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Synergistic Adaptive Mutations in the Hemagglutinin and Polymerase Acidic Protein Lead to Increased Virulence of Pandemic 2009 H1N1 Influenza A Virus in Mice

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Abstract

Influenza impressively reflects the paradigm of a viral disease in which continued evolution of the virus is of paramount importance for annual epidemics and occasional pandemics in humans. Because of the continuous threat of novel influenza outbreaks, it is essential to gather further knowledge about viral pathogenicity determinants. Here, we explored the adaptive potential of the influenza A virus subtype H1N1 variant isolate A/Hamburg/04/09 (HH/04) by sequential passaging in mice lungs. Three passages in mice lungs were sufficient to dramatically enhance pathogenicity of HH/04. Sequence analysis identified 4 nonsynonymous mutations in the third passage virus. Using reverse genetics, 3 synergistically acting mutations were defined as pathogenicity determinants, comprising 2 mutations in the hemagglutinin (HA[D222G] and HA[K163E]), whereby the HA(D222G) mutation was shown to determine receptor binding specificity and the polymerase acidic (PA) protein F35L mutation increasing polymerase activity. In conclusion, synergistic action of all 3 mutations results in a mice lethal pandemic H1N1 virus.

The first pandemic of the 21st century arose in 2009 with the outbreak of the pandemic influenza A virus (IAV) subtype H1N1 variant (H1N1v) virus. Although the majority of infected people experienced mild symptoms, there were numerous reports of severe cases. It is thus mandatory to improve our understanding of factors that could contribute to the emergence of more virulent pandemic H1N1v strains in the future.

Several studies that investigated the pathogenicity of different 2009 H1N1v isolates in ferrets, mice, and nonhuman primates revealed more effective virus propagation than did seasonal H1N1 strains [1–3]. However, there are also reports showing that the 2009 H1N1v strains are not highly pathogenic for mice [4–6]. Belser and colleagues [4] demonstrated that H1N1v viruses exhibit mild to moderate virulence in mice compared with pathogenic strains like the 1918 H1N1 influenza virus. Sequence analysis of the 2009 pandemic virus isolates unraveled the lack of known determinants of high pathogenicity, such as a polybasic cleavage site in the hemagglutinin (HA) or a lysine at position 627 of the polymerase basic 2 (PB2) protein (reviewed in [7, 8]). Even an introduction of some of these pathogenicity determinants, such as E627K and D701N in the PB2 protein, change of the premature stop codon in the polymerase basic 1 (PB1)–F2 protein, introduction of a K217E substitution in the

nonstructural 1 (NS1) protein, or an 11–amino acid C-terminal truncation of the NS1 protein, had, if any, only minimal impact on virulence [5, 9–11].

On the basis of these results, we wanted to know whether the pathogenicity of a 2009 H1N1v isolate could be enhanced during passaging of the virus in a mammalian host system. The assumption that lung-to-lung passaging would increase pathogenicity was driven by findings of several previous reports [12–14]. Numerous studies have identified mutations within the HA [15–18] and the polymerase subunits as mediators of increased pathogenicity upon passaging in mice [6, 16, 18–20]. Here we provide evidence that 3 lung-to-lung passages of the IAV strain A/Hamburg/04/09 (HH/04) in mice were sufficient to strongly increase viral pathogenicity due to 4 nonsynonymous adaptive mutations, located within the gene segments coding for the polymerase acidic (PA) subunit (F35L) of the polymerase complex, the HA(K163E and D222G), and the neuraminidase (NA)(T32I). Using reverse genetics, we demonstrated that a single amino acid change within the PA(F35L) enhanced virulence in mice and concomitantly increased the viral polymerase activity. The sole introduction of the D222G point mutation within the HA was sufficient to increase pathogenicity in mice as well, although additional introduction of the HA(K163E) mutation further increased virulence.

Taken together, our data demonstrate that only 3 passages in mice were sufficient to alter a low-pathogenic H1N1v virus to a highly mouse-pathogenic variant with higher replication capability, which was due to the acquisition of only 3 synergistically acting point mutations. These data clearly confirm the potential risk for the emergence of highly pathogenic human H1N1v strains.

Materials and Methods

Cells, Viruses, and Infection Conditions

A/Hamburg/04/2009 virus was obtained from the German National Reference Centre for Influenza (Brunhilde Schweiger, RKI). The virus was isolated from clinical specimens, and virus strains were passaged on Madin-Darby canine kidney (MDCK) cells. The infection procedure was performed as described elsewhere [21]. The mouse-adapted A/Hamburg/04/2009 (HH/04-3rd) was obtained upon passaging 3 times in mice lungs. MDCK cells were cultured in minimal essential medium (MEM; PAA Laboratories), whereas the human lung epithelial cell line A549 was cultivated in Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories). The porcine kidney epithelial (PK-15) cell line was cultured in MEM with 0.1 mmol/L nonessential amino acids (Sigma-Aldrich) and 1.0 mmol/L sodium pyruvate (PAA Laboratories), and the chicken embryonic fibroblast (DF-1) cells were cultured in DMEM with 0.1 mmol/L nonessential amino acids and 1.0 mmol/L sodium pyruvate. Cell-culture media were supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 100 U/mL penicillin (PAA Laboratories), and 0.1 mg/mL streptomycin (PAA Laboratories).

Generation of Recombinant Influenza Viruses

A set of 8 plasmids based on the bidirectional pHW2000 plasmid [22] was prepared for the influenza A/Hamburg/04/2009 virus by standard methods, allowing the rescue of recombinant wild-type (wt) and mutant viruses. Mutations in the gene segments were introduced by site-directed mutagenesis using the primers HA(K163E)(fwd:5′-caaagctcagcgaatcctacattaatg-3′), HA(K163E)(rev:5′-cattaatgtaggattcgctgagctttg-3′), HA(D222G)(fwd:5′-acccaaagtgaggggtcaagaagggagaa-3′), HA(D222G)(rev:5′-ttctcccttcttgacccctcactttgggt-3′), PA(F35L)(fwd:5′-aatggaacaacaagtgcctg-3′), and PA(F35L)(rev:5′-tgtgcatattgcagccaacttgttagtttcg-3′), NA(T32I)(fwd:5′-gaaacataatctcaatatggattagccactc-3′), NA(T32I)(rev:5′-gagtggctaatccatatagagattatgtttc-3′). Mutations in the PA gene segment of the SC35M virus were introduced by using the primers PA(F35L)(fwd:5′-gagacaaataaattggctgcaatatgcac-3′) and PA(F35L)(rev:5′-gtgcatattgcagccaatttatttgtctc-3′). The generation of the recombinant viruses was carried out as described elsewhere [21], and the presence and propriety of the desired mutations were confirmed by sequencing.

Whole Genome Sequence Analysis

Total RNA was isolated from MDCK cells (6 hours; multiplicity of infection, 5) by using an RNeasy-Mini-Kit (Qiagen) according to the manufacturer's protocol. Transcription of the complementary DNA (cDNA) was performed using RevertAid H-Minus First Strand cDNA Synthesis Kit (Fermentas) and primers specific for all influenza virus gene segments (5′-agcaaaagcagg-3′) according to the manufacturer's protocol. Whole genome sequencing was performed by the Institute of Hygiene, University hospital Muenster, Germany.

Plaque Titration

Supernatants of infected cells were collected at the time points indicated postinfection and used to assess the number of infectious particles (plaque titers) in the samples. Briefly, MDCK cells grown to 90% confluence in 6-well dishes were washed with phosphate-buffered saline (PBS; PAA Laboratories) and infected with serial dilutions of the collected supernatants in PBS/bovine albumin for 30 minutes at 37°C. The inoculum was aspirated, and cells were supplemented with 2 mL MEM/BA (medium containing 0.2% bovine serum albumin [Roth], 1 mmol/L $MgCl_2$ [Roth], 0.9 mmol/L $CaCl_2$ [Roth] and 100 U/mL penicillin, and 0.1 mg/mL streptomycin) containing 0.6% Agar (Oxoid), 0.3% DEAE-Dextran (Pharmacia Biotech), and 1.5% $NaHCO_3$ (Invitrogen); and incubated at 37°C, 5% CO_2, for 2–3 days.

Western Blot Assays

For Western blot analysis, cells were lysed in radioimmunoprecipitation assay buffer (25 mmol/L Tris-HCl [pH 8; Roth], 137 mmol/L NaCl [Roth], 10% glycerol [Roth], 0.1% sodium dodecylsulfate [Roth], 0.5% sodium deoxycholate [Roth], 1% octylphenoxypolyethoxyethanol [IGEPAL; Sigma-Aldrich], 2 mmol/L ethylenediaminetetraacetic acid [pH 8; Roth], 50 mmol/L sodium glycerophosphate [Roth], 20 mmol/L sodium pyrophosphate [Roth], 1 mmol/L sodium vanadate [Sigma-Aldrich], 5 µg/mL leupeptin [Roth], and 5 mmol/L benzamidine [Sigma-Aldrich]) at 4°C. Supernatants were cleared by centrifugation, and protein content was determined by the Bradford method. Protein expression was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blot. Antiserum samples against the influenza virus protein M1 and against the cellular extracellular signal-regulated kinase 2 were obtained from Santa Cruz Biotechnology.

Minireplicon Luciferase Assay

Luciferase-reporter gene assays were carried out as described elsewhere [21]. A549 cells were transfected with a plasmid encoding an antisense luciferase-reporter gene flanked by the influenza virus promoters. The RNA produced by this plasmid represents a viral RNA template and is expressed by an RNA-Pol I promoter. This plasmid was transfected in addition to Pol II–driven constructs expressing the viral polymerase genes PB1, PB2, NP of the recombinant (rec) HH/04 virus strain, and PA of either the HH/04 virus strain or the rec HH/04-PA(F35L) mutant virus strain and the corresponding SC35M constructs, respectively. As a negative control, cells were transfected without any PA expression construct. Cells were lysed 24 hours posttransfection, and luciferase activities were determined.

Mouse Experiments

All animal studies were performed in compliance with animal welfare regulations (permission number Az 8,87–50.10.36.09.007 by the State Agency for Nature, Environment and Consumer Protection [LANUV], Germany). Eight-week-old BALB/c mice were obtained from the animal-breeding facilities Harlan-Winkelmann. For infection of mice, the animals were anesthetized by intraperitoneal injection of 200 µL solution of ketamine (Ceva) and xylazin (Ceva; equal amounts of 2% xylazin solution and 10% ketamine solution were mixed 1:10 with PBS). Mice were infected by the intranasal route with the

indicated virus and plaque-forming units (PFU) in a 50 µL volume. In our experiments, due to protection of animal welfare restrictions, mice were killed upon a body weight loss of 25%.

For determination of lung virus titer, mice were killed at the times indicated, and lungs were collected in PBS. The samples were homogenized using a FastPrep-24 homogenizator (MP Biomedicals) with Lysing Matrix D (MP Biomedicals), and were adjusted with PBS to obtain a 10% tissue homogenate. The samples were centrifuged at 10 000 rpm for 5 minutes, and the supernatants were taken.

Structure Modeling

Images of PA proteins were created with PyMOL Molecular Graphics System, Version 1.1 (Schrödinger, LLC) with the help of the SWISS-MODEL Server [23] and the Protein Data Bank template 2w69 (Chain A+B) [24].

Results

Adaptation of the Human H1N1v Isolate HH/04 to Mice

To investigate the potential of HH/04 to adapt to a new mammalian host, the natural human isolate was passaged 3 times in mice, whereby the emerged virus was completely sequenced. Mice infected with a high dose of 10^6 PFU did not show any symptoms of disease, consistent with a marginal body weight loss of <10% during the observation period (Figure 1*A*, *filled circles*). After the first passage, only a single mutation in the HA(K163E) was detectable. The isolated HH/04-1st virus was used for infection in a next passage (Figure 1*A*, *open circles*). Monitoring of the body weight loss revealed no differences in comparison to HH/04, illustrating that the single HA mutation (K163E) alone has no impact on virulence. Sequencing of the second passage isolate (HH/04-2nd) revealed 3 additional nonsynonymous mutations within the HA, PA, and NA, and 1 synonymous mutation within the NA gene segment (Figure 1*B*). These 3 additional mutations indeed led to enhanced pathogenicity, as evidenced by a pronounced body weight loss of HH/04-2nd–infected mice (Figure 1*A*, *open squares*). Subsequent sequencing of the third passage isolate (HH/04-3rd) revealed that the previously emerged mutations were stable without any appearance of further mutations.

Increased Pathogenicity of the Mouse-Adapted HH/04-3rd Isolate

In contrast to the HH/04 virus, infection of mice with the HH/04-3rd–passage isolate resulted in increased body weight loss (Figure 2*A*) and enhanced lung virus titers (Figure 2*B*), which was accompanied by 80% lethality (Figure 2*C*). Our results clearly indicate that the almost apathogenic human HH/04 isolate can be adapted to a new mammalian host after only 2–3 lung-to-lung passages.

Mutations Within the Hemagglutinin and the PA Subunit of the Polymerase Complex Are Responsible for Enhanced Mouse Pathogenicity

We generated a recombinant 8 plasmid set for the recovery of recombinant HH/04 viruses. The identified nonsynonymous mutations were introduced separately or in combination into the genome of 9 recombinant HH/04 viruses, designated as rec HH/04, rec HH/04-3rd, and HH/04-HA(K163E); HH/04-HA(D222G); HH/04-PA(F35L) and HH/04-NA(T32I). Furthermore, the HH/04-HA(K163E+D222G) variant, containing both mutations in the HA, HH/04-HA(K163E+D222G) PA(F35L), and HH/04-HA(K163E+D222G) NA(T32I) mutants carrying the indicated changes were created. To show that the introduction of the 4 mutations in HH/04 reflects the phenotype of the 3rd-passage HH/04, the weight loss (Figure 3*A*) and lung virus titers (Figure 3*B*) of mice infected with HH/04, rec HH/04, HH/04-3rd, and rec HH/04-3rd were determined. Both parameters were almost identical when HH/04 was compared with rec HH/04 and HH/04-3rd to rec HH/04-3rd, respectively. Thus, the recombinant virus and the genetically engineered variant exhibited a similar replication behavior and pathogenicity as their natural counterparts.

To analyze the impact on virulence of each single adaptive mutation, mice were infected with rec HH/04 and 4 virus mutants, each containing the indicated single mutation (Figure 4*A*). By monitoring body weight loss, a strong contribution of the HA(D222G) and PA(F35L) mutations to increased virulence was detected. In contrast, the HA(K163E) and NA(T32I) mutations alone did not affect viral pathogenicity to a large extent. In contrast, examination of double- and triple-mutant viruses revealed a supplementary contribution of the HA(K163E), and just to a minor extent, of the NA(T32I) mutation to increased virulence (Figure 4*B*). In this low-dose infection experiment, the rec HH/04-HA(K163E+D222G) PA(F35L) virus indeed showed higher additive effects of the PA(F35L) mutation compared with the NA(T32I) mutation for virulence, although the NA(T32I) impact on virus virulence cannot completely be neglected.

PA(F35L)-Dependent Increase of Virulence Correlates With Enhanced Viral Polymerase Activity

As part of the heterotrimeric RNA-dependent RNA polymerase, the PA contributes to diverse functions such as endonuclease and protease activities, cap-binding, and cap-snatching, as well as viral RNA binding [24–28]. A likely explanation for enhanced pathogenicity of the HH/04-PA(F35L) variant could be that the PA mutation confers enhanced polymerase activity. We studied the polymerase activity of the HH/04-PA(F35L) virus in comparison to the rec HH/04 strain in a minireplicon assay, allowing the relative quantification of viral polymerase activity. Reconstitution of the polymerase complex with a PA containing the F35L mutation resulted in a 2.5-fold higher polymerase activity compared with the PA-wt protein (Figure 5*A*). This was not a strain-specific phenomenon because the same mutation in the background of the avian influenza virus A/Seal/Massachusetts/1/80 (H7N7) mouse-adapted strain (SC35M; Figure 5*B*) showed a similar result. To examine whether this enhanced polymerase activity improves the replication efficiency, different cell lines were infected with the rec HH/04 and rec HH/04-PA(F35L). Viral protein accumulation and virus propagation were examined by determination of the viral M1-protein synthesis and progeny virus titers. Upon infection with HH/04-PA(F35L), an increase of both viral protein amounts (Figure 5*C*) and virus titers (Figure 5*D*) was detected. This phenomenon was not dependent on the cell type used because infection of different cell lines resulted in a similar increase in polymerase activity if the F35L mutation was present in the PA. Taken together, these results clearly indicate a replication advantage conferred by the F35L mutation in the PA, which is most likely the molecular basis for enhanced virulence in PA(F35L)-infected mice.

Discussion

Adaptation is a major driving force in evolution, whereby beneficial mutations within the genome become manifest and result in adaptation to a novel host, accompanied by increased fitness and enhanced propagation. Taking this into account, it is likely that pandemic H1N1 viruses may acquire enhanced virulence caused by adaptive mutations during propagation and spreading among humans and/or pigs.

In the present study, the mouse-adapted virus strain showed increased pathogenicity illustrated by increased body weight loss and mortality, as well as increased replication capacity in mice lungs (Figure 2*A*–*C*). Four nonsynonymous mutations were identified via whole genome sequence analysis (Figure 1*B*). Among these, 3 mutations within the PA and the HA were recognized as a cause of the increased pathogenicity. The aspartic acid to glycine exchange D222G within the hemagglutinin of 2009 H1N1v viruses could be epidemiologically correlated to severe cases of illness in several countries [29–31]. In 1 out of 6 H1N1v-infected patients with primary severe respiratory failure treated at the intensive care unit of the university hospital Muenster in 2009, we were able to demonstrate that the HA(D222G) mutation arose simultaneously with the oseltamivir resistance mutation H275Y in the NA (data not shown). In this 31-year-old previously healthy male patient, virus isolated on day 1 after admission and initiation of the oseltamivir treatment (influenza A/Nordrhein-Westfalen/172/09, RKI) still contained the wt sequence (HA D222, NA H275) and was phenotypically oseltamivir sensitive. In a subsequent isolate obtained on day 5 after admission and completion of the oseltamivir therapy (influenza A/Nordrhein-Westfalen/173/09) and in all following isolates, the respective mutations were present and isolates were phenotypically oseltamivir resistant. This case clearly highlights the existing risk of further adaptation, leading to high virulent and nondrug-treatable virus variants. A D222G change in the HA protein of pandemic 2009 IAV led to broader binding range of alpha 2,3-linked sialyl receptor sequences [32, 33].

In mice, the D222G mutation is related to higher virus titers, elevated proinflammatory cytokine release in the lungs, and enhanced lethality [34]. Furthermore, similar H1N1v mice adaptation approaches detected the HA(D222G) mutation as well [35, 36], possibly indicating the necessity of an α 2,3-linked sialyl receptor binding specificity for efficient replication in mice [37]. It is conspicuous that mutations in the HA were most often accompanied by mutations within the polymerase complex, especially in the PA protein [35, 36, 38]. These data are in line with our results regarding the importance of the HA(D222G) mutation for the virulence of pandemic H1N1 viruses. Nevertheless, body weight loss of rec HH/04-HA(D222G)–infected mice was less severe as of rec HH/04-3rd–infected mice (Figure 4*B*), revealing the strong involvement of further mutations contributing to the increased virulence, which highlights synergistic effects of several mutations in combination.

The increase of virulence due to the HA(D222G) mutation is further enhanced by introduction of the HA(K163E) mutation, as indicated by the increased body weight loss of HA(K163E+D222G) double mutant–infected mice (Figure 4*B*). Similar to the amino acid at position 222 in the HA, the amino acid at position 163 is described as an antigenic site in H1-HA. Whereas amino acid 222 is located in the Ca epitope, amino acid 163 is closely located to the receptor-binding site in the Sa epitope [39, 40]. Additional introduction of either the PA or the NA mutation to the double-mutant virus revealed a stronger contribution of PA mutation to increased virulence as against the NA mutation, as it was expected from the single-mutant infection studies (Figure 4*A*).

During the virus life cycle, the PA protein is mainly responsible for transcription and replication of the viral RNA. The messenger RNA (mRNA) caps are snatched 10–14 nucleotides behind the 5′ cap from host pre-mRNAs by a distinctive, still not fully dissolved mechanism [41–44]. Recently, it was shown that the endonuclease activity of the polymerase complex is located in the N-terminal part of the PA subunit [24, 28]. These functions of the PA led us to the concept that enhanced body weight loss of mice could be due to more efficient virus propagation caused by increased polymerase activity. Indeed, the polymerase activity of PA(F35L) viruses outperformed 2 wild-type viruses of the H1 and H7 subtypes by 2- to 2.5-fold in a minireplicon assay (Figure 5*A* and 5*B*). Consistent with this finding, the increased polymerase activity also led to enhanced replication efficiency in cultured cells from different species.

The concept of optimized interactions among viral proteins and host cell factors as being partially responsible for adaptation to a new host was reported to be a determinant of host range [45]. At present, we cannot exclude that the PA(F35L) mutation improves the activity of the viral polymerase by enhancing its association with host cell factors in mice. However, given the result that the PA(F35L) variation increases viral polymerase activity in both human and murine cells (Figure 5*C* and 5*D*), we favor the hypothesis that this variation rather functions in a host-independent manner, possibly by improving the catalytic activity of the viral enzyme. Although precise and entire functions of PA still remain unclear, the PA is known to contribute to several steps during the replication process. Thus, numerous functions can be affected by the F35L mutation improving polymerase activity. The N-terminus of HH/04-PA displays amino acid 35 to be located in the α-helix 3, as shown in the computationally 3-dimensional (3D) structure prediction approach (Figure 6*A* and 6*B*).

This amino acid is closely located to histidine 41, which is part of the endonuclease active site and seems to be responsible for binding of divalent cations [24, 28]. The structure prediction of the PA(L35) protein reveals an unaltered protein structure and also no apparent change in the orientation of the marked residues. Sequence alignment of 2 representative IAV (H3N2 and H5N1) strains [24] reveals conserved sequences from amino acid 29 throughout the whole α-helix 3 of both strains, comparable to HH/04. Leucine at position 35 matches to the consensus sequence of influenza B viruses that also contain an identical H41 active site residue. Apart from that, a sequence alignment of human IAV strains (National Center for Biotechnology Information Influenza Virus Resource Database) reveals 8 pandemic 2009 H1N1 strains, comprising the amino acid leucine at position 35 within the PA. In addition to the 8 pandemic 2009 H1N1 strains, the mutation also emerged in 2 strains isolated in the forties and in 2 high-virulent H5N1 human isolates. These data clearly highlight the possibility of natural occurrence of the PA(F35L) mutation in humans, emphasizing the potential risk of the emergence of highly virulent pandemic H1N1 viruses in humans.

It would be interesting to know whether the emerged adaptive mutations originate de novo or were selected from preexisting quasispecies. Because the study was initiated with a natural isolate that was obtained by an oral cavity swap of an infected patient, the existence of quasispecies within the

inoculum cannot be totally ruled out. A closer look on the sequencing chromatograms of the HA (position 163) and NA (position 32) in the origin strain possibly reveals mixed populations within the inoculum (Figure 1*B*). However, similar to the natural isolate, enhanced pathogenicity in mice also was observed after 3 passages of the genetically defined recombinant HH/04, where quasispecies do not play a role (data not shown). Thus, although selection of quasispecies may occur, there is also de novo selection of variants.

In summary, this new HH/04-3rd variant is characterized by increased virulence primarily due to HA(K163E), HA(D222G), and the PA(F35L) mutations, which can be defined as pathogenicity determinants that are mainly responsible for enhanced virulence. Our data furthermore clearly illustrate that the examination of single-pathogenicity determinants for the assessment of enhanced viral pathogenicity can be somehow insufficient, highlighting the need to take the synergistic action of pathogenicity determinants for increased virulence into account. In these adaptation approaches, 2 events seem to have occurred for adaptation of pandemic 2009 H1N1v strains to mice. On the one hand, an optimized HA-dependent receptor binding specificity (probably leading to changes in attachment to different cell types [32, 37]), and on the other hand, increased replication efficiency (resulting in an enhanced virus propagation) is required for manifestation of an efficient infection.

Notes

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Potential conflicts of interest

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Footnotes

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References

1. Itoh Y, Shinya K, Kiso M, et al. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature 2009; 460:1021–5.
2. Maines TR, Jayaraman A, Belser JA, et al. Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and
mice. Science 2009; 325:484–7.
3. Munster VJ, de Wit E, van den Brand JM, et al. Pathogenesis and transmission of swine-origin 2009 A(H1N1) influenza virus in ferrets.
Science 2009; 325:481–3.
4. Belser JA, Wadford DA, Pappas C, et al. Pathogenesis of pandemic influenza A (H1N1) and triple-reassortant swine influenza A (H1)
viruses in mice. J Virol 2010; 84:4194–203.
5. Zhu H, Wang J, Wang P, et al. Substitution of lysine at 627 position in PB2 protein does not change virulence of the 2009 pandemic H1N1
virus in mice. Virology 2010; 401:1–5.
6. Zhou B, Li Y, Halpin R, Hine E, Spiro DJ, Wentworth DE. PB2 residue 158 is a pathogenic determinant of pandemic H1N1 and H5 influenza a viruses in mice. J Virol 2011; 85:357–65.
7. Chang LY, Shih SR, Shao PL, Huang DT, Huang LM. Novel swineorigin influenza virus A (H1N1): the first pandemic of the 21st century. J Formos Med Assoc 2009; 108:526–32.
8. Neumann G, Noda T, Kawaoka Y. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 2009; 459:931–9.
9. Hai R, Schmolke M, Varga ZT, et al. PB1-F2 expression by the 2009 pandemic H1N1 influenza virus has minimal impact on virulence in
animal models. J Virol 2010; 84:4442–50.
10. Hale BG, Steel J, Manicassamy B, et al. Mutations in the NS1 C-terminal tail do not enhance replication or virulence of the 2009 pandemic H1N1 influenza A virus. J Gen Virol 2010; 91:1737–42.
11. Herfst S, Chutinimitkul S, Ye J, et al. Introduction of virulence markers in PB2 of pandemic swine-origin influenza virus does not result in enhanced virulence or transmission. J Virol 2010; 84:3752–8.
12. Hirst GK. Studies on the mechanism of adaptation of influenza virus to mice. J Exp Med 1947; 86:357–66.
13. Narasaraju T, Sim MK, Ng HH, et al. Adaptation of human influenza H3N2 virus in a mouse pneumonitis model: insights into viral virulence, tissue tropism and host pathogenesis. Microbes Infect 2009; 11:2–11.
14. ScheiblauerH, Kendal AP, Rott R. Pathogenicity of influenza A/Seal/Mass/1/80 virus mutants for mammalian species. Arch Virol 1995; 140:341–8.
15. Brown EG, Liu H, Kit LC, Baird S, Nesrallah M. Pattern of mutation in the genome of influenza A virus on adaptation to increased virulence in the mouse lung: identification of functional themes. Proc Natl Acad Sci USA 2001; 98:6883–8.
16. Gabriel G, Dauber B, Wolff T, Planz O, Klenk HD, Stech J. The viral polymerase mediates adaptation of an avian influenza virus to
a mammalian host. P Natl Acad Sci USA 2005; 102:18590–5.
17. Keleta L, Ibricevic A, Bovin NV, Brody SL, Brown EG. Experimental evolution of human influenza virus H3 hemagglutinin in the mouse lung identifies adaptive regions in HA1 and HA2. J Virol 2008; 82:11599–608.
18. Song MS, Pascua PN, Lee JH, et al. The polymerase acidic protein gene of influenza a virus contributes to pathogenicity in a mouse
model. J Virol 2009; 83:12325–35.
19. Li J, Ishaq M, Prudence M, et al. Single mutation at the amino acid position 627 of PB2 that leads to increased virulence of an H5N1 avian influenza virus during adaptation in mice can be compensated by multiple mutations at other sites of PB2. Virus Res 2009; 144:123–9.
20. Rolling T, Koerner I, Zimmermann P, et al. Adaptive mutations resulting in enhanced polymerase activity contribute to high virulence of
influenza A virus in mice. J Virol 2009; 83:6673–80.
21. Mazur I, Anhlan D, Mitzner D, Wixler L, Schubert U, Ludwig S. The proapoptotic influenza A virus protein PB1-F2 regulates viral polymerase activity by interaction with the PB1 protein. Cell Microbiol 2008; 10:1140–52.
22. Hoffmann E, Krauss S, Perez D, Webby R, Webster RG. Eight-plasmid system for rapid generation of influenza virus vaccines. Vaccine 2002; 20:3165–70.

23. Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res 2003;
31:3381–5.
24. Dias A, Bouvier D, Crepin T, et al. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature 2009;
458:914–8.
25. Hara K, Schmidt FI, Crow M, Brownlee GG. Amino acid residues in the N-terminal region of the PA subunit of influenza A virus RNA polymerase play a critical role in protein stability, endonuclease activity, cap binding, and virion RNA promoter binding. J Virol 2006; 80:7789–98.
26. Maier HJ, Kashiwagi T, Hara K, Brownlee GG. Differential role of the influenza A virus polymerase PA subunit for vRNA and cRNA promoter binding. Virology 2008; 370:194–204.
27. Sanz-Ezquerro JJ, Zurcher T, de la Luna S, Ortin J, Nieto A. The aminoterminal one-third of the influenza virus PA protein is responsible for the induction of proteolysis. J Virol 1996; 70:1905–11.
28. Yuan P, Bartlam M, Lou Z, et al. Crystal structure of an avian influenza polymerase PA(N) reveals an endonuclease active site. Nature 2009; 458:909–13.
29. Anton A, Marcos MA, Martinez MJ, et al. D225G mutation in the hemagglutinin protein found in 3 severe cases of 2009 pandemic influenza A (H1N1) in Spain. Diagn Micr Infect Dis 2010; 67:207–8.
30. Chen H, Wen X, To KK, et al.Quasispecies of theD225G substitution in the hemagglutinin of pandemic influenza A(H1N1) 2009 virus from patients with severe disease in Hong Kong, China. J Infect Dis 2010; 201:1517–21.
31. Kilander A, Rykkvin R, Dudman SG, Hungnes O. Observed association between the HA1 mutation D222G in the 2009 pandemic influenza A(H1N1) virus and severe clinical outcome, Norway 2009–2010. Euro Surveill 2010; 15:19498.
32. Chutinimitkul S, Herfst S, Steel J, et al. Virulence-associated substitution D222G in the hemagglutinin of 2009 pandemic influenza
A(H1N1) virus affects receptor binding. J Virol 2010; 84:11802–13.
33. Liu Y, Childs RA, Matrosovich T, et al. Altered receptor specificity and cell tropism of D222G hemagglutinin mutants isolated fromfatal cases of pandemic A(H1N1) 2009 influenza virus. J Virol 2010; 84:12069–74.
34. Zheng B, Chan KH, Zhang AJ, et al. D225G mutation in hemagglutinin of pandemic influenza H1N1 (2009) virus enhances virulence in mice. Exp Biol Med 2010; 235:981–8.
35. Ilyushina NA, Khalenkov AM, Seiler JP, et al. Adaptation of pandemic H1N1 influenza viruses in mice. J Virol 2010; 84:8607–16.
36. Sakabe S, Ozawa M, Takano R, Iwastuki-Horimoto K, Kawaoka Y. Mutations in PA, NP, and HA of a pandemic (H1N1) 2009 influenza
virus contribute to its adaptation to mice. Virus Res 2011; 158:124–9.
37. Tate MD, Brooks AG, Reading PC. Receptor specificity of the influenza virus hemagglutinin modulates sensitivity to soluble collectins of the innate immune system and virulence in mice. Virology 2011; 413:128–38.
38. Ye J, Sorrell EM, Cai Y, et al. Variations in the hemagglutinin of the 2009 H1N1 pandemic virus: potential for strains with altered virulence phenotype? PLoS Pathog 2010; 6:e1001145.
39. Xu R, Ekiert DC, Krause JC, Hai R, Crowe JE, Wilson IA. Structural basis of preexisting immunity to the 2009 H1N1 pandemic influenza virus. Science 2010; 328:357–60.
40. Gerhard W, Yewdell J, Frankel ME, Webster R. Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies.
Nature 1981; 290:713–17.
41. Plotch SJ, Bouloy M, Ulmanen I, Krug RM. A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell 1981; 23:847–58.
42. Li ML, Rao P, Krug RM. The active sites of the influenza cap-dependent endonuclease are on different polymerase subunits. EMBO J 2001; 20:2078–86.
43. Fechter P, Mingay L, Sharps J, Chambers A, Fodor E, Brownlee GG. Two aromatic residues in the PB2 subunit of influenza A RNA polymerase are crucial for cap binding. J Biol Chem 2003;
278:20381–8.
44. Guilligay D, Tarendeau F, Resa-Infante P, et al. The structural basis for cap binding by influenza virus polymerase subunit PB2. Nat Struct Mol Biol 2008; 15:500–6.
45. Gabriel G, Herwig A, Klenk HD. Interaction of polymerase subunit PB2 and NP with importin alpha1 is a determinant of host range of influenza A virus. PLoS Pathog 2008; 4:e11.

Tables and Figures

Figure 1. H1N1v adapted to a new mammalian host upon passaging in mice lungs. A, Three 8-week-old BALB/c mice were intranasally infected with 10_6 PFU of HH/04, HH/04-1st, and HH/04-2nd, and body weight (6 SD) was monitored. B, Sequences of HH/04 and HH/04-3rd were compared, and identified mutations are depicted. Abbreviations: H1N1v, influenza A virus subtype H1N1 variant; HH/04, H1N1 variant isolate A/Hamburg/04/09; PFU, plaque-forming units.

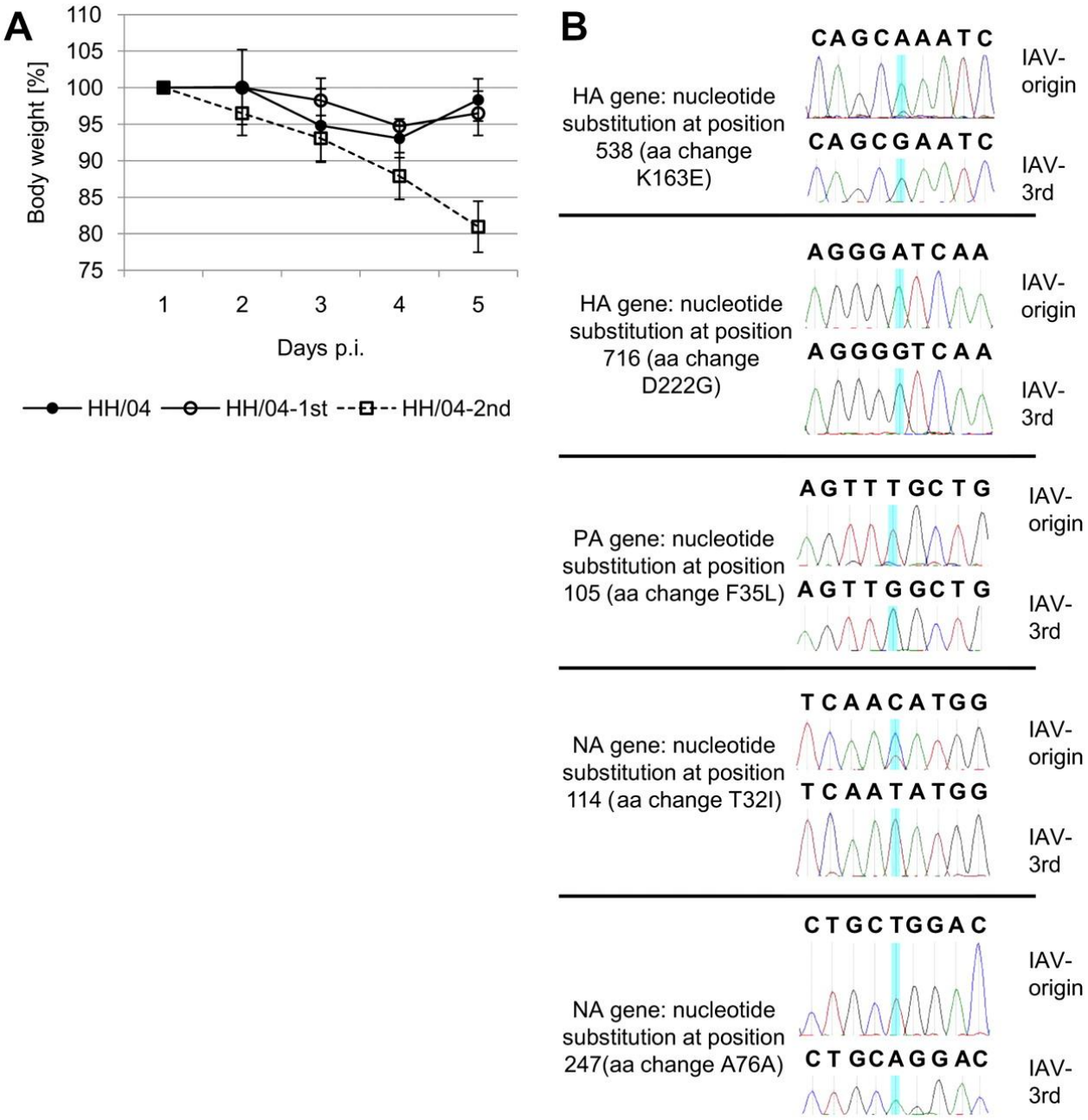

Figure 2. Mouse-adapted HH/04-3rd showed increased virulence. A–C, Five 8-week-old BALB/c mice were intranasally infected with 10_6 PFU of HH/04 and the HH/04-3rd, respectively. A, Body weight (6 standard deviation) of the infected mice was monitored over a time period of 5 days. B, Virus titers of infected lungs were determined 3 days postinfection in standard plaque titrations (mean titers, HH/04: 3.4 3 10_5 PFU/mL to HH/04-3rd: 1.6 3 10_6 PFU/ mL). Statistical significance was assessed by Student t test. *P , .05. C, Survival of the infected mice was monitored over a time period of 10 days.
Abbreviations: HH/04, H1N1 variant isolate A/Hamburg/04/09; PFU, plaque-forming units.

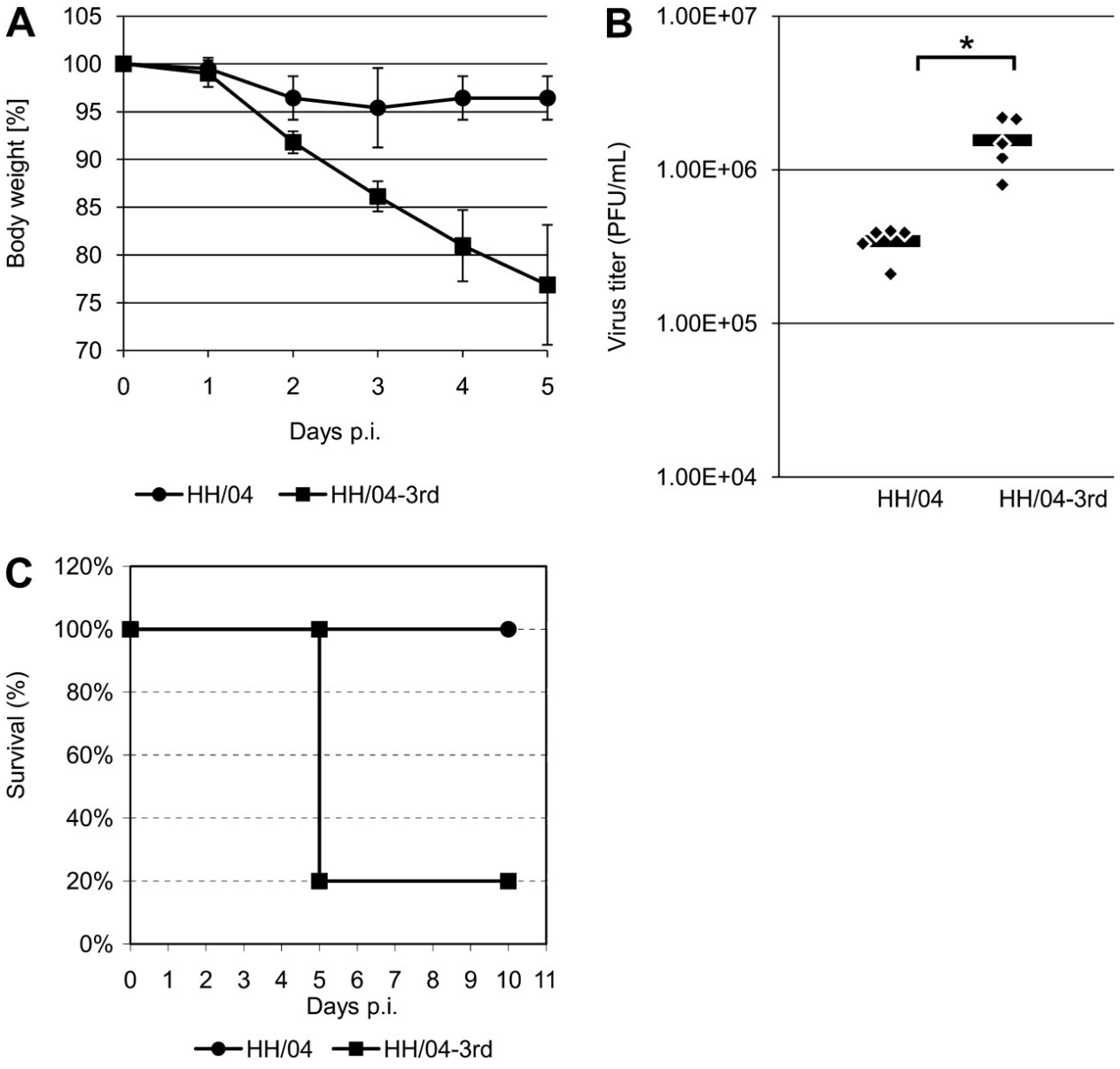

Figure 3. Comparison of recombinant (rec) and nonrecombinant virus strains. A, B, Four 8-week old BALB/c mice were infected intranasally with 5 3 10_4 PFU of HH/04, rec HH/04, HH/04-3rd and rec HH/04-3rd, respectively. A, Body weight of the infected mice was monitored over a time period of 6 days.
B, Virus titers of infected lungs were determined 6 days postinfection in standard plaque titrations. Statistical significance was assessed by Student t test. *P , .05. Abbreviations: PFU, plaque-forming units; HH/04, H1N1 variant isolate A/Hamburg/04/09.

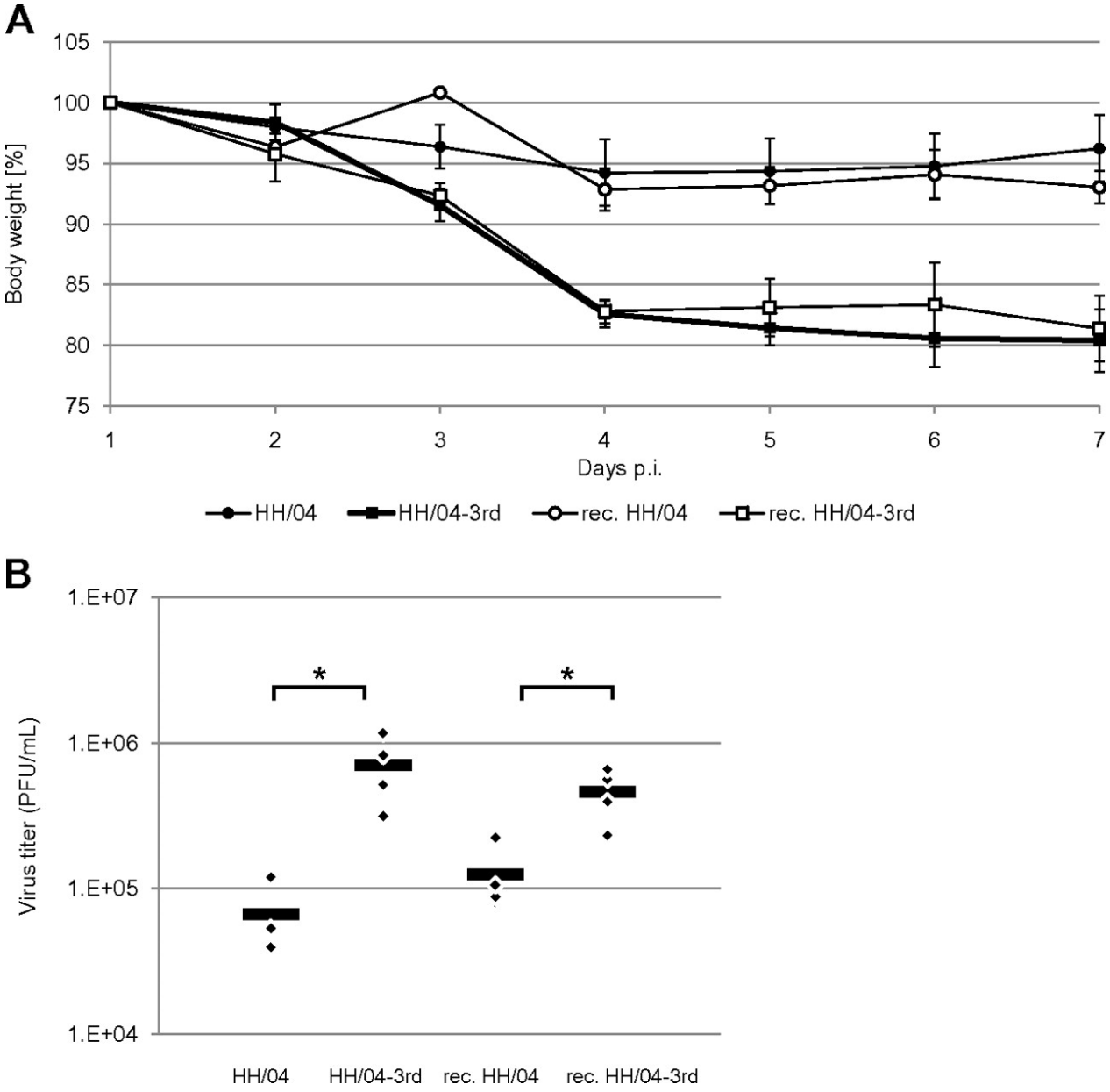

Figure 4. Impact of obtained mutations for enhanced virulence. Four 8-week-old BALB/c mice were infected intranasally with 2 3 10_5 PFU (A) and 1 3 10_2 PFU (B) of the indicated viruses and were monitored for body weight loss (6 SD) over a time period of (A) 15 and (B) 25 days. Abbreviation: PFU, plaque-forming units.

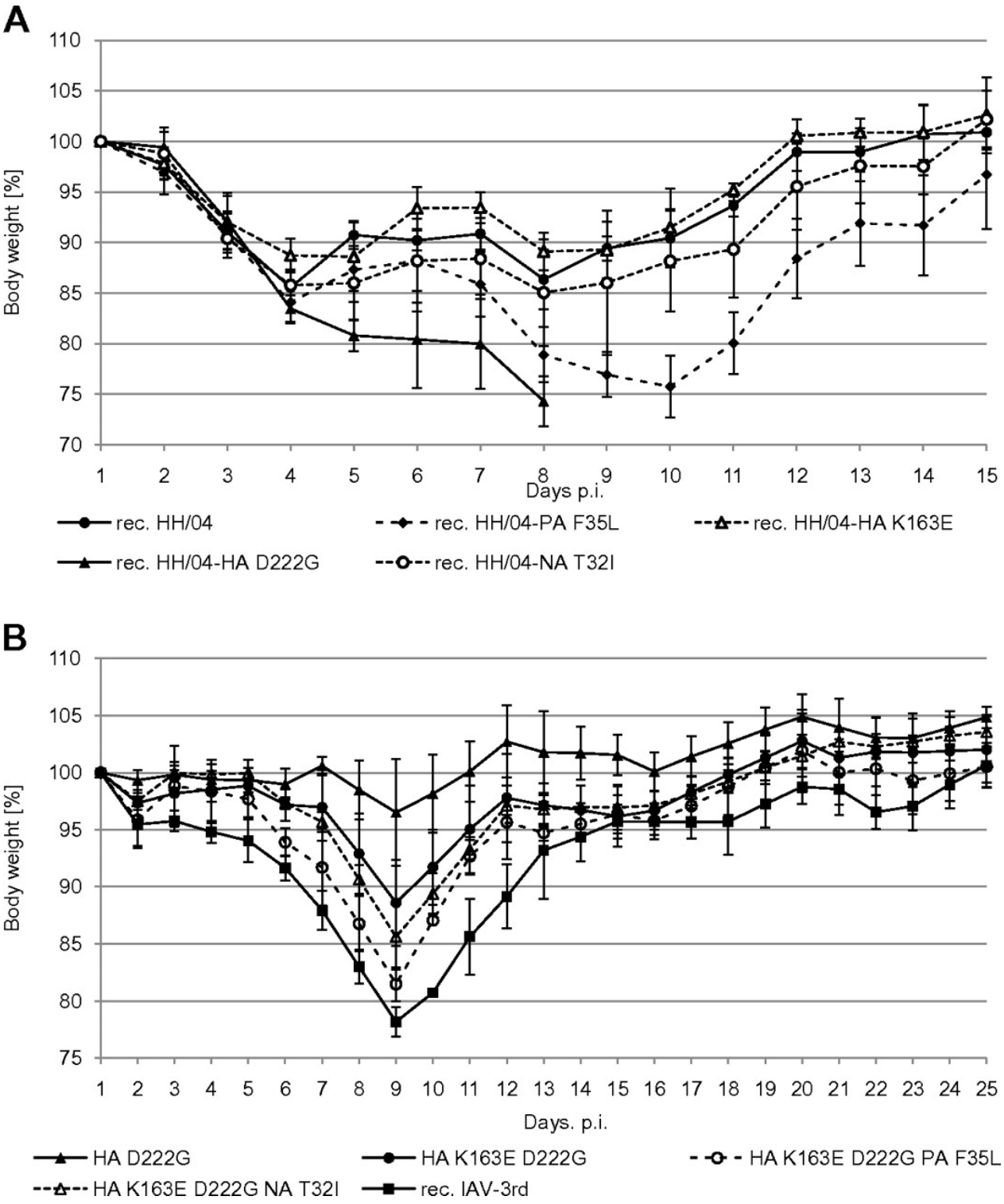

Figure 5. Increased replication ability of PA(F35L) mutant virus strain due to increased polymerase activity. A, B, Human lung epithelial cell line (A549) cells were transfected with a plasmid encoding an antisense luciferase-reporter gene flanked by influenza virus promoters.
The RNA produced by this plasmid represents a viral RNA template and is expressed via RNA-Pol I promoter. This plasmid was transfected in addition to Pol II–driven constructs expressing the viral polymerase genes PB1, PB2, and NP of (A) the recombinant (rec) HH/04 strain and PA of either the rec HH/04 strain or the rec HH/04-PA(F35L) mutant strain.
In (B), instead of the influenza rec HH/04 virus constructs, the corresponding constructs of the influenza A/Seal/Massachusetts/1/80 (H7N7) mouse-adapted virus strain were applied. As negative control, cells were transfected without any PA expression construct. Cells were lysed 24 hours posttransfection, and luciferase activities were determined. The value obtained with the PA-wt plasmid set was arbitrarily set to 100%. C, D, A549 cells, chicken embryonic fibroblast (DF-1) cells, porcine kidney epithelia cells (PK-15), and mouse pulmonary squamous cell carcinoma cells (ASB-XIV) were infected with a multiplicity of infection of 1 of either rec HH/04 or rec HH/04-PA(F35L). Eight hours postinfection, viral M1-protein accumulation was detected by Western blot analysis (C), and virus titers were determined in standard plaque titrations (D). A–D, Data show the representative result of at least 3 independent experiments. Statistical significance was assessed by Student t test.
*P , .05. A, C, D, Error bars represent SD. Abbreviations: PA, polymerase acidic; PB1, polymerase basic 1; PB2, polymerase basic 2; NP, nucleoprotein; HH/04, H1N1 variant isolate A/Hamburg/04/09; wt, wild type.

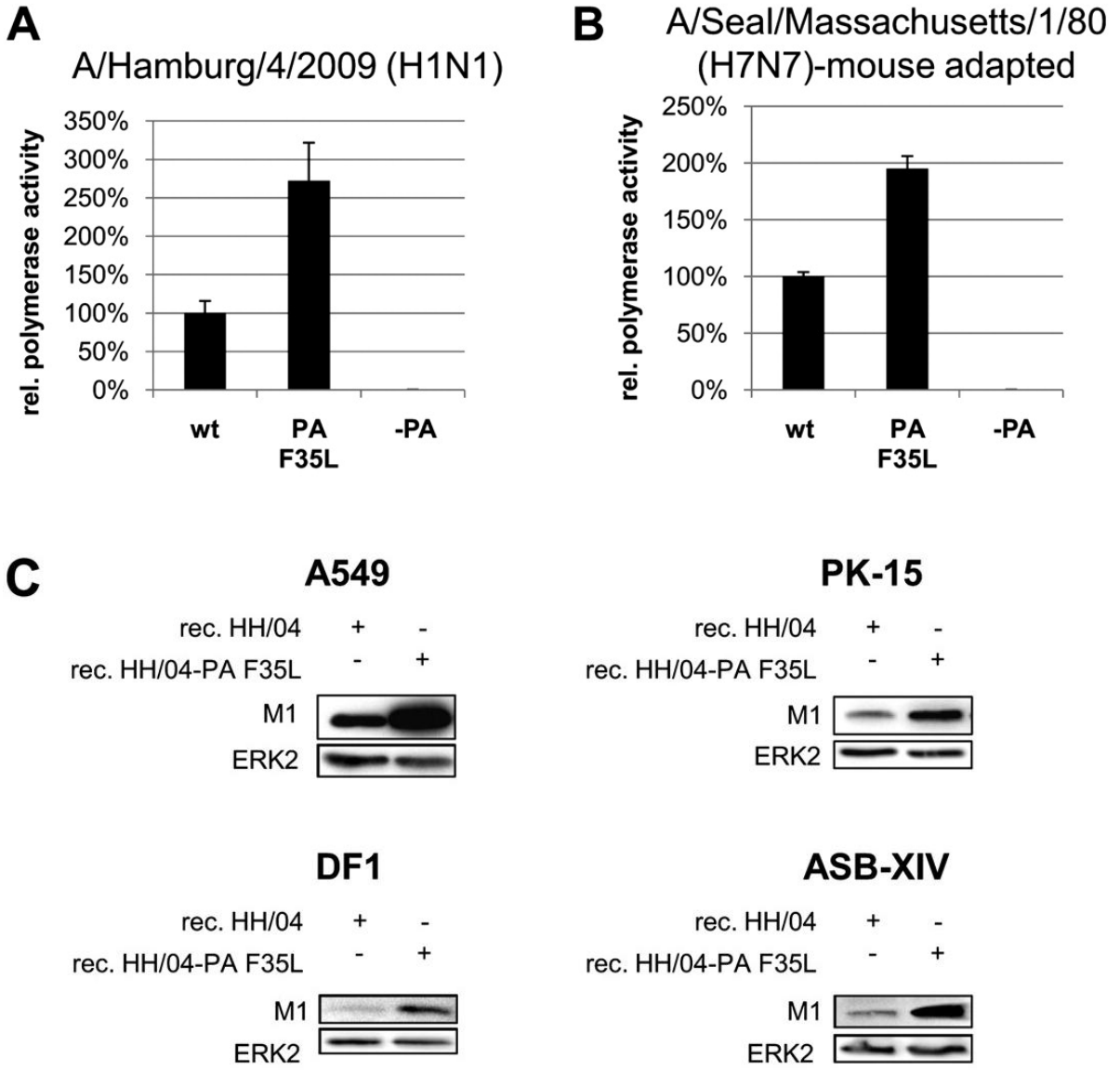

Figure 5 *(continued)*

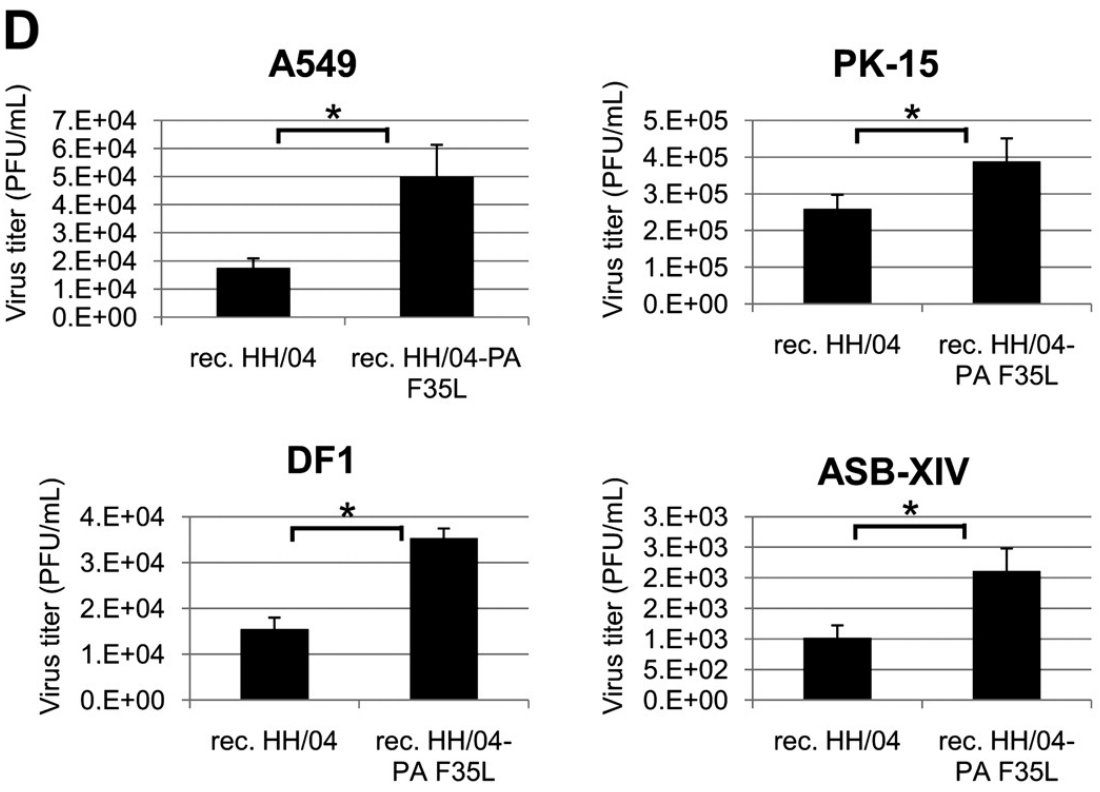

Figure 6. Three-dimensional structure models of the PA-N-terminal part. Ribbon representation of PA-F35 (A) and PA-L35 (B) N-terminal (1-196) protein structure model of HH/04 with a-helices in red, b-strands in yellow, and loops in green. A, B, The key active site residue (H41) and residues 35 are indicated in stick representation and are colored in black. Abbreviations: PA, polymerase acidic; HH/04, H1N1 variant isolate A/Hamburg/04/09.