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

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Summary

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, is demonstrated to be an anti-virulence 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 H9N2 wild type virus and H9N2 PA-X deficient virus. Loss of PA-X in H9N2 virus reduced apoptosis and had marginal effect on progeny virus output in human pulmonary adenocarcinoma (A549) cells. Without PA-X, PA was less able to suppress co-expressed green fluorescence protein (GFP) in human 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 anti-virulence role of PA-X in influenza viruses is virus-strain dependent.

Keywords: H9N2 influenza virus; PA-X; Pathogenicity
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 frame-shift in a +1 open reading frame (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; Katze et al., 1986b). Jagger et al. 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 remodeling (Jagger et al., 2012). 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, decreases viral replication and pro-inflammatory response in mice. The absence of PA-X in H9N2 virus also reduces 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. la) (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 an MOI of 1, and cell lysates were harvested at 12 hpi. We found that PA-X could be detected in H9N2 WT infected cells but not in H9N2-FS infected cells (Fig.1b).

**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 an MOI of 0.01, and the supernatants were collected and titrated at 6, 12, 24, 36, 48, 60, 72 and 84 h post infection (hpi). 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 H9N2WT viruses reached maximum virus output at around the same time (48 hpi) with comparable peak virus titers; viral titers 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 WT viruses at an MOI of 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 hpi and 6.59% at 12 hpi) than H9N2 WT virus (3.60% at 6 hpi and 10.73% at 12hpi) \((P < 0.05)\) (Fig.2c). Cells that were PI+ only, and annexin V+ PI+ showed no significant difference between H9N2-FS and H9N2 WT viruses. Overall, the data show that loss of PA-X in H9N2 virus has 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 wild type virus

To assess the effect of PA-X on pathogenicity, mice (15 per group) were intranasally inoculated at $10^6$ TCID$_{50}$ with each virus. Clinical signs, mortality and weight loss were monitored over 14 days. Three virus-infected mice a day were humanely killed at 3, 5, and 7 days post infection (dpi), and lungs were collected for virus titration. H9N2-FS virus infection resulted in no death, while 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 of H9N2 WT virus infected mice (Fig. 3b).

Histopathologically, H9N2-FS virus infected lung appeared nearly normal. However, in the H9N2 WT virus group, there were extensive vascular congestion, and cellular exudate (Fig. 3c). Viral titers of H9N2-FS virus infected lungs were 8-20 fold lower than those of H9N2 WT virus at 3, 5 and 7 dpi ($P < 0.05$) (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., 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 dpi. Interleukin-1β (IL-1β), interleukin-6 (IL-6), the mouse equivalent of human IL-8 (KC), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α or CCL3), tumor necrosis factor-alpha
(TNF-α) and interferon gamma (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 demonstrate that PA-X in H9N2 virus facilitates pathogenicity and up-regulated inflammatory response in mice.

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

Inhibition of host protein synthesis by influenza virus can hinder host anti-viral response and promote virus replication (Katze et al., 1986a; Katze et al., 1986b). 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 more than 20% when co-transfected with H9N2-FS PA, compared with H9N2 WT PA co-transfection (Fig.5a & b). Although the expression level of PA protein of H9N2-FS 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 suggest that loss of PA-X in H9N2 virus reduces 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 manifested as 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 in H9N2 virus is a virulence factor.

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 up-regulated inflammatory, apoptotic and T-lymphocyte
signaling pathways. Elevated virulence manifested as increased PA expression,
ribonucleoprotein polymerase activity and inflammatory response were the effects of
PA-X deletion in pH1N1 and highly pathogenic H5N1 avian influenza viruses (Gao et
al., 2015). PA-X is also an anti-virulence 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 down-regulated 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
anti-virulence 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 dpi. 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 but increased pathogenicity through enhanced host immune response.

Influenza virus infection can induce host shut off with rapid decline of host protein synthesis (Katze et al., 1986a; Katze et al., 1986b) to divert host resources towards viral replication. Inhibition of host protein synthesis also aids in dampening the anti-viral 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 made PA 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 less effectively inhibit host protein synthesis, 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.,
We speculate that the decrease in suppression of host protein synthesis exacerbates 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 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 (SPF) embryonated chicken eggs (Sun et al., 2011). 293T, MDCK, and A549 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Foster City, CA, USA) supplemented with 10% fetal...
bovine serum (FBS; Life Technologies), 100 units/ml of penicillin and 100 g/ml of
streptomycin.

Generation of recombinant viruses by reverse genetics

All eight gene segments have been previously amplified by reverse
transcription-PCR (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 UCC 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. PA ORF was unaltered in H9N2-FS. Rescued viruses
were detected using hemagglutination assays. The viruses were purified by sucrose
density gradient centrifugation, and viral RNA was extracted and analyzed by
RT-PCR, and each viral segment was sequenced to confirm sequence identity.

Viral titration and replication kinetics

Fifty % tissue culture infective dose (TCID_{50}) was determined in MDCK cells
using 10-fold serially diluted virus inoculated at 37°C and cultured for 72 h. The
TCID_{50} values were calculated by the method of Reed and Muench (Reed & Muench,
1938). MDCK and A549 cells were infected with viruses at an MOI of 0.01, overlaid
with serum-free DMEM containing 2μg/ml TPCK-trypsin (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 hpi. Virus titers were determined by TCID_{50} in MDCK
cells. Three independent experiments were performed.

**Mouse infections**

Fifteen mice (six week-old female BALB/c; Vital River Laboratory, Beijing, China) per group were anesthetized with Zoletil (tiletamine-zolazepam; Virbac S.A., Carros, France; 20 µg/g) and inoculated intranasally with 50 µl of 10^6 TCID_{50} of H9N2 diluted in phosphate-buffered saline (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 dpi for the determination of lung virus titers, histopathology and cytokine levels. Lungs were collected and homogenized in cold PBS. Virus titers were determined by TCID_{50}. 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 dpi was fixed in 10% phosphate-buffered formalin and processed for paraffin embedding. Each 5 µm section was stained with hematoxylin 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 or CCL3 and MCP-1 in lungs were determined by cytometric bead array
assays (BD Cytometric BEAD Array Mouse Inflammation Kit; BD Bioscience, San Diego, CA, USA). 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 analyzed on a BD FACS Array bioanalyzer (BD Bioscience). Data were analyzed using BD CBA Software (BD Bioscience). Each chemokine or cytokine was computed as pg/ml of homogenate.

**Cell death assays**

Virus infection assays were conducted in 6 well plates. Cells were seeded at a density of 1×10^6 cells/well for overnight incubation in infection media (cell growth media with 1% bovine serum albumin was used in place of FBS). Cells were then infected with virus at 1.0 MOI for 12 h. Cells pooled from the supernatant and monolayer were then harvested, washed and stained with FITC labeled annexin V and propidium iodide (PI) (Becton Dickinson, San Jose, CA) 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 analyzed on the FACS Calibur (BD Biosciences) with Flow Jo software (version 7.6.1). Cell death (apoptosis and necrosis) was defined as annexin-V^+ and PI^+, while apoptotic cells were annexin-V^+ only. Viable cells were considered as neither annexin-V nor PI positive.

**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% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Germany). Each PVDF membrane was blocked with 0.1% Tween 20 and 5% nonfat dry milk in Tris-buffered saline and subsequently incubated with a primary antibody. Primary antibodies were specific for influenza A virus PA (1:3000, GeneTex, USA), influenza A virus PA-X [diluted 1:2000, polyclonal rabbit antiserum against a H5N1 X-ORF derived peptide(CAGLPTKVSHTSPA), Genscript, China], influenza A virus PB1 (diluted 1:3000, Thermo Fisher Scientific, USA), GFP (1:1000, Abcam, UK), β-actin (1:1000, Santa Cruz, USA). The secondary antibody used was either horseradish peroxidase (HRP)-conjugated anti-mouse antibody or HRP-conjugated anti-rabbit antibody (diluted 1:10,000 Jackson ImmunoResearch USA), as appropriate. HRP presence was detected using a Western Lightning chemiluminescence kit (Amersham Pharmacia, Freiburg, Germany), following the manufacturer’s protocol.

Statistics

All statistical analyses were performed using GraphPad Prism Software Version 5.0 (GraphPad Software Inc., San Diego, CA, USA). The two treatment methods were compared by two-tailed Student’s t-test, and multiple comparisons were carried out by two-way analysis of variance (ANOVA) considering time and virus as factors. Differences were considered statistically significant at $P < 0.05$. All data are reported
as the mean ± standard deviation (SD).

Acknowledgments

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Figure legends

Figure 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 or PB1 or β-actin, as indicated, followed by AP-conjugated secondary antibodies.

Figure 2. Growth of H9N2 WT and FS viruses and induction of apoptosis. Virus growth curves of H9N2 WT and H9N2-FS viruses in MDCK cells (a) and A549 (b) cells over 84 h. (c) Cell death was determined in A549 cells by detection of annexin+ and/or PI+ at 6 and 12 hpi of H9N2 WT or H9N2-FS virus at 1.0 MOI. Representative dual-labeled quadrants of bivariant fluorescence dot plots showing the induction of apoptosis in infected cells. Apoptotic cells, positive for annexin V but not PI, were identified in the right lower quadrant. Cells positive for PI but not annexin V (indicative of necrosis) were identified in the right upper quadrant. MOCK = uninfected control cells. Each value represents the mean of three independent experiments performed in triplicates; error bars indicate standard deviations (SD). * indicates significant difference between FS virus and wild type virus (P < 0.05).

Figure 3. Pathogenicity of H9N2 WT and H9N2-FS viruses in mice. (a) Reduced survival (percentage) was found with H9N2 WT virus infected mice over a 14 day period (inoculation at day 0). (b) H9N2 WT virus infected mice showed significant
weight loss, unlike H9N2-FS virus and mock infected mice. Any mouse that lost more than 30% of its body weight was euthanized. (c) 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. Scale bars, 100 µm. (d) Mean of viral lung load ± SD was based on log_{10} TCID_{50} determination in MDCK cells. * indicates significant difference between H9N2 WT and H9N2-FS virus (P < 0.05). Means of the data of three mice per group are shown, and error bars are SDs.

Figure 4. Detection of cytokine/chemokine proteins in lungs of mice infected with H9N2 WT and H9N2-FS viruses. Mean cytokine/chemokine levels ± SDs are shown (n=3). * indicates significant difference between H9N2-FS and H9N2 WT (P < 0.05).

Figure 5. PA without PA-X is less able to suppress co-expressed GFP in 293T cells. (a) eGFP expression plasmid was co-transfected with PA plasmid derived from H9N2 WT or H9N2-FS viruses, or with mock plasmid (control pcDNA3.1). eGFP fluorescence was captured at 24 h post transfection. GFP fluorescence (green) is shown in the left panel, and merged with DAPI staining fluorescence is shown in the right panel. The GFP expression levels were quantified by fluorescence intensity. The fluorescence intensities were analyzed with Image-Pro-Plus (Media Cybernetics). Relative fluorescence intensity of each group as compared with control was shown as histograms. Values shown are means of the results of three independent experiments ± SDs. (b) PA and GFP protein expression were determined by Western blotting analysis using anti-PA and anti-GFP antibody. Anti-β-actin antibody was used as loading
control. Protein bands were quantified by densitometry. Protein levels of PA and GFP relative to β-actin are shown as histograms. Stronger expression of PA, in the absence of PA-X, derived from H9N2-FS virus did not lead greater inhibition of GFP expression. The results shown are representative data from three independent experiments. Error bars indicate SDs. * indicates significant difference between H9N2 WT and H9N2-FS virus ($P < 0.05$).
(a)

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(b)

- H9N1 WT
- H9N2- FS
- Mock

- PB1
- Actin
- PA-X