while exerting no effect on its replication. In swine influenza virus, Gong et al. [83] found that down-regulation of PA-X expression in swine H1N1 virus enhanced the activity of viral polymerase in mammalian cells as well as viral replication and virulence in mice. However, Xu et al. [39] showed that loss of PA-X expression decreased viral replication in PK15, PAM cells, and pigs, and also dampened the virulence of swine H1N2 virus in these animals.

Thus, these studies clearly suggest that the effects of PA-X on viral replication and virulence are strain or host specific. Considering the concerted role for PA-X and NS1 in blockading host gene expression [28, 31], we envisage that the precise impact of PA-X on viral growth and

Table 1 Effects of loss of PA-X expression on influenza A virus pathogenicity

Virus subtypes	Replication	RNP activity	Apoptosis	Inflammatory	PA accumulation	Virulence
H1N1(1918 pdm)	Have no effect in MDCK, eggs and mice [11]	–, [11]	Increase in mice [11]	Increase in mice [11]	–, [11]	Increase in mice [11]
H5N1(avian)	Increase in various mammalian and avian cells, and in mice, chickens, ducks [24, 35, 84]	Increase in 293T, CEF, DEF cells [24, 35, 84]	Increase in CEF, DEF, MDCK and A549 cells [24, 35, 84]	Increase in chickens, ducks and mice [24, 35, 84]	Increase in MDCK cells [35, 84) and A549 cells (35]	Increase in mice, chickens, ducks [24, 35, 84]
H1N1(2009 pdm)	Increase in A549 cells and mice [35]; decrease in Calu-3 cells and mice [33]; decrease in MDCK, A549 and mice [34]	Increase in 293T cells [35]; decrease in 293T cells [34]	Increase in A549 cells [33–35]	Increase in mice [35]	Increase in A549 cells [35]	Increase in mice [33, 35]; decrease in mice [34]
H9N2 (avian)	Have no effect in MDCK and A549 cells, decrease in mice [36]	–, [36]	Decrease in A549 cells [36]	Decrease in mice [36]	–, [36]	Decrease in mice [36]
H1N1 (swine)	Increase in MDCK, A549 cells and mice [83]	Increase in 293T cells [83]	–, [83]	–, [83]	–, [83]	Increase in mice [83]
H1N2 (swine)	Decrease in PK15, PAM cells and pigs [39]	–, [39]	–, [39]	Increase in PAM cells[39]	–, [39]	Decrease in pigs [39]

⁻Not carried out

pathogenicity may vary among different viral strains, depending on the specificity and activity of the NS1 protein in each strain.

Effects of the C-terminal 20 amino acids of PA-X on the pathogenicity of influenza virus

The X-ORF from the PA-X protein generally contains either 61 or 41 amino acids. The majority of IAV strains have a full-length 61-codon X-ORF, while around 25% of them (e.g., 2009 human pandemic H1N1 virus, and triple reassortant swine H1N1 viruses) carry a truncated form of PA-X, which contains the 41-codon X-ORF [15]. To investigate the biological significance of the length of PA-X in influenza virus infections, Gao et al. constructed a series of recombinant viruses carrying full or truncated versions of PA-X based on pandemic 2009 H1N1, avian H5N1 and H9N2 viruses and systemically compared their replication and pathogenicity profiles in mice. The results suggested that the last 233–252 amino acids increased viral replication and virulence, strengthened the viral-induced inflammatory response and apoptosis, and elevated the host-shutoff ability by the PA-X protein [32]. Using a molecular evolutionary approach, Xu et al. found that before 1985, all swine influenza viruses (SIVs) possessed full-length PA-X [85]. However, subsequently, the truncated forms of PA-X were continuously detected in SIVs and they gradually replaced the full-length PA-X to become the dominant PA-X phenotype in SIVs. To further explore the potential role of PA-X truncation in the adaptation of influenza virus to pigs, Xu et al. constructed PA-X-lengthened viruses based on the genetic backbone of an H1N2 SIV strain that encodes a truncated PA-X, and assessed their biological characteristics. In contrast to the Gao et al.'s study, Xu et al. found that compared with the whole length PA-X, SIV with truncated PA-X had higher pathogenicity and enhanced viral replication and transmissibility in pigs, and exerted a stronger inhibitory effect on IFN-I mRNA expression. Therefore, it seems likely that truncation of PA-X in SIV contributes to its adaptation in pigs, suggesting that an association between PA-X length and host specificity exists.

Host factors that interact with the PA-X protein

To facilitate viral infection and replication, viral proteins in IAV need to constantly interact with an array of cellular proteins and hijack the host pathways at the helm of cellular responses. Considering the essential role played by PA-X in modulating host responses and viral virulence, it is crucial to identify the host proteins interacting with PA-X to gain better understanding of how PA-X recruits the host cellular machinery for these functions. Using affinity purification and mass spectrometry and a high confidence threshold, Li et al. identified a total of 56 unique proteins physically interacting with PA-X from H5N1 virus in chicken cells [86]. Functional analysis of these proteins revealed the significant enrichment of biological processes, such as those associated with mitochondria and lipid transport, nucleosome assembly, and RNA processing. In addition, among the PA-X-interacting partners that were identified, GNB2L1, YWHAE, RPS20, RPS13, ARF1, and RPLP0 were annotated in the Gene Ontology term 'poly (A) RNA binding'. APOA1, ATP5B, NCL, NPM1, Thy1, and WDR1 were also reported to contribute to various types of viral infections. For example, APOA1 in hepatitis B virus (HBV) [87] and hepatitis C virus [88], ATP5B in HSV-1 [89]; NCL in influenza virus [90–93], dengue virus [94], parainfluenza virus type 3 [95], respiratory syncytial virus [96], Japanese encephalitis virus [97], Crimean-Congo hemorrhagic fever virus [98] and HIV [99, 100]; NPM1 in HIV [101], Thy1 in HBV [102], and WDR1 in Sendai virus [103]. However, this study only preliminarily described the proteins interacting with the PA-X protein overall in a chicken cell model, and further studies are needed to verify their interactions with PA-X and to investigate the underlying biological significance and mechanisms for these interactions. Moreover, to understand the potential molecular mechanism for PA-X-associated functioning in various hosts, it is also very important to investigate the host's interactome with PA-X in mammalian cells overall, such as in human or mouse cells.

Polymorphism in PA-X in influenza viruses as a threat to public health

AIVs with various HA subtypes (e.g., H1, H2, H3, H4, H5, H6, H7, H9, H10, and H11) have been circulating in domestic poultry in China [104]. Among them, currently, H5N1, H5N6, H9N2, and H7N9 AIVs are the most prevalent subtypes in many Chinese regions [105, 106]. It is noteworthy that all these subtypes not only cause substantial economic losses to poultry farming but also pose a potential threat to public health [106–112]. To find potential clues about the various effects of PA-X on the modulation of viral virulence, we systematically analyzed the PA-X polymorphisms in four prevalent AIV subtypes. To do this, we thoroughly compared all the available PA-X sequences from the Influenza Research Database (https://www.fludb.org/brc/home.spg?decorator=influenza) and the Global Initiative on Sharing Avian Influenza Data (GISAID) (https://www.gisaid.org/). Differences in the PA-X sequence in various hosts, and in avian and human isolates were separately analyzed. Specifically, the strains from 75 human H5N1, 589 avian H5N1, 19 human H5N6, 651 avian H5N6, 9 human H9N2,

917 avian H9N2, 813 human H7N9, and 437 avian H7N9 were included. Overall, we found that all of the viruses analyzed carry 61 amino acids in the X-ORF of their PA-X genes, and no clear-cut, host-specific genetic characteristics were identified in the avian and human isolates for all subtypes (Tables 2, 3). Next, we further analyzed the specific amino acid variations in the PA-X gene. As shown in Table 2, in the N-terminal of the PA-X gene, a total of 16 amino acid variations were identified in the different subtypes, including 20A, 27D, 37A, 57R, 58G, 61I, 63V, 68P, 70A, 94I, 96N, 100V, 101D, 115N, 129I, and 142K. Interestingly, the RNA endonuclease domain (P107 D108 × 10 E119 K134) was highly conserved among all the viral subtypes (Fig. 1) [40, 41]. Moreover, the helix 4 region (amino acids 85, 86, 91, 100, 114, and 186), which is important for the shutoff activity of the PA-X protein, is also relatively conserved (Fig. 1) [23], except for residue 100 (especially in the H7N9 influenza virus). Of note, the 51–74 amino acid motif in the PA-X protein, which is associated with host-shutoff ability [23], is highly variable and includes 57R, 58G, 61I, 63V, 68P, and 70A.

We further analyzed the genetic variation occurring in the C-terminal of the PA-X gene. We found that this region contains much higher variation (24.6%) than the N-terminal (8.38%). Specific variations occurred in positions 193N, 194P, 195R, 199R, 204G, 206K, 208Q, 209E, 210P, 213G, 215P, 218V, 228I, 248K, and 251K (Table 3). Interestingly, six conserved, basic amino acids (195R, 198K, 199R, 202K, 203K, and 206K) (Fig. 1), which were previously identified as contributing to host shutoff by the PA-X protein, also showed variations [26, 27] (e.g., 195R/K/N/S, 199R/K/T, and 206K/R/E/S/I polymorphisms in avian H9N2 viruses, 199R/K/I variations in avian H5N6 viruses, and 206K/T/N/I/E polymorphisms in avian H5N1 viruses). It will be

Table 2 Statistical analysis of the polymorphism at the N-terminal of PA-X gene

Sites[a]	Number of the analyzed strains and the sequence variation at these residues[b]							
	Human H5N1 (75 strains)	Avian H5N1 (589 strains)	Human H5N6 (19 strains)	Avian H5N6 (651 strains)	Human H9N2 (9 strains)	Avian H9N2 (917 strains)	Human H7N9 (813 strains)	Avian H7N9 (437 strains)
20A	5T (7%)	51T/1G (9%)	6T(32%)	552T (85%)	1T (11%)	19T (2%)	32T (4%)	5T (1%)
27D	25N/5S (40%)	126N/22S/2T/6V/1A (27%)	6S(32%)	548S/9N (86%)	0%	10N/1K/1S (1%)	1G (0.1%)	0%
37A	0%	1S/1T (0.3%)	12S (63%)	72S/1T (11%)	6S (67%)	243A/674S (74%)	813S (100%)	437S (100%)
57R	0%	12Q (2%)	0%	0%	0%	29Q/9K/1L (4%)	45Q (6%)	2Q (0.4%)
58G	10S (13%)	261S/1A (44%)	0%	0%	0%	19S/3N (2%)	0%	0%
61I	2M/5T (9%)	64M/17T/4V/1R/1W (15%)	12T (63%)	85T/4M (14%)	6T (67%)	691T/2K (75%)	811T (99%)	1A/434T (100%)
63V	2I/5A (9%)	39A/26I/14T/2L (14%)	12I/6A (95%)	554A/74I (96%)	6I (67%)	669I/3M/2L/1A (74%)	810I (99%)	437I (100%)
68P	2L (3%)	13Q/1H (2%)	0%	1L/1S (0.3%)	0%	11Q/3L/1A (2%)	806P/3L/2T/1S (0.7%)	0%
70A	2V (3%)	19V (3%)	0%	47V/1T (7%)	6V (67%)	154V/1M/1S/1T (17%)	95V (12%)	35V (8%)
94I	0%	3V/1T/1M/1L (1%)	0%	28V (4%)	0%	5L/2V (0.7%)	4V (0.5%)	0%
96N	0%	4S/1P/1T (1%)	0%	0%	0%	4H/1T (0.5%)	43H (5%)	93H (21%)
100V	2I (3%)	8A/1F/7I/ (3%)	0%	60A/1I (9%)	0%	82A/24I (12%)	41I (72%)	181A (41%)
101D	8E/10N (24%)	297E/34N/16G/1V (59%)	13E (68%)	79E/5G/5N (14%)	7E (70%)	870E/13Q/4K/3V (97%)	4E (100%)	435E (99%)
115N	2S (3%)	30D/8G/5S/3Y (8%)	0%	14D/3S (3%)	0%	23D/8S/8T/1H/1K/1Y (5%)	2D/1 (5%)	340N/93S/2D (22%)
129I	8T/2V (13%)	250T/19V/1A/1L/1M (46%)	0%	50T (8%)	0%	17M/5T/1L (3%)	4M/1T (0.6%)	3M/2T (1%)
142K	5R (7%)	51R/28N/1Q (14%)	7R (37%)	529R/21G (84%)	0%	52R/10N/1E (7%)	5E/2R (0.9%)	0%

[a]Indicates the original residues

[b]The number outside the brackets indicates the number of strains that carry certain kind of polymorphism; the number inside the brackets shows the variation rates relative to the original residues

Table 3 Statistical analysis of the polymorphism at the C-terminal of PA-X gene

Sites[a]	Number of the analyzed strains and the sequence variation at these residues[b]							
	Human H5N1 (75 strains)	Avian H5N1 (589 strains)	Human H5N6 (19 strains)	Avian H5N6 (651 strains)	Human H9N2 (9 strains)	Avian H9N2 (917 strains)	Human H7N9 (813 strains)	Avian H7N9 (437 strains)
193N	2S (3%)	140S/1T/1C (24%)	13S (68%)	92S (14%)	7S (78%)	793S/8R (87%)	813S (100%)	437S (100%)
194P	2L (3%)	49L/5Q (9%)	0%	5L (0.8%)	0%	92L/21Q/6R (13%)	398L/10Q (50%)	156L/8Q (38%)
195R	2K (3%)	12K (2%)	12K (63%)	92K (14%)	6K (67%)	670K/1N/1S (73%)	813K (100%)	437K (100%)
199R	2K (3%)	51K (9%)	12K (63%)	81K/1I (14%)	6K (67%)	672K/1T (73%)	811K (99%)	437K (100%)
204G	25D/6N/2S (44%)	235N/4S/1A (79%)	13D (68%)	97D/7N (16%)	9D (100%)	907D/1N (99%)	797D/1G/13N/2Y (100%)	437D (100%)
206K	0%	4T/1N/1I/1E (1%)	0%	2I/8R (2%)	0%	16R/2E/1S/1I (2%)	3R (0.4%)	1R (0.2%)
208Q	8L/2K (13%)	232L/1K/4P (39%)	0%	2K (0.3%)	0%	5H/5K/3L/1P (2%)	1E/3R (0.5%)	0%
209E	0%	4G/1V (0.8%)	5G (26%)	12G (2%)	1G (11%)	35G/5A/1Q/1V (4%)	30A/2G/3K/1V (4%)	2A (0.5%)
210P	1L (1%)	12L/2T/1S/17Q (5%)	0%	11L/8Q (3%)	0%	89Q/35L/1S (14%)	12Q (1%)	1Q (0.2%)
213G	10D/2Y (16%)	2A/1N/213D (37%)	0%	4D/3S (1%)	0%	19S/4C/3D (3%)	0%	0%
215P	13L/4Q (23%)	17Q/1R/1S/78L (16%)	6L (32%)	553L (85%)	1L (11%)	51L/3Q/1A (0.6%)	8L (1%)	1L (0.2%)
218V	4A (6%)	88A (15%)	0%	4A (0.6%)	0%	73A (8%)	0%	1A (0.2%)
228I	8T/2V (13%)	354T/7N (61%)	13T (68%)	98T (15%)	1P/8T (100%)	896T/3A/1N (98%)	813T (100%)	437T (100%)
248K	0%	1I/1Q/7R/1T (2%)	5R (26%)	63R (10%)	0%	130R (14%)	530R (65%)	181R (41%)
251K	5R (7%)	76R (13%)	6R (32%)	555R (85%)	0%	11R/1E/2M (2%)	0%	0%

[a]Indicates the original residues

[b]The number outside the brackets indicates the number of strains that carry certain kind of polymorphism; the number inside the brackets shows the variation rates relative to the original residues

fascinating to determine whether these genetic variations affect the host-shutoff ability and virulence of different viral subtypes.

Polymorphism comparisons of the PA-X proteins from different viral subtypes

To determine whether subtype-specific signatures exist in the PA-X sequence, we compared the variation rates for specific amino acids in PA-X among the different viral subtypes and some specific variations were found to be highly enriched in certain subtypes (Fig. 2). As shown in Fig. 2a, the variation rates at positions 37A, 61I, 101D, 193N, 195R, 199R, and 228I are higher in the H7N9 and H9N2 subtypes than in the H5N1 and H5N6 subtypes. Worth noting is that the variation rates were highest in the H7N9 viruses, reaching almost 100% in the seven sites (Fig. 2a). However, we also noticed that some amino acids (positions 27D, 58G, 129I, 208Q, 213G, and 215P), were more inclined to show variation in H5N1 and H5N6 than in H9N2 and H7N9 viruses, especially in H5N1 viruses (Fig. 2b). Interestingly, another small group of amino acids (20A, 142K, and 251K) showed higher variation rates than the other subtypes, particularly in avian H5N6 viruses (Fig. 2c). Therefore, based on these analyses generally, we found that the pattern of PA-X variation is very similar between H9N2 and H7N9 subtypes, and between H5N1 and H5N6 subtypes. In addition, some variations tended to be enriched in specific viral subtypes. The implementation of further studies investigating the role of PA-X polymorphism in modulating virulence and host-shutoff activity in different virus subtypes and different virus strains will shed further light on the potential mechanisms of pathogenicity in IAV.

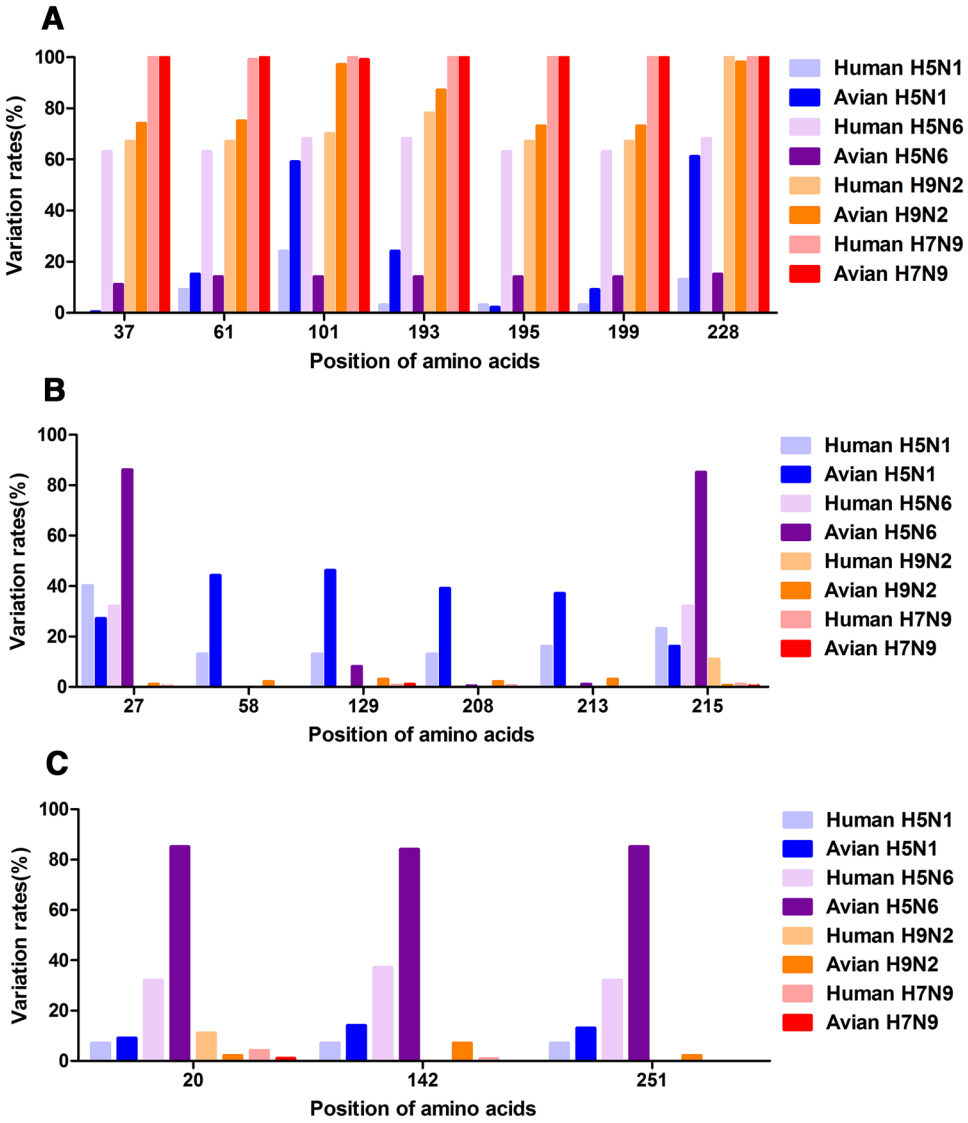

Fig. 2 Comparison of the polymorphism of the PA-X protein from viruses of different subtypes. The amino acids (for example, 37A) indicated in the figure are represented as the original residues. Therefore, the calculated mutation rates shown in this figure are compared with the original residues. **a** Positions of variation rate which are obviously higher in the H7N9 and H9N2 viruses than in H5N1 and H5N6 viruses. **b** Amino acids those were more inclined to variation in H5N1 and H5N6 viruses than in H9N2 and H7N9 viruses. **c** Small group amino acids that showed obviously higher variation rates than other subtype viruses, especially in avian H5N6 viruses

Future prospects

To ensure efficient translation of viral mRNAs while constraining host protein expression, IAV employs multiple host-shutoff mechanisms. The PA-X protein is a bona fide host-shutoff endonuclease with host-specific RNA destroying ability. Although accumulating evidence confirms that PA-X-associated host shutoff is related to viral adaptation and pathogenesis, the underlying mechanisms involved remain largely obscure. Further studies to characterize the biogenesis, characteristics, and mechanism of PA-X shutoff in different viral strains and various hosts should help us to unravel the complexity of the influenza virus–host-shutoff mechanisms. Moreover, although a strong connection has been established between PA-X accumulation and host-shutoff activity, further studies are needed to determine whether the C-terminal of PA-X causes nuclear accumulation through its interactions with other nuclear localization signal-containing proteins or whether it contains a functional NLS. Focus should also turn to elucidating the functional differences between the PA-X proteins from different circulating influenza strains, such as H7N9 AIV. Currently, low pathogenic avian influenza (LPAI) and high pathogenic avian influenza (HPAI) H7N9 viruses co-circulate in poultry. Therefore, it will also be very interesting to see whether the PA-X protein elicits similar effects for LPAI and HPAI H7N9 viruses in terms of modulating viral virulence and host responses in avian species and mammals. Finally, the essential role for PA-X in modulating viral replication and host innate immune and adaptive responses makes it highly feasible to explore PA-X as a target for drug development and vaccine design in influenza viruses.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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