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Amino acids substitutions in the PB2 protein of H7N9 influenza A viruses are important for virulence in mammalian hosts

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We tested the biological significance of two amino acid mutations in the PB2 protein (glutamic acid to lysine at position 627 and aspartic acid to asparagine at position 701) of A(H7N9) viruses for mammalian adaptation. Mutants were assessed for their viral polymerase activities, growth kinetics in mammalian and avian cells, and pathogenicity in mice. We found that lysine at position 627 and asparagine at position 701 in PB2 are essential for mammalian adaptation of A(H7N9) viruses.

Since February 2013, novel influenza A viruses of the H7N9 subtype [A(H7N9)] have infected more than 457 individuals, causing 167 deaths as of November 18th, 2014 (http://www.who.int/csr/don/18-november-2014-avian-influenza/en/). Phylogenetic analyses indicate that these viruses originated from reassortment events among different avian influenza viruses[1–7]. The HA and NA segments were derived from the H7N3 viruses and H7N9 viruses, respectively, and the other six segments (PB2, PB1, PA, NP, M, and NS) were derived from H9N2 viruses. Comprehensive studies have revealed that A(H7N9) viruses replicate efficiently in mammalian cells and show moderate transmissibility in ferret models[8–14]. These properties appear to be influenced by amino acid substitutions in the HA protein, such as HA-226L (H3 numbering), which confer binding affinity to human-type receptors[15], and in the PB2 protein, such as PB2-591K, PB2-627K, and PB2-701N, which is known to facilitate viral polymerase activity in mammals[16–20].

Almost all H7N9 viruses isolated from humans have an amino acid change, Q591K, E627K, or D701N, in their PB2 protein. Mok et al.[21] tested the importance of PB2-271A, PB2-591K, PB2-627K, and PB2-701N, using A/Shanghai/2/2013 as the backbone, and found that these amino acid changes contributed to mammalian adaptation. Here, we tested the contributions of PB2-627K and PB2-701N in the background of the prototype A/Anhui/1/2013 virus (Anhui/1)[8,22]. The PB2 proteins of Anhui/1 and A/Shanghai/2/2013 virus differ at amino acid position 292, which has been predicted to affect the host range of influenza viruses[23]. Hence, the PB2 proteins of the two viruses may differ in their replicative ability in mammalian cells. Zhang *et al.*[24] studied the importance of PB2-627K by using Anhui/1 virus but did not test the significance of PB2-701N in the background of this virus. For these reasons, we generated mutant proteins and viruses encoding PB2-627E/701D and PB2-627E/701N (mutations are underlined); the PB2-701N mutation was tested in combination with PB2-627E because the mammalian-adapting mutations PB2-627K and PB2-701N typically do not occur together.

Results and Discussion

PB2 is part of the viral replication complex; therefore, we evaluated viral polymerase activity in human alveolar adenocarcinoma epithelial A549 cells incubated at 37°C and chicken fibroblast DF-1 cells incubated at 39°C by using a luciferase-based mini-genome assay[22]. Cells were transfected with viral protein expression plasmids for NP, PB1, PA, and PB2 or its mutants, a plasmid expressing viral RNA encoding firefly luciferase, and pRL-null,

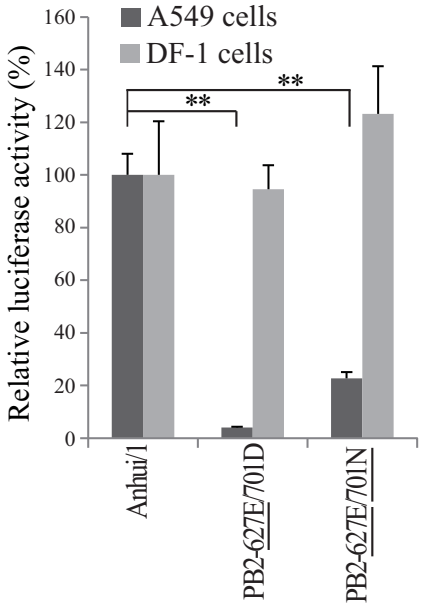

Figure 1 | Viral polymerase activity *in vitro*. Human A549 and avian DF-1 cells were transfected with plasmids encoding the PB1, PA, NP, and wild-type or mutant PB2, with a plasmid for the expression of a virus-like RNA encoding the firefly luciferase gene (driven by a human or an avian polymerase I promoter for use in human or avian cells, respectively), and with a control plasmid encoding Renilla luciferase. Cells were incubated for 24 h at 37°C (A549) or 39°C (DF-1), and then firefly and Renilla luciferase activities were measured by means of a dual-luciferase assay. Polymerase activity was calculated by normalization of the firefly luciferase activity to the Renilla luciferase activity. The data are shown as relative polymerase activities with SD (n = 3). The polymerase activity of the wild-type Anhui/1 PB2 was set to 100%. **, $P < 0.01$, according to a one-way ANOVA followed by a Dunnett's test.

which expresses Renilla luciferase, as a transfection control. At 24 h post-transfection, viral polymerase activities were measured by use of a dual-luciferase assay (Figure 1). In human A549 cells, the PB2-627E/701D mutation drastically decreased the viral polymerase activity compared with that of wild-type Anhui/1 PB2 ($P < 0.01$), whereas the simultaneous introduction of PB2-701N (PB2-627E/701N) partially restored the viral polymerase activity. In avian DF-1 cells, the viral polymerase activity was not statistically significantly affected by the mutations tested, although luciferase levels were somewhat increased by PB2-627E/701N. Hence, PB2-627K, and to a lesser extent PB2-701N, contribute to the high viral polymerase activity of A(H7N9) PB2 protein in human cells.

Next, we compared the viral growth kinetics of wild-type and mutant viruses in A549 and DF-1 cells incubated at 37 and 39°C, respectively. Cells were infected with viruses at a multiplicity of infection of 0.001, and virus titers in the cell culture supernatant were assessed at the indicated times (Figures 2a and 2b). In A549 cells, PB2-627E/701D virus was attenuated compared with Anhui/1, whereas the PB2-627E/701N virus replicated more efficiently than did Anhui/1 virus. In DF-1 cells, the PB2-627E/701N virus showed increased growth compared with the other two viruses tested. These results indicate that the PB2-K627E mutation reduced A(H7N9) virus replicative ability in human cells, and that this growth attenuation could be compensated for by the PB2-D701N mutation.

To assess the importance of PB2-627K, and PB2-701N *in vivo*, we compared virus titers in the nose tissues, lungs, brains, spleens, kidneys, livers, and colons of BALB/c mice infected intranasally with 10^6 plaque-forming units (PFU) of each virus (Table 1). PB2-627E/701D virus was significantly attenuated compared with wild-type Anhui/1; this attenuating effect was partially offset by the simultaneous introduction of PB2-701N (see PB2-627E/701N virus). None of the viruses tested here spread beyond the respiratory organs. We, next, evaluated viral pathogenicity in mice infected with different doses of virus (Figure 3). Wild-type Anhui/1 virus, which was prepared by use of reverse genetics and encodes an HA consensus sequence, killed all mice at the highest dose tested, resulting in a mouse lethal dose 50 (MLD_{50}) value of $10^{5.5}$ PFU. In contrast, PB2-627E/701D and PB2-627E/701N viruses did not kill mice infected with 10^6 PFU of virus (MLD_{50}, $> 10^{6.5}$ PFU), although PB2-627E/701N caused some weight loss. These results demonstrate that the PB2-K627E mutation drastically attenuate the virulence of A(H7N9) virus in mice and the additional PB2-D701N mutation partially recovers the virulence of the virus in mice. It should be noted that the MLD_{50} value of the Anhui/1 virus used in this study was substantially higher than that of the original Anhui/1 isolate (MLD_{50}: $10^{3.5}$ PFU/ml)[8]. This difference in MLD_{50} values is probably due to differences in the HA sequences because the virus generated in this study by using reverse genetics encodes the HA consensus sequence, whereas the original isolate possesses a mix of five different HA variants[8]. Also, we cannot exclude the possibility that sequence variations in the other seven viral segments might contribute to this difference.

The viral polymerase complex, which is composed of PB2, PB1, and PA, plays an essential role in the first step of mammalian adaptation. Alteration of the polymerase complex by an amino acid change

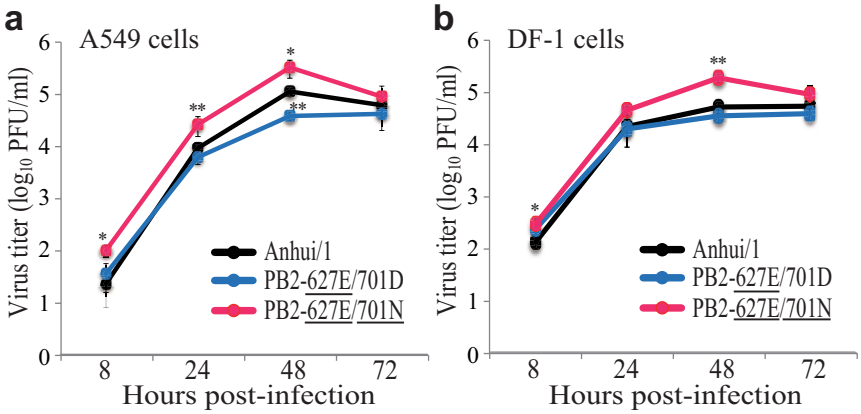

Figure 2 | Growth kinetics of mutant viruses *in vitro*. Human A549 (A) and avian DF-1 (B) cells were infected with the indicated viruses at a multiplicity of infection of 0.001 and incubated at 37°C or 39°C, respectively. The supernatants of the infected cells were collected at the indicated time points. Virus titers were determined by use of a plaque assay in MDCK cells. The virus titers are means ± SD (n = 3). * and **, $P < 0.05$ and $P < 0.01$, respectively, according to a one-way ANOVA followed by a Dunnett's test.

Table 1 | Virus titers in organs of infected mice [log_{10} PFU (mean ± SD)/g][a]

Virus	Day 3 post-infection		Day 6 post-infection	
	Nose tissue	Lung	Nose tissue	Lung
Anhui/1	6.16 ± 0.05	7.24 ± 0.09	5.46 ± 0.72	5.52 ± 0.47
PB2-627E/701D	3.25 ± 1.40*	4.85 ± 0.58**	Not detected	4.86 ± 0.36
PB2-627E/701N	6.20 ± 0.24	6.96 ± 0.17	4.38 ± 0.45	4.72 ± 0.09

[a]Six-week-old female BALB/c mice were intranasally inoculated with 10^6 PFU of viruses (in 50 µl). Three animals per group were euthanized on days 3 and 6 post-infection. Statistically significant differences compared with Anhui/1-infected mice were determined by use of a one-way ANOVA followed by a Dunnett's test (*, $P < 0.05$; **, $P < 0.01$).

or through reassortment enables a virus to replicate efficiently in a new host. Here, we demonstrated that the well characterized mammalian-adapting markers PB2-627K and PB2-701N are also important for the mammalian adaptation of the influenza virus A/Anhui/1/2013 (H7N9). PB2-627K and PB2-701N increased the viral polymerase activity in human cells compared with PB2-627E/701D, but did not affect that in avian cells. The virus carrying PB2-D701N replicated better than did wild-type Anhui/1 virus, which possesses PB2-627K, in both human and avian cells. PB2-627K and PB2-701N enhanced viral virulence in mice compared with PB2-627E/701D. While this study was in progress, Mok *et al.*[21] and Zhang *et al.*[24] published similar findings. By using A/Shanghai/2/2013, Mok *et al.*[21] reported that the viral polymerase activities of PB2-627E and PB2-701N were enhanced in human cells but decreased in avian cells compared with wild-type PB2. Similarly, Zhang *et al.*[24] showed that PB2-627K enhances viral polymerase activities in human cells but decreased these activities in avian cells. Although there is some variance in the details of the data from three different studies, collectively, they show the importance of the amino acid residues at positions 627 and 701 for mammalian adaptation of H7N9 viruses. Of note, a novel H10N8 virus recently isolated from a fatal human case was composed of populations encoding PB2-627E and PB2-627K[25]. PB2-627K thus appears to play an essential role in the mammalian adaptation of a wide range of influenza A viruses.

Methods

Cells. Madin-Darby canine kidney (MDCK) cells were cultured in Eagle's minimal essential medium (MEM) supplement with 5% newborn calf serum (NCS). Human embryonic kidney 293T cells, human alveolar adenocarcinoma epithelial A549 cells, and chicken fibroblast DF-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplement with 10% fetal calf serum (FCS). MDCK, 293T, and A549 cells were incubated at 37°C under 5% CO_2. DF-1 cells were incubated at 39°C under 5% CO_2.

Viruses and reverse genetics. Plasmid-based reverse genetics for virus generation was performed as previously described[22,26]. We used A/Anhui/1/2013 (H7N9; Anhui/1) as the backbone virus in this study. The titers of the stock viruses were determined by plaque assays in MDCK cells. All viruses were sequenced to confirm the absence of unwanted mutations. All experiments with A(H7N9) viruses were performed in enhanced biosafety level 3 (BSL3) containment laboratories at the University of Tokyo (Tokyo, Japan), which are approved for such use by the Ministry of Agriculture, Forestry and Fisheries, Japan.

Construction of plasmids. Mutations in the PB2 gene were generated by PCR amplification of the RNA polymerase I plasmid for the PB2 segment with primers possessing the desired mutations (primer sequences available upon request). To prepare plasmids for viral protein expression, the open reading frames of the PB2 genes were amplified by PCR with gene-specific primers (primer sequences available upon request). The PCR products were cloned into pCAGGS/MCS. All constructs were sequenced to confirm the absence of unwanted mutations.

Minigenome assay. A minigenome assay based on the dual-luciferase system was performed as previously reported[22]. Polymerase activity was calculated by standardization of the firefly luciferase activity to the Renilla luciferase activity. The polymerase activity of the wild-type was set to 100%.

Growth kinetics of virus in cell culture. Growth kinetic of viruses was assessed as previously described[22]. Briefly, A549 and DF-1 cells were infected with the indicated viruses at a multiplicity of infection (MOI) of 0.001. Cell culture supernatants including 0.25 µg/ml of trypsin were collected at 8, 24, 48 and 72 h post-infection and subjected to virus titration by use of plaque assays in MDCK cells.

Experimental infection of mice. Experimental infection of mice was performed as previously described[22]. Briefly, 4 six-week-old female BALB/c mice (Japan SLC) per group were intranasally inoculated with 10^3–10^6 plaque-forming units (PFU) of the indicated viruses. Body weight and survival were monitored daily for 14 days. For virological examinations, three mice infected with 10^6 PFU per group were euthanized at 3 and 6 days post-infection. The virus titers in the each tissue were determined by plaque assays in MDCK cells. All experiments with mice were performed in accordance with the University of Tokyo's Regulations for Animal Care

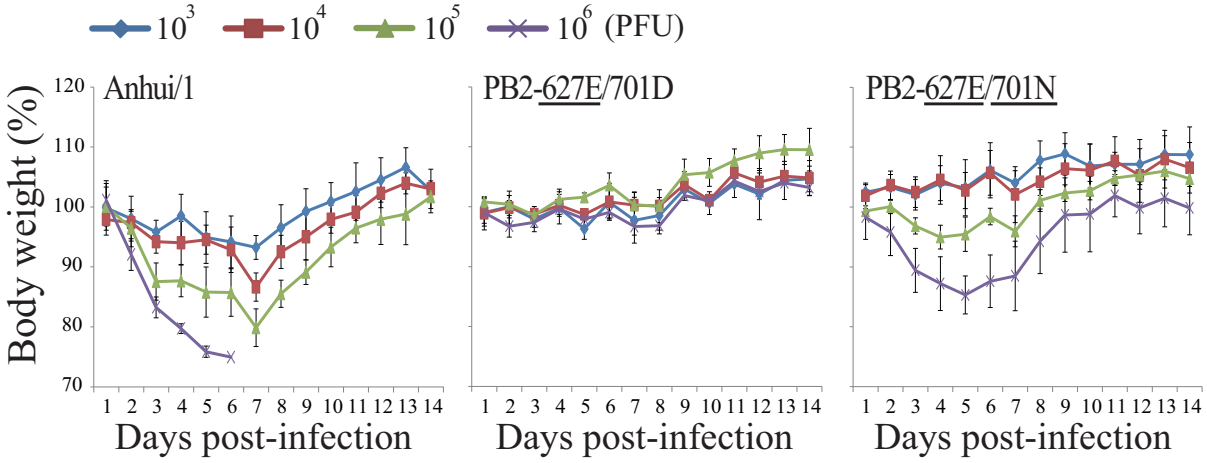

Figure 3 | Virulence of mutant viruses in mice. Four mice per group were intranasally inoculated with 10^3, 10^4, 10^5, or 10^6 PFU (each in 50 µl) of the indicated viruses. Body weight was monitored daily for 14 days. Mice that lost more than 25% of their baseline weight were euthanized. The values represent the average body weight compared with the baseline weight ± SD from four mice.

and Use and were approved by the Animal Experiment Committee of the Institute of Medical Science, the University of Tokyo.

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Author contributions

S. Yamayoshi, S.F., S. Yamada and Y.K. designed the study. S. Yamayoshi, S.F., S. Yamada, D.Z., S.M., R.U. and Y.T. performed the experiments. S. Yamayoshi, S.F., S. Yamada, D.Z., Y.T. and T.W. analyzed the data. S. Yamayoshi, G.N. and Y.K. wrote the manuscript. All authors reviewed the manuscript. S. Yamayoshi, S.F. and S. Yamada contributed equally to this work.

Additional information

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