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# Combination of PB2 271A and SR polymorphism at positions 590/591 is critical for viral replication and virulence of swine influenza virus in cultured cells and *in vivo*

Running title: PB2 271A and 590/591 SR for replication and virulence

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**Abstract**

Triple reassortant swine influenza viruses (SIVs) and 2009 pandemic H1N1 (pH1N1) virus contain an avian-origin PB2 with 271A, 590S, 591R and 627E. To evaluate the role of PB2 271A, 590S and 591R in replication and virulence of SIV, single- (1930-TX98-PB2-271T), double- (1930-TX98-PB2-590A591A) and triple-mutated (1930-TX98-PB2-271T590A591A) viruses were generated in the background of the H1N1 A/swine/Iowa/15/30 (1930) virus with an avian-origin PB2 from the triple reassortant A/swine/Texas/4199-2/98 (TX98) virus, called the parental 1930-TX98-PB2. Compared to parental, single- and double-mutated viruses, the triple-mutated virus replicated less efficiently in cell cultures and was attenuated in mice. These results suggest that a combination of 271A with 590/591 SR polymorphism is critical for pH1N1 and triple reassortant SIVs for efficient replication and adaptation in mammals.

**Key words:** swine influenza virus, PB2, 271A, 590/591 SR polymorphism, replication and virulence, cultured cells, *in vivo*

47 **Main text**

48 Both triple reassortant swine influenza viruses (SIVs) circulating in North American
49 swine herds and the 2009 pandemic H1N1 (pH1N1) virus contain an avian-origin PB2 with
50 271A, 590S, 591R and 627E (14, 18, 20). Avian-origin PB2 with 627E were introduced into
51 North American SIVs more than 10 years ago, and this avian-like signature (i.e., 627E) remains
52 stable rather than changing to the mammalian-like signature (i.e., 627K) (8). The pH1N1 viruses
53 have circulated in humans for more than 2 years and have been transmitted to other animal
54 species (1, 3, 11, 15), yet almost all of the isolates still contain the avian-like signature 627E
55 instead of the mammalian-like signature 627K in their PB2 proteins. The PB2 271A of pH1N1
56 contributes to enhance viral polymerase activity and growth in mammalian cells (2). Furthermore,
57 a recent study in cultured cells showed that the 590/591 (SR) polymorphism in the PB2 helps
58 pH1N1 overcome host restriction by enhancing its polymerase activity (9).

59 So far, no *in vivo* studies have been conducted to confirm the roles of the 590/591 (SR)
60 polymorphism or of PB2 271A of the pH1N1 and triple reassortant SIVs, so their effects on viral
61 replication and pathogenicity are poorly understood. In this study the 1930-TX98-PB2 used as
62 the parental virus was generated in the backbone of the classical H1N1 A/swine/Iowa/15/30
63 (1930) virus by replacing its swine-origin PB2 (containing 271T, 590G, 591Q and 627K) with an
64 avian-origin PB2 (containing 271A, 590S, 591R and 627E) from a triple reassortant
65 A/swine/Texas/4199-2/98 (TX/98) H3N2 virus; 1930-TX98-PB2 has been shown to be able to
66 induce severe disease and lung lesions in mice at low doses (8). To explore the potential roles of
67 these 3 amino acids in replication and virulence of SIVs, we generated recombinant viruses with
68 single (1930-TX98-PB2-271T), double (1930-TX98-PB2-590A591A) or triple (1930-TX98-
69 PB2-271T590A591A) amino acid substitutions and evaluated their replication and virulence

compared to the parental virus in cultured cells and *in vivo*. Our study demonstrates that a combination of 271A with 590/591 SR polymorphism (not single 271A or single 590/591SR) is critical for replication and virulence of SIV and compensates for the absence of 627K.

The single- (1930-TX98-PB2-271T), double- (1930-TX98-PB2-590A591A) and triple-mutated (1930-TX98-PB2-271T590A591A) viruses were generated in the background of the parental 1930-TX98-PB2 virus by substituting its TX98-PB2 with mutated PB2 with single, double, or triple amino acid substitutions (Supplementary Materials and Methods). Plaque assays showed that the double-mutated 1930-TX98-PB2-590A591A and triple-mutated 1930-TX98-PB2-271T590A591A formed smaller plaques than did either the parental 1930-TX98-PB2 or the single-mutated viruses (Figure 1A). The triple-mutated 1930-TX98-PB2-271T590A591A grew to significantly lower titers in MDCK cells than did the parental, single- and double-mutated viruses; no significant difference was observed between the parental and single- or double-mutated viruses (Figure 1B). Similarly, the triple-mutated viruses grew to significantly lower virus titers in human A549 cells than did the parental and double-mutated viruses (Figure 1C). The single-mutated virus had lower virus titers at 24h and 36h post infection (pi), but a similar titer at 48h pi when compared to the parental and double mutated viruses. In swine PK15 cells, the parental virus grew to significantly higher titers than the triple mutated virus did (Figure 1D). Significant differences were also observed between the single- and triple-mutated viruses at 12 and 36h pi, and between doubled- and triple-mutated viruses at 12 h pi. In conclusion, the triple-mutated virus replicates less efficiently in 3 different cell lines of human, canine and swine origin when compared to the parental, single- and double-mutated viruses.

To determine the effect of single, double and triple substitutions in the TX98-PB2 on replication and transcription rates of viral RNAs, the presence of viral vRNA, cRNA, and mRNA

was investigated by using primer extension assays to detect viral negative-strand and positive-strand RNAs derived from the PB1 gene segment as described previously (12) after virus infection of MDCK cells at a multiplicity of infection (MOI) of 3 (Supplementary Materials and Methods). Expression of the 3 viral RNA species of all 4 viruses appeared to increase over the course (4h, 8h and 12h) of infection (Figure 2A). In agreement with the growth curve, the viral RNA levels in cells infected with the triple-mutated 1930-TX98PB2-217T590A591A virus were the lowest of the 4 viruses throughout the course of infection (Figure 2A). Differences were observed in vRNA (1.2-2.4 fold), mRNA (1.4-2.7 fold) and cRNA (1.3-2.1 fold) levels when comparing the triple-mutated virus with parental, single- and double-mutated virus infected cells at indicated hours pi. Single- and double-mutated viruses produced comparable amounts of viral RNAs as the parental virus did (Figure 2A). These data suggest that SR polymorphism at position 590/591 and position 271A in the avian-origin PB2 are critical for replication and transcription of viral RNAs of SIVs.

To further understand how triple amino acid substitutions in PB2 affect virus replication, viral PB2 and PB1 proteins were examined by the western blot analysis after virus infection of MDCK cells at a MOI of 3 (Supplementary Materials and Methods). The amounts of both PB2 and PB1 proteins in cells infected with the triple-mutated 1930-TX98PB2-217T590A591A were the lowest of the 4 viruses at all tested hours post infection (Figure 2B), this is consistent with viral RNA levels shown in primer extension assays. The triple-mutated virus expressed 1.5 -3.1 fold less of the PB2 protein at indicated hours pi in infected cells than parental, single- and double-mutated viruses did (Figure 2B). Similarly, 1.1- 3.1 fold differences were observed in PB1 protein production by the triple-mutated virus when compared to the parental, single and double-mutated viruses. Notably, single- and double-mutated viruses expressed comparable

amounts of both PB1 and PB2 proteins as the parental virus did. Furthermore, both PB2 and PB1 proteins in these viruses were expressed at almost equal level (Figure 2B). Taken together, all these results indicate that triple amino acid substitutions seem to directly affect the expression of PB2 and PB1, resulting in decreased viral replication and transcription.

Mice infected with either $10^6$ $TCID_{50}$ of the parental or mutated viruses (Supplementary Materials and Methods) experienced body weight loss starting on 2 dpi and had clinical symptoms including ruffled fur, lethargy, anorexia and dyspnea. The triple-mutated 1930-TX98-PB2-271T590A591A virus caused less weight loss than did the other 3 viruses (Figure 3A); there was no significant difference among the weight loss caused by the parental and the single- or double-mutated viruses. The single-mutated 1930-TX98-PB2-271T and double-mutated 1930-TX98-PB2-590A591A viruses showed virulence similar to that of the parental virus (100% mortality rate). The triple-mutated 1930-TX98-PB2-271T590A591A was attenuated and caused onset of disease slower and less mortality (62.5%) than did the parental, single-, and double-mutated viruses (Figures 3B). These data indicate that the triple-mutated 1930-TX98-PB2-271T590A591A virus was significantly attenuated, consistent with the findings in cultured cells.

All viruses replicated very efficiently (virus titers range from $10^{5.8}$ to $10^{6.6}$ $TCID_{50}$/100μg) in mouse lungs without prior adaptation (Supplementary Figure 1A). The parental and mutated viruses induced pneumonia in infected mice (Supplementary Figure 1B and Supplementary Figure 2). The single-mutated 1930-TX98-PB2-271T and triple-mutated 1930-TX98-PB2-271T590A591A virus caused significantly fewer microscopic lung lesions in infected mice on 3 dpi than did the parental virus. On 5 dpi, the triple-mutated 1930-TX98-PB2-271T590A591A virus induced significantly fewer lung lesions in mice than the other viruses had; no significant

138 difference was observed among the groups inoculated with the parental, single-, or double-
139 mutated viruses (Supplementary Figure 1B and Supplementary Figure 2).

140 Two amino acids (627K or 701N) in the PB2 polymerase have been considered to be
141 important molecular markers for the adaptation of avian influenza viruses to mammals (4, 7, 10,
142 13, 16, 17). To date, most of pH1N1 isolates and currently circulating triple reassortant SIVs
143 possess an avian-origin PB2, which lacks these markers. Notably, both pH1N1 and triple-
144 reassortant SIVs replicate and are transmitted efficiently in human and swine populations. Our
145 previous study showed that a classical SIV containing avian-origin PB2 was able to infect pigs
146 and mice without prior adaption even if an E occupied position 627 (8). In the current study, a
147 high dose of the parental 1930-TX98-PB2 containing an avian PB2 induced 100% mortality in
148 mice, just as the 1930-TX98-PB2-627K does. All these data suggest that other residues in PB2
149 compensate for the lack of 627K and/or 701N.

150 PB2 271A has been shown to enhance the polymerase activity and virus replication of the
151 pH1N1 virus with an avian-like PB2 in cultured cell assays (2). Furthermore, PB2 271A
152 increased the polymerase activity of an avian influenza virus in human cells, but the avian
153 influenza virus with the PB2 271A did not cause significant weight loss and mortality in infected
154 mice (2). Our present study indicates that this single amino acid (i.e., PB2 271A) is insufficient
155 to affect viral replication and virulence of SIV in cultured cells and *in vivo*, consistent with
156 previous findings (2) .

157 A previous study showed that SR polymorphism at positions 590 and 591 in pH1N1 PB2
158 is important for efficient polymerase activity in cultured cells and enhances a reassortant
159 human/avian virus replication in human cells (9). Furthermore, the SR polymorphism increases
160 avian polymerase activity and enhances virus replication in human cells (9). This polymorphism

161 has been considered to be an adaptive strategy used by influenza viruses to overcome species-
162 specific restriction of replication; however, so far this has not been studied *in vivo*. Structural
163 analysis indicated that the 591R residue in pH1N1 PB2 affects both the shape and charge on the
164 surface of the PB2 protein, which may affect its interaction with other viral or host factors, thus
165 resulting in more efficient virus replication in mammals (19). Consequently, we hypothesized
166 that double substitutions (i.e., S590A and R591A) in the avian-origin TX98 PB2 might decrease
167 viral replication and attenuate the parental virus. However, the SR polymorphism seems not to be
168 very critical for replication of the parental 1930-TX98-PB2 virus. In addition, the double-
169 mutated virus had *in vivo* virulence similar to that of the parental and single-mutated viruses,
170 indicating that single (i.e., 271) and double substitutions (i.e., 590/591) are not enough to alter
171 the virulence of the parental virus.
172 The triple-mutated virus was attenuated in cultured cells and *in vivo*. Our results indicate
173 that a combination of the 3 residues (i.e., 271, 590, and 591) plays a critical role in virus
174 replication and virulence of SIVs and compensate for the lack of 627K or 701N in PB2. These 3
175 residues seem to directly contribute to PB2 polymerase activity, resulting in decreased virus
176 replication and growth dynamics in 3 different cell lines and in the mouse study. The 3 amino
177 acid substitutions in the PB2 seem to not affect interactions with host factors because the triple-
178 mutated virus exhibited a similar replication capacity in canine, swine and human cell lines.
179 Recent studies showed that the PB2 E627K or D701N substitutions in 2009 pH1N1 did not lead
180 to enhanced virulence in mice (5, 21) and in ferrets (5) or enhanced transmission in ferrets (5);
181 these substitutions actually cause attenuation of a reassortant virus that contains the 2009
182 pH1N1 influenza polymerase and NP, and the remaining four genes from a recent seasonal
183 H1N1 (A/New York/312/2001) virus in culture cells and mice (6). All these findings suggest that

the pH1N1 and currently circulating triple-reassortant SIVs use different strategies to achieve efficient replication and adaptation to mammals without the signature PB2 627K or 701N residues. The PB2 of the 2009 pH1N1showed 98.8% homology with the TX98 PB2 at the amino acid level, containing the avian-like signatures 627E and 701D. Our study demonstrates that a combination of PB2 271A with SR polymorphism at positions 590 and 591 in the genetic background of classical H1N1 SIV with an avian-origin PB2 is critical for viral replication and virulence of SIVs in cultured cells and *in vivo*; this suggests that *in vivo* virulence of pH1N1 might reside within the same amino acid positions of PB2. Taken together, the combination of 271A and 590/591 SR polymorphism in PB2 might be a novel strategy that is used by both pH1N1 and currently circulating triple reassortant SIVs for efficient replication and adaptation in mammals.

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## Figure legends

**Figure 1: Growth properties of the parental and mutated viruses**

(A) Plaque size in MDCK cells 2 days after infection. Growth curve (B) in MDCK cells that were infected with each virus at an MOI of 0.001, and in (C) A549 cells and (D) in PK15 cells that were infected with each virus at an MOI of 0.1. Each data point on the curve indicates the mean of 3 independent experiments, and error bars indicate the SEM (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

**Figure 2: Viral RNA accumulation and western blots**

MDCK cells were infected with the indicated viruses at an MOI of 3. (A) Total RNA was isolated at indicated time points and analyzed by performing primer extension assays. The 5S rRNA was used as a loading control. Quantification of relative RNA amount was calculated and normalized using ratios to 5S RNA. vRNA = viral RNA; mRNA = messenger RNA; cRNA = complementary RNA; rRNA = ribosomal RNA. (B) Infected cells were harvested and lysed at indicated time points and analyzed by conducting western blots using polyclonal antibodies against influenza PB2 and PB1. The GAPDH was used as a loading control. Quantification of relative PB2 and PB1 amount was calculated and normalized using ratios to GAPDH.

**Figure 3: Body weight changes and mortality of mice infected with the parental or mutated viruses**

a) Body weight changes, and b) survival rate of mice that were infected with either $10^6$ $TCID_{50}$ of the indicated viruses or mock-infected with 50 µL MEM (8 mice per group) (* $P < 0.05$).