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A PB1 T296R substitution enhance polymerase activity and confer a virulent phenotype to a 2009 pandemic H1N1 influenza virus in mice

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A R T I C L E   I N F O

A B S T R A C T

While the 2009 pandemic H1N1 virus has become established in the human population as a seasonal influenza virus, continued adaptation may alter viral virulence. Here, we passaged a 2009 pandemic H1N1 virus (A/Changchun/01/2009) in mice. Serial passage in mice generated viral variants with increased virulence. Adapted variants displayed enhanced replication kinetics in vitro and vivo. Analysis of the variants genomes revealed 6 amino acid changes in the PB1 (T296R), PA (I94V), HA (H3 numbering; N159D, D225G, and R226Q), and NP (D375N). Using reverse genetics, we found that a PB1-T296R substitution found in all adapted viral variants enhanced viral replication kinetics in vitro and vivo, increased viral polymerase activity in human cells, and was sufficient for enhanced virulence of the 2009 pandemic H1N1 virus in mice. Therefore, we defined a novel influenza pathogenic determinant, providing further insights into the pathogenesis of influenza viruses in mammals.

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Introduction

A novel swine-origin H1N1 reassortant influenza virus (2009 pH1N1) emerged in April of 2009 and caused the first pandemic of the 21st century (Garten et al., 2009). Although the pandemic ended in August of 2010, the 2009 pH1N1 virus has continued to circulate among humans as a seasonal influenza virus.

Prolonged circulation of 2009 pH1N1 viruses in the human population may result in adaptive changes that impinge upon viral virulence. Mice are a well-established mammalian model for studying influenza A virus pathogenicity (Cheng et al., 2014a; Deng

et al., 2015; Fan et al., 2009; Hatta et al., 2001; Jiao et al., 2008; Manicassamy et al., 2010; Metreveli et al., 2014; Shinya et al., 2004; Song et al., 2015; Tumpey et al., 2004, 2005). While mice are not naturally infected with influenza viruses, influenza strains can be experimentally adapted to mice (Chen et al., 2015; Yu et al., 2015). Mouse-adapted influenza viruses have been shown to have enhanced virulence (Chen et al., 2015; Cheng et al., 2014a; Sang et al., 2015; Yu et al., 2015), possess an increased ability to infect alveolar cells, and can cause lethal pneumonitis.

Here, we generated mutants of the 2009 pH1N1 virus through serial lung-to-lung passages in mice. Serial passage of the 2009 pH1N1 virus in mice resulted in variant viruses that displayed a marked increase in virulence. We identified a novel amino acid change within the PB1 protein that is sufficient for increased viral pathogenicity for mice. In vitro and in vivo analyses demonstrated that this substitution combination enhanced RNA polymerase activity and viral replication. Therefore, we defined a novel

influenza pathogenic determinant, providing further insights into the pathogenesis of influenza viruses in mammals.

Materials and methods

Ethics statement

The protocol of the study was conducted in accordance with guidelines of animal welfare of World Organization for Animal Health. All experimental protocol were approved by the Review Board Military Veterinary Research Institute of the Academy of Military Medical Sciences.

Facilities

Studies using the 2009 pandemic H1N1 influenza virus and derivative mutants 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 (SYXK-2009-045).

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. 293T cells were cultured in DMEM supplemented with 10% FCS, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine. A549 cells were cultured in DMEM supplemented with 2 mM glutamine, 10% FCS, 100 IU/ml penicillin, and 100 mg/ml streptomycin. The 2009 pandemic H1N1 influenza virus A/Changchun/01/2009 (abbreviated as XD), was obtained from the Military Veterinary Research Institute of Academy of Military Medical Sciences. Stock virus was 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 use. The GenBank accession numbers corresponding to each of the eight XD viral gene segments are JN032403–JN032410.

Adaptation of 2009 pandemic H1N1 influenza virus in mice

Three mouse-adapted variants of the XD virus were derived from three independent series of sequential lung-to-lung passages of virus in mice as described previously (Cheng et al., 2014a; Yu et al., 2015). Briefly, 4–6-week-old female BALB/c mice (Merial-Vital Laboratory Animal Technology Co., Ltd., Beijing, China) were inoculated intranasally with 50 μl of allantoic fluid containing the XD H1N1 virus under light isoflurane anesthesia. Lungs were harvested and homogenized 48 h after infection. The disrupted lung tissue was centrifuged to remove debris and 50 μl of the supernatant was used to inoculate the next naïve mouse in the series. Virus was passaged mouse-to-mouse eighteen times in three independent lines. After the eighteenth passage in each series of mice, viruses present in the final lung homogenates was cloned once by plaque purification in MDCK cells as described previously (Song et al., 2009). The cloned viruses were independently amplified in the allantoic cavities of 10-day-old chicken eggs for 60 hours at 37 °C to prepare virus stocks. Three plaque-purified mouse-adapted variants of the original XD virus were obtained for further characterization and named UIXD-P18-1, UIXD-P18-2, and UIXD-P18-3.

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 as described previously (Cheng et al., 2014b). 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). Sanger sequencing methodology was used to sequence the PCR-amplified viral gene segments. DNA sequences were analyzed and compared to the parental 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 XD or UIXD-P18-2 viral genes between the ribozyme and polymerase I promoter sequence. A site-directed mutagenesis kit (Invitrogen, www.invitrogen.com) was used to introduce specific mutations in the viral genome. The plasmids used for transfection were prepared using the QIAfilterTM Plasmid Midi kit (QIAGEN). Recombinant viruses were rescued by transfection in 293 T cells as previously described (Gao et al., 2009; Li et al., 2005), and propagated in MDCK cells. All constructs were sequenced to ensure the absence of unwanted mutations.

In vivo experiments

The 50% mouse lethal dose (MLD$_{50}$) of the parental XD virus, the three mouse-adapted isolates, 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) as described previously (Cheng et al., 2014a). 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 (Reed and Muench, 1938) after a 14-day observation period and are expressed as EID$_{50}$.

To evaluate the impact of individual amino acid substitutions alone or in combination on morbidity and mortality, we inoculated groups of five mice with 10^6 EID$_{50}$ of the indicated influenza viruses and recorded weight loss and mortality for 14 dpi. To measure the replicative capacity of each virus in the lungs, cohorts of mice were intranasally inoculated with 10^4 EID$_{50}$ of each indicated virus. On days 3 and 5 post-infection, three mice per group were euthanized. 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, and virus titers of each strain were expressed as mean log$_{10}$ EID$_{50}$/g ± 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$_{50}$ of each indicated virus 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 (Reed and Muench,

1938) and expressed as mean $\log_{10}EID_{50}/g \pm SD$. 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 vitro

MDCK cells and A549 cells were infected with the 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, and 72 h after infection and stored at $-80\ ^\circ C$. Virus titer was determined by end-point titration in MDCK cells. Virus titers of each virus in different time point were expressed as mean $\log_{10}$ $TCID_{50}$/ml $\pm$ SD.

Minigenome assay for polymerase activity

Polymerase activity was measured using a minigenome assay as previously described (Cheng et al., 2014a; Song et al., 2011). In brief, sub-confluent monolayers of 293T 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 incubation at 37 $^\circ C$, 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.

Statistical analysis

Data were analyzed by two-way analysis of variance using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA). When a significant effect was observed, pairwise comparisons were performed using the Bonferroni post-hoc test.

Results

Generation of virulent mouse-adapted 2009 pH1N1 variants

Previous studies have shown that the novel 2009 pH1N1 virus is nearly avirulent in mice (MLD_{50} of 6 $\log_{10}$ EID_{50} for A/California/04/2009(H1N1)) (Itoh et al., 2009; Maines et al., 2009). In order to generate mouse-adapted 2009 pH1N1 viral variants, we serially passaged the 2009 pH1N1 XD virus in mice. Following serial passage, three mouse-adapted variants were selected for further characterization. At an infecting dose of 10^6 EID_{50}, mice infected with the mouse-adapted variants (UIXD-P18-1, UIXD-P18-2, and UIXD-P18-3) displayed rapid weight loss and universally succumbed to infection by day 6 post-infection (Fig. 1A). In contrast, 4/5 mice infected with the parental XD virus recovered from the infection (Fig. 1A).

Serial passage of the XD virus in mice produced variant viruses with substantially increased virulence in mice as indicated by marked reductions in MLD_{50} values. The XD virus MLD_{50} was 6.25 $\log_{10}$ EID_{50}, whereas the MLD_{50} of the UIXD-P18-1, UIXD-P18-2, and UIXD-P18-3 viruses were 3.75 $\log_{10}$ EID50, 3.25 $\log_{10}$ EID_{50}, and 4.25 $\log_{10}$ EID_{50}, respectively (Table 1). These results show that the serial passage of the XD virus in mice resulted in substantially increased virulence.
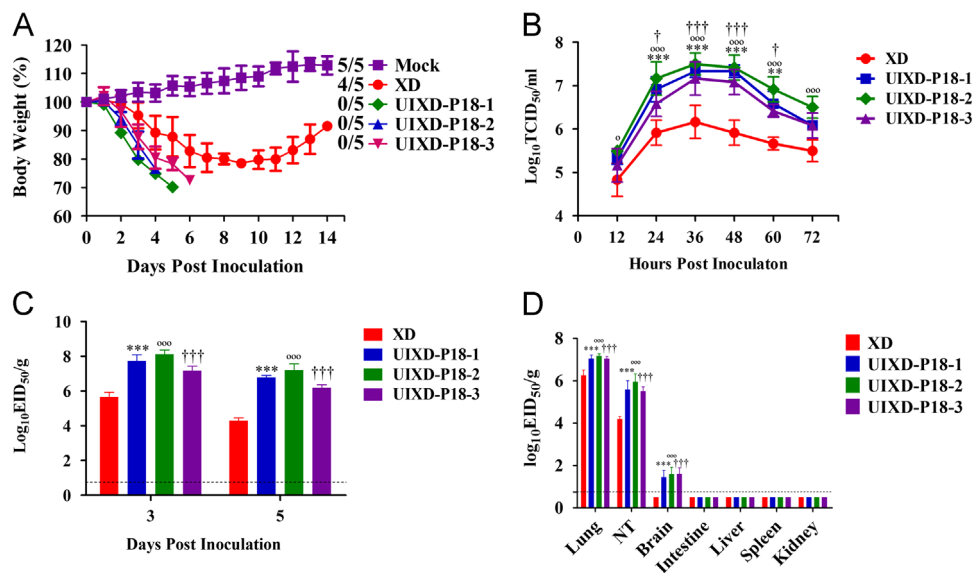

Fig. 1. Mouse-adapted pH1N1 viruses show increased virulence in mice and enhanced replicative capacity. (A) Mice ($n=5$) were inoculated intranasally with 50 μl containing 10^6 EID_{50} of mouse-adapted viruses (UIXD-P18-1, UIXD-P18-2 and UIXD-P18-3), or the parental wild-type H1N1 virus (XD). Mock-inoculated mice served as a control group. Animals that lost more than 25% of their pre-infection weight were euthanized. Body weights of inoculated mice were recorded daily, and are presented 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 indicate the proportion of surviving mice per group. (B) MDCK cells were inoculated at a multiplicity of infection of 0.01 $TCID_{50}$/cell with the parental wild-type H1N1 virus (XD) or a mouse-adapted virus (UIXD-P18-1, UIXD-P18-2, or UIXD-P18-3). Supernatants were collected at the indicated time points and titrated in MDCK cells by $TCID_{50}$. (C) Mice ($n=3$) were inoculated intranasally with 10^4 EID_{50} of the parental wild-type H1N1 virus (XD) or a mouse-adapted virus (UIXD-P18-1, UIXD-P18-2, or UIXD-P18-3). Viral loads in the lungs were determined at 3 and 5 days post-infection by EID_{50}. Results are expressed as $\log_{10}$ EID_{50}/g of tissue. The dotted line indicates the limit of detection. (D) Mice ($n=3$) were infected with 10^6 EID_{50} of the parental wild-type H1N1 virus (XD) or a mouse-adapted virus (UIXD-P18-1, UIXD-P18-2, or UIXD-P18-3). On day 3 post-infection, viral loads in the lungs, nasal turbinates (NT), brains, intestines, livers, spleens, and kidneys were titrated by EID_{50}. Results are expressed as $\log_{10}$ EID_{50}/g of tissue. The dotted line indicates the limit of detection. In each panel, the average of three replicates is shown with error bars indicating the standard deviation. *, °, †, indicate $p < 0.05$ when comparing UIXD-P18-1, UIXD-P18-2, and UIXD-P18-3 with XD respectively. **, °°, ††, indicate $p < 0.01$ when comparing UIXD-P18-1, UIXD-P18-2, and UIXD-P18-3 with XD respectively. ***, °°°, †††, indicate $p < 0.001$ when comparing UIXD-P18-1, UIXD-P18-2, and UIXD-P18-3 with XD respectively. Statistical analysis was performed using a two-way ANOVA.

Table 1
MLD$_{50}$ of wide-type virus, mouse-adapted variants, and recombinant viruses in BALB/c mice and amino acid substitutions identified in indicated viruses.

Virus[a]	PB1	PA	HA[b]			NP	MLD$_{50}$ (log$_{10}$EID$_{50}$)
	296	94	159	225	226	375	
XD	T	I	N	D	R	D	6.25
UIXD-P18-1	R	V	N	G	Q	N	3.75
UIXD-P18-2	R	V	D	G	Q	N	3.25
UIXD-P18-3	R	V	N	D	Q	N	4.25
rXD	T	I	N	D	R	D	6
rUI182	R	V	D	G	Q	N	3
rXD-PB1-296R	R	I	N	D	R	D	3
rXD-PA-94V	T	V	N	D	R	D	5
rXD-HA-159D/225G/ 226Q	T	I	D	G	Q	D	4.75
rXD-NP-375N	T	I	N	D	R	N	5

[a] XD, A/Changchun/01/2009 (H1N1); UIXD-P18-1, UIXD-P18-2, and UIXD-P18-3, mouse-adapted variants; rXD, recombinant XD virus; rUI182, recombinant virus carrying 8 genes from UIXD-P18-2; rXD-PB1-296R, recombinant virus carrying the PB1-T296R substitution in the backbone of XD; rXD-PA-94V, recombinant virus carrying the PA-I94V substitution in the backbone of XD; rXD-HA-159D/225G/ 226Q, recombinant virus carrying the HA-N159D, D225G, and R226Q substitutions in the backbone of XD; rXD-NP-375N, recombinant virus carrying the NP-D375N substitution in the backbone of XD.
[b] H3 numbering.

The mouse-adapted viruses replicate more efficiently in vitro and in vivo

We next evaluated the replicative ability of the parental XD virus and mouse-adapted variants in MDCK cells (Fig. 1B). *In vitro* growth kinetics revealed that the UIXD-P18-1, UIXD-P18-2, and UIXD-P18-3 viruses grew faster and achieved higher titers than the parental XD virus (Fig. 1B). UIXD-P18-2 grew to the highest titer and yielded approximately 22-fold more virus than XD in MDCK cells by 36 h post-inoculation (Fig. 1B). To determine if the mouse-adapted H1N1 strains also replicate more efficiently than wild type virus *in vivo*, we inoculated mice with 10^4 EID$_{50}$ of each virus and analyzed virus titers in the lungs on day 3 and 5 post-infection (Fig. 1C). Each of the three mouse-adapted viral variants was present at higher titers in the lungs of mice on day 3 and day 5 post-inoculation when compared to the parental XD virus (Fig. 1C). These data show that the enhanced virulence of the mouse-adapted H1N1 viruses correlates with increased viral replication *in vitro* and *in vivo*.

The mouse-adapted viruses replicate to higher titers in respiratory tissues and acquired the ability to replicate in the brain in mice

We then asked whether the increased virulence of the mouse-adapted H1N1 strains was due to an expansion of the tropism of the adapted variants. Mice were inoculated intranasally with 10^6 EID$_{50}$ of the parental XD virus or one of the three mouse-adapted variant viruses (UIXD-P18-1, UIXD-P18-2, or UIXD-P18-3). On day 3 post-infection, the lungs, nasal turbinates (NT), brains, intestines, livers, spleens, and kidneys were collected and the viral load in each tissue was determined (Fig. 1D). Virus was recovered from the lungs and NT of mice inoculated with the parental XD virus, but was not detected in any other organ tested (Fig. 1D). In contrast, virus from mice inoculated with each of the three mouse-adapted H1N1 variants was recovered from the lungs and NT at higher titers when compared to mice inoculated with the parental XD virus and was also recovered from the brains of mice on day 3 post-infection (Fig. 1D). These data demonstrate that mouse-adapted H1N1 variants replicate to higher titers in respiratory tissues when compared to the parental XD virus and acquired the ability to replicate to detectable levels in the brain.

Sequence analysis

We sequenced the complete genome of the mouse-adapted viruses to identify the mutations responsible for increased virulence (Table 1). We focused our analysis on mutations that encoded amino acid substitutions. Analysis of the variant virus genomes revealed one amino acid change in the PB1 (T296R), one amino acid change in the PA (I94V), three amino acid changes in HA (H3 numbering; N159D, D225G, and R226Q), one amino acid changes in the NP (D375N) (Table 1).

To investigate whether the amino acid changes observed in mouse-adapted H1N1 strains generated in this study have been identified in 2009 pandemic H1N1 isolates, we queried 2009 pH1N1 sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). We identified seven isolates (frequency of substitution is 7/10,346) possessing a D residue at HA position 159 (H3 numbering), eighty-seven isolates (frequency of substitution is 87/10,346) possessing a G residue at HA position 225 (H3 numbering), ten thousand two hundred and seventy-three isolates (frequency of substitution is 10,273/10,346) possessing a Q residue at HA position 226 (H3 numbering), and eight isolates (frequency of substitution is 8/5040) possessing a N residue at NP position 375 (Table 2). The PB1-T296R and PA-I94V substitutions were not identified in any H1N1 strains deposited in GenBank (Table 2).

PB1-T296R substitution enhances viral replication in vitro

We used reverse genetics to generate stocks of the parental XD virus (rXD), the most virulent mouse-adapted UIXD-P18-2 virus (rUI182), and recombinant viruses incorporating one or more amino acid substitutions identified in the UIXD-P18-2 virus to assess the individual contribution of amino acid changes to the increased virulence of the mouse-adapted 2009 pH1N1 virus. As expected, the rUI182 virus grew to higher titers than the parental rXD virus in MDCK cells (Fig. 2A) and A549 cells (Fig. 2B). Recombinant viruses harboring a PA-I94V substitution, a NP-D375N substitution, or the HA-N159D/D225G/R226Q substitutions displayed modest enhancements in viral replication when compared to the rXD parental virus in MDCK cells (Fig. 2A) and A549 cells (Fig. 2B). In contrast, a recombinant virus possessing the PB1-T296R substitution phenocopied the mouse-adapted rUI182 virus, suggesting that an arginine residue at PB1 position 296 was sufficient for increased replicative capacity in MDCK cells (Fig. 2A) and A549 cells (Fig. 2B).

PB1-T296R substitution enhance viral replication in vivo

We then evaluated the *in vivo* replicative capacity of each recombinant virus. Mice were inoculated with 10^4 EID$_{50}$ of each virus and virus titers in the lungs were measured on days 3 and 5 post-infection (Fig. 3A). The rUI182 mouse-adapted virus

Table 2
Web search for the amino acid changes observed in mouse-adapted H1N1 strains generated in this study have been identified in 2009 pH1N1 isolates.

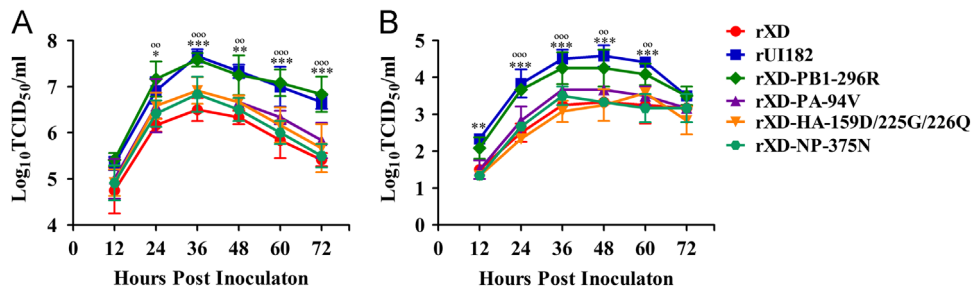| Segment	Position	Amino acid	Frequency of substitution (no. of strains with the substitution/total no. of strains)
PB1	296	R	0/ 4708
PA	94	V	0/ 4935
HA	159[a]	D	7/10,346
	225[a]	G	87/10,346
	226[a]	Q	10,273/10,346
NP	375	N	8/5040

[a] H3 numbering.

Fig. 2. Effects of amino acid substitutions on *in vitro* replication kinetics. The parental XD 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) MDCK cells or (B) A549 cells were inoculated at a multiplicity of infection of 0.01 $TCID_{50}$/cell with rXD, rUI182, rXD-PB1-296R, rXD-PA-94V, rXD-HA-159D/225G/226Q, or rXD-NP-375N. Supernatants were collected at the indicated time points and titrated in MDCK cells by $TCID_{50}$. In each panel, the average of three replicates is shown with error bars indicating the standard deviation. *, °, †, #, &, indicate $p < 0.05$ when comparing rUI182, rXD-PB1-296R, rXD-PA-94V, rXD-HA-159D/225G/226Q, and rXD-NP-375N with rXD respectively. **, °°, ††, ##, &&, indicate $p < 0.01$ when comparing rUI182, rXD-PB1-296R, rXD-PA-94V, rXD-HA-159D/225G/226Q, and rXD-NP-375N with rXD respectively. ***, °°°, †††,###, &&&, indicate $p < 0.001$ when comparing rUI182, rXD-PB1-296R, rXD-PA-94V, rXD-HA-159D/225G/226Q, and rXD-NP-375N with rXD respectively. Statistical analysis was performed using a two-way ANOVA.

replicated to higher titers than the parental rXD on both day 3 and 5 post-inoculation (Fig. 3A). A recombinant virus incorporating the PB1-T296R substitution alone replicated to titers comparable to the rUI182 mouse-adapted virus, suggesting that PB1-T296R was primarily responsible for the increased *in vivo* replicative capacity of the mouse-adapted virus (Fig. 3A). In contrast, recombinant viruses harboring a PA-I94V substitution, HA-N159D/D225G/R226Q substitutions, or a NP-D375N substitution displayed only modestly increased lung viral titers when compared to the parental rXD virus (Fig. 3A). These data show that a PB1-T296R substitution increases the replicative capacity of the parental XD virus *in vivo*.

PB1-T296R substitution enhance virulence and expand the tropism of 2009 pandemic H1N1 virus in mice

Cohorts of mice were infected with 10^6 EID_{50} of the recombinant parental virus rXD, the recombinant mouse-adapted virus rUI182, or a recombinant virus possessing one or more of the amino acid substitutions identified in the mouse-adapted virus. Infected mice were followed for 14 days to evaluate weight loss and survival. Mice inoculated with rXD, rXD-PA-94V, rXD-HA-159D/225G/226Q, or rXD-NP-375N showed modest body weight loss and partial lethality of the infected cohort (Fig. 3B). In contrast, mice infected with rUI182 and rXD-PB1-296R rapidly lost weight and succumbed to infection by day 4 post-infection (Fig. 3B). We determined the rXD virus MLD_{50} was 6 log_{10} EID_{50}, and the MLD_{50} of the rUI182, rXD-PB1-296R, rXD-PA-94V, rXD-HA-159D/225G/226Q, and rXD-NP-375N viruses were 3 log_{10} EID_{50}, 3 log_{10} EID_{50}, 5 log_{10} EID_{50}, 4.75 log_{10} EID_{50}, and 5 log_{10} EID_{50}, respectively (Table 1). The mouse lethal doses (MLD_{50}) of the rXD-PB1-296R were reduced 1000-fold compared to the parental virus. These data demonstrate that the PB1-T296R substitution alone was sufficient to confer increased virulence to the parental rXD virus.

We next sought to identify the amino acid substitutions that conferred expanded tissue tropism to the mouse-adapted virus. Mice were inoculated intranasally with 10^6 EID_{50} of rXD, rUI182, rXD-PB1-296R, rXD-PA-94V, rXD-HA-159D/225G/226Q, and rXD-NP-375N, 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. 3C). The recombinant virus possessing a PB1-T296R substitution replicated to higher titers in the lungs and NT when compared to the rXD and the other recombinant viruses and was present at levels comparable to the mouse-adapted rUI182 virus (Fig. 3C). Additionally, the recombinant virus possessing a PB1-T296R substitution was recovered from the brain, indicating the PB1-T296R substitution is

sufficient to confer extrapulmonary replication in the brain (Fig. 3C). Taken together, these data show that the PB1-T296R substitution enhances virulence and expands the tropism of 2009 pandemic H1N1 virus in mice.

PB1-T296R substitution enhances viral polymerase activity

The enhanced replication of viruses possessing PB1-T296R substitution *in vitro* and *in vivo* suggested that this amino acid substitution could impact viral polymerase activity. We applied a mini-genome reporter assay to assess the impact of the PB1-T296R, PA-I94V, or NP-D375N substitutions on the transcription and replication of viral RNAs (vRNAs) in human 293T cells. The PB1-T296R substitution increased polymerase activity approximately 1.8-fold (Fig. 4). In contrast, the NP-D375N substitution did not increase polymerase activity (Fig. 4). It is worth noting that the PA-I94V substitution also increased polymerase activity approximately 2.7-fold (Fig. 4). Because we do not exclude that the increase of polymerase activity is caused by expression level difference of PB1, this result suggests that the enhanced replication of viruses harboring PB1-T296R substitution *in vitro* and *in vivo* may be in part due to enhanced viral polymerase activity.

Discussion

In 2009, a novel H1N1 influenza A virus emerged and caused a pandemic. Continuing evolution of circulating 2009 pH1N1 viruses in the human population may additionally result in changes to viral virulence. The specific amino acid substitutions that are likely to enhance virulence in mammals are not well known. Although amino acid substitutions in the avian (PB2 and PA)/human (PB1) RNA-dependent RNA polymerase (RDRP) that enhance the replicative capacity and transmission of 2009 pH1N1 viruses have been reported previously (e.g., PB2-G590S/Q591R) (Mehle and Doudna, 2009; Yamada et al., 2010; Zhang et al., 2012), additional evolutionary changes will likely be acquired as the virus adapts to humans.

Serial passage of influenza viruses in animals has been widely used to identify adaptive mutations that result in enhanced viral pathogenicity (Gabriel et al., 2005; Hensley et al., 2009). For example, an amino acid substitution at position 225 of the HA protein has been identified in 2009 pH1N1 isolates from severely ill patients, though its role in viral virulence is uncertain (Chen et al., 2010). PB2-E158G is a novel pathogenic determinant of influenza A viruses in the mouse model in a previous study(Zhou et al., 2011). Remarkably, PB2-E158G substitutions also altered the pathotypes of two avian H5 viruses in mice, indicating that this
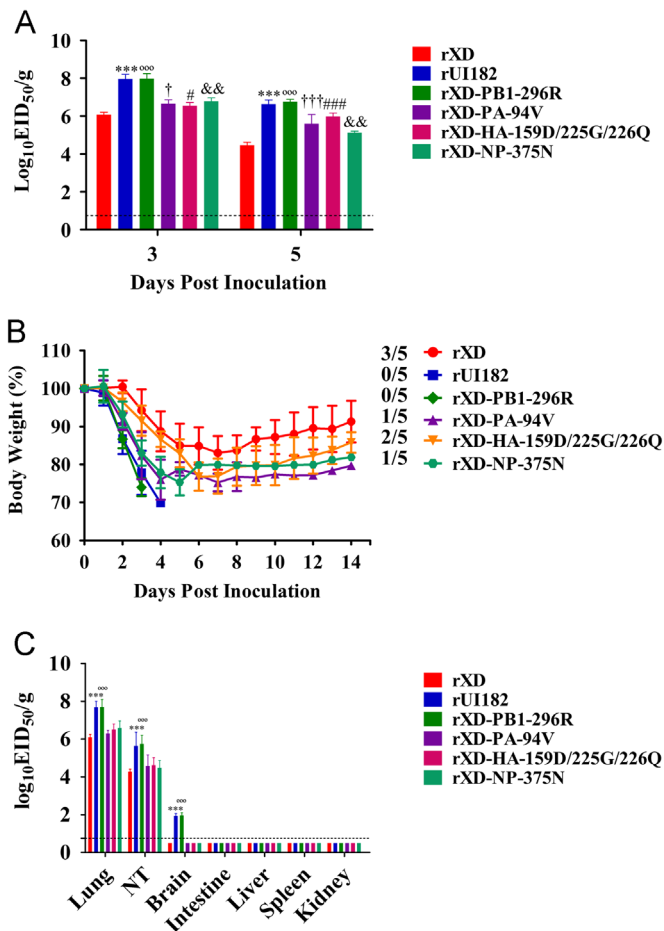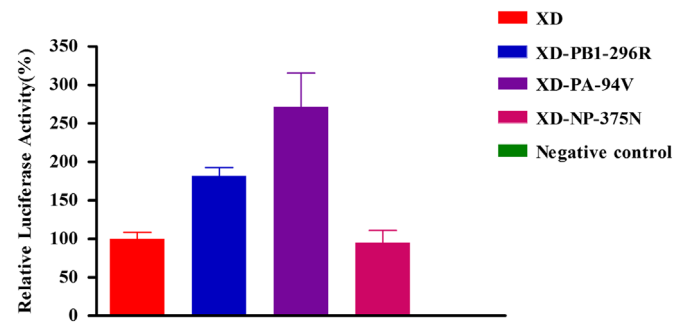
Fig. 4. Viral polymerase activity of RNP complexes. 293T cells were transfected with a luciferase reporter plasmid (paviPoll-T-Luc) that expresses a negative-sense virus-like RNA encoding a destabilized firefly luciferase enzyme, and one plasmid for each of the proteins in the polymerase complex (NP, PB1, PB2, and PA). 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 averages of triplicate experiments are shown. Error bars represent the standard deviation (SD).

Fig. 3. Effects of amino acid substitutions on *in vivo* replication kinetics, virulence, and tropism. The parental XD 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) Mice ($n=6$) were inoculated intranasally with 10^4 EID$_{50}$ 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 ($n=5$) 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) Mice ($n=3$) were inoculated intranasally with 10^6 EID$_{50}$ 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 each panel, the average of three replicates is shown with error bars indicating the standard deviation. *, °, †, #, &, indicate $p < 0.05$ when comparing rUI182, rXD-PB1-296R, rXD-PA-94V, rXD-HA-159D/225G/226Q, and rXD-NP-375N with rXD respectively. **, °°, ††, ##, &&, indicate $p < 0.01$ when comparing rUI182, rXD-PB1-296R, rXD-PA-94V, rXD-HA-159D/225G/226Q, and rXD-NP-375N with rXD respectively. ***, °°°, †††,###, &&&, indicate $p < 0.001$ when comparing rUI182, rXD-PB1-296R, rXD-PA-94V, rXD-HA-159D/225G/226Q, and rXD-NP-375N with rXD respectively. Statistical analysis was performed using a two-way ANOVA.

residue impacts genetically divergent influenza A viruses (Zhou et al., 2011). In addition, some naturally occurring mutations in the PA gene are contributors to increased virulence of 2009 pandemic influenza virus in mice (Sun et al., 2014).

Our data demonstrates that a single amino acid substitution in the PB1 protein (T296R) was sufficient to confer increased viral virulence to the 2009 pH1N1 virus. The enhanced replication of viruses possessing PB1-T296R substitution *in vitro* and *in vivo* suggested that this amino acid substitution could impact viral polymerase activity. Consistent with enhanced growth kinetics *in vivo* and *in vitro*, the PB1-T296R amino acid substitution increased polymerase activity in a luciferase-based mini genome assay (Fig. 4). Taken together, our data show that the PB1-T296R

substitution alone enhances viral virulence, polymerase activity and viral replication kinetics of a pH1N1 virus.

Adaptation is considered to drive evolution by conferring mutations that enhance viral fitness. In our study, we found that an amino acid substitution (PB1-T296R) was the primary virulence determinant responsible for the increased pathogenicity of 2009 pH1N1 virus in mice. Since the 2009 pH1N1 virus containing PB1-T296R replicated efficiently in mammalian cells and the RDRP activity of this virus was enhanced in human kidney cells, we speculate that PB1-T296R substitution might enhance 2009 pH1N1 replication in other mammalian species, although additional work in other animal models, such as ferrets or primates, are needed to better assess the impact of the PB1-T296R substitution on pH1N1 virulence in humans. These findings define PB1-T296R as a pH1N1 virulence determinant and emphasize the importance of surveillance efforts to track the emergence of viral variants with amino acid substitutions that are known to affect viral virulence in animal models. Ultimately, defining the molecular features associated with altered virulence may facilitate the early recognition of variant viruses with epidemic potential.

Acknowledgments

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