Conserved Features of the PB2 627 Domain Impact Influenza Virus Polymerase Function and Replication

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ABSTRACT

Successful replication of influenza virus requires the coordinated expression of viral genes and replication of the genome by the viral polymerase, composed of the subunits PA, PB1, and PB2. Polymerase activity is regulated by both viral and host factors, yet the mechanisms of regulation and how they contribute to viral pathogenicity and tropism are poorly understood. To characterize these processes, we created a series of mutants in the 627 domain of the PB2 subunit. This domain contains a conserved “P[F/P]AAAPP” sequence motif and the well-described amino acid 627, whose identity regulates host range. A lysine present at position 627 in most mammalian viral isolates creates a basic face on the domain surface and confers high-level activity in humans compared to the glutamic acid found at this position in avian isolates. Mutation of the basic face or the P[F/P]AAAPP motif impaired polymerase activity, assembly of replication complexes, and viral replication. Most of these residues are required for general polymerase activity, whereas PB2 K586 and R589 were preferentially required for function in human versus avian cells. Thus, these data identify residues in the 627 domain and other viral proteins that regulate polymerase activity, highlighting the importance of the surface charge and structure of this domain for virus replication and host adaptation.

IMPORTANCE

Influenza virus faces barriers to transmission across species as it emerges from its natural reservoir in birds to infect mammals. The viral polymerase is an important regulator of this process and undergoes discrete changes to adapt to replication in mammals. Many of these changes occur in the polymerase subunit PB2. Here we describe the systematic analysis of a key region in PB2 that controls species-specific polymerase activity. We report the importance of conserved residues that contribute to the overall charge of the protein as well as those that likely affect protein structure. These findings provide further insight into the molecular events dictating species-specific polymerase function and viral replication.

The potential of influenza virus to cause recurring pandemics makes it a significant threat to human health. The three major influenza pandemics of the last century, occurring in 1918, 1957, and 1968, caused significant morbidity and mortality, with the 1918 pandemic virus claiming an estimated 600,000 lives in the United States and a total of 50 million lives globally (1, 2). More recently, a new pandemic virus emerged in 2009 (3, 4). Whereas the 2009 virus transmitted readily in people, symptoms were fairly mild, and pathogenicity was comparable to that of seasonal influenza virus (5). Thus, a major cause for concern is the emergence of a new pandemic virus with both high transmissibility and pathogenicity.

Pandemic viruses emerge from their natural reservoir in migratory aquatic birds, often by first infecting domesticated animals such as pigs and chickens before ultimately infecting humans. This is accompanied by adaptive changes in the virus through mutation, reassortment of its eight genomic RNA segments, or both. Genetic changes in the viral RNA-dependent RNA polymerase have been critical to the emergence of the last four pandemics, and it has been known for over 30 years that the polymerase plays a key role in regulating this process of cross-species transmission (6). The polymerase is a heterotrimer containing the subunits PB1, PB2, and PA. The polymerase assembles with the viral nucleoprotein (NP) and genomic RNA to form higher-order ribonucleoprotein (RNP) complexes. RNPs are templates for genome transcription via a “cap-snatching” process and genome replication (reviewed in reference 7). RNPs are also the native form by which genome segments are packaged into the virion.

Polymerases derived from avian viruses generally function poorly in human cells, leading to restricted infection and pathogenicity (8), possibly due to the presence of a restriction factor in human cells that impairs their function (9). A single mutation in the PB2 subunit, conversion of the avian-signature glutamic acid at position 627 to the human-signature lysine, is sufficient to overcome restriction in human cells and restore replication in animal models (8–12). It is possible that interactions with enhancing factors may also contribute to adaptation at position 627 (13). Several additional adaptive mutations have been identified in PB2. The 2009 pandemic virus retained the avian-signature PB2 residue E627 but acquired a T271A mutation and the so-called SR polymorphism, PB2 G590S and Q591R, both of which increase polymerase activity and virus replication in mammalian cells (14–16). The PB2 D701N mutation also increased replication in mice and may be associated with increased replication in humans (17, 18). Importantly, these adaptive mutations have been found to enhance virulence and/or transmission of influenza virus in ani-

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mal experiments (8, 10, 15–17, 19–25). The mechanism of restriction of avian-derived polymerases in mammalian cells is not well understood, yet all circulating human isolates have acquired adaptive mutations that evade restriction and facilitate high-level polymerase activity and virus replication.

Many of the host-range-determining residues are clustered at the C terminus of PB2. Crystal structures identified a discretely folded “627 domain” along with a downstream bipartite nuclear localization sequence (NLS) that engages the importin- α family of nuclear import receptors (26–28). Examination of the 627 domain revealed that residue 627 is exposed on the surface of PB2 within a flexible loop that partially encircles the preceding α -helix, approximating the shape of the ϕ symbol to form a novel “phi-loop” (ϕ -loop) fold that lacks structural homology to other protein structures (28). Predicted surface electrostatics showed that residue 627 occurs within a positively charged region formed by a series of highly conserved basic residues (27, 28). Comparison of the structures of mammalian and avian PB2 revealed no significant difference in the domain architecture but rather a dramatic change in the surface charge potential dependent upon the amino acid at position 627; a lysine at position 627 creates a contiguous basic face that is disrupted if a glutamic acid is present (27). Mutagenic analysis had previously shown that the maintenance of a basic charge at this position was important for polymerase function in human cells, suggesting that this basic face plays a significant role in adaptation to human cells (9). Studies of the PB2 627 domain of the 2009 pandemic H1N1 influenza virus yielded structures nearly identical to those of previously reported isolates (15). Notably, the SR polymorphism caused a minor change in surface topology and partially neutralized the E627 residue that is retained in the 2009 virus, supporting a model wherein this polymorphism enhances activity by restoring the basic charge to the 627 domain surface and slightly altering the domain conformation (14, 15). Consistent with this, it has been reported that the domain charge of the 627 domain is not the only determinant for this region regulating polymerase function in mammalian hosts (29). As the virus exploits multiple strategies to adapt to replication in human cells, a deeper analysis of the features within the 627 domain may provide further insights into the structural role of PB2 as well as the mechanisms by which PB2 escapes host restriction.

To better understand the function of PB2 in polymerase activity and host range determination, we explored the role of the highly conserved residues that contribute to the basic patch on the surface of the PB2 627 domain and a proline-rich amino acid motif, “P[P/F]AAAPP,” located immediately upstream of residue 627. A panel of PB2 mutants that disrupt the basic face and the P[P/F]AAAPP motif were tested for their ability to support polymerase activity and virus replication. We identified critical residues required for general polymerase function as well as specific residues on the basic face that are preferentially required in human but not avian cells. Thus, our results have identified key features within the 627 domain essential for function, providing further evidence for the importance of this domain in affecting species tropism.

MATERIALS AND METHODS

Cells. 293T, A549, HeLa, DF1, MDBK, and MDCK cells were maintained in complete Dulbecco’s modified Eagle’s medium (DMEM) supple-

mented with 10% fetal bovine serum (FBS). Calu-3 cells were maintained in DMEM/F-12 medium supplemented with 20% defined FBS. LMH cells were grown in DMEM/F-12 medium supplemented with 5% FBS. All cells were grown at 37°C in 5% CO₂.

Plasmids. Plasmids used throughout were described previously (9). Briefly, the polymerase proteins and NP were derived from A/WSN/33. Epitope-tagged PB2-hemagglutinin (HA) and NP-V5 were expressed as C-terminal fusions. Plasmid pTM Δ PB2 expresses viral RNAs (vRNAs) for all influenza virus genes except PB2. The bidirectional expression construct pBD-PB2 expresses both vRNA and mRNA. The vNA-Luc reporter plasmids encode a minus-sense luciferase gene flanked by untranslated regions (UTRs) from NA that is expressed from a polymerase I promoter and terminator (30). Mutants were created by PCR-based strategies and confirmed by sequencing. The PB2 nuclear localization signal (NLS) mutant (K738Q, K752N, and R755Q) was based on a previously characterized mutant (26).

Transfections. 293T, DF1, and LMH cells were transfected with TransIT-LT1 or TransIT-2020, and HeLa cells were transfected with TransIT-HeLaMonster transfection reagent (Mirus Bio).

Polymerase activity assays. 293T and DF1 cells were transfected in triplicate with plasmids encoding PA, PB1, PB2-HA, NP, and the vNA-Luc reporter plasmid. Cells were lysed 24 to 48 h later in cell culture lysis reagent, and luciferase activity was measured by using the luciferase assay system (Promega). PB1, PB2, PA, and NP expressions were confirmed by Western blotting.

Immunoprecipitations. Cells were transfected with plasmids expressing PA, PB1, PB2-HA, NP-V5, and the luciferase reporter and lysed 24 to 48 h posttransfection in coimmunoprecipitation (co-IP) buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.5% NP 40). Clarified lysates were precleared and subjected to immunoprecipitation with the indicated antibodies. Immunocomplexes were captured on protein A Dynabeads (Invitrogen), washed extensively, and analyzed by Western blotting.

Immunofluorescence. HeLa cells were seeded onto coverslips and transfected as described above for the polymerase activity assay. Cells were fixed with 3% formaldehyde at 24 to 48 h posttransfection and blocked in 3% bovine serum albumin (BSA). PB2-HA was detected by anti-HA.11b followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibodies. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

Rescue of recombinant viruses. Plasmids expressing PA, PB1, and NP were reverse transfected alongside plasmids pTM Δ PB2 and pBD-PB2 into a coculture of 293T and MDBK cells. Twenty-four hours later, the medium was replaced with virus growth medium (VGM) (DMEM, 0.2% BSA, 25 mM HEPES buffer, and 1 μ g/ml tosyl phenylalanyl chloromethyl ketone [TPCK] trypsin). Viruses were harvested after an additional 24 to 72 h and amplified in MDBK cells. The titers of all viruses were determined in MDCK cells by using a protocol where cells are overlaid with medium containing 1.2% Avicel (catalog number RC581; FMC BioPolymer) (31). Viral RNA was isolated from the rescued viruses, and reverse transcription-PCR (RT-PCR) was conducted on the PB2 gene. Mutations in the PCR products were confirmed by sequencing and/or digestion with restriction enzymes targeting silent restriction sites incorporated during mutagenesis.

Multicycle replication assays. Cells were infected in triplicate in VGM at the indicated multiplicity of infection (MOI). Aliquots were taken throughout the infection, and titers were determined by a plaque assay. Infections were performed at 37°C for LMH cells and 33°C for Calu-3 cells.

Statistics. Data represent means $\pm$ standard deviations ($n \geq 3$). Mean activities for polymerase activity assays were determined and normalized to wild-type (WT) activity. The error was propagated to yield a normalized standard deviation. Where indicated, comparisons between groups were performed by a two-tailed Student *t* test.

RESULTS

PB2 aa 627 and the conserved PFAAAPP motif are important for polymerase function. The crystal structures of the PB2 627 domain (amino acids [aa] 538 to 693) from a human influenza virus isolate (PB2 627K) and an avian-like mutant (PB2 K627E) revealed a compact structure with six α -helices connected to five β -sheets by a flexible linker (27). This linker contains the important host-range-determinant residue 627 positioned in the solvent-exposed ϕ -loop (Fig. 1A) (27, 28). Since the homologous structures of the PB2 627 domain containing either Lys627 or Glu627 did not reveal any conformational differences but rather a difference in the surface charge, we generated a deletion mutant lacking aa 627 (PB2 Δ 627) to test the importance of this position without disrupting the positive surface charge. Polymerase activity assays were performed in cells expressing a luciferase-based viral reporter construct, NP, and the polymerase subunits PB1, PB2, and PA derived from A/WSN/33 (9, 30). As expected, polymerase with WT PB2 displayed significant amounts of activity in both human (293T) and avian (DF1) cells, whereas the avian-signature PB2 K627E was selectively impaired in human cells (9, 11, 14, 32, 33). In contrast, deletion of residue 627 completely ablated function in both human and avian cells. Western blotting confirmed equivalent expression of each PB2 variant, suggesting that residue 627 in PB2 is necessary for polymerase function (Fig. 1B).

The local region containing the ϕ -loop is similar in influenza A, B, and C viruses (27). Alignment of the amino acid sequences of this region (aa 610 to 641) from influenza A viruses isolated from different host species revealed a unique, highly conserved, proline-rich (P[P/F]AAAPP) motif between aa 620 and 626, including the newly identified isolates from little yellow-shouldered bats (Fig. 1A). This motif begins in α -helix 6 of the 627 domain immediately upstream of the ϕ -loop. We generated PB2 mutants with changes in the proline and phenylalanine residues within the motif (positions 620, 621, 625, and 626) and an additional point mutation at the conserved glutamine at position 628 just after the motif. The function of PB2 mutants was tested in polymerase activity assays. Mutation of P620 and F621 completely abolished polymerase activity in human and avian cells. Western blotting showed that the mutants were expressed at levels similar to those of the WT, suggesting that these residues are essential for PB2 function (Fig. 1C). Additionally, the other RNP proteins PB1, PA, and NP were expressed equivalently, excluding the possibility that the differences in polymerase activity resulted from changes in the levels of these proteins. The remaining mutations in the P[P/F]AAAPP motif and at residue 628 were well tolerated. PB2 P625A and Q628A displayed activity similar to that of the WT, whereas the P626A mutant possessed ~50% of the activity of the WT. Thus, despite their conservation, these residues are not essential for PB2 function in the minimalist polymerase activity assay.

The conserved basic face on the 627 domain of PB2 is important for polymerase function. The electrostatic surface potential calculations from the structure of the PB2 627 domain revealed that the domain presents a large, positively charged face surrounding residue 627 in proteins derived from human viruses, while this charge is disrupted by the introduction of the avian-signature PB2 E627. The positively charged face is composed of highly conserved basic residues that are present on the surface of the 627 domain (Fig. 2A) (27). To understand the contribution

FIG 1 Residue 627 and the P[P/F]AAAPP motif of the PB2 627 domain are important for polymerase activity. (A) Alignment of the conserved P[P/F]AAAPP motif of α -helix 6, the ϕ -loop, and β -sheet 1 from A/WSN/33 PB2 with consensus sequences compiled from human H1N1 isolates (from pre-2009 and the 2009 pandemic), swine H1N1 isolates, avian H5N1 isolates, and little yellow-shouldered bat isolates from 2009. (B and C) Polymerase activity assays were performed in human (293T) or chicken (DF1) cells expressing PB1, PA, NP, a vNA-luciferase reporter gene, and WT or mutant PB2-HA ($n = 3 \pm$ standard deviation). PB1, PB2, PA, and NP expressions were detected by Western blotting. Data are normalized to WT values.

FIG 2 Conserved basic residues on the surface of the PB2 627 domain are important for polymerase function. (A) Electrostatic surface potential of the PB2 627 domain (PDB accession number 2VY7) highlighting conserved residues that contribute to the basic face surrounding amino acid 627. (B and C) Polymerase activity assays were performed in human (293T) and chicken (DF1 or LMH) cells with the indicated PB2 mutants. RNP proteins were detected in cell lysates by Western blotting. Data are normalized to WT values ($n = 3 \pm$ standard deviation). PB2 K586A R589A mutants were modeled on the 627 domain (accession number 2VY7), and the predicted surface electrostatic charges for WT and mutant domains are shown.

that these basic residues make toward polymerase activity, and possibly toward species-specific function, the 627 domain was systematically mutated to remove some of the positive charge by making conservative substitutions to alanine. The resultant mutants were tested in a polymerase activity assay (Fig. 2B). As before, polymerase reconstituted with WT PB2 supported high levels of activity in both human and chicken cells, whereas PB2 K627E was active in chicken cells but severely restricted in human cells. The PB2 R646A and R650A mutants displayed dramatically reduced polymerase activity in these assays, with levels approaching background levels. The R597A, R604A, and R692A mutations reduced polymerase activity in both human and chicken cells, to less than 10% and 25% of the WT activity, respectively. This is similar to our previous findings that showed that the PB2 K627A mutant possessed ~25% of WT activity (9). A final pair of mutations, PB2 K586A and R589A, possessed a pronounced species-specific reduction in polymerase activity. The K586A mutant had at least a 10-fold reduction in polymerase activity in human cells but only a 2-fold reduction in chicken cells. Similarly, the activity of the R589A mutant had at least a 2-fold reduction in polymerase activity in human cells but no change in activity in chicken cells. Western blotting showed that PB1, PA, NP, and WT or mutant PB2 were all expressed at similar levels.

We confirmed these observations by creating a K586A R589A double mutant and performing activity assays in another chicken cell line (LMH) (Fig. 2C). Again, mutants retained activity in chicken cells but displayed a significant reduction in human cells. The double mutant was no more impaired than either single mutant, suggesting that residues 586 and 589 contribute to PB2 function in a similar fashion. Western blotting confirmed that all of the mutants were expressed at similar levels. In general, chicken cells were more tolerant of mutations in the basic face of PB2. Of those that showed activity, the basic face mutants displayed higher levels of activity in chicken than in human cells. This was particularly true for residues 586 and 589; modeling of the PB2 K586A R597A mutations onto the structure of the 627 domain removed a significant region of positive charge from the predicted electrostatic surface (Fig. 2C). However, chicken cells are not simply a more permissive environment for all polymerase activity, as mutations in the P[F/P]AAAPP motif displayed no cell type preference. Thus, mutagenic analysis identified key residues surrounding amino acid 627 that are essential for general polymerase activity (R597, P620, F621, R646, and R650), those that are conserved but not required for high-level activity (P625, P626, and Q628), and those that preferentially contribute to polymerase function in human cells (K586, R589, R597, R604, and R692).

PB2 mutants localize to the nucleus. The influenza virus polymerase functions in the nucleus of infected cells (34, 35). The polymerase subunits and NP are actively transported into the nucleus by host nuclear import factors that recognize nuclear localization signals (NLS) on the viral proteins. PB2 contains a bipartite NLS located at the extreme C terminus that is recognized by members of the importin- α family of import adaptors (26). The adaptive mutation D701N resides in this region and plays a role in the binding of PB2 to mammalian importins (36). Furthermore, structural studies of the PB2 C terminus indicate that the 627 domain is proximal to and can interact with the NLS (27). We therefore assessed the subcellular localization of our mutants to determine whether changes in the 627 domain alter nuclear localization. Polymerase activity was reconstituted in HeLa cells as de-

FIG 3 PB2 627 domain mutants localize to the nucleus. PB1, PA, NP, a vNA-luciferase reporter gene, and the indicated PB2-HA proteins were expressed in HeLa cells. The subcellular localization of PB2 (green) was determined by immunofluorescent detection. A PB2 NLS mutant containing disruptions in both regions of the bipartite nuclear localization signal (K738Q, K752N, and R755Q) was included. The nucleus is stained with DAPI (blue). DIC, differential interference contrast.

scribed above, and subcellular distribution was assessed by immunofluorescence (Fig. 3). WT PB2 localized to the nucleus, colocalizing with DAPI staining, whereas PB2 with a mutated NLS was dispersed in the cytoplasm. All of our mutants, regardless of functionality, localized to the nucleus similarly to the WT. Thus, the defects in polymerase function are not due to a failure to enter the nucleus, and neither the structure imposed by the conserved P[P/F]AAAPP motif nor the charge of the 627 domain surface is essential for nuclear import.

Failure of polymerase mutants to assemble RNPs. Current models suggest that PB1 and PA are transported into the nucleus as a dimer, whereas PB2 is imported by itself (37). Following nuclear import, the polymerase subunits assemble into the trimeric holoenzyme. The holoenzyme further assembles with genomic RNA and NP to form higher-order vRNPs. Defects or the intentional perturbation of any of these assembly steps can impair polymerase activity (9, 11, 38–40). Polymerase activity was reconstituted with the P[P/F]AAAPP or basic face mutants, and immunoprecipitation assays were used to test whether impaired polymerase activity resulted from defects in the assembly process. Interactions between PB1 and PB2 were used to measure trimer formation. The WT and all of the PB2 mutants coprecipitated similar amounts of PB1, suggesting that neither the P[F/P]AAAPP motif (Fig. 4A) nor the basic face of the 627 domain (Fig. 4B) is required for stable interactions with PB1. This is consistent with structural and functional analyses localizing the primary PB1–PB2 interface to the extreme N terminus of PB2 (41, 42). A second PB1 binding interface has been identified at the C terminus of PB2, and this too appears to be unaffected by 627 domain mutations (43).

vRNP assembly was monitored by assessing coprecipitation of PB2 by NP. In stark contrast to trimer formation, PB2 mutants with impaired polymerase activity failed to assemble vRNPs as efficiently as the WT. NP coprecipitated significantly smaller amounts of PB2 K627E from human cells than the WT (Fig. 4A and B), in agreement with previous findings (9, 11, 12, 40). The amount of coprecipitated PB2 was directly related to polymerase activity for all of the P[F/P]AAAPP and basic face mutants. For example, NP coprecipitated very small amounts of the severely

impaired PB2 P620A and F621A mutants, whereas intermediate amounts were coprecipitated by NP for the PB2 K586A and R589A mutants with intermediate levels of polymerase activity. When these experiments were performed in chicken cells, where the PB2 K586A, R589A, and K627E mutants displayed significant amounts of activity, the mutants formed polymerase trimers and associated with NP at levels indistinguishable from those of the WT (Fig. 4C).

Polymerase mutations reduce viral replication. Our polymerase activity assays identified mutants with general defects in polymerase function (R597, P620, F621, R646, and R650) as well as those that retain high levels of activity in avian cells but are restricted in human cells (K586A and R589A). To determine the impact of these mutations on virus replication, we rescued mutant viruses and performed multicycle replication assays. Chicken cells were infected with virus encoding the P[F/P]AAAPP mutants (Fig. 5A). Both WT and the avian-signature PB2 K627E viruses replicated to high levels. P[F/P]AAAPP mutants with high polymerase activity supported high levels of virus replication; viruses encoding PB2 P625A, P626A, or K627E all replicated with kinetics and titers comparable to those of the WT, while the PB2 Q628A mutant was slightly attenuated. Conversely, mutants with low polymerase activity displayed obvious defects in replication. We were unable to rescue virus encoding PB2 F621A. To our surprise, PB2 P620A, which possessed ~0.1% of the polymerase activity of the WT, produced virus, although its replication was severely impaired, reaching titers only 2 to 3 logs lower than those of the WT. Sequencing of PB2 cDNA from the PB2 P620A virus confirmed that this virus was a mutant and had not reverted during replication (data not shown). Thus, residues 620 and 621 play critical roles in both polymerase activity and virus replication.

Replication assays were subsequently performed with basic face mutants. Mutant viruses encoding PB2 K586A, R589A, R597, K627E, or R692A all replicated to high levels in chicken cells, similarly to the WT (Fig. 5B). Notably, all of these mutants possessed levels of polymerase activity at least 25% of that of the WT (Fig. 2B). Replication by the PB2 R646A virus was highly attenuated in both chicken (Fig. 5B) and human (Fig. 6) cells, in agreement with

FIG 4 Defective PB2 mutants fail to form vRNP complexes. Polymerase activity was reconstituted with the indicated PB2 proteins, and cell lysates were subjected to immunoprecipitation of PB2 (anti-HA) to assess polymerase trimer formation and NP (anti-V5) to assess vRNP formation. Coprecipitating proteins and input levels were detected by Western blotting. (A and B) P[F/P]AAAPP mutants (A) and basic face mutants (B) were precipitated from human (293T) cells. (C) Select basic face mutants were analyzed in chicken (LMH) cells.

its severe defect in polymerase activity. However, this mutant produced a relatively high viral titer given its dramatic defect in polymerase activity (Fig. 2B). We were unable to rescue the other severe polymerase mutants PB2 R597A and R650A. To determine if the species-specific defects in the polymerase activity of PB2

FIG 5 627 domain mutants reduce virus replication. Multicycle replication kinetics were determined for P[F/P]AAAPP (A) and basic face (B) mutant viruses in avian cells. Recombinant viruses encoding WT or mutant PB2 were used to infect LMH cells (MOI = 0.01). Virus yield at the indicated time points was determined by a plaque assay ($n = 3$ independent infections $\pm$ standard deviation).

K586A, R597A, and K627E also manifest species-specific changes in replication, infections were performed again in human lung epithelial cells. WT virus replicated to high levels, whereas the prototypical PB2 K627E mutant carrying the avian-signature change replicated with titers approximately 5- to 10-fold lower than those of the WT (Fig. 6). In contrast, WT and PB2 K627E titers were indistinguishable in infections performed in chicken cells. Similarly, PB2 K586A and R589A viruses displayed replication defects in human cells, with titers reduced up to 30-fold (Fig. 6), but no detectable change in viral titers from chicken cells. The defective replication of these three mutants was significantly dif-

FIG 6 Species-specific restriction of basic face mutant virus replication in human cells. Viruses encoding WT or mutant PB2 were used to initiate infections in human Calu-3 cells (MOI = 0.001). Virus yield at the indicated time points was determined by a plaque assay ($n = 3 \pm$ standard deviation). *, the titer for PB2 K586A was below the limit of detection at 8 hpi.

ferent from the replication of the WT from 16 h postinfection (hpi) onward ($P < 0.005$ by t test). Together, our replication data showed that PB2 mutants with general defects in polymerase function (i.e., P620A, F621A, and R650A) did not replicate or were severely impaired, whereas the species-specific defects in polymerase activity of PB2 K586A and R589A were recapitulated as species-specific defects in replication in human but not avian cells. Furthermore, the unexpected replication of the PB2 P620A and R646A viruses highlights the importance of understanding polymerase activity in the context of a viral infection in addition to the minimalist polymerase activity assay.

DISCUSSION

Structures of the PB2 C terminus containing the 627 domain revealed a highly conserved basic surface surrounding residue 627 in human influenza A virus isolates (15, 27, 28). This surface charge is disrupted by the presence of the avian-signature glutamic acid at position 627 while maintaining a nearly identical structure. A key element of this structure is the conserved P[F/P]AAAPP motif preceding residue 627. To further understand the importance of the conserved structure and charge of this domain, and their role in species-specific polymerase function, we disrupted both by mutating conserved residues and tested their effect on polymerase activity and virus replication. Our results show that perturbing the structure by mutating P620 or F621 dramatically impaired polymerase function and virus replication. These residues are in the α -helix before residue 627 and cause a slight kink in the helix, suggesting that they are critical for the correct positioning of residue 627 and conformation of the ϕ -loop. The results also demonstrate that most of the conserved basic residues are important for function, as mutation severely reduced polymerase activity and attenuated virus replication. Specifically, residues R597, R604, R646, and R650 were required for general polymerase ac-

tivity, whereas residues K586 and R589 are preferentially required for activity in human but not avian cells. These findings highlight the importance of the unique fold of the 627 domain and the basic nature of the entire surface, not only amino acid 627.

The species-specific requirement for PB2 K586 and R589 is similar to the well-characterized species-specific requirement for PB2 K627 (9, 11, 21, 32). For each of these residues, the presence of a basic charge is more important in human cells than in avian cells. Residues 586 and 589 cluster together in the structure adjacent to residues 627 and 591, a site recently identified as being important for the adaptation of the 2009 pandemic virus to mammals (14, 15). PB2 from the 2009 pandemic virus encodes the avian-signature residue E627. Acquisition of an arginine at residue 591, part of the so-called SR polymorphism, causes a slight change in surface topology and partially neutralizes the charge of the glutamic acid in these proteins. Restoration of the basic face by introducing either arginine, as in the 2009 virus, or lysine, as is the case for a primary human H5N1 isolate (A/Indonesia/UT3006/2005), led to increased polymerase activity, virus replication, and pathogenicity (14, 15). Residues 588 and 636 are also localized to this same area of the protein and have been bioinformatically and experimentally identified as positions affecting species-specific polymerase function and viral host range (29, 32, 44). The proximity of residues 586, 588, 589, 591, 627, and 636 in the structure together with the new functional data reported here show that this larger area is vital for species-specific regulation.

Multiple other evolutionary strategies that adapt avian polymerases to replication in mammalian hosts have been identified (8, 10, 14–16, 21, 45–47). Nonetheless, the molecular mechanisms governing restriction in mammals remain unclear. We and others have shown that restricted polymerases fail to assemble RNPs (9, 11, 40, 48). RNPs are assembled in the nucleus from the polymerase trimer, NP, and genomic RNA. The 627 domain is near the NLS for PB2, and it has been suggested that restricted PB2 cannot utilize the appropriate mammalian importin- α during nuclear import (36, 49). However, none of the restricted PB2 proteins display defects in nuclear localization (Fig. 3) (9, 12, 50). Moreover, while biochemical analysis has shown that the adaptive PB2 D701N mutation increases the affinity for importin- α and may explain its enhanced activity in humans, this was not the case for residue 627, as both PB2 K627 and PB2 E627 bound with similar affinities (51). Thus, the role of importins in restriction, during either import or an undefined postimport step, is unclear.

Once in the nucleus, a *trans*-acting polymerase is thought to replicate genomic RNA by using preexisting RNPs as a template and assemble RNPs *de novo* by binding free NP and the nascent genome (52). NP interacts directly with both PB1 and PB2. Assembly failure of restricted polymerases is not due to a disruption of the PB2-NP interface, as restricted PB2 binds NP at levels similar to those of active PB2 (9, 40). It has been suggested that assembly failure may result from limiting amounts of genomic RNA, possibly due to reduced enzymatic activity and genome replication of the restricted polymerase (40). This possibility could explain our data showing that defective polymerases fail to assemble RNP (Fig. 4), independent of whether the defects are species specific. However, this does not appear to be the case for species-specific restricted polymerase. Restriction does not block intrinsic polymerase activity, as both PB2 K627 and E627 polymerases purified from human cells possess similar levels of *in vitro* polymerase activity (53, 54). This agrees with more recent results showing

that WT or PB2 K627E polymerases function equivalently in human cells when provided short vRNA templates that do not require NP or RNP assembly for replication (54). Thus, these data support a model where the restriction phenotype does not result from intrinsic differences in enzymatic activity but rather from a specific defect in RNP assembly.

The polymerase mutants PB2 P620A and R646A possessed exceedingly low levels of activity in the polymerase activity assay yet supported moderate to high levels of virus replication. While the polymerase activity assay is a useful proxy for measuring polymerase function, it does not fully recapitulate polymerase function during a viral infection (55). This suggested that additional viral factors could modulate polymerase activity during infection and potentially overcome the defects detected in the polymerase activity assay. Both NP and nuclear export protein (NEP) have been shown to drive polymerase activity away from transcription toward replication (55, 56). NS1 is also known to modulate polymerase activity (57, 58), associate with viral RNPs (59, 60), and disrupt host mRNA processing and export (61, 62), all of which could contribute to the restored polymerase activity. We also cannot exclude the possibility that pseudorevertant mutations arose in the replicating PB2 P620A or R646A mutant. We confirmed the presence of the desired mutation in these recombinant viruses, but there may be additional second-site suppressor mutations in other viral genes that restore polymerase activity and contribute to increased replication. In both of these scenarios, mutations that would otherwise impair polymerase activity may be buffered by other viral factors and/or compensatory changes permitting the replication machinery to explore an expanded evolutionary space as it adapts to a new host (63).

The mechanism of RNP assembly and polymerase restriction is likely to involve a complex interplay between viral and host factors that remains to be determined. The data reported here expand the region of PB2 that is involved in this process. Given the central role that the polymerase plays during infection, an understanding of these events will be important for predicting the replication potential and pathogenicity of emerging influenza viruses.

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