Structural and Functional Characterization of K339T Substitution Identified in the PB2 Subunit Cap-binding Pocket of Influenza A Virus*§

Yong Liu†**, Kun Qin†, Geng Meng‡**, Jinfang Zhang‡*, Jianfang Zhou‡, Guangyu Zhao‡, Ming Luo***, and Xiaofeng Zheng†§

From the ‡ State Key Lab of Protein and Plant Gene Research and § Department of Biochemistry and Molecular Biology, School of Life Sciences, Peking University, Beijing 100871, China, † Chinese National Influenza Center, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 155 Changbai Road, Beijing 102206, China, ** Department of Microbiology, University of Alabama, Birmingham, Alabama 35294, and † State Key Lab of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing 100071, China.

Background: Amino acid changes in PB2 are associated with evolution of influenza virus.

Results: K339T substitution in PB2cap reduces the cap binding affinity, polymerase activity, RNA synthesis activity, and murine mortality.

Conclusion: Substitution in PB2cap modulates the polymerase activity and virulence by regulating the cap binding activity.

Significance: We identified and characterized an emerging K339T substitution in PB2cap.

Influenza virus RNA-dependent RNA polymerase is a heterotrimer composed of PA, PB1, and PB2 subunits. RNA-dependent RNA polymerase is required for both transcription and replication of influenza viral RNA taking place in the nucleus of infected cells. A “cap-snatching” mechanism is used to generate a 5′-capped primer for transcription in which the cap-binding domain of PB2 (PB2cap) captures the 5′ cap of the host pre-mRNA. Our statistical analysis of PB2 sequences showed that residue Lys339 located in the cap-binding pocket of H5N1 PB2cap was gradually replaced by Thr339 over the past decade. To understand the role of this amino acid polymorphism, we solved the crystal structures of PB2cap with or without a pre-mRNA cap analog, m7GTP, in the presence of Lys339 or Thr339. The structures showed that Lys339 contributes to binding the γ-phosphate group of m7GTP, and the replacement of Lys339 by Thr eliminates this interaction. Isothermal titration calorimetry analysis showed that Thr339 attenuated the PB2cap binding activity in vitro compared with Lys339. Further functional studies confirmed that Thr339-PB2-containing ribonucleoprotein complex has a reduced influenza polymerase activity and RNA synthesis activity, and a reconstituted H5N1 virus containing the Thr339 substitution exhibited a lower virulence to mice but more active replication in Madin-Darby canine kidney cells. The K339T substitution in the cap-binding pocket of PB2 modulates the polymerase activity and virulence by regulating the cap binding activity. It is informative to track variations in the cap-binding pocket of PB2 in surveillance of the evolution and spread of influenza virus.

Both seasonal and pandemic influenza can cause severe illness and death in humans and farm animals. Virus variants emerge frequently and pose a constant health threat to humans. What changes in viral genes will result in viral evolutionary advantages, such as change of virulence and more efficient replication and transmissibility, is an intensive topic. Human infections by avian influenza A virus (H5N1) and the recent pandemic H1N1 influenza A virus 2009 (pH1N1) originating from swine are examples for transmission of animal influenza viruses to humans. Recent reports have shown that an adapted H5N1 virus with mutations in HA and PB2 can be transmitted between ferrets (1, 2). And the new substitution S590G/R591Q in PB2 of pH1N1 with the avian signature Glu627 could rescue the polymerase activity and viral replication (3).

The genome of influenza virus consists of eight ribonucleoprotein complexes that encapsidate eight viral genomic RNA segments. Like other negative-stranded RNA viruses, the viral RNA polymerase of influenza virus is always packaged in the infectious virion as a complex with the nucleoprotein (4–7). The RNA-dependent RNA polymerase of influenza virus is composed of PA, PB1, and PB2 subunits. The heterotrimeric polymerase is required for RNA transcription and replication that take place in the nucleus during influenza virus infection. Influenza virus accomplishes its transcription by using a cap-snatching mechanism (8). After the ribonucleoprotein complexes enter the nucleus, the 5′ cap of the host pre-mRNA in the nucleus is captured by the cap-binding domain of PB2 (PB2cap). The cap, together with 10–13 nucleotides downstream of the cap, is cleaved off by the N-terminal cap-dependent endonuclease of PA (9, 10). This 5′-capped oligonucleotide is then used as the primer for initiation of viral transcription by PB1. This

* This work was supported by the National High Technology and Development Program of China 973 Programs (Grant 2010CB911800), National Natural Science Foundation of China (Grant 30930020), International Centre for Genetic Engineering and Biotechnology (Project CRP/CHN09-01), and a major research project for infectious disease (Project 2013ZX10004611-003).

† This article contains supplemental Figs. S1–S4 and Table S1.

The atomic coordinates and structure factors (codes 4ENF, 4EQK, and 4ESS) have been deposited in the Protein Data Bank (http://wwpdb.org/).

1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed: School of Life Sciences, Peking University, Beijing 100871, China. Tel: 86-10-6275-5712; Fax: 86-10-6276-5913; E-mail: xiaofengz@pku.edu.cn.
New Substitution Site in Influenza Virus PB2

process is termed “cap snatching.” Residues 318–483 of PB2 are defined as the cap-binding domain that binds the cap of the host pre-mRNA (11). The C terminus of PB2 (amino acids 535–759), on the other hand, is involved in nucleoprotein binding and nuclear import (12, 13), whereas residues 1–37 at the N-terminal region were found to interact with PB1 (14).

The reported complex structure of PB2cap (A/Victoria/3/75[H3N2]) with bound cap analog m7GTP shows a 13 residue-containing pocket that is essential for cap binding (11). His357, Phe404, Glu431, and Lys376 in the pocket are essential for binding to the 7-methylated guanine base of m7GTP. The phosphate groups of m7GTP are fixed by Asn429, His432, Lys339, and Arg355, and the latter two residues form a positively charged surface on the pocket edge.

It has been reported that amino acid changes in PB2, such as T271A, K627E, and D701N, are related to adaptation of avian influenza virus to humans and virulence (15–25). In this study, we found that the residue at position 339 in the cap-binding pocket displays a significant and widespread amino acid polymorphism. Our structural comparison showed that PB2 Lys339 has a significant conformational change during cap binding. Surface charge analysis showed that substitution with Thr339 eliminates the interactions with the γ-phosphate group in m7GTP originally made by Lys339. Measurements of cap binding by isothermal titration calorimetry (ITC), overall polymerase activity, and specific RNA synthesis activity in mammalian cells confirmed that residue Thr339 attenuates the cap binding activity. Furthermore, a recombinant H5N1 virus containing Thr339 showed a lower virulence to mice but more active replication in MDCK cells.

MATERIALS AND METHODS

Cell Culture—Human A549 and human embryonic kidney (293T) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen), and MDCK cells were in minimum essential medium (Invitrogen), respectively, supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 10 mM HEPES, 100 units ml−1 penicillin, and 100 μg ml−1 streptomycin (Invitrogen). Chicken DF-1 cells were maintained in DMEM supplemented with 10% FBS at 39 °C.

Plasmid Construction—The cDNAs encoding amino acids 318–483 of PB2 were cloned from influenza A/Puerto Rico/8/34 (H1N1), influenza A/California/08/2009 (H1N1), influenza A/Hong Kong/1/68 (H3N2), and influenza A/bar-headed goose/Qinghai/15c/2005 (H5N1), respectively, followed by insertion into the pET28a vector (Novagen) by NdeI and Xhol sites. Eight gene segments of PR8-H1N1 and 2005-H5N1 were cloned into the bidirectional expression plasmid pHW2000 for rescuing the recombinant viruses as described previously (26). The cDNAs of PB1, PB2, PA, and NP from PR8-H1N1 were cloned into plasmid pCDNA3.1+ (Invitrogen), respectively. The plasmid pPOLI-NA-RT expressing the NA vRNA (A/WSN/33) was used as a template for RNA synthesis. Mutations of PB2-T339K and K627E in 2005-H5N1, PB2-K339T in PR8-H1N1, and PB2-K339T and R355T in 2009-pH1N1 were generated using a QuikChange™ site-directed mutagenesis Kit (Stratagene) and verified by DNA sequencing. The avian promoter-containing reporter plasmid poll-Gluc (Poll-Gluc) was constructed by replacing the human promoter of poll-Gluc with the synthetic avian promoter (27).

Protein Preparation—All the His tag fusion proteins were overexpressed and purified in Escherichia coli strain Rosetta (DE3). The expressions of the proteins were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (Sigma) at 18 °C for 16 h. After purification with a nickel affinity HiTrap chelating HP column (GE Healthcare), the proteins were digested with thrombin at room temperature for 0.5 h and then applied to a nickel affinity column again to remove the His tag and undigested proteins. The proteins were further purified by Superdex 75 gel filtration chromatography (GE Healthcare) and desalted in buffer containing 10 mM Tris-HCl, pH 8.0 and 200 mM NaCl with a HiTrap desalting column (GE Healthcare).

Crystallography—The purified proteins were concentrated to 10 mg ml−1 and incubated with 5 mM m7GTP (Sigma) on ice for 1 h. The initial crystallization screening for both native proteins and m7GTP-bound proteins from the four subtypes was performed by the sitting drop vapor diffusion method at 20 °C using kits including PEG-Ion Screen, Crystal Screen, Crystal Screen 2, Index, and Natrix (Hampton Research). Further crystal optimizations were carried out using the Additive Screen kit (Hampton Research). Finally, appropriate crystals that yielded better diffraction qualities were obtained by the hanging drop vapor diffusion method at 20 °C in the following conditions. Native PR8-H1N1 PB2cap was from 0.2 M KNO3, 0.1 M HEPES, pH 7.5, 20% (w/v) PEG 3350, 8% (v/v) 1,4-butanediol, 10 mg ml−1 protein; 1968-H3N2 PB2cap complex with m7GTP was from 0.2 M LiNO3, 0.1 M Tris-HCl, pH 8.0, 18% (w/v) PEG 3350, and 10 mg ml−1 protein; and 2005-H5N1 PB2cap with m7GTP was from 0.1 M (NH4)2SO4, 0.1 M MgCl2, 0.05 M MES, pH 5.6, 22% PEG 8000, and 6 mg ml−1 protein.

Data Collection and Structure Determination—X-ray diffraction data were collected on beamline BL17U at Shanghai Synchrotron Radiation Facility (China) with a wavelength of 0.9795 Å. The crystals of 2005-H5N1 PB2cap with m7GTP and native PR8-H1N1 PB2cap were flash cooled and maintained at 100 K in cooled nitrogen gas during data collection. In the case of 1968-H3N2 PB2cap with m7GTP, 20% (w/v) glycerol was used as the cryoprotectant. The data were processed with HKL2000 and solved by the molecular replacement method using the CCP4 suite with the x-ray structure of influenza A/Victoria/3/1975(H3N2) PB2cap (Protein Data Bank code 2VQZ) as a search model. The 424-loop of native PR8-H1N1 PB2cap was rebuilt by ARP/WARP. Structure refinements of the three structures were performed by Coot (28), REFMAC5 (29), and TLS motion (30). The detailed data collection statistics are presented in supplemental Table S1.

ITC Analysis—ITC measurements were performed at 20 °C with an ITC200 titration calorimeter (MicroCal Inc.). The wild-type and mutant PB2cap protein samples were purified as described above and dialyzed with buffer containing 150 mM NaCl and 10 mM HEPES, pH 7.4. 180–200 μM protein in the cell was titrated with 2 mM m7GTP in the syringe, and 1 μl was

3 The abbreviations used are: ITC, isothermal titration calorimetry; MDCK, Madin-Darby canine kidney; vRNA, viral RNA; Gluc, Gaussia luciferase; TCID50, median tissue culture infective dose; W, water.
injected followed by 29 injections of 2 μl. Data were fitted to a single binding site model and analyzed to obtain the parameter $K_a$ using the Origin 7.0 program.

**Polymerase Activity Assay**—Polymerase activity of the ribonucleoprotein complex was detected and quantified using *Gaussia* luciferase (Gluc) system as described previously (31). The reporter plasmid pGLuc-Gluc consisting of the Gluc ORF flanked by the noncoding regions of influenza NS segment was co-transfected in human A549 cells with PB1, PB2, PA, and NP that were cloned, respectively, into the bidirectional expression plasmid pHW2000. Cells transfected with the reporter plasmid pHW2000 empty vector were used as the negative control. The polymerase activity of 2005-H5N1 ribonucleoprotein complex was also detected in DF-1 cells at 39 °C. Gluc activity in supernatants was analyzed 24 h post-transfection using the BioLux *Gaussia* luciferase assay kit (New England Biolabs). All the experiments were performed in triplicate.

**Primer Extension Assays**—The recombinant plasmids pcDNA-PB2, pcDNA-PB1, pcDNA-NP, and pPOLI-NA-RT were co-transfected with pcDNA-PB2 containing Thr$^{339}$ or Lys$^{339}$ into human kidney 293T cells for RNA synthesis. The total RNA was isolated using TRizol reagent (Kangwei) at 12 and 24 h post-transfection. Two NA gene-specific primers (32) and a 55 rRNA primer (33) were labeled with $\gamma$-$32$P]ATP (PerkinElmer Life Sciences) for reverse transcription. The primer extension assays were performed at 42 °C for 1.5 h using the GoldScript cDNA synthesis kit (Invitrogen). The products were analyzed in 7% polyacrylamide gels containing 7M urea and detected by autoradiography. The lanes corresponding to vRNA, mRNA, cRNA, and 5 S rRNA were quantified with ImageQuant software (GE Healthcare).

**Generation of Recombinant Viruses and Virus Titration**—Recombinant 2005-H5N1 viruses containing Lys$^{339}$ and Thr$^{339}$ were generated by reverse genetics as described previously (26). pHW2000 plasmids containing eight influenza gene segments were co-transfected into a 293T/MDCK co-culture monolayer. The propagated viruses were ascertained by sequencing, and the polymerase activity of recombinant 2005-H5N1 viruses was assayed. The 13 residues were cloned, respectively, over the past decade (Fig. 1B). In 2003, Thr$^{339}$ was detected for the first time in avian and human-isolated H5N1. Lys$^{339}$ was changed to Thr in 57.3 and 48.9% isolates, respectively, over the past decade (Fig. 1A). When the occupancy of Lys and Thr was calculated for a total of 9246 sequences with unambiguous host annotation (Table 1). The 13 residues are Phe$^{323}$, Phe$^{325}$, Lys$^{339}$, Arg$^{355}$, His$^{357}$, Glu$^{361}$, Phe$^{363}$, Lys$^{376}$, Phe$^{404}$, Glu$^{406}$, Asn$^{429}$, Met$^{431}$, and His$^{375}$, and subtypes covered by the analysis include avian H9N2, avian H7N2, avian H5N1, human-isolated H5N1, human H1N1, and human H3N2. Most of the 13 residues were highly conserved except Lys$^{339}$ and Arg$^{355}$ showed polymorphisms in certain subtypes.

In H1N1, H3N2, H9N2, and influenza B virus, residue 339 is predominantly a Lys. However, this location frequently has a Gly (57.1%) in avian H7N2 (Table 1). The most noticeable variation at position 339 was found in avian and human-isolated H5N1. Lys$^{339}$ was changed to Thr in 57.3 and 48.9% isolates, respectively, over the past decade (Fig. 1A). When the occupancy of Lys and Thr was calculated for a total of 1017 sequences of avian H5N1, the rate of Lys$^{339}$ presence decreased, whereas that of Thr$^{339}$ increased from years 2003 to 2011 (Fig. 1B). In 2003, Thr$^{339}$ was detected for the first time in avian H5N1. Rapidly, the rate of Thr$^{339}$ reached 86.7% in the isolates of 2006. And from 2006 to 2011, Thr$^{339}$ stably remained at the rate of ~80% of the avian H5N1 isolates. Correlated changes were also observed in the human-isolated H5N1 sequences (Fig. 1C). A similar pattern was found in H7N2, another avian influenza A virus (supplemental Fig. S2A). In this case, Lys$^{339}$ was gradually replaced by Gly$^{339}$ from 2004 to 2007. Other amino acid substitutions were identified in minor cases.

Besides residue 339, an amino acid polymorphism at residue 355 was recognized in human H1N1 (Table 1). The PB2 sequences of H1N1 were first grouped into five periods: years 1918–1977, 1978–1991, 1995–1999, 2000–2008, and 2009–2011. During the first four periods, residue 355 was changed successively from Arg to Lys, Asp, or Thr (supplemental Fig. S2B). Between 2009 and 2011, the majority of the sequences in the database corresponded to pH1N1, and this position reverts to Arg$^{355}$ or Thr$^{339}$ at high frequency. This observation is consistent with the notion that PB2 of pH1N1 is derived from a relatively ancient avian-isolated H1N1 strain containing Arg$^{355}$ during the reassortment event (34–36).

**Statistical Analysis of Residues in the Cap-binding Pocket of PB2**—The previously reported structure of the PB2$^{cap}$ bound with m$^7$GTP shows that 13 key residues are involved in binding m$^7$GTP (11) (supplemental Fig. S1). We performed a statistical analysis of the 13 residues using a total of 9246 sequences with unambiguous host annotation (Table 1). The 13 residues are Phe$^{323}$, Phe$^{325}$, Lys$^{339}$, Arg$^{355}$, His$^{357}$, Glu$^{361}$, Phe$^{363}$, Lys$^{376}$, Phe$^{404}$, Glu$^{406}$, Asn$^{429}$, Met$^{431}$, and His$^{375}$, and subtypes covered by the analysis include avian H9N2, avian H7N2, avian H5N1, human-isolated H5N1, human H1N1, and human H3N2. Most of the 13 residues were highly conserved except Lys$^{339}$ and Arg$^{355}$ showed polymorphisms in certain subtypes.

In H1N1, H3N2, H9N2, and influenza B virus, residue 339 is predominantly a Lys. However, this location frequently has a Gly (57.1%) in avian H7N2 (Table 1). The most noticeable variation at position 339 was found in avian and human-isolated H5N1. Lys$^{339}$ was changed to Thr in 57.3 and 48.9% isolates, respectively, over the past decade (Fig. 1A). When the occupancy of Lys and Thr was calculated for a total of 1017 sequences of avian H5N1, the rate of Lys$^{339}$ presence decreased, whereas that of Thr$^{339}$ increased from years 2003 to 2011 (Fig. 1B). In 2003, Thr$^{339}$ was detected for the first time in avian H5N1. Rapidly, the rate of Thr$^{339}$ reached 86.7% in the isolates of 2006. And from 2006 to 2011, Thr$^{339}$ stably remained at the rate of ~80% of the avian H5N1 isolates. Correlated changes were also observed in the human-isolated H5N1 sequences (Fig. 1C). A similar pattern was found in H7N2, another avian influenza A virus (supplemental Fig. S2A). In this case, Lys$^{339}$ was gradually replaced by Gly$^{339}$ from 2004 to 2007. Other amino acid substitutions were identified in minor cases.

Besides residue 339, an amino acid polymorphism at residue 355 was recognized in human H1N1 (Table 1). The PB2 sequences of H1N1 were first grouped into five periods: years 1918–1977, 1978–1991, 1995–1999, 2000–2008, and 2009–2011. During the first four periods, residue 355 was changed successively from Arg to Lys, Asp, or Thr (supplemental Fig. S2B). Between 2009 and 2011, the majority of the sequences in the database corresponded to pH1N1, and this position reverts to Arg$^{355}$ or Thr$^{339}$ at high frequency. This observation is consistent with the notion that PB2 of pH1N1 is derived from a relatively ancient avian-isolated H1N1 strain containing Arg$^{355}$ during the reassortment event (34–36).

**Conformational Differences between the Apo- and m$^7$GTP-bound PB2 Cap-binding Domain in the Presence of Lys$^{339}$ or Thr$^{339}$**—To clarify the structural features of the cap-binding pocket, PB2$^{cap}$ from four subtypes of influenza A virus, PR8-
H1N1 (A/Puerto Rico/8/34, seasonal, Lys339), 2009-pH1N1 (A/California/08/09, pandemic, Lys339), 1968-H3N2 (A/Hong Kong/1/68, pandemic, Lys339), and avian 2005-H5N1 (A/bar-headed goose/Qinghai/15c/2005, pandemic, Thr 339), were crystallized in the absence and presence of m7GTP. Three crystal structures are reported here: the apoPR8-H1N1 PB2cap at 1.32-Å resolution (Fig. 2B), the 1968-H3N2 PB2 cap with m7GTP at 1.95-Å resolution (Fig. 2A), and the 2005-H5N1 PB2cap with m7GTP at 1.80-Å resolution (Fig. 3A). All the structures were solved by the molecular replacement method using the 2.3-Å x-ray structure of influenza A/Victoria/3/1975 (H3N2) PB2cap with m7GTP (Protein Data Bank code 2VQZ) as a search model (11). Data collection, phasing, and refinement statistics are listed in supplemental Table S1.

As reported previously (11), PB2cap has a highly conserved core of four H9251-helices partially enclosed by three H9252-sheets and a 16-residue loop (424-loop) (Fig. 2A and supplemental Fig. S1).

### Table 1

| Subtype                  | Amino acid frequencies |
|--------------------------|------------------------|
| Avian H9N2 (n = 244)     | Phe323                 |
|                          | Arg355                 |
|                          | His357                 |
|                          | Lys361                 |
|                          | Gln363                 |
|                          | Lys376                 |
|                          | Phe404                 |
|                          | Arg429                 |
|                          | Met431                 |
|                          | His432                 |
| Avian H7N2 (n = 154)     | Leu1                   |
|                          | Thr583                 |
|                          | Met14                  |
|                          | Gln4                  |
|                          | Arg4                  |
|                          | Asn2                  |
|                          | Ala1                  |
|                          | Val1                  |
| Avian H5N1 (n = 1017)    | Leu1                   |
|                          | Thr92                  |
|                          | Met4                  |
|                          | Arg2                  |
|                          | Lys28                  |
|                          | Gln5                  |
|                          | Arg3                  |
|                          | Asn1                  |
|                          | Ser1                  |
|                          | Tyr1                  |
| Human-isolated H5N1      | Leu1                   |
| (n = 188)                | Arg2                  |
|                          | Asn1                  |
|                          | Thr785                 |
|                          | Lys88                 |
|                          | Asn79                 |
|                          | Ile2                  |
|                          | Ser1                  |
|                          | Tyr1                  |
| Human H1N1 up to 2008    | Leu1                   |
| (n = 1018)               | Met2                  |
|                          | Asn2                  |
|                          | Thr234                 |
|                          | Ser1                  |
|                          | Ser2                  |
|                          | Tyr3                  |
| Human H1N1 since 2009    | Leu1                   |
| (n = 3503)               | Met2                  |
|                          | Asn2                  |
|                          | Thr234                 |
|                          | Ser1                  |
|                          | Ser2                  |
|                          | Tyr3                  |
| Human H5N2 (n = 366)     | Gln                     |
|                          | Lys                    |
|                          | Ser36                 |
|                          | Gly2                  |
|                          | Met1                  |
| Human B virus (n = 366)  | Leu1                   |
|                          | Phe                    |
|                          | Asn1                  |
|                          | Thr36                 |
|                          | Gly2                  |
|                          | Met1                  |
|                          | Phe                    |
|                          | Gln                    |
|                          | Ser                    |
|                          | Met                    |
|                          | Tyr                    |

| TABLE 1 (continued)      |

| Subtype                  | Amino acid frequencies |
|--------------------------|------------------------|
| Avian H9N2 (n = 244)     | Phe323                 |
|                          | Arg355                 |
|                          | His357                 |
|                          | Lys361                 |
|                          | Gln363                 |
|                          | Lys376                 |
|                          | Phe404                 |
|                          | Arg429                 |
|                          | Met431                 |
|                          | His432                 |
| Avian H7N2 (n = 154)     | Leu1                   |
|                          | Thr583                 |
|                          | Met14                  |
|                          | Gln4                  |
|                          | Arg4                  |
|                          | Asn2                  |
|                          | Ala1                  |
|                          | Val1                  |
| Avian H5N1 (n = 1017)    | Leu1                   |
|                          | Thr92                  |
|                          | Met4                  |
|                          | Arg2                  |
|                          | Lys28                  |
|                          | Gln5                  |
|                          | Arg3                  |
|                          | Asn1                  |
|                          | Ser1                  |
|                          | Tyr1                  |
| Human-isolated H5N1      | Leu1                   |
| (n = 188)                | Arg2                  |
|                          | Asn1                  |
|                          | Thr785                 |
|                          | Lys88                 |
|                          | Asn79                 |
|                          | Ile2                  |
|                          | Ser1                  |
|                          | Tyr1                  |
| Human H1N1 up to 2008    | Leu1                   |
| (n = 1018)               | Met2                  |
|                          | Asn2                  |
|                          | Thr234                 |
|                          | Ser1                  |
|                          | Ser2                  |
|                          | Tyr3                  |
| Human H1N1 since 2009    | Leu1                   |
| (n = 3503)               | Met2                  |
|                          | Asn2                  |
|                          | Thr234                 |
|                          | Ser1                  |
|                          | Ser2                  |
|                          | Tyr3                  |
| Human H5N2 (n = 366)     | Gln                     |
|                          | Lys                    |
|                          | Ser36                 |
|                          | Gly2                  |
|                          | Met1                  |
| Human B virus (n = 366)  | Leu1                   |
|                          | Phe                    |
|                          | Asn1                  |
|                          | Thr36                 |
|                          | Gly2                  |
|                          | Met1                  |
|                          | Phe                    |
|                          | Gln                    |
|                          | Ser                    |
|                          | Met                    |
|                          | Tyr                    |

* Consensus amino acids involved in cap binding are shown in three-letter amino acid codes with superscript position numbers.

* n = number of sequences in the group.

* An amino acid code followed by a number in parentheses indicates the number of sequences containing the variant amino acid in the corresponding position.
As represented by the structure of 1968-H3N2 PB2\textsubscript{cap} with m\textsuperscript{7}GTP, 13 highly conserved residues constitute the cap-binding pocket (Fig. 2D and supplemental Fig. S1). His\textsuperscript{357}, Phe\textsuperscript{404}, Glu\textsuperscript{361}, and Lys\textsuperscript{376} are essential for interactions with 7-methylguanine of m\textsuperscript{7}GTP. The \(\alpha\)- and \(\beta\)-phosphate groups are fixed by Asn\textsuperscript{429} (in 424-loop) and His\textsuperscript{432}. Meanwhile, the positively

FIGURE 2. Crystal structure of the PB2\textsubscript{cap} with and without m\textsuperscript{7}GTP. A, a ribbon diagram of the structure of 1968-H3N2 PB2\textsubscript{cap} in complex with m\textsuperscript{7}GTP. The overall structure is in bright orange with m\textsuperscript{7}GTP colored by element. The four \(\alpha\)-helices in the core are labeled and the prominent 424-loop is marked. B, a ribbon diagram of the structure of cap-free PR8-H1N1 PB2\textsubscript{cap} (dark blue). C, a structural alignment for the 424-loop from the structures in A and B. D, a structural comparison of the cap-binding pocket between 1968-H3N2 PB2\textsubscript{cap} and PR8-H1N1 PB2\textsubscript{cap}. Side chains are colored for 1968-H3N2 PB2\textsubscript{cap} and PR8-H1N1 PB2\textsubscript{cap}. Hydrogen bonds between m\textsuperscript{7}GTP and 1968-H3N2 PB2\textsubscript{cap} side chains are indicated by dotted lines. E, upon cap binding, the side chain of His\textsuperscript{357} makes room for m\textsuperscript{7}GTP by a 42° rotation, and Lys\textsuperscript{339} closes to interact with the \(\gamma\)-phosphate group and with the hydroxyl group OH(2) in the ribose. F, distribution of the water molecules in the cap-binding pocket of m\textsuperscript{7}GTP-bound 1968-H3N2 (bright orange) and that in cap-free PR8-H1N1 (dark blue). m\textsuperscript{7}GTP is shown as a line model with dotted surface contour, and water molecules are labeled as W1–W8. The structure models were prepared with PyMOL (50).

FIGURE 3. Crystal structure of 2005-H5N1 Thr\textsuperscript{339}-PB2\textsubscript{cap} with bound m\textsuperscript{7}GTP. A, molecule A in the crystallographic asymmetric unit of 2005-H5N1 PB2\textsubscript{cap} in complex with m\textsuperscript{7}GTP is shown as a gray ribbon diagram with m\textsuperscript{7}GTP as a stick model colored by element. B, a structural comparison of the cap-binding pocket between 1968-H3N2 PB2\textsubscript{cap} (bright orange) and 2005-H5N1 Thr\textsuperscript{339}-PB2\textsubscript{cap} (gray). Side chains and m\textsuperscript{7}GTP are colored by element, and hydrogen bonds between m\textsuperscript{7}GTP and 1968-H3N2 PB2\textsubscript{cap} side chains are indicated by dotted lines. In the other three molecules in the asymmetric unit, the \(\gamma\)-phosphate group assumes the same conformation as that of m\textsuperscript{7}GTP in 1968-H3N2 PB2\textsubscript{cap}. C, the calculated electrostatic surface of PB2\textsubscript{cap}. The electrostatic surface potentials were calculated with PyMOL (positive in blue and negative in red). The model of PB2\textsubscript{cap} with Thr\textsuperscript{339} was built from 1968-H3N2 PB2\textsubscript{cap} using PyMOL. m\textsuperscript{7}GTP is presented as a stick model.
charged Lys\textsuperscript{339} and Arg\textsuperscript{355} interact with the \(\gamma\)-phosphate group (Fig. 2D).

The apo form of PR8-H1N1 PB\(_{2}\text{cap}\) provides the structural details prior to host pre-mRNA binding (Fig. 2B). This is the only cap-free structure obtained so far. Six water molecules (W1–W6) occupy the location where m\(^7\)GTP would have bound, and two water molecules (W7 and W8) are still buried among Glu\textsuperscript{361}, Lys\textsuperscript{376}, and Gln\textsuperscript{406} as reported previously in the structure of PB\(_{2}\text{cap}\) with m\(^7\)GTP (Fig. 2F) (11). In the cap-binding pocket, the conformation of the 424-loop and residues Lys\textsuperscript{339} and His\textsuperscript{357} change dramatically upon cap binding (Fig. 2, C and E). Before binding the cap, the 424-loop is unstable and shows a more opened conformation due to the absence of interactions between m\(^7\)GTP and Asn\textsuperscript{429} in comparison with the cap-bound structure. The side chain of His\textsuperscript{357} has a tilt of 42° that keeps the 7-methylguanine binding site closed. Lys\textsuperscript{339} points outward due to the absence of interactions with the \(\gamma\)-phosphate group of m\(^7\)GTP. On the contrary, the side chains of Glu\textsuperscript{361}, Lys\textsuperscript{376}, and Phe\textsuperscript{404} are unchanged. The side chains of Tyr\textsuperscript{360} and Glu\textsuperscript{362} also show conformational changes, although they are not directly involved in cap binding.

In 2005-H5N1 PB\(_{2}\text{cap}\), the residue at position 339 is Thr, which replaced the positively charged basic residue Lys\textsuperscript{339} that interacts with the \(\gamma\)-phosphate group found in other PB\(_{2}\)s (Fig. 3, A and B). The side chain of Thr is shorter than that of Lys and uncharged, resulting in disorder of the \(\gamma\)-phosphate group. The positively charged electrostatic surface formed by Lys\textsuperscript{339} and Arg\textsuperscript{355} is also disrupted by Thr\textsuperscript{339} (Fig. 3C).

In the structure of 2005-H5N1 PB\(_{2}\text{cap}\) with m\(^7\)GTP, two conformational forms of m\(^7\)GTP are present in four independent molecules in the crystallographic asymmetric unit. In molecule A, the \(\gamma\)-phosphate group of m\(^7\)GTP is rotated by 90° and interacts with residue Asn\textsuperscript{429} with which \(\alpha\)– and \(\beta\)-phosphate groups also interact (Fig. 3, A and B). In the other three molecules, the \(\gamma\)-phosphate groups assume the same conformation as that of m\(^7\)GTP bound in 1968-H3N2 PB\(_{2}\text{cap}\) that has Lys\textsuperscript{339}.

A structural model of 1968-H3N2 PB\(_{2}\text{cap}\) with Thr\textsuperscript{355} was built from the structure of 1968-H3N2 PB\(_{2}\text{cap}\) with m\(^7\)GTP.

**FIGURE 4. Comparisons of the cap binding capacity.** m\(^7\)GTP binding by different PB\(_{2}\text{cap}\) subtypes was examined by ITC analysis. For every titration figure, the raw data are shown in the top and the integrated data shown with continuous lines representing the fit of the data to a one-site binding model are in the bottom.

The association constant (\(K_a\)) values of Lys\textsuperscript{339} or Thr\textsuperscript{339}–PB\(_{2}\text{cap}\) from different subtypes for binding m\(^7\)GTP are compared. The bars in the histogram represent the range of the data (the maximum minus the minimum).
Similar to the disruption by Thr\textsuperscript{339} in 2005-H5N1 PB\textsubscript{2}\text{cap}, the positively charged surface formed by Lys\textsuperscript{339} and Arg\textsuperscript{355} is disrupted by Thr\textsuperscript{355}. Interestingly, when Arg is gradually replaced by Lys, Asn, and Thr at position 355, the side chain becomes shorter and lesser charged (supplementary Fig. S2B). It is worth mentioning that in influenza B virus a conserved residue equivalent to residue 355 in PB\textsubscript{2} of influenza A virus is Gly\textsuperscript{357}, which has no side chain (Table 1). Similar to replacing Lys\textsuperscript{339} by Thr\textsuperscript{339} in 2005-H5N1 PB\textsubscript{2}\text{cap}, substitutions of Arg\textsuperscript{355} by other residues may reduce interactions with the \(\gamma\)-phosphate group of m\textsuperscript{7}GTP.

Thr\textsuperscript{339}-PB\textsubscript{2}\text{cap} Has a Reduced Cap Binding Affinity in Comparison with Lys\textsuperscript{339}-PB\textsubscript{2}\text{cap}—The structural analyses suggest that the change from Lys to Thr at position 339 weakens interactions with the cap by PB\textsubscript{2}. To investigate whether the change indeed has an effect on the cap binding activity, the m\textsuperscript{7}GTP binding activity by Thr\textsuperscript{339}-PB\textsubscript{2}\text{cap} and Lys\textsuperscript{339}-PB\textsubscript{2}\text{cap} as represented by 2005-H5N1, PR8-H1N1, and 2009-pH1N1, respectively, was measured by ITC assays at 20 °C at pH 7.4. The results showed that the change of Lys\textsuperscript{339} to Thr reduced the binding affinity to m\textsuperscript{7}GTP. The derived association constant (\(K_a\)) of m\textsuperscript{7}GTP to Lys\textsuperscript{339}-PB\textsubscript{2}\text{cap} is at least 2-fold higher than that to Thr\textsuperscript{355}-PB\textsubscript{2}\text{cap} (Fig. 4). These observations confirmed that the polymorphism at position 339 correlates with the PB\textsubscript{2} cap binding affinity and that Thr\textsuperscript{339} reduces the in vitro m\textsuperscript{7}GTP binding affinity of PB\textsubscript{2}\text{cap}.

The binding affinity of Arg\textsuperscript{355}-PB\textsubscript{2}\text{cap} to m\textsuperscript{7}GTP was also compared with Thr\textsuperscript{355}-PB\textsubscript{2}\text{cap} within the human H1N1 subtype. As expected, the results showed that Arg\textsuperscript{355}-PB\textsubscript{2}\text{cap} also has a 2-fold higher m\textsuperscript{7}GTP binding affinity than Thr\textsuperscript{355}-PB\textsubscript{2}\text{cap} (supplemental Fig. S3).

Thr\textsuperscript{339}-PB\textsubscript{2}\text{cap} Shows a Reduced Polymerase Activity and RNA Synthesis Activity in Comparison with Lys\textsuperscript{339}-PB\textsubscript{2}\text{cap} in Mammalian Cells—The H5N1 virus A/bar-headed goose/Qinghai/15c/2005 is the first highly pathogenic avian influenza virus isolated from migratory waterbird in Qinghai Lake, West China in 2005. This strain carries Thr at PB\textsubscript{2} position 339 and a mammalian adaptive marker of Lys at PB\textsubscript{2} position 627. An A/bar-headed goose/Qinghai/15c/2005-like virus caused an outbreak in aquatic birds initiated in 2005 and then was transmitted into Africa, resulting in large scale outbreaks in poultry in Nigeria. As a species-specific signature, Glu\textsuperscript{627} generally predominates in avian viruses, whereas Lys\textsuperscript{627} predominates in human viruses, and the avian signature Glu\textsuperscript{627} in PB\textsubscript{2}attenuates polymerase activity (37, 38). However, the 2009 pandemic H1N1 isolates and approximately two-thirds of the human-isolated H5N1 viruses contain the avian-like Glu\textsuperscript{627} (3, 39).

The polymerase activity of both wild-type Thr\textsuperscript{339}-PB\textsubscript{2} and mutant Lys\textsuperscript{339}-PB\textsubscript{2} from A/bar-headed goose/Qinghai/15c/2005 (H5N1) was quantitated using a Gluc system in human A549 cells (31). With either wild-type Lys\textsuperscript{627} or avian-like Glu\textsuperscript{627} in PB\textsubscript{2}, the mutant Thr\textsuperscript{339}-PB\textsubscript{2} reduced the polymerase activity by 65% (Fig. 5B). These results
demonstrate that replacement of Lys by Thr at position 339 indeed decreases the polymerase activity of influenza virus. We also determined the polymerase activity of 2005-H5N1 in DF-1 cells. Unexpectedly, with either Lys627 or Glu627, the polymerase activity of Thr339-PB2 was more active than that of the Lys339 mutant (supplemental Fig. S4).

Primer extension assays were performed at 12 and 24 h post-transfection in 293T cells to demonstrate whether K339T in PB2 influences viral transcription (Fig. 5, C and D). Quantification of mRNA over vRNA showed that replacement of Lys339 by Thr in PR8-H1N1 decreased the mRNA level at both time points, which is consistent with the result of polymerase activity analysis.

Thr339-virus Has a Reduced Mortality in Mice but More Active Replication in MDCK Cells—To determine the contribution of the polymorphism at amino acid 339 to viral virulence in mammals, recombinant 2005-H5N1 viruses containing Thr339 or Lys339 were generated by reverse genetics as described previously (26). Six-week-old female BALB/c mice were anesthetized with isoflurane and intranasally inoculated with 25 μl of PBS containing 10-fold serial dilutions of recombinant viruses (1 × 10^2, 1 × 10^3, and 1 × 10^4 TCID₅₀) using four mice per dilution. The negative control was infected with 25 μl of PBS. The mortality was monitored continuously for 14 days. The survival rate of the group of mice inoculated with wild-type Thr339-containing virus is significantly higher compared with the Lys339-virus-infected group especially with lower virus titers in initial inoculums (Fig. 6A). This result indicates that wild-type Thr339-containing virus is less virulent and induces a lower mortality in mice.

We monitored the growth kinetics of the recombinant 2005-H5N1 viruses containing Thr339 or Lys339 in MDCK cells for 48 h. The result showed that wild-type Thr339-virus replicated more actively than Lys339-virus in MDCK cells especially after 36 h (Fig. 6B).

DISCUSSION

Both natural selection and human intervention can cause mutation in influenza A virus, and an influenza pandemic usually occurs when new virus signatures emerge (3, 39). Changes in influenza virus polymerase will alter the polymerase activity and influence viral fitness in certain hosts. Species-specific res-
idue 627 in PB2 has been studied extensively and was shown to be related to the polymerase activity, virus replication, transmission, and mortality in mammals. More importantly, the E627K mutation has been frequently observed in avian H5N1 viruses crossing the species barrier and causing human death (37, 39). Recent studies found that substituting Glu627 in PB2 with Lys changes its interaction with importin-a1 and importin-a7 as well as the viral nucleoprotein in a species-specific manner (13, 18, 40–42). Other host range determinants in PB2 such as amino acids at positions 271 (23) and 701 (24, 25) are also hypothesized to relieve the restriction and recover the polymerase activity by interacting with cellular factors in new host cells (43).

Here we analyzed the amino acid frequencies by using the 9246 full-length PB2 sequences annotated with human and avian hosts from the NCBI Influenza Virus Resource database. The frequency of the PB2 sequence with Lys or Thr at the 339 position showed a significant trend in isolates from years 2004 to 2007 that have a similar sample size per year. The increase of Thr339-PB2-containing H5N1 virus could have been strengthened.

The parallel increase of Thr339-PB2-containing H5N1 virus isolated from human infections suggests that the K339T substitution in PB2 does not reduce the probability of human infection by avian H5N1 virus. However, different from the observation in human A549 cells, Thr339 polymerase showed an increased activity in avian DF-1 cells (Fig. 5 and supplemental Fig. S4). Whether some host factors function together with the residue at 339 in PB2 remains to be examined.

The residue at 339 in PB2, which is involved in cap snatching, is directly related to transcription. Here we showed that Thr339 in PB2 reduced mRNA and protein synthesis in mammalian cells. Unexpectedly, Thr339-H5N1 virus showed more efficient replication than Lys339-virus in MDCK cells. How this residue influences viral replication!s also need to be investigated further.

The relative levels of different viral functional groups need to be balanced for optimal fitness in corresponding hosts. On the premise of survival and reproduction, parasites tend to be less virulent to the host for long term coexistence (44, 45). For example, T271A in PB2 that was believed to contribute to the 2009 H1N1 influenza pandemic has been shown to enhance viral replication in mammalian cells but does not significantly increase viral pathogenicity in mice (23). The retained substitution K339T in PB2 reduces mRNA and protein synthesis in mammalian cells but does not significantly increase viral pathogenicity in mice (23). The retained substitution K339T in PB2 has made the influenza A virus evolve toward lower mortality and more active replication, which actually could benefit viral fitness. In contrast, virulence-enhancing mutations usually show low popularity, for example I504V and H357N in PB2, the latter of which is located in the PB2 cap-binding pocket (21, 46).

Previous reports show that Lys355 and Arg355 in PB2 are correlated with high pathogenicity in mice, and Gln355 is correlated with low pathogenicity (47–49). Here we found that the residue at position 355 in H1N1 PB2 also changed from the basic residue arginine to threonine (supplemental Fig. S2B). Our ITC assays showed that the cap binding activity of R355T also decreased similar to that of K339T (supplemental Fig. S3). This observation is supported by the structure model that showed a similar reduction of the positive charge in the K339T change (Fig. 3C). By analogy, Gly339 in H7N2 PB2 and Gly357 in influenza B virus may affect the polymerase activity through the same mechanism. These amino acid changes that occurred in PB2 may also be advantageous to the fitness of viruses in their respective hosts.

In conclusion, we identified that substitution of Lys339 by Thr attenuates the cap binding affinity, polymerase activity, and mRNA production level in mammalian cells and leads to a lower mortality rate in mice but increases the virus replication. The polymorphism at this position was unveiled with viruses that were isolated from both avian and human hosts without host bias in our statistics. The retained K339T substitution suggests that influenza virus might fit better with this substitution, and K339T substitution could be a new strategy utilized by influenza virus to increase its viral fitness in different hosts.

Acknowledgments—We thank Drs. Robert Webster and Henju Marjuki at St. Jude’s Children’s Research Hospital and Dr. Yuqing Liang at Emory University for stimulating discussions; Dr. Ervin Fodor at University of Oxford and Yongmei Qin at Peking University for providing the plasmids pPOLI-NA-R7 and pCDNA3.1+; and Dr. Xuemei Li for helping with primer extension assay. We also thank the staff at beamline BL17U of the Shanghai Synchrotron Radiation facility and beamline 1W2B of the Beijing Synchrotron Radiation Facility for assistance with data collection.

REFERENCES

1. Imai, M., Watanabe, T., Hatta, M., Das, S. C., Ozawa, M., Shinya, K., Zhong, G., Hanson, A., Katsura, H., Watanabe, S., Li, C., Kawakami, E., Yamada, S., Kiso, M., Suzuki, Y., Maher, E. A., Neumann, G., and Kawaoka, Y. (2012) Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature 486, 420–428

2. Herfst, S., Schrauwen, E. J., Linster, M., Chutinimitkul, S., de Wit, E., Munster, V. J., Sorrell, E. M., Bestebroer, T. M., Burke, D. F., Smith, D. J., Rimmelzwaan, G. F., Osterhaus, A. D., and Fouchier, R. A. (2012) Airborne transmission of influenza A/H5N1 virus between ferrets. Science 336, 1534–1541

3. Mehle, A., and Doudna, J. A. (2009) Adaptive strategies of the influenza virus polymerase for replication in humans. Proc. Natl. Acad. Sci. U.S.A. 106, 21312–21316

4. Noda, T., Sagara, H., Yen, A., Takada, A., Kida, H., Cheng, R. H., and Kawaoka, Y. (2006) Architecture of ribonucleoprotein complexes in influenza A virus particles. Nature 439, 490–492

5. Coloma, R., Valpuesta, J. M., Arranz, R., Carrascosa, J. L., Ortín, J., and Martin-Benito, J. (2009) The structure of a biologically active influenza virus ribonucleoprotein complex. PLoS Pathog. 5, e1000491

6. Ng, A. K., Zhang, H., Tan, K., Li, Z., Liu, J. H., Chan, P. K., Li, S. M., Chan, W. Y., Au, S. W., Joachimiak, A., Walz, T., Wang, J. H., and Shaw, P. C. (2008) Structure of the influenza virus A H5N1 nucleoprotein: implications for RNA binding, oligomerization, and vaccine design. FASEB J. 22, 3638–3647

7. Ye, Q., Krug, R. M., and Tao, Y. J. (2006) The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA. Nature 444, 1078–1082

8. Plutchnick, S. J., Boulou, M., Ulanman, I., and Krug, R. M. (1981) A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell 23, 847–858

9. Dias, A., Bouvier, D., Crépin, T., McCarthy, A. A., Hart, D. J., Baudin, F., Cusack, S., and Ruigrok, R. W. (2009) The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature 458, 914–918
New Substitution Site in Influenza Virus PB2

10. Yuan, P., Bartlam, M., Lou, Z., Chen, S., Zhou, J., He, X., Lv, Z., Ge, R., Li, X., Deng, T., Fodor, E., Rao, Z., and Liu, Y. (2009) Crystal structure of an avian influenza polymerase PA(N) reveals an endonuclease active site. Nature 458, 909–913

11. Guilligay, D., Tarendeau, F., Resa-Infante, P., Coloma, R., Crepin, T., Sehr, P., Lewis, J., Ruigrok, R. W., Ortiz, J., Hart, D. J., and Cusack, S. (2008) The structural basis for cap binding by influenza virus polymerase subunit PB2. Nat. Struct. Mol. Biol. 15, 500–506

12. Tarendeau, F., Boudet, J., Guilligay, D., Mas, P. J., Bougault, C. M., Boulo, S., Baudin, F., Ruigrok, R. W., Daigle, N., Ellenberg, J., Cusack, S., Simorre, J. P., and Hart, D. J. (2007) Structure and nuclear import function of the C-terminal domain of influenza virus polymerase PB2 subunit. Nat. Struct. Mol. Biol. 14, 229–233

13. Ng, A. K., Chan, W. H., Choi, S. T., Lam, M. K., Lau, K. F., Chan, P. K., Au, S. W., Fodor, E., and Shaw, P. C. (2012) Influenza polymerase activity correlates with the strength of interaction between nucleoprotein and PB2 through the host-specific residue KE627. PLoS One 7, e36145

14. Sugiyama, K., Obayashi, E., Kawaguchi, A., Suzuki, Y., Tame, J. R., Nagata, K., and Park, S. Y. (2009) Structural insight into the essential PB1–PB2 subunit contact of the influenza virus RNA polymerase: EMBO J. 28, 1803–1811

15. Bogs, J., Kalhoff, D., Veits, J., Pavlova, S., Schweemmle, M., Mänz, B., Mettenleiter, T. C., and Stech, J. (2011) Reversion of PB2–627E to -627K enhances transcription by enhancing cap-1 RNA binding activity. Acta Virol. 55, 1069–1069

16. Liu, Q., Qiao, C., Marjuki, H., Bawa, B., Ma, J., Guillossou, S., Webby, R. J., Richt, J. A., and 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

17. Zhang, S., Wang, Q., Wang, J., Mizumoto, K., and Toyoda, T. (2012) Two mutations in the C-terminal domain of influenza virus RNA polymerase PB2 enhance transcription by enhancing cap-1 RNA binding activity. Biochim. Biophys. Acta 1819, 78–83

18. Hudjietz, B., and Gabriel, G. (2012) Human-like PB2 627K influenza virus polymerase activity is regulated by importin-α1 and -α7. PLoS Pathog. 8, e1002488

19. Schat, K. A., Bingham, J., Butler, J. M., Chen, L. M., Lowther, S., Crowley, J. S., Baudin, F., Ruigrok, R. W., Daigle, N., Ellenberg, J., Cusack, S., Simorre, J. P., and Hart, D. J. (2011) Reversion of PB2–627E to -627K improves transcription of influenza virus RNA polymerase play a critical role in protein stability, endonuclease activity, cap binding, and virion RNA promoter binding. J. Virol. 86, 2126–2132

20. J. Virol. 80, 779–7798

21. Vijaykrishna, D., Poorn, L. L., Zhu, H. C., Ma, S. K., Li, O. T., Cheung, C. L., Smith, G. J., Peiris, J. S., and Guan, Y. (2010) Reassortment of pandemic H1N1/2009 influenza A virus in swine. Science 328, 1529

22. Smith, G. J., Vijaykrishna, D., Bahl, J., Lyncott, S. J., Worobey, M., Pybus, O. G., Ma, S. K., Cheung, C. L., Raghvani, J., Bhatt, S., Peiris, J. S., Guan, Y., and Rambaut, A. (2009) Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A pandemic. Nature 459, 1122–1125

23. Garten, R. J., Davis, C. T., Russell, C. A., Shu, B., Lindstrom, S., Balish, A., Sessions, W. M., Xu, X., Skepner, E., Deyde, V., Okomo-Adhiambo, M., Gubareva, L., Barnes, J., Smith, C. B., Emery, S. L., Hillman, M. J., Rivaillet, P., Smagala, J., de Graaf, M., Burke, D. F., Foucher, R. A., Pappas, C., Alpuerce-Aranda, C. M., López-Gatell, H., Olivera, H., López, I., Myers, C. A., Faix, D., Blair, P. J., Yu, C., Keene, K. M., Dotson, P. D., Jr., Boxrud, D., Sambol, A. R., Abid, S. H., St George, K., Bannerman, T., Moore, A. L., Stringer, D. J., Blevins, P., Demollier-Harrison, G. J., Ginsberg, M., Kriner, P., Waterman, S., Smole, S., Guevara, H. F., Belongia, E. A., Clark, P. A., Beatrice, S. T., Donis, R., Katz, J., Finelli, L., Bridges, C. B., Shaw, M., Jennigan, D. B., Uyeki, T. M., Smith, D. J., Klimov, A. I., and Cox, N. J. (2009) Antigenic and genetic characteristics of swine-origin 2009 A/H1N1 influenza viruses circulating in humans. Science 325, 197–201

24. Subbarao, E. K., London, W., and Murphy, B. R. (1993) A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J. Virol. 67, 1761–1764

25. Chen, G. W., Chang, S. C., Mok, C. K., Lo, Y. L., Kung, Y. N., Huang, J. H., Shih, Y. H., Wang, J. Y., Chiang, C. C., and Shih, S. R. (2006) Genomic signatures of human versus avian influenza A viruses. Emerg. Infect. Dis. 12, 1353–1360

26. Hatta, M., Gao, P., Hallmann, P., and Kawaoka, Y. (2001) Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 293, 1840–1842

27. Gabriel, G., Herwig, A., and Klenk, H. D. (2008) Interaction of polymerase subunit PB2 and NP with importin-α1 is a determinant of host range of influenza A virus. PLoS Pathog. 4, e11

28. Gabriel, G., Klenge, K., Ortega, A., Thiele, S., Hudjietz, B., Arman-Kalceck, G., Sauter, M., Shimidt, T., Rother, F., Baumgarte, S., Keiner, B., Hartmann, E., Bader, M., Brownlee, G. G., Fodor, E., and Klenk, H. D. (2011) Differential use of importin-α isoforms governs cell tropism and host adaptation of influenza virus. Nat. Commun. 2, 156

29. Foeglein, Á., Loucaides, E. M., Mura, M., Wise, H. M., Barclay, W. S., and Digard, P. (2011) Influence of PB2 host-range determinants on the in

from eight plasmids. Proc. Natl. Acad. Sci. U.S.A. 97, 6108–6113
tranuclear mobility of the influenza A virus polymerase. *J. Gen. Virol.* **92**, 1650–1661

44. Reperant, L. A., Kuiken, T., Grenfell, B. T., Osterhaus, A. D., and Dobson, A. P. (2012) Linking influenza virus tissue tropism to population-level reproductive fitness. *PLoS One* **7**, e43115

45. Alizon, S., Hurford, A., Mideo, N., and Van Baalen, M. (2009) Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. *J. Evol. Biol.* **22**, 245–259

46. Rolling, T., Koerner, I., Zimmermann, P., Holz, K., Haller, O., Staeheli, P., and 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

47. Katz, J. M., Lu, X., Tumpey, T. M., Smith, C. B., Shaw, M. W., and Subbarao, K. (2000) Molecular correlates of influenza A H5N1 virus pathogenesis in mice. *J. Virol.* **74**, 10807–10810

48. Chen, H., Bright, R. A., Subbarao, K., Smith, C., Cox, N. J., Katz, J. M., and Matsuoka, Y. (2007) Polygenic virulence factors involved in pathogenesis of 1997 Hong Kong H5N1 influenza viruses in mice. *Virus Res.* **128**, 159–163

49. Lee, M. S., Deng, M. C., Lin, Y. J., Chang, C. Y., Shieh, H. K., Shiau, J. Z., and Huang, C. C. (2007) Characterization of an H5N1 avian influenza virus from Taiwan. *Vet. Microbiol.* **124**, 193–201

50. DeLano, W. L. (2010) *The PyMOL Molecular Graphics System*, Version 1.3, Schrödinger, LLC, New York