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PB2-E627K and PA-T97I substitutions enhance polymerase activity and confer a virulent phenotype to an H6N1 avian influenza virus in mice

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ABSTRACT

H6N1 avian influenza viruses (AIVs) may pose a potential human risk as suggested by the first documented naturally-acquired human H6N1 virus infection in 2013. Here, we set out to elucidate viral determinants critical to the pathogenesis of this virus using a mouse model. We found that the recombinant H6N1 viruses possessing both the PA-T97I and PB2-E627K substitutions displayed the greatest enhancement of replication in *vitro* and in *vivo*. Polymerase complexes possessing either PB2-E627K, PA-T97I, and PB2-E627K/PA-T97I displayed higher virus polymerase activity when compared to the wild-type virus, which may account for the increased replication kinetics and enhanced virulence of variant viruses. Our results demonstrate that PB2-E627K and PA-T97I enhance the ability of H6N1 virus to replicate and cause disease in mammals. Influenza surveillance efforts should include scrutiny of these regions of PB2 and PA because of their impact on the increased virulence of H6N1 AIVs in mice.
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Introduction

H6 subtypes of influenza viruses are the most abundantly detected influenza virus subtype, and are associated with a broader host range than any other influenza subtype (Munster et al., 2007; Spackman et al., 2005). The first human case of H6N1 AIV infection have been reported in Taiwan in 2013 (Myers et al., 2007). A recent report also demonstrated that some H6 viruses could transmit between guinea pigs by direct contact (Wang et al., 2014). Taken together, the reported case of human infection with an H6 subtype virus, the ability of some H6 viruses to transmit between mammals, and a lack of pre-existing immunity to H6 influenza viruses in humans suggest that H6 AIVs may pose a pandemic threat. Sporadic infection of mammalian species with avian influenza viruses provides an opportunity for avian viruses to adapt to a new host environment and potentially acquire adaptive changes leading to the emergence of a pandemic virus. Therefore, a more complete understanding of the molecular features involved in the host range restriction and mammalian adaptation of H6 AIVs should be further studied.

Mice provide an excellent model in which to study of the mechanisms of influenza virus adaptation (Blazejewska et al., 2011; Gabriel et al., 2005; Hatta et al., 2001; Jiao et al., 2008; Li et al., 2005; Liu et al., 2014; Llompart et al., 2014; Lu et al., 1999; Memoli et al., 2009; Metreveli et al., 2014; Qi et al., 2012; Song et al., 2009; Tarnow et al., 2014; Tumpey et al., 2005; Watanabe et al., 2013; Zaraket et al., 2013; Zhang et al., 2012a). In a previous study, to identify changes that are associated with the adaptation of H6N1 AIV to mammalian hosts, we had serially passaged a mallard origin

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H6N1 virus in mice, and after sequential passage of this virus in mice, we obtained two mouse-adapted viruses, which named MA-P8M1 and MA-P8M3. In this study, we found that the MA-P8M3 displayed increased virulence and a novel amino acid substitution combination (PB2-E627K and PA-T97I) in the viral RNA-dependent RNA polymerase (RDRP) complex that was sufficient for increasing the pathogenicity of the H6N1 AIVs. In *vitro* and in *vivo* analyses demonstrated that this substitution combination significantly enhanced RNA polymerase activity and viral replication. Therefore, this combination of adaptive mutations in the PA and PB2 gene contribute to mammalian adaptation of H6N1 AIVs.

Results

Mouse adapted H6N1 variant is high pathogenic for mice

We compared the pathogenicity of the parental virus WT H6N1 SJ/275 virus with the mouse-adapted variant MA-P8M3 in mice. Mice were infected intranasally with 10^4 EID_{50} or 10^5 EID_{50} doses of WT SJ/275 or MA-P8M3, and were followed for 14 days for body weight loss and mortality. A group of mock-infected mice were included as a control. Mice that lost $>25\%$ of their initial body weight were considered moribund and were euthanized. At doses of 10^5 EID_{50} and 10^4 EID_{50}, mice infected with WT SJ/275 survived infection and did not show any appreciable weight loss (Fig. 1), whereas mice infected with 10^5 EID_{50} and 10^4 EID_{50} of the mouse-adapted MA-P8M3 variant rapidly lost weight and succumbed to infection (Fig. 1). Based on these data, we found that serial passage of WT SJ/275 resulted in substantially increased virulence in mice.

Sequence analysis

We identify three adaptive mutations in the genomes of the MA-P8M3. The MA-P8M3 viruses had an E→K substitution at PB2 position 627, and a T→I substitution amino at PA position 97, and an N→T substitution at HA position 394.

PB2-E627K and PA-T97I substitutions enhance viral replicationin MDCK cells

The PB2, PA, and HA gene segments from the MA-P8M3 virus and the PB1, NP, NA, M, and NS gene segments from the WT SJ/275 virus were cloned into plasmids for reverse genetics in order to produce recombinant viruses and determine the contribution of each amino acid substitution to the enhanced virulence of the mouse-adapted H6N1 viruses. We first compared the in *vitro*

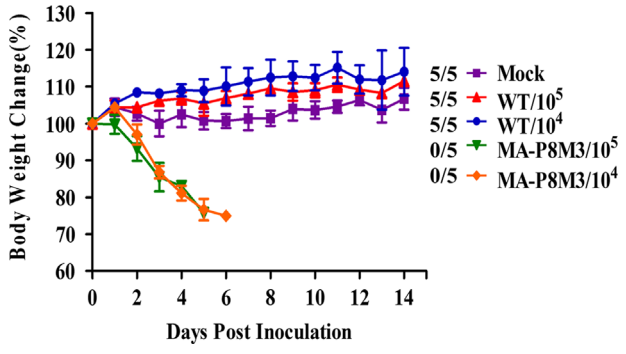

Fig. 1. Increased virulence of mouse-adapted H6N1 virus in mice. Mice ($n=5$) were inoculated intranasally with 50 μl containing 10^4 or 10^5 EID_{50} of mouse-adapted viruses (MA-P8M3) or the parental wild-type H6N1 virus (WT) or mock inoculated (Mock; $n=5$), and animals that lost more than 25% of their pre-infection weight were euthanized. Morbidity was examined by recording the body weights of inoculated mice daily, and it is represented as a percentage of the weight on the day of inoculation (day 0). The average of each group is shown. The numbers in the graphs indicated the numbers of surviving mice per group.

Table 1
Comparison of amino acid and MLD_{50} differences between wild-type SJ/275 and recombinant H6N1 viruses.

Virus[a]	PB2 627	PA 97	HA 394	MLD_{50}
WT SJ/275	E	T	N	>6.5
rSJ/275-P8M3-PB2	K	T	N	4.5
rSJ/275-P8M3-PA	E	I	N	>6.5
rSJ/275-P8M3-HA	E	T	T	>6.5
rSJ/275-P8M3-PB2/PA	K	I	N	3.25
rSJ/275-P8M3-PB2/HA	K	T	T	5
rSJ/275-P8M3-PA/HA	E	I	T	5.5
rSJ/275-P8M3-PB2/PA/HA	K	I	T	3.5

[a] WT SJ/275, A/Mallard/SanJiang/275/2007(H6N1); MA-P8M3, mouse-adapted SJ/275; We constructed the WT SJ/275-PB2, the WT SJ/275-PB1, the WT SJ/275-PA, the WT SJ/275-HA, the WT SJ/275-NP, the WT SJ/275-NA, the WT SJ/275-M, the WT SJ/275-NS, the MA-P8M3-PB2, the MA-P8M3-PA, and the MA-P8M3-HA plasmids, respectively. These plasmids were used for transfection to rescue the recombinant viruses in this study. rSJ/275-P8M3-PB2, recombinant carrying the PB2 gene from MA-P8M3 and remaining 7 genes from WT SJ/275; rSJ/275-P8M3-PA, recombinant carrying the PA gene from MA-P8M3 and remaining 7 genes from WT SJ/275; rSJ/275-P8M3-HA, recombinant carrying the HA gene from MA-P8M3 and remaining 7 genes from WT SJ/275; rSJ/275-P8M3-PB2/PA, recombinant carrying the PB2 and PA genes from MA-P8M3 and remaining 6 genes from WT SJ/275; rSJ/275-P8M3-PB2/HA, recombinant carrying the PB2 and HA genes from MA-P8M3 and remaining 6 genes from WT SJ/275; rSJ/275-P8M3-PA/HA, recombinant carrying the PA and HA genes from MA-P8M3 and remaining 6 genes from WT SJ/275; rSJ/275-P8M3-PB2/PA/HA, recombinant carrying the PB2, PA, and HA genes from MA-P8M3 and remaining 5 genes from WT SJ/275.

replication kinetics of the WT SJ/275 virus to three recombinant viruses generated by reverse genetics: one incorporating the PB2 E627K substitution (rSJ/275-P8M3-PB2), one incorporating the PA T97I substitution (rSJ/275-P8M3-PA), and one incorporating the HA N394T substitution (rSJ/275-P8M3-HA) (Table 1). The recombinant viruses all replicated with kinetics similar to that of the WT SJ/275 virus, although the rSJ/275-P8M3-PB2 virus achieved higher titers at 24 h post-inoculation (Fig. 2A).

We then generated recombinant viruses with different combinations of mouse-adaptive amino acid substitutions to determine if there was a combinatorial effect of multiple substitutions on viral growth kinetics in MDCK cells (Table 1). Whereas the recombinant virus with only the PB2-E627K substitution replicated with similar kinetics as the WT SJ/275 virus in MDCK cells (Fig. 2A), the presence of both the PB2-E627K and PA-T97I amino acid substitutions, either with or without the HA-N394T substitution, resulted in increased viral titers in MDCK cells when compared to the WT SJ/275 virus (Fig. 2C). Recombinant viruses that did not harbor the PB2-E627K substitution but possessed the PA-T97I or HA-N394T substitution either alone or in combination showed similar growth kinetics to the WT SJ/275 virus. Taken together, these data indicate that PB2-E627K in conjunction with PA-T97I enhanced viral replication in MDCK cells (Fig. 2A and C).

PB2-E627K and PA-T97I substitutions enhance viral replication in mice

We then evaluated the in *vivo* replicative capacity of each recombinant virus by inoculating mice with 10^4 EID_{50} of each virus and analyzing virus titers in the lungs on days 3 and 5 post-infection (Fig. 3A). The WT SJ/275 virus was not detected in the lungs of inoculated mice at day 3 or 5 post-infection (Fig. 3A). Highest viral titers in the lung at both time points were found in mice infected with recombinant viruses possessing both PB2-E627K and PA-T97I (Fig. 3A). Recombinant viruses possessing either the PB2-E627K or PA-T97I substitution in isolation resulted

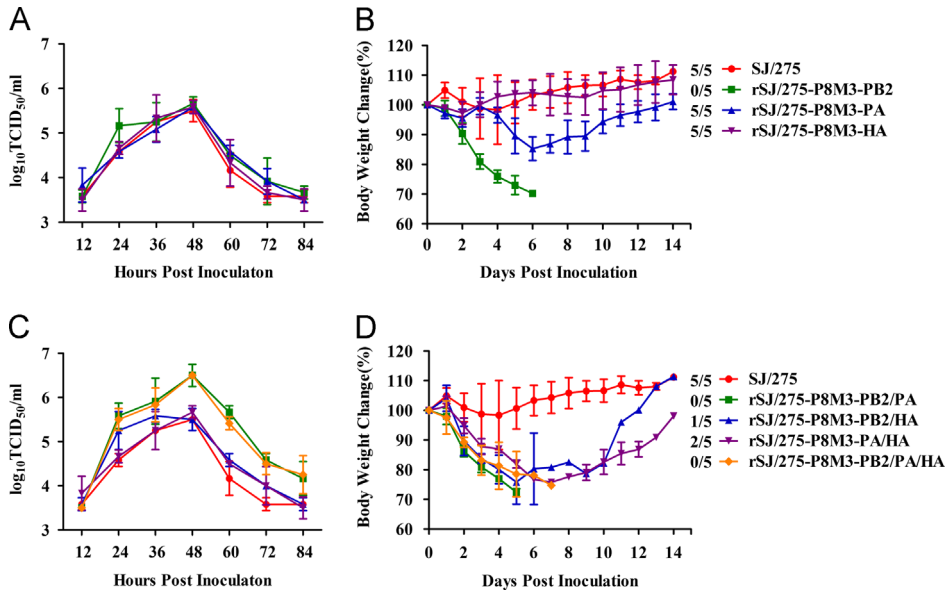

Fig. 2. Effects of amino acid mutations for replication and virulence of SJ/275. The parental SJ/275H6N1 virus and recombinant viruses possessing one or more of the amino acid substitutions identified in mouse-adapted viral variants were generated by reverse genetics. A-B) The parental SJ/275H6N1 virus (SJ/275) and recombinant viruses possessing PB2-E627K (rSJ/275-P8M3-PB2), PA-T97I (rSJ/275-P8M3-PA), and HA-N394T (rSJ/275-P8M3-HA) were generated by reverse genetics. A) Each virus was inoculated at a multiplicity of infection of 0.01 $TCID_{50}$/cell onto MDCK cells. Supernatants were collected at the indicated time points and titrated in MDCK cells by $TCID_{50}$. B) Mice ($n=5$) were infected with 10^6 EID_{50} of the indicated virus and followed for weight loss. The numbers in the graphs indicated the numbers of surviving mice per group. C-D) The parental SJ/275H6N1 virus (SJ/275) and recombinant viruses possessing PB2-E627K and PA-T97I (rSJ/275-P8M3-PB2/PA), PB2-E627K and HA-N394T (rSJ/275-P8M3-PB2/HA), PA-T97I and HA-N394T (rSJ/275-P8M3-PA/HA), and PB2-E627K, PA-T97I, and HA-N394T (rSJ/275-P8M3-PB2/PA/HA) were generated by reverse genetics. C) Each virus was inoculated at a multiplicity of infection of 0.01 $TCID_{50}$/cell onto MDCK cells. Supernatants were collected at the indicated time points and titrated in MDCK cells by $TCID_{50}$. D) Mice ($n=5$) were infected with 10^6 EID_{50} of the indicated virus and followed for weight loss. The numbers in the graphs indicated the numbers of surviving mice per group.

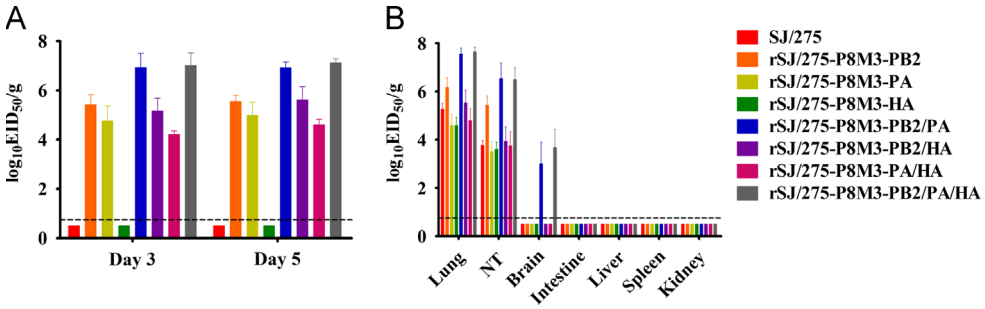

Fig. 3. Effects of amino acid mutations for replication and tropism of SJ/275 in *vivo*. A) Mice ($n=6$) were inoculated intranasally with 10^4EID_{50} of the indicated virus. Viral loads in the lungs of infected mice ($n=3$/time point) were determined at 3 and 5 dpi in eggs by EID_{50}. Results are expressed as $\log_{10}$ EID_{50}/g of tissue. The dotted indicates the lower limit of detection of infectious virus. B) Mice ($n=3$) were inoculated intranasally with 10^6EID_{50} of the indicated virus. Viral loads in the lungs, nasal turbinates (NT), brains, intestines, livers, spleens, and kidneys were determined at 3 dpi in eggs by EID_{50}. The dotted indicates the lower limit of detection of infectious virus.

in lung viral titers approximately 10-fold less than viruses harboring both substitutions together regardless of whether the viruses also included the HA-N394T substitution (Fig. 3A). The recombinant virus possessing the HA-N394T substitution alone was not detectable in the lungs of mice on days 3 or 5 post-infection (Fig. 3A).

PB2-E627K and PA-T97I substitutions enhance virulence and expand the tropism of an H6N1 virus in mice

Cohorts of mice were infected with 10^6 EID_{50} of WT SJ/275 or a recombinant virus harboring one of the mouse-adapting amino acid substitutions and were followed for 14 days to evaluate weight loss and survival (Fig. 2B). Mice inoculated with WT SJ/275 and rSJ/275-P8M3-HA lost little weight and survived infection (Fig. 2B). In contrast, mice infected with rSJ/275-P8M3-PB2 rapidly lost weight and succumbed to infection by day 6 post-infection (Fig. 2B). The rSJ/275-P8M3-PA virus elicited appreciable weight loss in mice, but was not lethal (Fig. 2B). We determined the WT SJ/275 virus MLD_{50} was >6.5 $\log_{10}$ EID_{50}, and the MLD_{50} of the rSJ/275-P8M3-PB2, rSJ/275-P8M3-PA, and rSJ/275-P8M3-HA viruses were 4.5 $\log_{10}$ EID_{50}, >6.5 $\log_{10}$ EID_{50}, and >6.5 $\log_{10}$ EID_{50}, respectively (Table 1), indicating that PB2-E627K resulted in increased virulence in mice.

Mice were inoculated with 10^6 EID_{50} of WT SJ/275 or recombinant viruses harboring different combinations of the mouse-adaptive amino acid substitutions and were followed for weight loss and survival. In contrast to mice inoculated with WT SJ/275 which lost little weight and survived infection, infection of mice with any recombinant virus possessing PB2-E627K resulted in rapid weight loss and significant mortality (Fig. 2D). In addition including the PA-T97I and/or HA-N394T amino acid substitutions with PB2-E627K in the recombinant viruses did not significantly alter the pattern of weight loss or survival when compared to viruses harboring PB2-E627K alone, demonstrating that the

PB2-E627K substitution is the dominant contributor to viral virulence in the mouse-adapted viruses (Fig. 2D).

We determined the MLD_{50} of the rSJ/275-P8M3-PB2/PA, rSJ/275-P8M3-PB2/HA, rSJ/275-P8M3-PA/HA, and rSJ/275-P8M3-PB2/PA/HA viruses were 3.25 $\log_{10}$ EID_{50}, 5 $\log_{10}$ EID_{50}, 5.5 $\log_{10}$ EID_{50}, and 3.5 $\log_{10}$ EID_{50}, respectively (Table 1), indicating that the PA-T97I and PB2-E627K substitutions displayed the greatest enhancement of virulence in mice.

We sought to map the expanded tissue tropism of the adapted viruses using recombinant viruses possessing one of the three amino acid substitutions. Mice were first inoculated intranasally with 10^6 EID_{50} of WT SJ/275 or a recombinant virus possessing either PB2-E627K, PA-T97I, or HA-N394T, and virus replication in the lungs, nasal turbinates (NT), brains, intestines, livers, spleens, and kidneys of infected animals was determined on day 3 post-infection (Fig. 3B). The recombinant virus possessing PB2-E627K replicated to higher titers in the lungs and NT when compared to the WT SJ/275 virus and the PA-T97I and HA-N394T viruses (Fig. 3B). However, none of the recombinant viruses possessing single amino acid substitutions was detected in the brains 3 days post-infection (Fig. 3B). When recombinant viruses possessing combinations of mouse-adapting amino acid substitutions were evaluated, viruses harboring PB2-E627K and PA-T97I were recovered from the brain, indicating a requirement for both amino acid substitutions for extrapulmonary replication in the brain (Fig. 3B). Taken together, these data show that the PB2-E627K substitution is a prerequisite for enhanced pathogenicity of the H6N1 virus in mice, but that additional adaptive changes in the PA protein can further enhance replicative capacity and expand viral tropism.

The combination of PB2-E627K and PA-T97I enhances viral polymerase activity to a greater extent than PB2-E627K or PA-T97I alone

The enhanced replication of viruses possessing PB2-672K and/or PA-T97I in *vitro* and in *vivo* suggested that these amino acid substitutions could impact viral polymerase activity. We applied a mini-genome reporter assay to assess the impact of mouse-adaptive changes in the PB2 and PA polymerase proteins on the transcription and replication of viral RNAs (vRNAs) in human 293T cells. We found that the PB2-E627K substitution alone increased polymerase activity about 57-fold and PA-T97I alone increased the polymerase activity about 14-fold when compared to WT SJ/275 polymerase activity (Fig. 4). A polymerase complex possessing both PB2-E627K and PA-T97I substitutions increased polymerase activity about 75-fold compared to WT SJ/275 polymerase activity (Fig. 4). These results suggest that the enhanced replication of viruses harboring both PB2-E627K and PA-T97I in *vitro* and in *vivo* is in part due to enhanced polymerase activity.

Discussion

H6 viruses isolated from waterfowl have also shown the capacity to infect humans via experimental inoculation (Beare and Webster, 1991), and the first naturally-acquired human case of H6N1 has recently been reported in Taiwan, raising public health concern (Yuan et al., 2013). However, the mechanisms of mammalian adaptation and interspecies transmission of H6N1 avian influenza viruses remain poorly understood.

Here we have identified several amino acid substitutions that substantially enhance the polymerase activity of an H6N1 virus in mammalian cells, increase replication kinetics in *vitro* and in *vivo*, and result in increased viral virulence and expanded tissue tropism. In this report, we document that the PB2-E627K substitution was

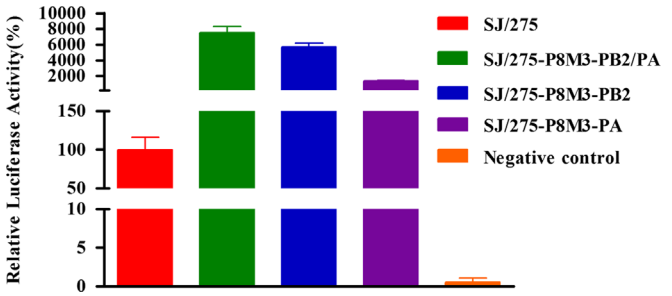

Fig. 4. Viral polymerase activity of RNP complexes. 293T cells were transfected with a luciferase reporter plasmid (paviPolI-T-Luc) that expresses a negative-sense virus-like RNA encoding a destabilized firefly luciferase enzyme, and one plasmid for each of the proteins in the polymerase complex (NP, PB1, PB2, and PA). Transfection conditions in which plasmids encoding an adaptive substitution at PB2-627 and/or PA-97 were included in place of the wild-type plasmids are indicated. Cells were also cotransfected with a Renilla luciferase expression plasmid to control for transfection efficiency. Thirty-six hours post-transfection, firefly and Renilla luciferase levels were measured, and Renilla expression was used to normalize the data. The temperature used for the polymerase activity study is 37 °C. The averages of triplicate experiments are shown. Error bars represent the standard deviation (SD).

necessary to enhance the replicative capacity and pathogenicity of an H6N1 virus in a mouse model. In our study, we have also shown that this mutation in PA contributes to the enhancement of polymerase activity of an avian H6N1 virus in mammalian cells, and is essential for mammalian host adaptation. PA-T97I substitution enhance the virulence of the H6N1 AIVs in a mouse model (Fig. 2B and Fig. 3A) but not greatly (the MLD_{50} of rSJ/275-P8M3-PA is >6.5 log10EID_{50}), although polymerase activity of this mutant was markedly enhanced compared to wild type H6N1, unlike the role of PA-T97I substitution in H5N2 AIVs (Song et al., 2009). A similar observation was also made using 2009 pandemic H1N1 viruses, in which introduction of virulence markers E627K, D701N, and E677G amino acid substitutions in the PB2 protein increased polymerase complex reporter activity, but did not enhance viral virulence in mice and ferrets (Herfst et al., 2010). While a H6N1 mutant containing PA-T97I alone was not lethal in mice at an infecting dose of 10^6 EID_{50}, a mutant virus containing both PB2-E627K and PA-T97I had a much stronger influence on the pathogenicity of H6N1 AIVs, suggesting a cooperative effect of these adaptive changes within the polymerase complex.

We investigated the mechanisms responsible for the increased pathogenicity rendered by the combination of PB2-E627K and PA-T97I in *vitro* and in *vivo*. We found that viruses containing only a single adaptive amino acid substitution replicated to titers similar to that of the WT H6N1 virus in MDCK cells (Fig. 2A). However, the virus containing PB2-E627K and PA-T97I replicated to ten-fold higher titers than the WT H6N1 virus, demonstrating a synergistic relationship of these adaptive changes on H6N1 replication in mammalian cells (Fig. 2C). It is not clear that although the E627K mutant virus showed high pathogenicity in mouse study, it only showed mild increase in titer at 24 h post-infection in cell culture. Considering that the presence of both the PB2-E627K and PA-T97I amino acid substitutions, resulted in obvious increased viral titers in MDCK cells when compared to the WT SJ/275 virus, the PA-T97I substitution may be prerequisite for the increased viral replicative capability that rendered by the PB2-E627K in MDCK cells.

Amino acid substitutions in the viral polymerase complex, a heterotrimer composed of three subunits, PB2, PB1, and PA, represent one of the major adaptive mechanisms that avian influenza viruses exploit for efficient replication, transmission, and pathogenesis in mammals (Gao et al., 2009; Herfst et al., 2010; Llompart et al., 2014; Ma et al., 2011; Ozawa et al., 2011; Shinya et al., 2004; Steel et al., 2009; Subbarao et al., 1993; Sun et al., 2014; Suzuki et al., 2014; Van Hoeven et al., 2009; Zhang

et al., 2012b). All three subunits, PB2, PB1, and PA, are required for both transcription and replication of influenza virus. PB2 includes the capped-RNA recognition domain, PB1 carries the polymerase active site, and PA holds the endonuclease active site and has critical roles in endonuclease activity of the influenza virus polymerase (Braam et al., 1983; Dias et al., 2009; Horisberger, 1980; Nagata et al., 2008; Yuan et al., 2009). We subsequently focused on the impact of adaptive amino acid substitutions on the function of the viral RNA-dependent RNA polymerase (RDRP) using a luciferase-based mini-genome assay to better understand how the PB2-E627K/PA-T97I combination led to enhanced production of infectious virus particles during in *vitro* and in *vivo* replication. Although the RDRP containing PB2-E627K or PA-T97I alone showed higher luciferase expression compared to wild-type H6N1, the RDRP containing the PB2-E627K/PA-T97I combination had the highest (75-fold) luciferase expression (Fig. 4). Importantly, the RDRP activity of polymerase complex proteins with single or combined amino acid substitutions closely correlated with the replication of viruses harboring those amino acid substitutions in MDCK cells and murine lungs (Fig. 2A and C and Fig. 3A). Collectively, our data show that these PB2 and PA amino acid substitutions strongly contribute to the adaptation of H6N1 AIVs in mammals.

In conclusion, we demonstrated that a combination of the PB2-E627K and PA-T97I amino acid substitutions identified in the H6N1 mouse-adapted strains comprised the primary virulence determinant responsible for the increased pathogenicity and adaptation of H6N1 AIVs in mice. Our data suggest that the increased pathogenicity of viruses harboring both PB2-E627K and PA-T97I amino acid substitutions is due to increased activity of the polymerase complex which translates to improved replication in mammalian cells.

Materials and methods

Facilities

Studies with H6N1 AIVs were conducted in a biosecurity level 3 laboratory approved by the Military Veterinary Research Institute of the Academy of Military Medical Sciences. All animal studies were approved by the Review Board of Military Veterinary Research Institute of the Academy of Military Medical Sciences.

Cells and virus

Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine. 293 T cells were cultured in DMEM supplemented with 10% FCS, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine. The H6N1 virus A/Mallard/SanJiang/275/2007 (abbreviated as SJ/275), was isolated from an anal swab of a mallard in Sanjiang natural reserve of Heilongjiang province, China (Cheng et al., 2014). Stock viruses were grown in the allantoic cavities of 10-day-old chicken eggs for 60 h at 37 °C, and aliquots were stored at −80 °C until used. The GenBank accession numbers corresponding to each of the eight SJ/275 viral gene segments are KJ021045-KJ021052.

Virus titration in eggs and MDCK cells

Virus titers in virus stocks and homogenized organ samples were determined by end-point titration in eggs and/or MDCK cells. For end-point viral titration in eggs, ten-fold serial dilutions of each sample were inoculated into eggs. Sixty hours after inoculation, fluid from the allantoic cavity was collected and tested for the ability to agglutinate chicken erythrocytes as an indicator of virus replication. Infectious virus titers were reported as $\log_{10}$ EID_{50} /ml or $\log_{10}EID_{50}$/g, and were calculated from 3 replicates by the method of Reed–Muench (Matumoto, 1949). For end-point viral titration on MDCK cells, ten-fold serial dilutions of each sample were inoculated onto MDCK cell monolayers grown in a 96 well culture plate. One hour after inoculation, the monolayer was washed with phosphate-buffered saline (PBS), and cultured in 100 μl of DMEM supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 2 μg/ml TPCK-treated trypsin. Seventy-two hours after inoculation, supernatants of infected cell cultures were tested for agglutinating activity using chicken erythrocytes as an indicator of virus replication. Infectious virus titers were calculated from 3 replicates by the method of Reed–Muench (Matumoto, 1949), and expressed as $\log_{10}$ $TCID_{50}$/ml.

RNA isolation, PCR amplification, and sequencing

Viral RNA was isolated from the allantoic fluid of inoculated eggs using the RNeasy Mini kit (QIAGEN, Germantown, MD) according to the manufacturer's protocol. Reverse transcription of viral RNA and subsequent PCR were performed using primers specific for each gene segment (sequences available upon request). PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Germantown, MD) according to the manufacturer's protocol. Viral gene segments were sequenced by the Beijing Genomics Institute (Beijing, China). DNA sequences were analyzed and compared to the parental SJ/275 virus using the Lasergene sequence analysis software package (DNASTAR, Madison, WI).

Rescue of viruses by reverse genetics

We used an eight-plasmid reverse genetics system for virus rescue as described previously (Li et al., 2005). We inserted the cDNA derived from SJ/275 or MA-P8M3 viral genes between the ribozyme and polymerase I promoter sequence. The plasmids used for transfection were prepared using the QIAfilter™ Plasmid Midi kit (QIAGEN). Recombinant viruses rSJ/275-P8M3-PB2, rSJ/275-P8M3-PA, rSJ/275-P8M3-HA, rSJ/275-P8M3-PB2/PA, rSJ/275-P8M3-PB2/HA, rSJ/275-P8M3-PA/HA, and rSJ/275-P8M3-PB2/PA/HA were rescued in 293 T cells using reverse genetics as previously described (Gao et al., 2009; Li et al., 2005), and propagated in MDCK cells. All the constructs were completely sequenced to ensure the absence of unwanted mutations.

In vivo experiments

The 50% mouse lethal dose (MLD_{50}) of the parental SJ/275 virus and the recombinant viruses was measured using groups of three female 4–6-week-old BALB/c mice (Merial-Vital Laboratory Animal Technology Co., Ltd., Beijing, China). Mice were intranasally inoculated with 50 μl of 10-fold serial dilutions of each indicated influenza virus in PBS under isofluorane sedation, with doses ranging from 10^1–10^6 EID_{50}. Survival and body weight changes were recorded daily for 14 days post-infection (dpi). Animals that showed signs of severe disease and weight loss $>25\%$ of their initial body weight were considered moribund and were humanely sacrificed. MLD_{50} values were calculated by the Reed–Muench method (Matumoto, 1949) after a 14-day observation period and expressed as EID_{50}.

To evaluate the impact of individual amino acid substitutions alone and in combination on morbidity and mortality, we inoculated groups of five mice with 10^6 EID_{50} SJ/275 or recombinant H6N1 variant viruses harboring different adaptive amino acid substitutions and recorded weight loss and mortality for 14 dpi.

Mice were additionally intranasally inoculated with 10^4 EID_{50} of the indicated viruses to measure the replicative capacity of recombinant viruses as compared to the parental SJ/275 virus in the lungs. Three mice in each group were euthanized at 3 and 5 dpi. The lungs were collected, homogenized, and tissue debris was removed by low-speed centrifugation. Virus titers of each sample were determined by endpoint titration in eggs as described above, and virus titers of each strain were expressed as mean $\log_{10}EID_{50}/g \pm$ standard deviation (SD). To evaluate the tropism and replication capacity of each virus in *vivo*, we euthanized three mice on 3 days after inoculation with 10^6 EID_{50} SJ/275 or recombinant H6N1 viruses and harvested the lungs, nasal turbinates (NT), brains, intestines, livers, spleens, and kidneys. Organs were homogenized in 1 ml of PBS and viral titers in each of the organs were determined by titration in chicken eggs. Titers were calculated by the Reed–Muench method (Matumoto, 1949) and expressed as mean $\log_{10}EID_{50}/g \pm$ standard deviation (SD). The limit of virus detection was 0.75 $\log_{10}EID_{50}/g$. For calculation of the mean, samples with a virus titer of < 0.75 $\log_{10}EID_{50}/g$ were assigned a value of 0.

Analysis of replication kinetics in MDCK cells

MDCK cells were infected with indicated influenza viruses at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$ (50% tissue culture infectious dose)/cell. After incubation, the cells were washed and overlaid with DMEM containing 2 μg/ml TPCK-treated trypsin. Supernatants were collected 12, 24, 36, 48, 60, 72, and 84 h after infection and stored at −80 °C. Virus titer was determined by endpoint titration in MDCK cells. Virus titers of each virus in different time points were expressed as mean $\log_{10}$ $TCID_{50}/ml \pm$ standard deviation (SD).

Minigenome assay for polymerase activity

Polymerase activity was measured using a minigenome assay as previously described (Song et al., 2011), and the temperature used for the polymerase activity study is 37 °C. In brief subconfluent monolayers of 293 T cells were transfected with a luciferase reporter plasmid (paviPolI-T-Luc) together with the pTK-RL (Promega) and pcDNA3.1+ plasmid constructs encoding the polymerase genes PB2, PB1, PA, and NP using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. pTK-RL expresses Renilla luciferase and was used as an internal transfection control for the dual luciferase assay. After eight hours, the medium was replaced with DMEM containing 10% FCS. Cell extracts were harvested 36 h post-transfection and luciferase activity was assayed by using the Luciferase assay system (Promega). The assay was standardized against the Renilla luciferase activity. Experiments were performed in triplicate.

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