Amino Acid Changes in the Influenza A Virus PA Protein That Attenuate Avian H5N1 Viruses in Mammals

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ABSTRACT

The influenza viral polymerase complex affects host tropism and pathogenicity. In particular, several amino acids in the PB2 polymerase subunit are essential for the efficient replication of avian influenza viruses in mammals. The PA polymerase subunit also contributes to host range and pathogenicity. Here, we report that the PA proteins of several highly pathogenic avian H5N1 viruses have attenuating properties in mammalian cells and that the attenuating phenotype is conferred by strain-specific amino acid changes. Specifically, lysine at position 185 of A/duck/Vietnam/TY165/2010 (TY165; H5N1) PA induced strongly attenuating effects *in vitro* and *in vivo*. More importantly, the introduction of the arginine residue commonly found at this position in PA significantly increased the viral polymerase activity of TY165 in mammalian cells and its virulence and pathogenicity in mice. These findings demonstrate that the PA protein plays an important role in influenza virulence and pathogenicity.

IMPORTANCE

Highly pathogenic influenza viruses of the H5N1 subtype cause severe respiratory infections in humans, which have resulted in death in nearly two-thirds of the patients with laboratory-confirmed cases. We found that the viral PA polymerase subunit of several H5N1 viruses possesses amino acid changes that attenuate virus replication in mammalian cells (yet the H5N1 viruses possessing these mutations are highly pathogenic in mice). Specifically, we found that an arginine-to-lysine substitution at position 185 of an H5N1 virus PA protein significantly affected that virus's virulence and pathogenicity in mice. The PA protein thus plays a role in the pathogenicity of highly pathogenic H5N1 influenza viruses.

Highly pathogenic avian H5N1 influenza viruses pose a potential threat to human health, evidenced by more than 650 human infections with these viruses, resulting in 358 deaths as of 24 January 2014 (<http://www.who.int>). Multiple studies have established that the influenza viral polymerase complex, composed of the polymerase subunits PB1, PB2, and PA, is a determinant of pathogenicity and host range, including that of highly pathogenic avian H5N1 viruses (1–13). Several residues in PB2, including 627K (11–13), 701N (1, 2), 591R/K (3, 5, 9), 271A (8, 9), and 158G (10), are known to contribute to the adaptation of avian influenza viruses to mammalian hosts, suggesting a major role for PB2 in influenza virus pathogenicity and host adaptation. Recently, we identified three novel pathogenicity markers in avian H5N1 virus PB2 proteins (PB2-147T/339T/588T) that can compensate for the mammal-adapting function of PB2-627K (unpublished data).

The PA subunit of the polymerase complex has been also linked to influenza pathogenicity and adaptation to new host species (14–20). The PA protein of the 2009 pandemic H1N1 viruses contributed to the enhanced polymerase activity of avian influenza viruses in mammalian cells, which was mediated by several residues not typically found at those amino acid positions of the influenza virus PA protein (15). Mutations in PA acquired through viral passages in mice also contributed to the enhanced replication and pathogenicity of a 2009 pandemic H1N1 (19), a low-pathogenicity avian H5N2 (16), and the A/Puerto Rico/8/34 (H1N1) virus (21). A mutation detected in the PA protein of an H7N7 virus isolated from a fatally infected individual cause a greater increase in polymerase activity in human than in avian cells (20), suggesting that PA contributes to influenza virus host range. In the spring of 2013, novel influenza viruses of the H7N9

subtype emerged in China, and these viruses have caused 384 human infections as of 10 March 2014, 72 of which had fatal outcomes (<http://www.healio.com>). We recently demonstrated that several amino acid changes in PA that occurred on the evolutionary path to H7N9 viruses contributed to the virulence of the novel viruses (17). Moreover, the PA protein also contributes to the high virulence of avian H5N1 influenza viruses in ducks (18). Collectively, these data demonstrate a role for PA in influenza virus pathogenicity.

During our recent study that identified novel pathogenicity markers in the PB2 protein of avian H5N1 viruses (i.e., 147T/339T/588T [unpublished data]), we also noticed a strongly attenuating effect by the PA gene in mammalian cells. In this study, we expanded on this preliminary finding and characterized the PA genes of several avian H5N1 viruses. We identified several amino acids in PA that strongly attenuated viral polymerase activity in mammalian cells. In particular, lysine at position 185 of PA significantly attenuated avian H5N1 virus replication *in vitro* and *in vivo*.

Received 18 April 2014 Accepted 12 September 2014

Published ahead of print 17 September 2014

Editor: A. García-Sastre

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doi:10.1128/JVI.01081-14

MATERIALS AND METHODS

Cells. Madin-Darby canine kidney (MDCK) cells were maintained in Eagle's minimal essential medium (MEM) containing 5% newborn calf serum and antibiotics. Human embryonic kidney cells (293T) and chicken fibroblasts (DF-1) were grown in Dulbecco's modified essential medium (DMEM) with 10% fetal calf serum and antibiotics. All cells were maintained at 37°C with 5% CO₂.

Viruses. The H5N1 A/duck/Vietnam/TY165/2010 (TY165), A/duck/Vietnam/LS1349/2011 (LS1349), and A/chicken/Vietnam/QT517/2009 (QT517) viruses were isolated from dead (TY165 and LS1349) or healthy (QT517) animals in Vietnam between 2009 and 2011. A/chicken/Vietnam/NCVD5/2003 (VD5), A/Muscovy duck/Vietnam/NCVD18/2003 (VD18), and A/Vietnam/1203/2004 (VN1203) viruses have been previously described (12, 22). All H5N1 viruses used for this study were generated by using reverse genetics (23) and were amplified in MDCK cells in a biosafety level 3 containment laboratory approved for such use by the Centers for Disease Control and Prevention and the U.S. Department of Agriculture.

Plasmid construction and reverse genetics. The reverse genetics systems for VD5, VD18, VN1203, and TY165 are described in previous reports (12, 22–24); minireplicon systems for LS1349 and QT517 were generated similarly. To generate mutant viruses, the RNA polymerase I plasmids encoding the PA segment were mutated by means of site-directed mutagenesis PCR and mutant viruses were produced by use of reverse genetics as described by Neumann et al. (23). To generate plasmids for the synthesis of the influenza virus polymerase and NP proteins, the PB1, PB2, NP, and wild-type and mutant PA genes of the respective viruses were inserted into the pCAGGS/BsmBI protein expression vector (25, 26). All constructs were sequenced to ensure the absence of unwanted mutations. The plasmids pPol(Hu)-VD5(50)M(50)-Luc and pPol(Ck)-VD5(50)M(50)-Luc (encoding the luciferase reporter gene from the viral M segment under the control of human and chicken RNA polymerase I promoters, respectively) were constructed as described previously (26, 27).

Minireplicon assay. Human 293T cells were transfected by using TransIT-LT1 (Mirus, Madison, WI) with 0.1 µg each of the constructs encoding PB1, PB2, NP, and wild-type or mutant PA, together with 0.025 µg of pPol(Hu)-VD5(50)M(50)-Luc and 0.01 µg of pGL4.74[hRluc/TK] (an internal control encoding *Renilla* luciferase; Promega, Madison, WI). Avian DF-1 cells were transfected by using Lipofectamine LTX reagent (Invitrogen, Carlsbad, CA) with the same set of plasmids except for pPol(Hu)-VD5(50)M(50)-Luc (possessing a human RNA polymerase I promoter), which was replaced with pPol(Ck)-VD5(50)M(50)-Luc (possessing a chicken RNA polymerase I promoter). The transfected cells were incubated at 33°C and 37°C for 293T cells or at 37°C and 41°C for DF-1 cells. At 48 h posttransfection, cells were lysed and luciferase activity was determined by using the dual-luciferase system detector kit according to the manufacturer's protocol (Promega). The luciferase activity values were normalized to the *Renilla* activity. The data presented are the averages of three independent experiments ± standard deviations.

Virus replication in Calu-3 and DF-1 cells. Confluent Calu-3 and DF-1 cells were infected with wild-type or PA mutant H5N1 viruses at a multiplicity of infection (MOI) of 1×10^{-4} or 2×10^{-5} , respectively, and incubated for 1 h at 37°C. One hour later, cells were washed twice and then further incubated in DMEM-F12 (Calu-3) or DMEM (DF-1) containing 0.3% bovine serum albumin and tosylsulfonphenylalanyl chloromethyl ketone (TPCK) trypsin (2.0 µg/ml) at 33°C and 37°C for Calu-3 cells or at 37°C and 41°C for DF-1 cells; although the viruses used in this study possess a hemagglutinin (HA) that is cleaved by ubiquitous proteases, we added trypsin to ensure similar cleavage efficiencies for all viruses. Aliquots of supernatants were harvested for virus titration at various time points postinfection (p.i.). Virus titers at each time point were determined by use of plaque assays in MDCK cells. Values are presented as the averages of the triplicate wells ± standard deviations from one experiment.

Mouse experiments. Four- to 6-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were used for these experiments. To determine the survival of infected mice, 3 mice per virus-infected group were anesthetized with isoflurane and inoculated intranasally with the doses indicated below in a 50-µl volume. The mice were monitored daily for 14 days and checked for changes in body weight and mortality. Animals were euthanized when they lost more than 25% of their initial body weight. For virus replication in organs, groups of mice (9 per group) were infected intranasally with the doses of virus indicated below. Three mice in each group were euthanized on days 2, 4, and 6 p.i. Organs (brains, lungs, nasal turbinates, kidneys, and spleens) and nasal washes were collected for virus titration by using plaque assays in MDCK cells. The data shown are the mean virus titers ± standard deviations.

Biosafety consideration. This study was approved by the local Institutional Biosafety Committed (IBC); in addition, the Alternate Responsible Official of the University of Wisconsin—Madison Select Agent Program and NIAID evaluated this study and concluded that it does not involve dual-use research of concern (DURC).

RESULTS

The PA proteins of several H5N1 influenza viruses attenuate the activity of the viral polymerase complex in human cells. Recently, we characterized an avian H5N1 influenza virus isolated from the lungs of a dead duck in Vietnam in 2010 (A/duck/Vietnam/TY165/2010 [TY165]) (unpublished data). This virus was highly pathogenic in mice, a property that we mapped to three novel pathogenicity markers (147T/339T/588T) in the viral PB2 polymerase subunit that could substitute for the mammal-adapting function of PB2-627K (11, 12). Interestingly, the TY165 PA protein significantly reduced the polymerase activities of two avian H5N1 influenza viruses that did not encode PB2-627K or PB2-147T/339T/588T (A/chicken/Vietnam/NCVD5/2003 [VD5] and A/Muscovy duck/Vietnam/NCVD18/2003 [VD18]) in minireplicon assays in human cells; conversely, the VD5 and VD18 PA proteins increased the activity of the TY165 polymerase complex. On the basis of these findings, we speculated that the TY165 PA protein attenuates the polymerase activity of avian H5N1 influenza viruses in human cells, perhaps to counteract the high replicative ability conferred by mutations such as PB2-627K or PB2-147T/339T/588T.

To test this hypothesis, we first asked whether other avian H5N1 influenza viruses with known pathogenicity markers in PB2 encode attenuating PA proteins. To determine this, we selected A/duck/Vietnam/LS1349/2011 (LS1349), which was identified through our surveillance activities in Vietnam, is highly pathogenic in mice, and encodes the PB2-147T/339T/588T markers (our unpublished findings). We also tested A/chicken/Vietnam/QT517/2009 (QT517), another virus isolated through our surveillance activities in Vietnam, which is highly pathogenic in mice (our unpublished data). QT517 encodes PB2-147T/339M; these two residues were found in another H5N1 virus with high replicative ability in human cells (unpublished data). LS1349 and QT517 are genetically unrelated and belong to the H5 HA clades 2.3.2.1 and 2.3.4.1, respectively (unpublished data).

First, we combined the PA protein of the test viruses (i.e., TY165, LS1349, and QT517) with the PB2, PB1, and NP proteins of VD5 or VD18, which were used in our recent study that led to the identification of the attenuating properties of TY165 PA (unpublished data). We tested the activity of the recombinant replication complexes by using minireplicon assays in human embry-

FIG 1 Attenuating properties of avian H5N1 PA proteins in human cells. 293T cells were transfected with PB2, PB1, PA, and NP protein expression plasmids, together with a minigenome encoding the luciferase gene [pPol(Hu)-VD5(50)M(50)-Luc] and an internal control plasmid encoding *Renilla* luciferase (pGL4.74[hRluc/TK]). Transfected cells were incubated at 37°C and 33°C for 48 h and then lysed. Luciferase activity was determined by using the dual-luciferase system kit. The relative polymerase activity was normalized to that of wild-type polymerase complexes. The data are presented as the averages of three independent experiments $\pm$ standard deviations. (A) Polymerase activities of TY165, LS1349, and QT517 PA proteins tested with VD5 or VD18 PB2, PB1, and NP proteins. (B) Polymerase activities of VD5 and VD18 PA proteins tested with TY165, LS1349, and QT517 PB2, PB1, and NP proteins.

onic kidney (293T) cells at 37°C and 33°C (the temperatures of the lower and upper respiratory tract of humans). The TY165, LS1349, and QT517 PA proteins significantly reduced the activity of the VD5 and VD18 replication complexes at both temperatures (Fig. 1A). Conversely, the VD5 and VD18 PA proteins significantly enhanced the activity of the TY165, LS1349, and QT517

replication complexes, an effect that was more pronounced at 33°C than at 37°C (Fig. 1B). Hence, the TY165, LS1349, and QT517 PA proteins tested here strongly attenuated the avian H5N1 polymerase activity in human cells.

Identification of mutations that confer attenuating properties to PA. To identify the amino acids in the TY165, LS1349, and

TABLE 1 Comparison of the TY165, LS1349, and QT517 PA protein sequences with those of the VD5 and VD18 viruses and with the consensus sequence of avian H5N1 PA proteins^a

Amino acid position	Amino acid					Consensus amino acid of avian H5N1 influenza viruses
	LS1349	TY165	QT517	VD5	VD18	
20	T	T	A	A	A	A
27	S	S	D	D	D	D
<u>44</u>	V	V	I	V	V	V
63	A	A	V	V	V	V
<u>85^b</u>	T	T	<u>A</u>	T	T	T
142	R	R	K	K	K	K
<u>185</u>	R	<u>K</u>	R	R	R	R
<u>231</u>	A	A	T	A	A	A
<u>241^b</u>	C	C	<u>Y</u>	C	C	C
<u>263</u>	T	T	<u>K</u>	T	T	T
272	E	E	D	D	D	D
<u>305</u>	Y	Y	<u>F</u>	Y	Y	Y
352	D	D	E	E	E	E
<u>372</u>	<u>D</u>	E	E	E	E	E
<u>379</u>	I	V	V	V	V	V
<u>401</u>	R	R	<u>K</u>	R	R	R
<u>538</u>	<u>V</u>	E	E	E	E	E
<u>573</u>	I	I	<u>V</u>	I	I	I
614	T	T	N	N	N	N
631	S	S	G	G	G	G

^a Shown are positions at which the TY165, LS1349, and/or QT517 PA proteins differ from the VD5 and VD18 PA proteins, and at which avian H5N1 viruses encode a highly conserved amino acid (shared by > 85% of isolates). Strain-specific amino acid differences are underlined.

^b Not tested in this study because our unpublished data suggest that the PA gene of an H5N1 virus carrying these two residues does not confer attenuating properties in mammalian cells.

FIG 2 Identification of attenuating amino acid changes in avian H5N1 PA proteins in human cells. Mini-replicon experiments were carried out as described in the legend for Fig. 1. (A) Polymerase activities of TY165 PA proteins possessing a mutation shared with LS1349 (Table 1). (B) Polymerase activities of TY165, LS1349, and QT517 PA proteins possessing strain-specific mutations (Table 1). (C) Polymerase activities of VD18 and VN1203 PA proteins encoding the attenuating PA-R185K mutation.

QT517 PA proteins that attenuated the activity of the viral polymerase complex in mammalian cells, we compared the amino acid sequences of the respective PA proteins with those of VD5 and VD18 and with avian H5N1 virus PA sequences. Because we were originally seeking to identify attenuating amino acid changes of broad significance (i.e., those affecting a number of different PA proteins), we focused on positions in which >85% of the isolates encoded the same amino acid. At positions 20, 27, 63, 142, 272, 352, 614, and 631, the amino acids of the TY165 and LS1349 PA proteins differed from those of VD5, VD18, and most other avian H5N1 PA proteins (Table 1). For these amino acid positions, we mutated the TY165 PA protein to encode the amino acid commonly found among avian H5N1 viruses at that particular position (for example, we generated a TY165-PA-T20A mutant). In addition, we identified strain-specific amino acid differences (underlined in Table 1) and replaced them with the consensus amino acid at that particular position. All strain-specific amino acid changes were tested in the genetic background in which they were found; for example, we generated a mutant TY165 PA protein possessing the K185R mutation, a mutant LS1349 PA protein possessing the D372E mutation, and a mutant QT517 PA protein encoding F305Y. Two strain-specific residues (PA-85A and PA-241Y in QT517) were not tested in this study because our previous studies showed that the PA gene of an H5N1 virus carrying these two amino acids does not confer attenuating properties (unpublished data).

We then tested the activity of the mutant and wild-type polymerase complexes by using mini-replicon assays in human 293T cells at 37°C and 33°C. Most of the mutations tested did not increase the TY165 polymerase activity (Fig. 2A), suggesting that the particular amino acid change was not critical for TY165, LS1349, and QT517 PA attenuation or may only exhibit an effect in combination with other mutations. The TY165-PA-D352E mutant caused a 2- to 3-fold increase in polymerase activity compared with TY165, indicating that the PA-352D residue encoded by the TY165 and LS1349 viruses may contribute to PA attenuation. In contrast, the strain-specific mutations of PA-K185R (TY165), -D372E (LS1349), and -F305Y and -V573I (both QT517) increased the activity of the respective replication complexes at 37°C and/or 33°C 6- to 50-fold (Fig. 2B); these mutations did not affect the PA expression levels as assessed by Western blotting (data not shown). These data indicate that the attenuating properties of the TY165, LS1349, and QT517 PA proteins are not conferred by an amino acid change that is shared among these viruses; rather, all three PA proteins encode strain-specific attenuating mutations.

Because the PA-K185R mutation had a significantly stronger effect on the polymerase activity than any of the other mutations tested, we selected it for further studies. Introduction of the PA-185K residue (found in TY165-PA) into the PA proteins of VD18 and A/Vietnam/1203/2004 (VN1203, a highly pathogenic H5N1 virus that we have characterized extensively [12]) decreased their

FIG 3 Replicative ability of TY165 and TY165-PA-K185R viruses in human cells. Calu-3 cells were infected with viruses at a multiplicity of infection of 1×10^{-4} and incubated at 33°C and 37°C. Aliquots of the supernatants were collected at the indicated time points and titrated in MDCK cells by use of plaque assays. Values are presented as the averages of three wells $\pm$ standard deviations from one experiment.

FIG 4 Survival and body weight changes of mice infected with wild-type or mutant viruses. BALB/c mice (three/group) were inoculated intranasally with the indicated doses of TY165 and TY165-PA-K185R (A), VD18 and VD18-PA-R185K (B), or VN1203 and VN1203-PA-R185K (C) viruses. Infected animals were monitored daily for body weight changes and survival. The numbers of animals that survived infection are shown.

polymerase activities significantly (Fig. 2C). These data further demonstrate the strongly attenuating effect of PA-185K on avian H5N1 viral polymerase activity in mammalian cells.

Growth properties of viruses encoding PA-185R or -185K in human cells. To understand the role of PA-185R/K in influenza virus replication in cultured cells, we tested the growth properties of wild-type and mutant TY165 viruses in human airway epithelial cells (Calu-3) at 37°C and 33°C. At 37°C, the TY165 and TY165-PA-185R viruses were comparable in terms of their growth kinetics in Calu-3 cells (Fig. 3B); in contrast, the mutant virus expressing PA-185R replicated more efficiently than the wild-type virus at the lower temperature of 33°C (Fig. 3A). These data further suggest that PA-185K plays an attenuating role in H5N1 virus replication in human cells, at least at 33°C.

Effect of PA-185R/K on the pathogenicities of avian H5N1 viruses in mice. To assess the significance of PA-185R/K for

influenza virus replication *in vivo*, we generated wild-type TY165 and mutant TY165-PA-K185R viruses. Each virus was intranasally inoculated into three BALB/c mice at a dose of 10^2 PFU of virus. We chose a low infectious dose because TY165 is highly pathogenic in mice—the dose required to kill 50% of infected animals (50% mouse lethal dose [MLD₅₀]) is 1 PFU (unpublished data)—and replacement of the potentially attenuating PA-185K residue could further increase the pathogenicity. Infected mice were observed daily for weight loss and survival. At the low infectious dose of 10^2 PFU, TY165-infected mice experienced moderate weight loss and died by day 10 postinfection (Fig. 4A). Compared with TY165, the mutant TY165-PA-K185R virus caused more body weight loss and earlier death in infected mice (Fig. 4A), consistent with the increased polymerase activity of the TY165-PA-K185R replication complex in mammalian cells (Fig. 2). Given that the

FIG 5 Replication of TY165 and TY165-PA-K185R viruses in mice. Nine mice per group were intranasally inoculated with 10^2 PFU of TY165 and TY165-PA-K185R viruses. Three mice were euthanized on days 2, 4, and 6 postinfection. Lungs, nasal turbinates, spleens, kidneys, brains, and nasal washes were collected and virus titers were determined by means of plaque assays in MDCK cells. Data shown are the mean virus titers with standard deviations. The *P* values were calculated by using Student's *t* test, comparing the virus titers between the wild-type and mutant viruses detected in the indicated organs. **, *P* < 0.001; *, *P* < 0.05.

MLD₅₀ of TY165 virus is 1 PFU, we could not determine an MLD₅₀ value for the more pathogenic TY165-PA-K185R virus.

To further test the significance of PA-185R/K, we also compared the mouse pathogenicities of the wild-type VD18 and VN1203 viruses (both of which encode PA-185R) with those of the respective PA-R185K mutant viruses (Fig. 4B and C). VD18 is of intermediate pathogenicity in mice (the MLD₅₀ is 10^4 PFU [12]); given that the PA-R185K mutation was expected to attenuate this virus, we infected mice with high doses (10^6 , 10^5 , and 10^4 PFU) of wild-type and mutant VD18 viruses. At a dose of 10^6 PFU, all mice infected with VD18 or VD18-PA-R185K virus succumbed to their infection (Fig. 4B). However, at the lower dose of 10^5 PFU, animals infected with the wild-type virus died by day 9 postinfection, whereas those infected with the mutant PA-R185K virus survived (Fig. 4B). The attenuating effect of PA-R185K was also observed at the dose of 10^4 PFU because animals infected with VD18-PA-R185K did not experience weight loss, whereas wild-type VD18 virus caused moderate weight loss and death in one mouse (Fig. 4B). VN1203 is highly pathogenic in mice (MLD₅₀, 1 PFU [12]), prompting us to infect mice with doses of 10^3 , 10^2 , and 10^1 PFU of wild-type or mutant virus, respectively. Overall, VN1203-PA-R185K was slightly less pathogenic in mice than the wild-type virus (Fig. 4C); however, this effect was less pronounced than that observed with the TY165 and VD18 viruses, presumably

because the PB2-627K mutation in VN1203 (a known determinant of avian influenza virus replication in mammals [12]) masked the attenuating effect of the PA-R185K mutation. Collectively, these findings demonstrate the attenuating effect of PA-185K on avian H5N1 pathogenicity in a mouse model.

Effect of PA-185R/K on the replication of H5N1 viruses in mice. Next, we compared the replication kinetics of TY165 and VD18 wild-type and mutant viruses in mice; we did not test the wild-type and mutant VN1203 viruses due to the small differences in pathogenicity observed for these viruses in mice (see previous paragraph and Fig. 4C). Briefly, nine mice each were intranasally infected with 10^2 or 10^5 PFU of wild-type or mutant TY165 or VD18 virus, respectively. Three mice from each group were euthanized on days 2, 4, and 6 postinfection, and lungs, nasal turbinates, kidneys, spleens, brains, and nasal washes were collected for virus titration in MDCK cells. Mutant TY165-PA-K185R virus replicated to higher titers than wild-type virus in lungs on day 2 (Fig. 5A), in nasal turbinates and nasal washes on all days tested (Fig. 5B and F), in spleens on day 4 (Fig. 5C), and in kidneys on day 6 (Fig. 5D) postinfection. Moreover, TY165-PA-K185R virus replicated to appreciable titers in mouse brains on days 4 and 6 postinfection, whereas wild-type TY165 virus was not isolated from brain (Fig. 5E). These data demonstrate that the PA-185K residue in TY165 attenuates this virus in mice. Likewise, the VD18-PA-R185K mu-

FIG 6 Replication of VD18 and VD18-PA-R185K viruses in mice. Nine mice per group were intranasally inoculated with 10^5 PFU of VD18 and VD18-PA-R185K viruses. Three mice were euthanized on days 2, 4, and 6 postinfection. Lungs, nasal turbinates, spleens, kidneys, brains, and nasal washes were collected and virus titers were determined by means of plaque assays in MDCK cells. Data shown are the mean virus titers with standard deviations. The *P* values were calculated by using Student's *t* test, comparing the virus titers between the wild-type and mutant viruses detected in the indicated organs. **, *P* < 0.001; *, *P* < 0.05.

tant virus replicated less efficiently than wild-type VD18 (encoding PA-185R) in lungs, nasal turbinates, nasal washes, and brains on day 6 postinfection (Fig. 6A, B, E, and F), in spleens on day 2 postinfection (Fig. 6C), and in kidneys on all days postinfection tested (Fig. 6D), further demonstrating the attenuating effect of PA-185K on avian H5N1 virulence in mice. Although no significant differences were detected between the growth curves of TY165 and TY165-PA-K185R virus in human cells at 37°C, the virulence of these viruses in mice differed. This finding suggests that in addition to its role in viral RNA replication and transcription, PA may have additional functions in the viral life cycle, for example, in the regulation of host responses to infection.

Replication properties of 185R/K viruses in avian cells. Our data demonstrated that the PA-185K residue in TY165 attenuates avian H5N1 virus polymerase activity in human cells and reduces virulence and pathogenicity in mice. To determine whether this mutation also has a fitness effect in avian species, we compared the polymerase activities of the wild-type and mutant TY165 PA proteins in minireplicon assays in chicken DF-1 fibroblasts at 37°C and at 41°C, which is the body temperature of birds. The TY165-PA-K185R mutation did not affect the polymerase activity at 41°C but caused a slight increase in activity at 37°C (Fig. 7A), comparable to the increase detected in human cells at 37°C (Fig. 2).

We also tested the growth properties of the wild-type and mutant TY165 viruses in DF-1 cells at 37°C and 41°C. Cells were

infected at an MOI of 2×10^{-5} . Supernatants were collected at various time points and virus titers were determined by use of plaque assays in MDCK cells. Replacement of PA-185K (encoded by TY165) with PA-185R (commonly found at this position) did not affect the viral replication kinetics in avian cells at 37°C but caused a slight reduction in virus titers at 41°C (Fig. 7B); in contrast, the PA-185R mutation conferred increased replicative properties in human cells at 33°C (Fig. 3). These data suggest that the PA-185K residue encoded by TY165 does not significantly attenuate an H5N1 virus in avian cells at 33°C and 37°C, as well as in mice.

DISCUSSION

In this study, we identified several mutations in avian H5N1 PA proteins with an attenuating effect in mammalian cells, namely, PA-185K, -305F, -372D, and -573V. In fact, all three avian H5N1 PA proteins tested (derived from the TY165, LS1349, and QT517 viruses) had an attenuating effect in minireplicon assays in human cells. This effect was more significant at 33°C than at 37°C and may facilitate virus replication at the lower temperatures of the upper respiratory tract. Temperature-sensitive mammalian-attenuating mutations in PA may therefore be more widespread than previously thought but may be difficult to identify because of their strain-specific nature. This strain specificity is in contrast to the

FIG 7 Replicative abilities of TY165 and TY165-PA-K185R viruses in avian cells. (A) Minireplicon assays in avian DF-1 cells. DF-1 cells were transfected with PB2, PB1, PA, and NP protein expression plasmids, together with a minigenome encoding the luciferase gene [pPol(Ck)-VD5(50)M (50)-Luc] and an internal control plasmid encoding *Renilla* luciferase (pGL4.74[hRLuc/TK]). Transfected cells were incubated at 37°C and 41°C for 48 h and then lysed. Luciferase activity was determined by using the dual-luciferase system kit. The relative polymerase activity was normalized to that of wild-type polymerase complexes. The values are presented as the averages of triplicate wells $\pm$ standard deviations from one experiment. (B) Viral growth curves in avian DF-1 cells. DF-1 cells were infected with viruses at an MOI of 2×10^{-5} and incubated at 37°C and 41°C. Aliquots of the supernatants were collected at the indicated time points and titrated in MDCK cells by use of plaque assays. Values are presented as the averages of three wells $\pm$ standard deviations from one experiment.

pathogenicity markers PB2-627K and PB2-147T/339T/588T, which have been found in many different highly pathogenic H5N1 viruses and even in viruses of other subtypes (for PB2-627K).

Lysine at position 185 of PA reduced avian H5N1 polymerase activity in minireplicon assays in human cells and pathogenicity in mice. Conversely, replacement of PA-185K with the amino acid commonly found at this position (i.e., PA-185R) increased avian H5N1 virus polymerase activity in human cells and avian H5N1 virus pathogenicity in mice. The amino acid at position 185 of PA is highly conserved among human and avian influenza A viruses. Our inspection of $>10,000$ avian and $>10,000$ human influenza A virus PA sequences (obtained from the Influenza Research Database [<http://www.fludb.org>]) revealed only 3 human and 37 avian PA proteins encoding PA-185K. Interestingly, two of the three human viruses encoding PA-185K are avian influenza viruses isolated from humans (A/Guangxi/1/2005 [H5N1] and A/Guangzhou/333/1999 [H9N2]); we do not know whether the PA-185K mutation emerged during replication in humans or was already encoded by the avian predecessors of these viruses. Among the avian influenza virus PA proteins encoding PA-185K, seven originated from highly pathogenic avian H5N1 viruses.

It is interesting that all avian H5N1 influenza viruses encoding PA-185K were isolated in or after 2005. At around that time, many avian H5N1 viruses acquired mutations that facilitate replication in mammals, such as PB2-627K or PB2-147T/339T/588T (unpublished data). We therefore speculate that PA-185K may counter-

balance the strong polymerase activity conferred by mammal-adapting mutations in PB2. However, not all avian H5N1 viruses possessing PA-185K encode these marker amino acids in PB2, and TY165 (encoding PA-185K) and TY165-PA-185R replicated to similar levels in avian DF-1 cells, suggesting that the PA-185K was not selected in avian species to counterbalance the high replicative ability conferred by mutations in PB2.

The crystal structures of the N-terminal portions of several influenza virus PA proteins have been determined (28–30). The amino acid at position 185 is prominently exposed at the surface and is part of a turn that connects helices $\alpha 6$ and $\alpha 7$ (28–30); a mutation at this position may affect the interaction with helix $\alpha 1$. The replacement of arginine (commonly found at this position) with the smaller lysine residue (found in TY165) could affect the interaction of PA with other viral proteins and/or host proteins. Moreover, the attenuating effect of PA-185K was significantly greater at 33°C than at 37°C, suggesting that the lower temperature may restrict the flexibility of the amino acid at position 185, which could be critical for its function.

In addition to PA-185K, our data also suggest that the PA-305F, -372D, and -573V residues attenuate H5N1 polymerase complexes in mammalian cells at 33°C and/or 37°C. The PA-305F (QT517) and PA-372D (LS1349) mutations are extremely rare among human and avian influenza virus PA proteins. Only three human and six avian influenza viruses encode PA-305F; among these, there are two human and three H5N1 viruses. At position

372, aspartic acid has not been detected among human virus PA proteins and is present in only 5 avian virus PA proteins (two of which are avian H5N1 influenza viruses). At position 573, 14 avian influenza viruses encode PA-573V; 5 of these isolates are highly pathogenic avian H5N1 viruses, 2 of which also encode PA-305F. Note that among the viruses encoding the attenuating PA-185K, -305F, -372D, and -573V mutations, there are several highly pathogenic H5N1 viruses; the biological significance of this observation is not yet clear.

In summary, we identified several amino acids in influenza A virus PA proteins that do not significantly affect viral polymerase activity in an avian cell line at 41°C but appreciably attenuate avian H5N1 viruses in mice.

ACKNOWLEDGMENTS

We thank Susan Watson for scientific editing. We thank Kelly Moore and Lisa Burley for technical assistance.

This work was supported by the NIAID-funded Center for Research on Influenza Pathogenesis (CRIP; HHSN266200700010C), by a Grant-in-Aid for Specially Promoted Research, by the Japan Initiative for Global Research Network on Infectious Diseases from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, by ERATO, Japan, by the Strategic Basic Research Programs of Japan Science and Technology Agency, Japan, and by a National Institute of Allergy and Infectious Diseases Public Health Service research grant.

REFERENCES

- Gabriel G, Dauber B, Wolff T, Planz O, Klenk HD, Stech J. 2005. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. *Proc. Natl. Acad. Sci. U. S. A.* 102:18590–18595. <http://dx.doi.org/10.1073/pnas.0507415102>.
- Li Z, Chen H, Jiao P, Deng G, Tian G, Li Y, Hoffmann E, Webster RG, Matsuoka Y, Yu K. 2005. Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. *J. Virol.* 79:12058–12064. <http://dx.doi.org/10.1128/JVI.79.18.12058-12064.2005>.
- Mehle A, Doudna JA. 2009. Adaptive strategies of the influenza virus polymerase for replication in humans. *Proc. Natl. Acad. Sci. U. S. A.* 106:21312–21316. <http://dx.doi.org/10.1073/pnas.0911915106>.
- Neumann G, Kawaoka Y. 2006. Host range restriction and pathogenicity in the context of influenza pandemic. *Emerg. Infect. Dis.* 12:881–886. <http://dx.doi.org/10.3201/eid1206.051336>.
- Yamada S, Hatta M, Staker BL, Watanabe S, Imai M, Shinya K, Sakai-Tagawa Y, Ito M, Ozawa M, Watanabe T, Sakabe S, Li C, Kim JH, Myler PJ, Phan I, Raymond A, Smith E, Stacy R, Nidom CA, Lank SM, Wiseman RW, Bimber BN, O'Connor DH, Neumann G, Stewart LJ, Kawaoka Y. 2010. Biological and structural characterization of a host-adapting amino acid in influenza virus. *PLoS Pathog.* 6:e1001034. <http://dx.doi.org/10.1371/journal.ppat.1001034>.
- Kawaoka Y, Krauss S, Webster RG. 1989. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *J. Virol.* 63:4603–4608.
- Naffakh N, Tomoiu A, Rameix-Welti MA, van der Werf S. 2008. Host restriction of avian influenza viruses at the level of the ribonucleoproteins. *Annu. Rev. Microbiol.* 62:403–424. <http://dx.doi.org/10.1146/annurev-micro.62.081307.162746>.
- Bussey KA, Bousse TL, Desmet EA, Kim B, Takimoto T. 2010. PB2 residue 271 plays a key role in enhanced polymerase activity of influenza A viruses in mammalian host cells. *J. Virol.* 84:4395–4406. <http://dx.doi.org/10.1128/JVI.02642-09>.
- Liu Q, Qiao C, Marjuki H, Bawa B, Ma J, Guilloisou S, Webby RJ, Richt JA, Ma W. 2012. 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. *J. Virol.* 86:1233–1237. <http://dx.doi.org/10.1128/JVI.05699-11>.
- Zhou B, Li Y, Halpin R, Hine E, Spiro DJ, Wentworth DE. 2011. PB2 residue 158 is a pathogenic determinant of pandemic H1N1 and H5 influenza A viruses in mice. *J. Virol.* 85:357–365. <http://dx.doi.org/10.1128/JVI.01694-10>.
- Hatta M, Gao P, Halfmann P, Kawaoka Y. 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science* 293:1840–1842. <http://dx.doi.org/10.1126/science.1062882>.
- Hatta M, Hatta Y, Kim JH, Watanabe S, Shinya K, Nguyen T, Lien PS, Le QM, Kawaoka Y. 2007. Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. *PLoS Pathog.* 3:1374–1379. <http://dx.doi.org/10.1371/journal.ppat.0030133>.
- Subbarao EK, London W, Murphy BR. 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. *J. Virol.* 67:1761–1764.
- Mehle A, Dugan VG, Taubenberger JK, Doudna JA. 2012. Reassortment and mutation of the avian influenza virus polymerase PA subunit overcome species barriers. *J. Virol.* 86:1750–1757. <http://dx.doi.org/10.1128/JVI.06203-11>.
- Bussey KA, Desmet EA, Mattiacci JL, Hamilton A, Bradel-Tretheway B, Bussey HE, Kim B, Dewhurst S, Takimoto T. 2011. PA residues in the 2009 H1N1 pandemic influenza virus enhance avian influenza virus polymerase activity in mammalian cells. *J. Virol.* 85:7020–7028. <http://dx.doi.org/10.1128/JVI.00522-11>.
- Song MS, Pascua PN, Lee JH, Baek YH, Lee OJ, Kim CJ, Kim H, Webby RJ, Webster RG, Choi YK. 2009. The polymerase acidic protein gene of influenza A virus contributes to pathogenicity in a mouse model. *J. Virol.* 83:12325–12335. <http://dx.doi.org/10.1128/JVI.01373-09>.
- Yamayoshi S, Yamada S, Fukuyama S, Murakami S, Zhao D, Uraki R, Watanabe T, Tomita Y, Macken C, Neumann G, Kawaoka Y. 2014. Virulence-affecting amino acid changes in the PA protein of H7N9 influenza A viruses. *J. Virol.* 88:3127–3134. <http://dx.doi.org/10.1128/JVI.03155-13>.
- Song J, Feng H, Xu J, Zhao D, Shi J, Li Y, Deng G, Jiang Y, Li X, Zhu P, Guan Y, Bu Z, Kawaoka Y, Chen H. 2011. The PA protein directly contributes to the virulence of H5N1 avian influenza viruses in domestic ducks. *J. Virol.* 85:2180–2188. <http://dx.doi.org/10.1128/JVI.01975-10>.
- Ilyushina NA, Khalkov AM, Seiler JP, Forrest HL, Bovin NV, Marjuki H, Barman S, Webster RG, Webby RJ. 2010. Adaptation of pandemic H1N1 influenza viruses in mice. *J. Virol.* 84:8607–8616. <http://dx.doi.org/10.1128/JVI.00159-10>.
- de Wit E, Munster VJ, van Riel D, Beyer WE, Rimmelzwaan GF, Kuiken T, Osterhaus AD, Fouchier RA. 2010. Molecular determinants of adaptation of highly pathogenic avian influenza H7N7 viruses to efficient replication in the human host. *J. Virol.* 84:1597–1606. <http://dx.doi.org/10.1128/JVI.01783-09>.
- Rolling T, Koerner I, Zimmermann P, Holz K, Haller O, Staeheli P, Kochs G. 2009. Adaptive mutations resulting in enhanced polymerase activity contribute to high virulence of influenza A virus in mice. *J. Virol.* 83:6673–6680. <http://dx.doi.org/10.1128/JVI.00212-09>.
- Kim JH, Hatta M, Watanabe S, Neumann G, Watanabe T, Kawaoka Y. 2010. Role of host-specific amino acids in the pathogenicity of avian H5N1 influenza viruses in mice. *J. Gen. Virol.* 91:1284–1289. <http://dx.doi.org/10.1099/vir.0.018143-0>.
- Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez DR, Donis R, Hoffmann E, Hobom G, Kawaoka Y. 1999. Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl. Acad. Sci. U. S. A.* 96:9345–9350. <http://dx.doi.org/10.1073/pnas.96.16.9345>.
- Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, Hatta M, Muramoto Y, Tamura D, Sakai-Tagawa Y, Noda T, Sakabe S, Imai M, Hatta Y, Watanabe S, Li C, Yamada S, Fujii K, Murakami S, Imai H, Kakugawa S, Ito M, Takano R, Iwatsuki-Horimoto K, Shimajima M, Horimoto T, Goto H, Takahashi K, Makino A, Ishigaki H, Nakayama M, Okamatsu M, Takahashi K, Warshawer D, Shult PA, Saito R, Suzuki H, Furuta Y, Yamashita M, Mitamura K, Nakano K, Nakamura M, Brockman-Schneider R, Mitamura H, Yamazaki M, Sugaya N, Suresh M, Ozawa M, Neumann G, Gern J, Kida H, Ogasawara K, Kawaoka Y. 2009. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. *Nature* 460:1021–1025. <http://dx.doi.org/10.1038/nature08260>.
- Li C, Hatta M, Nidom CA, Muramoto Y, Watanabe S, Neumann G, Kawaoka Y. 2010. Reassortment between avian H5N1 and human H3N2 influenza viruses creates hybrid viruses with substantial virulence. *Proc. Natl. Acad. Sci. U. S. A.* 107:4687–4692. <http://dx.doi.org/10.1073/pnas.0912807107>.

26. Chu C, Fan S, Li C, Macken C, Kim JH, Hatta M, Neumann G, Kawaoka Y. 2012. Functional analysis of conserved motifs in influenza virus PB1 protein. *PLoS One* 7:e36113. <http://dx.doi.org/10.1371/journal.pone.0036113>.
27. Fan S, Macken CA, Li C, Ozawa M, Goto H, Iswahyudi Nidom CA, Chen H, Neumann G, Kawaoka Y. 2013. Synergistic effect of the PDZ and the p85beta-binding domains of the NS1 protein in virulence of an avian H5N1 influenza A virus. *J. Virol.* 87:4861–4871. <http://dx.doi.org/10.1128/JVI.02608-12>.
28. Dias A, Bouvier D, Crepin T, McCarthy AA, Hart DJ, Baudin F, Cusack S, Ruigrok RW. 2009. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. *Nature* 458:914–918. <http://dx.doi.org/10.1038/nature07745>.
29. DuBois RM, Slavish PJ, Baughman BM, Yun MK, Bao J, Webby RJ, Webb TR, White SW. 2012. Structural and biochemical basis for development of influenza virus inhibitors targeting the PA endonuclease. *PLoS Pathog.* 8:e1002830. <http://dx.doi.org/10.1371/journal.ppat.1002830>.
30. Kowalinski E, Zubieta C, Wolkerstorfer A, Szolar OH, Ruigrok RW, Cusack S. 2012. Structural analysis of specific metal chelating inhibitor binding to the endonuclease domain of influenza pH1N1 (2009) polymerase. *PLoS Pathog.* 8:e1002831. <http://dx.doi.org/10.1371/journal.ppat.1002831>.