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Influenza A viruses with different amino acid residues at PB2-627 display distinct replication properties *in vitro* and *in vivo*: revealing the sequence plasticity of PB2-627 position

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Abstract

Sequence analyses of influenza PB2 sequences indicate that the 627 position almost exclusively contains either lysine (K) or glutamic acid (E), suggesting a high sequence constraint at this genetic marker. Here, we used a site-directed random mutagenesis method to demonstrate that PB2-627 position has a high sequence plasticity. Recombinant viruses carrying various amino acid residues at this position are viable in cell cultures. These PB2-627 mutants showed various polymerase activities and replication kinetics in mammalian and avian cells as well as pathogenicity in mice. Serially passaging these mutants in MDCK cells generated some compensatory PB2 mutations that can restore polymerase activities of the PB2-627 mutants. Of these, PB2-D309N was identified as a novel one. Besides showing that influenza virus can tolerate a wide range of amino acid residues at the PB2-627 position, this study also demonstrates a potential strategy to identify novel mutations that can enhance viral polymerase.

Keywords

Influenza; Random mutagenesis; PB2; Polymerase; PB2-627

Introduction

Viral polymerase of influenza A viruses is important for modulating virus replication kinetics, host specificity and pathogenicity (Gabriel et al., 2005; Naffakh et al., 2008; Zhu et al., 2012). Among the three viral polymerase subunits, PB2 is the largest one and it has binding sites for both PB1 and NP at its N-terminus and C-terminus, respectively (Poole et al., 2004). It contains nuclear localization signal (NLS) and is transported independently into

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the nucleus (Huet et al., 2010; Mukaigawa and Nayak, 1991; Tarendeau et al., 2007). Functionally, PB2 binds to the 5′ cap of the host mRNA for obtaining a cap primer for viral transcription initiation. Several genetic markers of PB2 are associated with host adaptation. There has been evidence showing that PB2-E627K mutation is one of the genetic markers leading to enhanced polymerase activity, host adaptation and enhanced virulence (Hatta et al., 2001; Labadie et al., 2007; Massin et al., 2001; Shinya et al., 2004; Subbarao et al., 1993). In general, avian PB2 has a Glu (E) residue at position 627 and replacing this amino acid residue with a Lys (K) can enhance the polymerase activity of an avian vRNP in mammalian cells. This makes PB2-E627K mutation as one of the most important markers for predicting the fitness of an avian virus in mammal hosts. Studies further show that this 627K/E polymorphism can affect viral RNA synthesis (Paterson et al., 2014) and the binding of vRNP to cellular importin (Hudjetz and Gabriel, 2012). On the other hand, the PB2 D701N mutation also alters the binding ability of PB2 to cellular importin, thereby affecting the nuclear localization pattern of PB2 (Boivin and Hart, 2011; Gabriel et al., 2008). Recently, PB2-590 and 591 residues found in the pandemic H1N1/2009 virus were shown to enhance viral replication and virus virulence in mammalian hosts (Liu et al., 2012; Mehle and Doudna, 2009).

In the case of PB2 sequences available from public databases, the majority of influenza viral PB2 proteins possess either a K or E at this position (99.6%, total number of analyzed viral sequences =19,469; personal observations). It is therefore highly logical to study PB2 proteins, vRNPs or recombinant viruses carrying this PB2-627K/E polymorphism. However, it is evident that there is a lack of systematic mutagenic studies of this important position. In addition, there is a small and yet significant number of influenza PB2 segments that have a valine (V) at this position (Davidson et al., 2013; Golender et al., 2008). It is not known whether vRNPs with an atypical amino acid residue at this position behave differently from those carrying a K or E at the same position. In particular, it is of our interest to determine whether introducing atypical amino acid residues at the PB2-627 position would lead to viable viruses and whether such mutations would affect the viral polymerase activity and viral fitness.

Here, we applied a random mutagenesis method to create a number of influenza viruses with different point mutations at the PB2-627 position. These PB2 mutants are characterized both *in vitro* and *in vivo* conditions. In addition, their polymerase activities were also determined by using a reporter gene assay for influenza polymerase. Our results indicate that the phenotypes of these viruses and genetic stabilities of these mutations are different.

Results

Generation of recombinant viruses with mutation at PB2-627

The primary objective of this study is to determine the sequence plasticity of PB2-627 position. A laboratory-adapted strain (A/Puerto Rico/8/1934; PR8) is used as the prototype virus of this study. Recombinant viruses were generated by a plasmid-based reverse genetic system (Hoffmann et al., 2000) (Fig. 1). An inverse PCR technique was used to introduce random mutations at the PB2-627 in the PB2 expression plasmid as described in the Materials and Methods section (Dominy and Andrews, 2003; Valetti and Gilardi, 2013).

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Previous studies have demonstrated that viral particles with a defective polymerase gene can be generated by reverse genetics (de Wit et al., 2006; Ozawa et al., 2011), indicating the formation of virions in transfected human embryonic kidney 293T cells is independent of the functionality of influenza viral polymerase complex. Infectious mutants generated from transfected 293T cells were amplified in Madin-Darby canine kidney (MDCK) cells and 10-day-old chicken embryonated eggs, followed by plaque purification using MDCK cells (hereafter called as “mammalian system”) and chicken fibroblast DF-1 cells (hereafter called as “avian system”), respectively.

In the mammalian virus culturing system, a total of 41 purified viral clones were randomly selected from three independent transfection experiments. These mutants were found to contain a total of 7 different amino acid residues, including Ala (A), Ile (I), K, Leu (L), Arg (R), Ser (S) and Val (V), at the PB2-627 position (Table 1). In contrast, 17 plaque purified clones cultured in the avian system were selected for further characterization. These recombinants were obtained from a single transfection experiment and were found to contain A, Cys (C), K and Glu (Q) at the PB2-627 position (Table 1). Overall, 9 different PB2 mutant viruses were rescued, with PB2-627A and PB2-627K mutants derived from both mammalian and avian systems. Apart from the introduced mutations, no mutation was detected in other PB2 regions. These results suggest that there is high sequence plasticity at the PB2-627 position and that a K or E residue at this position is not absolutely essential for viruses to propagate in cell lines.

Effect of PB2-627 on polymerase activity of vRNPs in mammalian and avian cells

The PB2-627 K/E polymorphism is known to modulate viral polymerase activities in mammalian and avian cells. To study whether the identified PB2-627 mutations would affect viral polymerase activities, the PB2 segment from each of these recombinant viruses was individually cloned and tested for its activities in two luciferase reporter assays (1 for mammalian cells and 1 for avian cells) (Li et al., 2009). Recombinant vRNPs of these viruses, together with a control vRNP carrying PB2-627E, were reconstituted in mammalian 293T cells and avian DF-1 cells. The transfected cells were incubated at 37°C and 39°C, which mimic the core temperatures of human and chicken hosts, respectively.

In transfected 293T cells incubated at 37°C, vRNPs with PB2-627K and PB2-627E had the highest and lowest polymerase activities, respectively (Fig. 2A). With the exception PB2-627R and PB2-627A mutants, all vRNP mutants (I, L, S, Q and C) caused various degrees of reduction of polymerase activities. At 39°C, all the vRNPs had further reduction of polymerase activity compared with the wild-type level (PB2-627K), but the overall pattern of these reductions was similar to those observed at 37°C. In contrast, there were less variations of viral polymerase activity observed in the DF-1 cells (Fig. 2B). At 37°C, vRNPs carrying I, R, S, V, C and E at PB2-627 positions were found to be less active in the reporter assays, but these mutants still had at least 50% of the wild-type activity. At 39°C, the majority of these vRNPs had activities comparable to those observed at 37°C. Interestingly, 2 of these vRNP mutants (PB2-627A and 627C) were found to have enhanced polymerase activities at 39°C, although these were not significantly different from the wild-type level. It

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is important to note that PB2-627A and PB2-627C were those identified from recombinant viruses rescued in avian cells (Table 1).

It is interesting to note that the polymerase activities of these PB2 mutants in transfected 293T cells are positively correlated to the isoelectric point (pI value) of the introduced amino acid residues (37°C, R^2=0.72; 39°C, R^2=0.76). This suggests that the polymerase activity of vRNP can be influenced by the net charge of PB2-627 residue in 293T cells. The more positive the charge at PB2-627 residue is, the higher the polymerase activity will be. However, this correlation was not observed in vRNPs constituted in DF-1 cells at 37°C or 39°C (37°C, R^2=0.01; 39°C R^2=0.02), suggesting the correlation between pI value of PB2-627 amino acid residue and viral polymerase activity is host or cell type specific.

Overall, vRNPs with different amino acids at PB2-627 showed different polymerase activities in different host cells. It was also observed that temperature can affect viral polymerase activity and this response is cell type dependent.

Effect of PB2-627 on growth kinetics of recombinant viruses in mammalian and avian cells

To determine whether the identified PB2-627 mutations would have effects on virus replications, growth kinetics of these recombinant viruses were characterized in mammalian MDCK and avian DF-1 cells at two different temperatures. Viral growth kinetics of mutants obtained from the mammalian system, i.e. K (wild-type), A, I, L, R, S and V, were first characterized in MDCK cells at 37°C and 39°C. At 37°C, all recombinant viruses had growth rates similar to the wild-type level (Fig. 3A). However, at 39°C, PB2-627L mutant was found to have a significantly reduced growth kinetic and its titer at 72h post-infection was about 2 logs lower than that of the PB2-627K virus (Fig. 3B). In addition, PB2-627A, 627S and 627V mutants were also found to have moderate but significant reductions in viral titers compared with the PB2-627K virus (Fig. 3B). Recombinant PB2 mutants generated in the avian system (i.e. K (wild-type), C and Q) and an additional site-directed PB2 control mutant carrying the avian PB2-627E marker were also characterized in the MDCK cells in the same manner. At 37°C, both PB2-627Q and PB2-627E mutants were found to have slightly reduced replication kinetics than the wild-type PB2-627K virus (Fig. 3C). The effects of these mutations on virus replication kinetics were more pronounced when infected cells were incubated at 39°C. In these experimental conditions, PB2-627Q and PB2-627C mutants were found to have about 1 and 2 log reductions, respectively, in viral titers, compared with the wild-type level (Fig. 3D).

To investigate the effects of these mutations on virus replication in avian cells, representative mutants generated from the mammalian (A, K, L, R and V) and avian (K, C and Q) systems were characterized in avian DF-1 cells (Fig. 4). Of the viruses generated from the mammalian system, the PB2-627R mutant had the most significant retardation in growth rates compared with the wild-type levels at both 37°C and 39°C (Figs. 4A and 4B). Interestingly, the PB2-627L mutant, which had a reduced replication rate in MDCK cells, was found to have a significant increase of virus titer compared with the wild-type at both temperatures. The growth kinetics of PB2-627A and PB2-627V mutants were found to be similar to the wild-type. Of the mutants generated from the avian system, viruses PB2-627K,

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PB2-627C and PB2-627Q had similar replication kinetics at both temperatures, whereas the PB-627E control virus had the highest replication kinetic in DF-1 cells.

The above results overall demonstrate that influenza viral polymerase activity might not necessarily correlate to virus replication kinetics. Mutants that have highly reduced viral polymerase activities might have robust virus replication rates in cells, whereas some mutants that have moderate polymerase activities were found to have highly reduced virus replications (e.g. PB2-627L vs PB2-627S in MDCK cells at 39°C). Nonetheless, mutants derived from the mammalian system generally had larger variations in virus titers in DF-1 cells (Figs. 4A), but their titers in MDCK cells at 37°C were found to be comparable (Fig. 3A). In contrast, mutants derived from the avian system generally had larger variations in virus titers in infected MDCK cells at 39°C (Fig. 3D), but their titers in DF-1 cells at the same incubation temperature were found to be similar (Fig. 4D).

Some recombinant viruses are less virulent in mice and are genetically unstable

To determine the virulence of these mutants in mice, all recombinant viruses rescued from the mammalian and avian systems together with the PB2-627E control mutant were used to infect mice via intranasal route with a dose of 1000 $TCID_{50}$ (~3 MLD_{50} of wild-type virus).

The wild-type PB2-627K virus was found to be the most virulent in mice and PB2-627I, 627R and 627E mutants were found to be more virulent than the other PB2 mutants in terms of weight loss and mortality rate (Figs. 5A and 5B). Mice infected with PB2-627K, 627I, 627R and 627E mutants (N=6 per group) had the most dramatic weight loss at day 3 post-infection (Fig. 5A) and met the experimental endpoint (>30% of weight loss) at or before day 8 post-infection (Fig. 5B). In contrast, mice infected with other mutant viruses (PB2-627L, 627A, 627S, 627V, 627C and 627Q) had only moderate, if any, weight loss at the early post-infection time points. However, these infected mice started to have weight loss at day 5 post-infection and about 70–100% of these mice reached the experimental endpoint at the second week of post-infection (Figs. 4A and B).

Some of the representative PB2 mutants were selected for further characterization in mice. Mice were initially infected by the wild-type PB2-627K virus or its mutant (PB2-627I, 627R, 627C or 627E) with a dose of 1000 $TCID_{50}$ and the infected mice were checked for lung virus titers at day 3 and day 6 post-infection (N=3 per group). It was observed that PB2-627C mutant, which is less virulent in mice, replicated less well than the PB2-627K virus in mouse lungs (Fig. 5C). Of the 3 mutants that have virulence comparable to the wild-type virus, PB2-627I and PB2-627R mutants yielded lung virus titers similar to the wild-type at both the studied time points. In contrast, PB2-627E, which initially had a wild-type level at day 3 post-infection, produced less virus titers than the wild-type virus in mouse lungs at day 6 post-infection.

Based on the weight loss and mortality rates observed in the infected mice (Fig. 5A and B), these PB2 mutants generated from our random mutagenesis can generally be classified into two groups: 1) those that can cause rapid weight loss and death in the first week post-infection; and 2) those that can cause mild weight loss in the first week but death in the second week. To determine whether these PB2 mutations are still genetically stable after a

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single passage in mice, the PB2 genes of these viruses were sequenced at the stage where the mice were close to the experimental endpoints (day 6 post-infection for PB2-627K, 627I and 627R; day 12–14 post-infection for PB2-627L, 627S, 627V, 627A, 627C and 627Q; N=2 per virus). The 627I and 627R mutations, together with the wild-type PB2 627K residue, were found to be stable in mice and no mutation was detected at this position at the experimental endpoint. Strikingly, samples from mice infected with PB2-627L, 627S, 627A, 627C and 627Q were all found to have mutations at the PB2 627 position and each of these samples were mixtures of viruses containing an E, a K or the introduced mutation at the position. For mice infected with PB2-627V mutant, the samples were found to contain mixture of viruses carrying the introduced mutation or an E at this position. The detection of K and/or E at PB2-627 might also explain why mice infected with the second group of mutants (PB2-627L, 627S, 627V, 627A, 627C and 627Q) died in the second week post-infection.

Genetic stability of mutations at PB2-627 residues in MDCK cells

In order to further investigate the genetic stability of these PB2 627 mutations, the PB2 mutants were serially passaged in MDCK cells at 37°C and 39°C in duplicate with initial infection at MOI of 0.01. The PB2 genes of these mutants were sequenced after 10 serial passages. Interestingly, all PB2 mutations were found to be genetically stable in all experiments. None of these mutants has PB2-627E or 627K mutation as observed in the above *in vivo* experiments. Instead, some of the mutants were found to acquire additional mutations in other PB2 regions (Table 2). In general, mutants passaged at 39°C were found to be more likely to have additional mutations than those passaged at 37°C. It is also interesting to note that some of these mutations could be identified more than once, either from independent passages of the same mutant or from passages of different PB2-627 mutants. Mutations A152V and Q591K were reproducibly found in progeny viruses of PB2-627V and PB2-627E mutants, respectively, at different incubation temperatures, whereas mutations D309N, I504V and D701N were reproducibly found in different PB2 mutants passaged at a specific temperature.

To determine the impacts of these five reproducible mutations on viral polymerase activity, they were individually introduced into the corresponding PB2-627 mutants. These double mutants were then characterized by the viral polymerase reporter assay as described above. Four of the selected mutations (D309N, I504V, R591K and D701N) were found to enhance the viral polymerase activity compared with the corresponding controls (Fig. 6). In particular, PB2-627 mutants carrying D309N or I504V were found to have polymerase activities that are higher than the wild-type level (PB2-627K). However, the introduction of PB2-A152V to the PB2-627V mutant was found to have no effect on viral polymerase activity.

PB2-D309N mutation alone can enhance polymerase activity of mammalian and avian vRNPs

Among the 4 compensatory mutations that can restore or partially restore the polymerase activity of above PR8 PB2-627 mutants, there has been no previous report on the biological functions of PB2-D309N (see Discussion). In order to investigate the effect of this mutation

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alone on the polymerase activity, the D309N mutation was introduced into wild-type PB2 of PR8 by site-directed mutagenesis. The polymerase activity of the mutant vRNP was compared with the wild-type vRNP at 37°C and 39°C. The result indicated that PB2-D309N mutation alone can enhance the polymerase activity of PR8 vRNP at 37°C and 39°C (Fig. 7A).

To test whether the PB2-D309N mutation can also stimulate the viral polymerase activity of an avian vRNP, this mutation was introduced into the PB2 of an avian strain (A/Quail/HK/G1/1997, G1; H9N2). The polymerase activity of the mutant G1 vRNP was compared with the wild-type G1 vRNP at 37°C and 39°C (Fig. 7B). Similar to the result of PR8 vRNPs, the PB2-D309N mutation enhance the polymerase activity of G1 vRNP by about two folds at 37°C. At 39°C, the polymerase activity of the mutant vRNP also enhanced, albeit with a lower enhancement compared to that in the PR8 background. These results demonstrated that PB2-D309N mutation can enhance polymerase activity of human and avian vRNPs.

Discussion

PB2-627 is a well-known genetic marker for host adaptation, virulence and transmission (Hatta et al., 2001; Herfst et al., 2012; Imai et al., 2012; Naffakh et al., 2008; Steel et al., 2009; Subbarao et al., 1993). However, most of the studies have focused on PB2-627K and 627E, which are the prevailing amino acids at this position in human and avian viruses, respectively. Using a targeted random mutagenesis approach, we created a panel of viable viruses with different amino acid residues at the PB2-627 position (Table 1). Surprisingly, our results demonstrated that the influenza viral polymerase can accommodate a wide range of amino acids residues at this position (i.e. acidic, basic, aliphatic, sulfur-containing and hydroxyl amino acid residues). These mutants exhibited distinct properties in different experimental settings and the mutations were at least stable in the *in vitro* conditions.

The viral polymerase reporter assays showed that PR8 vRNP possessing PB2-627K had the highest polymerase activity and the control mutant with an avian signature at the PB2 627 position (PB2-627E) had the lowest polymerase activity in human 293T cells (Fig. 2A). This is in agreement with previous findings in that PB2-E627K mutation enhances polymerase activity in mammalian cells (Labadie et al., 2007; Moncorge et al., 2010; Naffakh et al., 2000). The novel PB2 mutants generated from this work were found to have polymerase activities in between those PB2-627K and PB2-627E in 293T cells. Previous studies of the PB2-627K/E polymorphism showed that a PB2-K627E mutation disrupts a prominent basic surface patch located at the PB2-627 domain (Tarendeau et al., 2008). It is possible that the surface charge difference of the 627-domain of PB2 is a determining factor of polymerase activity. This hypothesis is supported by our observations that the viral polymerase activity in 293T cells is positively correlated to the pI value of the introduced amino acid mutation. However, this correlation was not observed from data deduced from avian DF-1 cells. These findings agree with previous findings that the host restriction due to PB2-627K/E polymorphism is observed in mammalian, but not in avian, cells (Labadie et al., 2007; Mehle and Doudna, 2008).

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In this study, a few PB2-627 mutants were found to have distinct properties under certain experimental settings. We sequenced the PB2, PB1, PA and NP genes of plaque-purified mutant that were subsequently found to have interesting phenotypes. Specifically, mutants that have pathogenicity comparable to the wild-type virus (PB2-627R, 627E and 627I) and mutants that have the high (PB2-627A) or low (PB2-627C, 627S) polymerase activities were selected for the additional sequencing analyses. No mutation was identified in the studied mutants, suggesting that our observations were primarily due to the introduced mutations.

Amongst these PB2-627 mutants, PB2-627R had the highest polymerase activity in 293T cells but it had the lowest activity in DF-1 cells. Mutations selected by the avian system (e.g. PB2-627A, 627C and 627Q) were found to have polymerase activities similar to the wild-type PB2-627K in DF-1 cells at 39°C, but they had variable activities in 293T cells. These results suggest that some of the PB2-627 mutations might affect the viral polymerase in a conditional manner. The mechanism accounts for their unique properties are yet to be determined. Nevertheless, this panel of mutants would be a useful tool to study the role of PB2-627 position in some specific functions, such as vRNA promoter binding, of influenza viral polymerase.

The growth kinetics of these viruses were not fully correlated to their polymerase activities, suggesting that viral polymerase activity is normally not a rate-limiting factor for virus replication. However, viruses that were found to have much reduced replication kinetics also showed reduced polymerase activities at the corresponding conditions (e.g., PB2-627C in mammalian cells at 39°C and PB2-627R in avian cells). We also noted that the PB2-627 mutants generated from the mammalian system yielded more variable titers in DF-1 cells at 37°C. PB2-627 mutants generated from the avian system cultured in MDCK cells at 39°C displayed similar behaviours (Figs. 3 and 4). We reason that when the mammalian-derived PB2-627 mutants were cultured in a condition very different from the original culturing condition (i.e. DF-1 cell at 39°C vs MDCK cells at 37°C), some of these mutants might not achieve robust virus replications in such “unfavorable” conditions. This possible explanation can also apply to PB2 mutants generated from the avian system. These findings support the hypothesis that different hosts might have different tolerances to or preferences for amino acids at this PB2 position. It is of interest to use the same study approach to generate PB2-627 avian virus mutants to test this hypothesis and validate our findings in future. In addition, we do not exclude the possibility that our observations may also be affected by the cell type used in our models.

Of the 58 plaque purified clones generated in the study, none of these recombinant viruses were found to contain E at the PB2-627 position (Table 1). This result is rather surprising and does not entirely agree with observations found in nature. In addition, our results indicate that the PB2-627E control mutant achieved robust virus replications in our experiments (Figs. 3 and 4). PB2-627E mutant alone can form distinct viral plaques in standard plaque assays, indicating that the failure of detection of PB2-627E mutant was not an artefact caused by the clone isolation method. One, however, should note that viral polymerase with PB2-627E was shown to have the lowest polymerase activity in the luciferase reporter assays. It is possible that the poor polymerase activity of PB2-627E mutant is the limiting factor that accounts for our observations in the virus rescue work.

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For other mutations that were not detected in the rescued viruses, it is possible that mutants with these mutations might have poor plaque forming ability and cannot be isolated by plaque purification. Alternatively, mutants with these mutations might also have highly reduced polymerase activities and could not complete with other mutants in the initial virus cultures. Other study approaches, such as use of limiting dilution methods for clone isolation and increase sampling size in the initial screening, may be useful to test the above hypotheses.

Although the PB2-627E mutation might reduce virus polymerase activity, the amino acid clearly has an unknown advantage for influenza virus in infected cells. Both *in vitro* and *in vivo* data revealed that the mutant virus with PB2-627E does not compromise as much as some other mutants do in terms of replication ability and pathogenicity. We sequenced the whole vRNP complex and no compensatory mutation was found in the PB2-627E mutant. For all PB2 mutants that caused delayed disease onset in mice (PB2-627L, S, V, A, C and Q), they were found to have generated progeny viruses with PB2-627E in the lungs. The potential benefits of acquiring PB2-627E mutation in these mutants are not known. With the results obtained from our *in vitro* data, we speculate that the acquisition of PB2-627E mutation might have benefits other than regulating viral RNA polymerase activity itself. For example, PB2 is known to have effect on degradation of host RNA polymerase II. Further characterization of this panel of PB2 mutants might help to understand the role of PB2-627 in the virus lifecycle. Nonetheless, our data demonstrated that there is a much more stringent amino acid requirement at this position in the *in vivo* conditions. This might also explain why animal influenza viruses found in nature predominantly have an E, if not K, at this position.

Of the six mutants that could generate PB2-627E mutation in mice, five also generated progeny viruses with PB2-627K mutation in the same infected mice. It is not known whether these PB2-627E and PB2-627K mutations occurred independently or sequentially. Previous studies have demonstrated that the PB2-E627K mutation can occur in mice (Bogs et al., 2011). These previous results highly suggest that the mutations of our PB2 mutants in mice occurred in a sequential manner, with the mutants first acquiring a PB2-627E mutation, followed by a PB2-E627K mutation. The PB2-627I and PB2-627R mutants had virulence similar to PB2-627E in mice. No mutation was detected in mice infected with PB2-627I and PB2-627R at the experimental endpoint. Besides, it is interesting to note that PB2-627I mutant, which does not carry a charged amino acid side chain at the PB2-627, was also shown to have virulence comparable to PB2-627E, 627R and 627-K viruses. This indicates that a charged amino acid side-chain at this position is not absolutely essential to achieve a robust replication in mice.

In addition to the amino acid change at PB2-627 position, the RNA sequence of the PB2 gene may also have a role in modulating the viral fitness. Most of the PB2-627 mutants tested consist of two (I, L, R, S, V and A) or even three (C) nucleotide changes at the codon compared to the wild-type PB2-627K or the avian signature PB2-627E (Supplementary table). It is not known whether these RNA sequence changes would have effects on the secondary structure of the PB2 vRNA segment, which may affect the phenotype of a virus (Witteveldt et al., 2014).

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Contrary to our *in vivo* findings, all of the introduced PB2-627 mutations were found to be stable *in vitro* and no mutation at this position was detected after 10 serial passages in MDCK cells. This demonstrates that the selection pressures from these *in vitro* cultures to the PB2-627 position were different from those imposed from *in vivo* conditions. We, however, detected a number of point mutations at other PB2 positions in the passaged mutants. Of the five reproducible mutations, four (309N, 504V, 591K and 701N) are compensatory mutations that could completely or partially restore viral polymerase activities. PB2 591 and 701 positions are well-known markers for enhancing viral polymerase activities in mammalian cells. The PB2-D701N mutation can increase the binding of PB2 to importin-α1 in mammalian cells, leading to enhanced viral polymerase activity (Gabriel et al., 2005; Gabriel et al., 2008; Zhou et al., 2013). The PB2-Q591R mutation can compensate for the absence of PB2-627K in mammalian adaptation (Liu et al., 2012; Mehle and Doudna, 2009; Yamada et al., 2010). The PB2-I504V mutation was found to have the strongest stimulatory effects on the viral polymerase activities. This mutation has previously been shown to have a role in regulating viral polymerase activity, viral virulence and host RNA polymerase II degradation (Llompart et al., 2014), but the relationship between these events is yet to be fully understood. In contrast, little is known about the biological function of PB2-309 position. The residue is close to the cap binding domain of PB2, but none of the resolved PB2 protein structures can provide information regarding this position. We are also not aware of any biological or biochemical studies at this position. Knowing the fact that the PB2-D309N mutation can be repeatedly found in two different PB2-627 mutants and the mutation can significantly enhance viral polymerase activity, in both human and avian vRNPs, further investigation of this PB2-D309N mutation is warranted.

This study not only characterizes a number PB2-627 mutants, but also demonstrates the combine use of site-directed random mutagenesis and reverse genetics techniques (Dominy and Andrews, 2003; Hoffmann et al., 2001; Valetti and Gilardi, 2013) to generate recombinant influenza viruses with random mutations at the position of interest. With this approach, much effort can be saved for generating multiple mutant viruses with different amino acids by site mutagenesis. In addition, our results suggest that viruses with “suboptimal” viral polymerase activities attempt to restore their fitness by introducing mutations at other vRNP regions. Although it is not known whether these compensatory mutations directly cooperate with the PB2-627 residue in the virus lifecycle, we reason that our approach is a good strategy to identify novel mutations that can enhance viral polymerase.

In summary, our results demonstrate that influenza virus can tolerate a wide range of amino acids at the PB2-627 position. Viruses with mutations at this position displayed different properties *in vitro* and *in vivo*. The stability of these PB2-627 mutations *in vitro* and *in vivo* is different, indicating that the selection pressures imposed by the *in vitro* and *in vivo* conditions on the PB2-627 position are different and that influenza virus use different strategies to restore the viral fitness in these conditions. Further characterization of these PB2 mutants and their derivatives might help to better understand some specific biological events of influenza viral polymerase.

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Materials and methods

Cell cultures and viruses

MDCK cells, 293T cells, specific pathogen free embryonated chicken eggs and DF-1 cells were used in this study. MDCK cells and 293T cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Life Technologies). DF-1 cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS. Influenza viruses of PR8 and G1 backgrounds were generated by reverse genetics technique as described (Hoffmann et al., 2000).

Generation of recombinant viruses with random amino acid at PB2-627

Introduction of random mutations at the 627 position of PR8-PB2 was done by site-directed random mutagenesis by PCR (Valetti and Gilardi, 2013). The pHW2000-PR8-PB2 plasmid was amplified using inverse PCR with degenerated forward primer (5′-CCANNNCAAAGTAGAATGCAGTTCTCC, N= any base) and reverse primer (5′-TGGAGCGGCTGCGAAGGGAAG), followed by blunt-end ligation, to introduce the random mutation at PB2-627 position (Dominy and Andrews, 2003). The repaired circular PB2 plasmid DNA was gel purified using a gel extraction kit (Qiagen). The mutated PB2 plasmid preparation was transfected, together with the other 7 plasmids encoding the remaining influenza viral segments, into 293T cells to generate recombinant PR8 mutants by reverse genetics technique (Hoffmann et al., 2000). The progeny viruses were harvested 48 hours post-transfection and were inoculated into MDCK cells (mammalian system) or 10-day-old embryonated chicken eggs (avian system). The infected cells and eggs were incubated for 72 hours at 37 °C. The progeny viruses from the infected MDCK cells and eggs were plaque purified in MDCK cells and DF-1 cells, respectively. Well isolated plaques were picked and cultured in the corresponding cells. The full-length PB2 gene of each plaque purified progeny virus was sequenced by standard dideoxy sequencing.

Luciferase reporter assay

The polymerase activities of vRNPs carrying different PB2 627 mutations in avian (DF1) and human (293T) cells were determined by luciferase reporter assays (Li et al., 2009). Plasmid mixtures were prepared by mixing pcDNA plasmids expressing PB2 (wide type or its mutants), PB1, PA and NP proteins together with pPolI-Luc-NS and pMax-GFP. For determination of polymerase activity in avian DF-1 cells, the promoter of the pPolI-Luc-NS plasmid was replaced with an avian promoter. The plasmid mixtures were transfected into cells seeded on a 96-well culture plate and transfected cells were incubated at 37°C or 39°C for 48 hours. The transfected cells were then lysed by Steady-Glo luciferase assay substrate solution (Promega) for 5 minutes. The luciferase activities were determined by the luminescence measured with a luminometer (PerkinElmer) and the data were normalized with expression level of GFP (Li et al., 2009).

Viral growth kinetics assay

MDCK or DF-1 cells were grown on 24-well culture plates to confluence. The mammalian and avian cells were infected by the viruses at MOI of 0.01 and 0.1 respectively. After 1

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hour of viral adsorption, the virus inoculum was removed and the cells were briefly washed with acidic buffer (0.9% NaCl solution of pH 2) to inactivate any free, unattached viral particles (Yen et al., 2005). The cells were then washed with PBS and replenished with virus culture medium (MEM supplement with 1 μg/ml TPCK trypsin for MDCK cells and DMEM supplement with 0.2% FBS and 1 μg/ml TPCK trypsin for DF-1 cells). The cells were incubated at 37°C or 39°C. Samples were harvested at different post-infection time points as indicated. Viral titers of the harvested samples were determined by standard plaque assays using MDCK cells.

In vivo pathogenicity study

Female BALB/c mice of 4–6 week old were used for the *in vivo* pathogenicity studies. Mice were infected with 1000 $TCID_{50}$ of rescued viruses (N=6 per group) via intranasal route. Infected mice were monitored and weighed for 14 consecutive days after infection. Mice with weight loss of more than 30% of the initial body weight were humanely euthanized.

To determine the lung viral titer of the infected mice, they were infected with the rescued viruses as describe above. The mice were humanely euthanized at day 3 or 6 post-infection (N=3 per group). The harvested lungs were homogenized in 1 ml of ice-cold PBS. The viral titer of the lung homogenate was determined by standard $TCID_{50}$ assay using MDCK cells.

Two mice from each group were euthanized when they are close to the experimental endpoint (day 6 post-infection for PB2-627K, 627I and 627R; day 12–14 post-infection for PB2-627L, 627S, 627V, 627A, 627C and 627Q). The lungs were dissected and were homogenized. Viral RNA was extracted from the lung homogenates with the viral RNA extraction kit (Qiagen). DNA sequences of the full-length PB2 genes of the progeny viruses obtained from the lung homogenates were determined by standard dideoxy sequencing.

Serial passage of recombinant viruses with PB2-627 mutants

MDCK cells were infected by wild-type PR8 or its PB2 mutants at MOI of 0.01. The infected cells were incubated at 37°C or 39°C for 72 hours before harvesting the virus cultures. The harvested cultures and their diluted samples (1:10 and 1:100 dilutions) were inoculated into MDCK cells. Virus cultures from cells infected with the most diluted samples that still showed cytopathic effects were selected for further serial passaging. Progeny viruses of each PB2 mutant were harvested at the 10^{th} passage and the full-length PB2 of each passaged mutant was determined by standard dideoxy sequencing.

Statistical analysis

Unless otherwise specified, data obtained in the experiments were expressed as mean values of results of three individual experiments (±standard deviation). Data were analyzed by Student's t-test. The difference was considered significant with $p < 0.05$.

Supplementary Material

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Highlights

1. Influenza A virus has a high sequence plasticity at the PB2-627 position.
2. The selection pressures imposed by in vitro and in vivo conditions on this position are different.
3. A PB2-D309N mutation can enhance viral polymerase activity.

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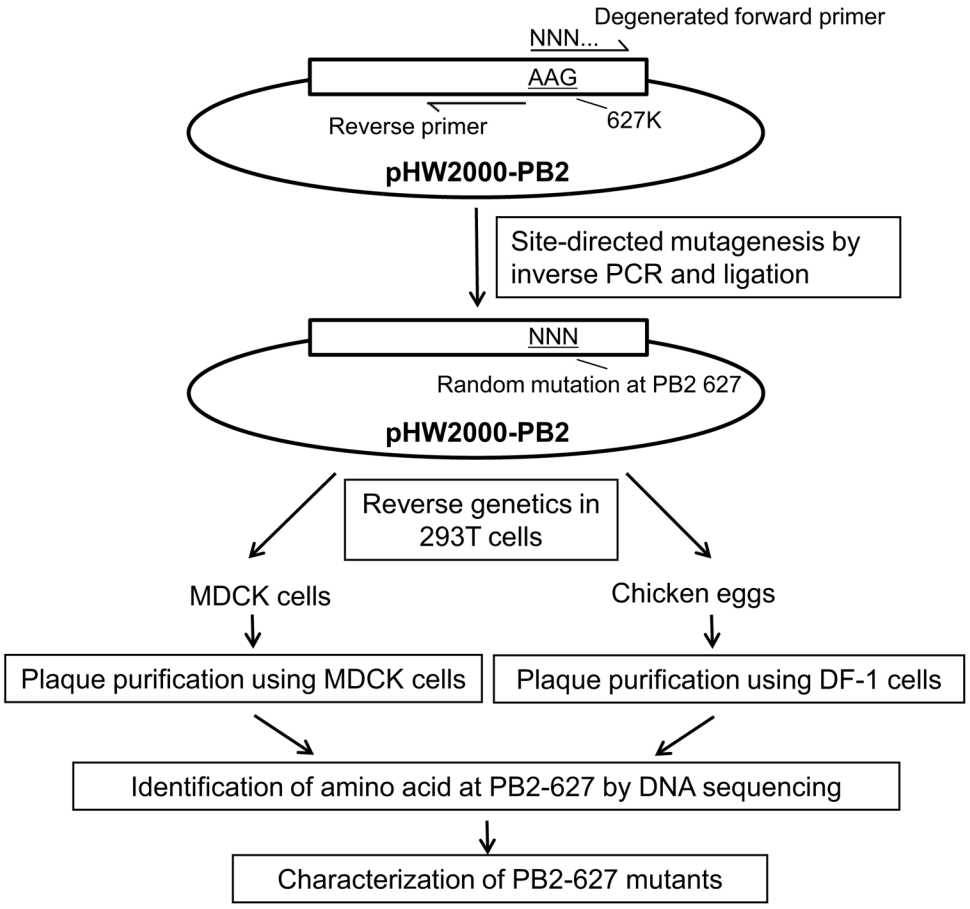

Fig. 1.

Schematic diagram showing the methodology of generating recombinant viruses with random mutations at PB2-627.

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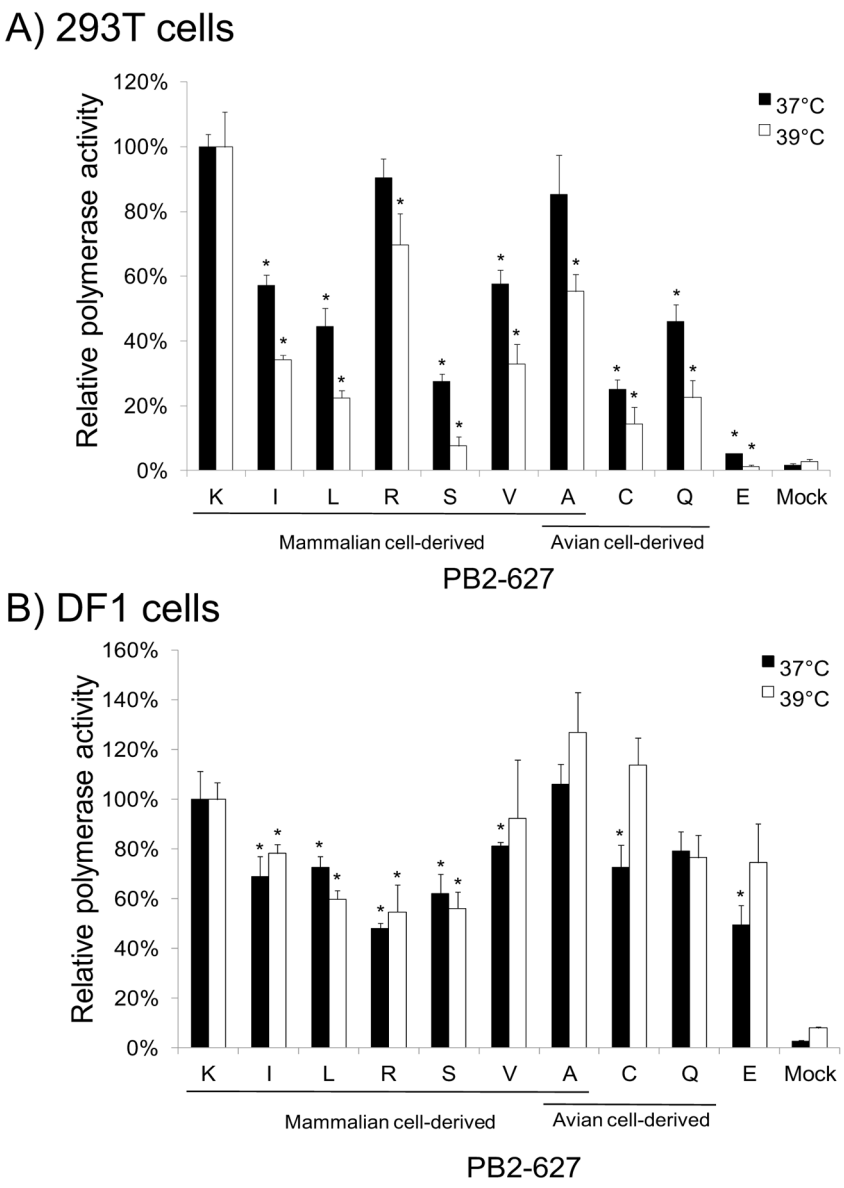

Fig. 2.

Effect of PB2-627 mutations on relative polymerase activity of PR8 vRNP complexes in mammalian and avian cells at different temperatures. Mutated vRNP complexes were reconstituted in (A) mammalian 293T cells and (B) avian DF-1 cells at 37°C (black) and 39°C (white). Polymerase activities were determined by luciferase reporter assays and were normalized by GFP expression. The normalized results are expressed as mean relative polymerase activity in relative to wild-type PR8 vRNP (PB2-627K) at the corresponding cell type and temperature. Mock represents the negative control without vRNP complexes. Error bars represent one standard deviation (N=3, * $p<0.05$, by t-test).

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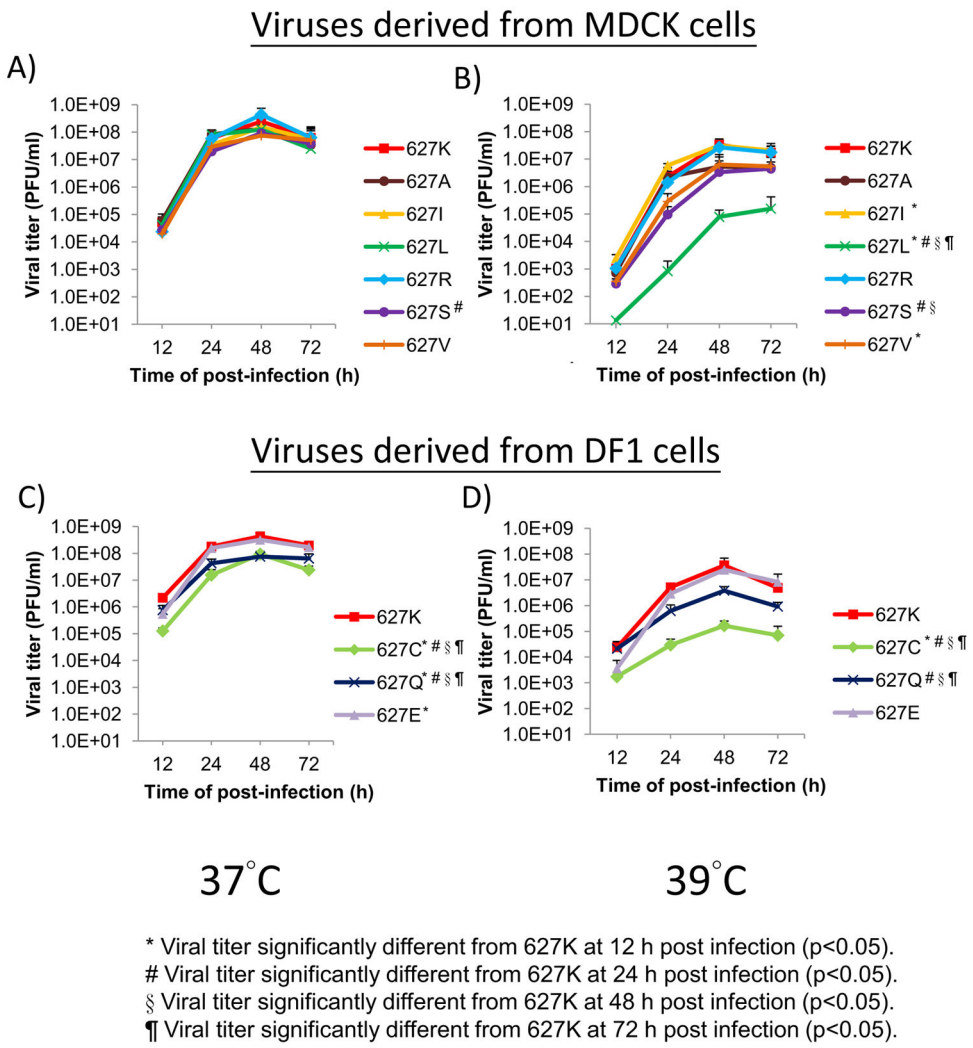

Fig. 3.

Effect of PB2-627 mutations on growth kinetics of recombinant PR8 viruses in MDCK cells at different temperatures. MDCK cells were infected by PB2-627 mutants derived from the mammalian system (A and B) or the avian system (C and D) at MOI of 0.01. The cells were incubated at 37°C (A and C) or 39°C (B and D). Supernatants were harvested at 12 h, 24 h, 48 h and 72 h post-infection, and were subjected to standard plaque assay with MDCK cells for determination of viral titers. Error bars represent one standard deviation (N=3). Significant differences as compared with the wild-type virus are indicated by * for 12 h, # for 24 h, § for 48 h and ¶ for 72 h post-infection ($p<0.05$, by t-test).

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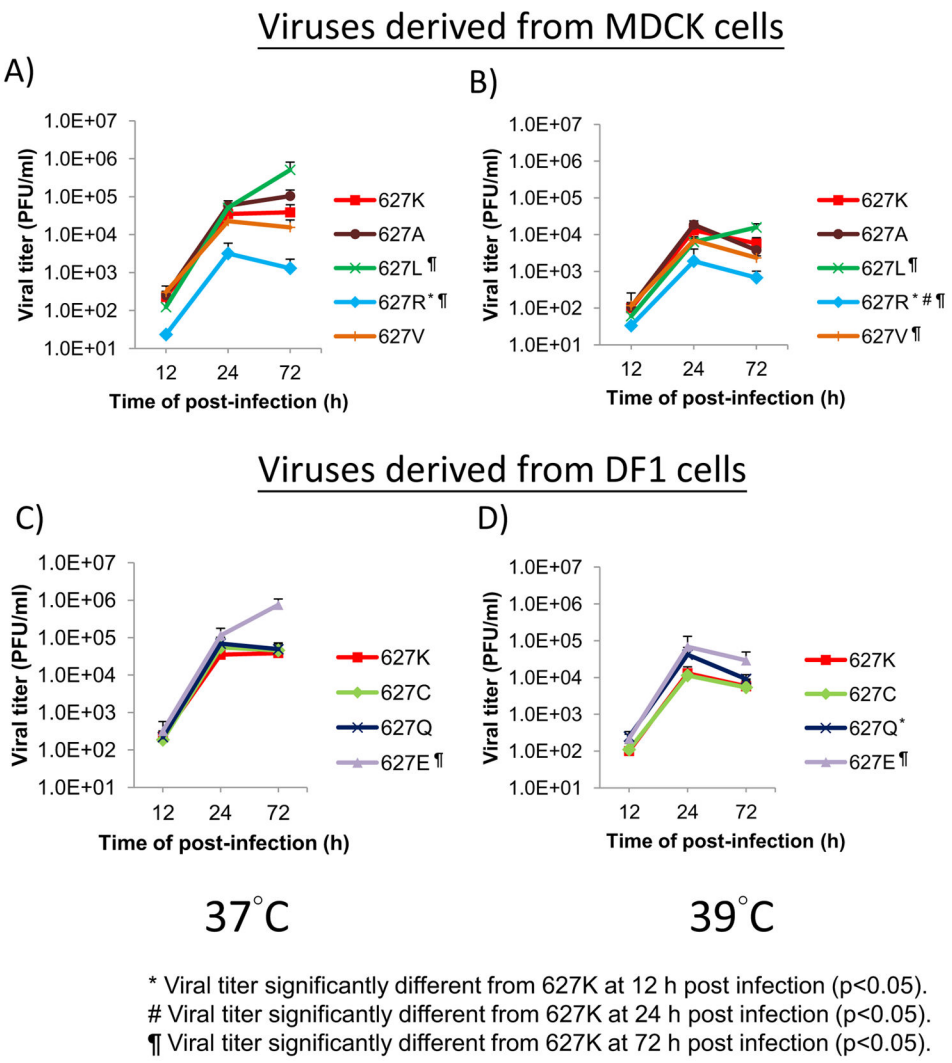

* Viral titer significantly different from 627K at 12 h post infection ($p<0.05$).
\# Viral titer significantly different from 627K at 24 h post infection ($p<0.05$).
¶ Viral titer significantly different from 627K at 72 h post infection ($p<0.05$).

Fig. 4.

Effect of PB2-627 mutations on growth kinetics of recombinant PR8 viruses in DF-1 cells at different temperatures. DF-1 cells were infected by PB2-627 mutants derived from the mammalian system (A and B) or the avian system (C and D) at MOI of 0.1. The cells were incubated at 37°C (A and C) or 39°C (B and D). Supernatants were collected at 12 h, 24 h and 72 h post-infection, and were subjected to standard plaque assay with MDCK cells for determination of viral titers. Error bars represent one standard deviation (N=3). Significant differences as compared with the wild-type virus are indicated by * for 12 h, # for 24 h and ¶ for 72 h post-infection ($p<0.05$, by t-test).

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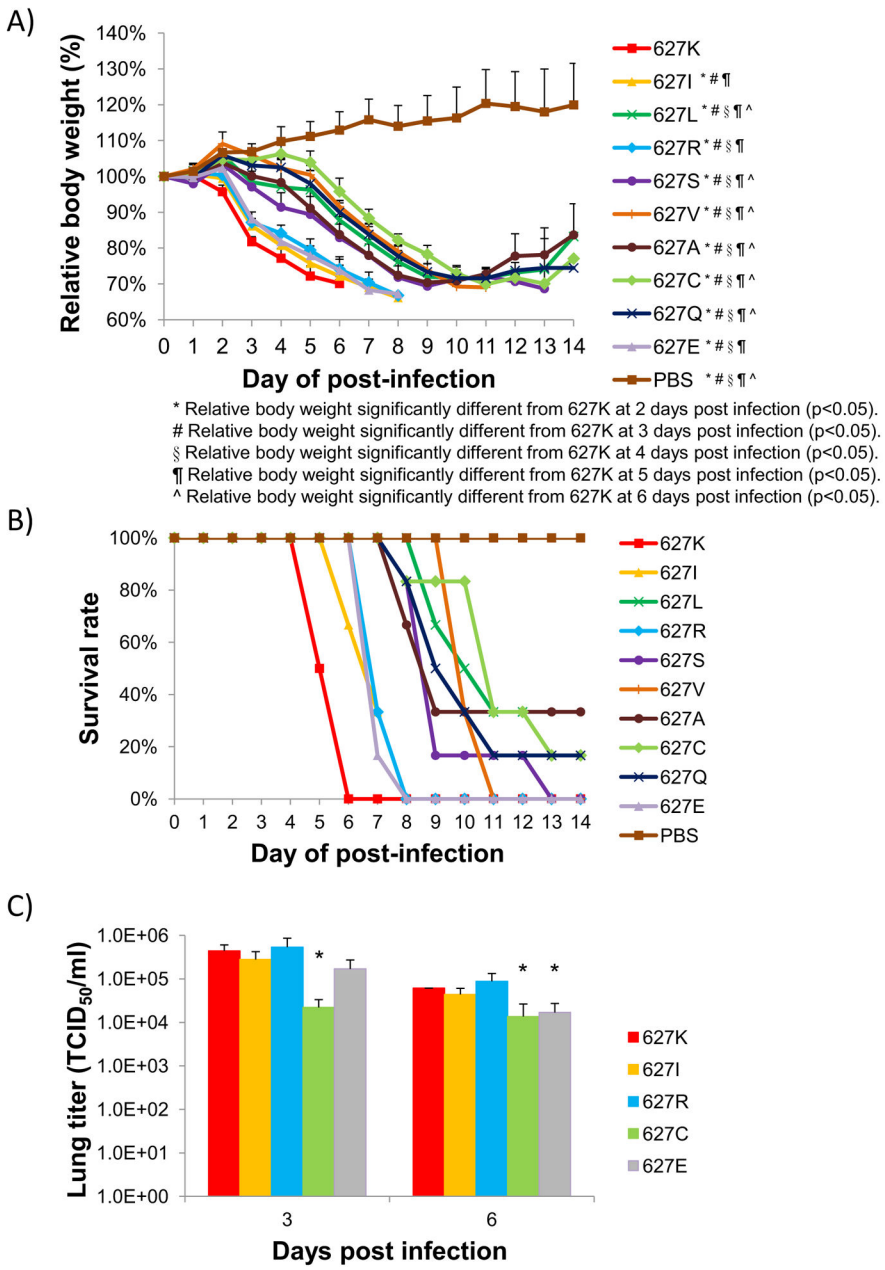

Fig. 5.

Effect of PB2-627 mutations on virulence of recombinant PR8 viruses in mice. Six BALB/c mice per group were infected by PB2-627 mutants via intranasal route at a dose of 1000 $TCID_{50}$ per mouse. Mice inoculated with PBS were used as uninfected control. (A) Body weight of the mice were measured daily for 14 days and are presented as relative to their initial body weight. Error bars represent one standard deviation (N=6). Significant differences as compared with the wild-type virus infected mice are indicated by * for day 2 post-infection, # for day 3 post-infection, § for day 4 post-infection, ¶ for day 5 post infection and ^ for day 6 post-infection ($p<0.05$, by t-test). (B) Survival rate of six mice per group was also recorded. Mice with weight loss of more than 30% of the initial body weight

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were humanely euthanized. (C) Three mice per group were infected by the recombinant viruses with different amino acids at PB2-627 position via intranasal route at a dose of 1000 $TCID_{50}$ per mouse. The mice were humanely euthanized on day 3 or day 6 post-infection. Their lungs were collected and were homogenized in ice-cold PBS. Viral titers of the whole lung homogenates were determined by standard $TCID_{50}$ assay with MDCK cells. Error bars represent one standard deviation (N=3). Significant differences as compared with the wild-type virus infected mice are indicated by * ($p<0.05$, by t-test).

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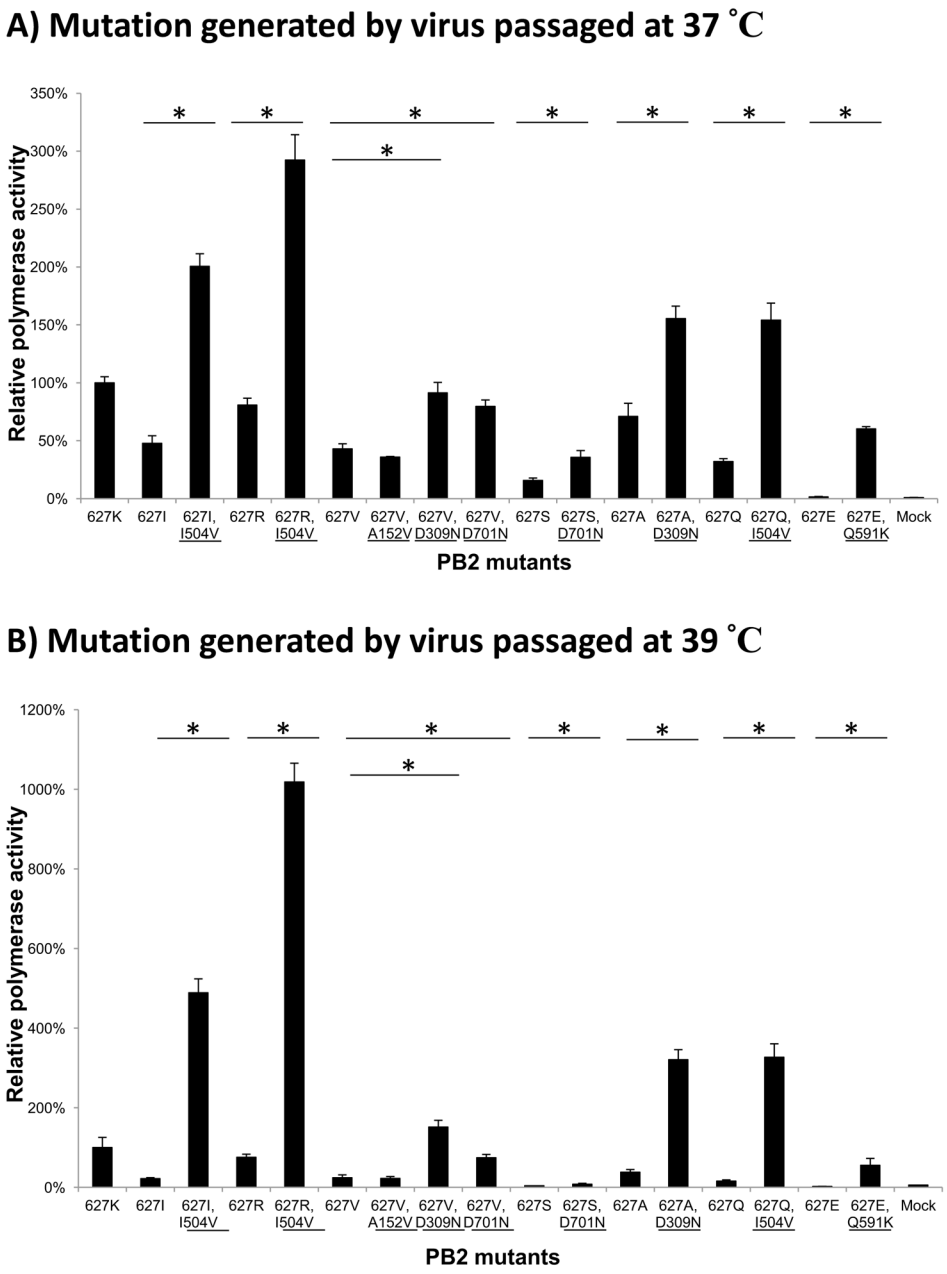

Fig. 6.

Effect of additional mutations on the polymerase activity of PB2-627 mutants. PB2 mutations that were reproducibly found in passaged PB2 mutants were introduced into the corresponding PB2-627 mutants by site mutagenesis to generate PB2 double mutants. The double mutants were reconstituted in 293T cells at 37°C (A) and 39°C (B). Polymerase activities were determined by luciferase reporter assays and were normalized by GFP expression. The normalized results are expressed as mean relative polymerase activity in relative to wild-type PR8 vRNP (PB2-627K) at the corresponding temperature. Mock represents the negative control without vRNP complexes. Error bars represent one standard deviation (N=3, * $p<0.05$, by t-test).

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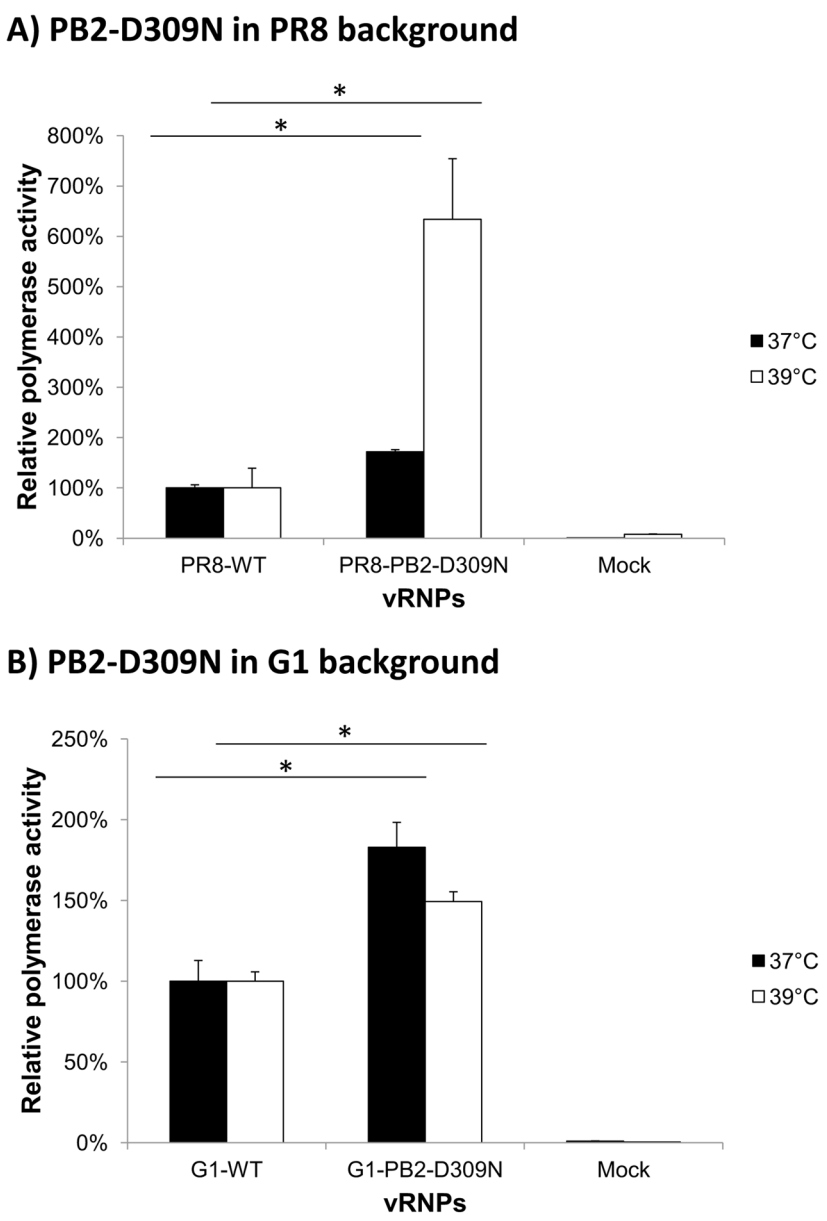

Fig. 7.

Effect of PB2-D309N mutation on the polymerase activity in vRNPs of different genetic backgrounds. PB2-D309N mutation was introduced into PR8 (human H1N1; A) or G1 (avian H9N2; B) vRNP. The wild-type and mutant vRNPs were reconstituted in 293T cells at 37°C (black) and 39°C (white). Polymerase activities were determined by luciferase reporter assay and were normalized by GFP expression. The normalized results are expressed as mean relative polymerase activity in relative to wild-type vRNPs at the corresponding temperature. Mock represents the negative control without vRNP complexes. Error bars represent one standard deviation (N=3, * $p<0.05$, by t-test).

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Table 1

PB2-627 mutants generated in this study

Cell	PB2-627 mutation	Number (%)
MDCK	A	7 (17.1%)
	I	2 (4.9%)
	K	3 (7.3%)
	L	8 (19.5%)
	R	18 (43.9%)
	S	1 (2.4%)
	V	2 (4.9%)
DF-1	A	2 (11.8%)
	C	3 (17.6%)
	K	8 (47.1%)
	Q	4 (23.5%)

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Table 2

PB2 mutations identified in PB2-627 mutants after 10 serial passages in MDCK cells

PB2-627 mutant	Additional PB2 mutations after serial passages	
	37 °C	39 °C
627K	Nil	Nil
627I	Nil	D153N, I504V
627L	Nil	R436K
627R	E487G	I504V
627S	D701N	Nil
627V	A152V*, D701N*, D740N	A152V, D309N
627A	Nil	D309N, R349K
627Q	Nil	I504V
627C	Nil	M81K, T106A
627E	Q591K*	Q591K*

* Mutations reproducibility found in passaged PB2 mutants.