Reassortment between Seasonal H1N1 and Pandemic (H1N1) 2009 Influenza Viruses Is Restricted by Limited Compatibility among Polymerase Subunits

Cássio Pontes Octaviani,1 Hideo Goto,1 and Yoshihiro Kawaoka1,2,3,4,*

Division of Virology, Department of Microbiology and Immunology,1 and Department of Special Pathogens, International Research Center for Infectious Diseases, The Institute of Medical Science, The University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan;2 ERATO Infection-Induced Host Responses Project, Japan Science and Technology Agency, Saitama 332-0012, Japan;3 and Influenza Research Institute, Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, Madison, Wisconsin 537114

Received 7 May 2011/Accepted 1 June 2011

Reassortment is important for influenza virus evolution and the generation of novel viruses with pandemic potential; however, the factors influencing reassortment are still poorly understood. Here, using reverse genetics and a replicon assay, we demonstrated that a mixed polymerase complex containing a pandemic (H1N1) 2009 influenza virus PB2 on a seasonal H1N1 virus background has reduced polymerase activity, leading to impaired virus viability. Adaptation of viruses containing the mixed polymerase complex resulted in compensatory mutations in PB1. Taken together, our results identify the cooperation between PB2 and PB1 as an important restricting factor for reassortment of influenza viruses.

Influenza A viruses are enveloped viruses of the family Orthomyxoviridae and have a genome composed of eight single-stranded, negative-sense RNA segments. This segmented genome allows reassortment, which is an important mechanism for the generation of new viruses, such as those that sporadically cause pandemics in humans (6).

Influenza pandemics are caused by the introduction of a virus with a hemagglutinin (HA) subtype that is new to human populations, which can result from reassortment (15). However, at least the three last pandemic viruses, i.e., the 1957 “Asian influenza,” the 1968 “Hong Kong influenza,” and the pandemic (H1N1) 2009 influenza viruses, contained, besides the HA gene, one or more polymerase subunits from a non-human source (4, 11, 14), which suggests the importance of these genes for the generation of pandemic viruses through reassortment. However, limited compatibility among polymerase subunits from different viruses is also a restricting factor for reassortment (2, 7, 8).

The emergence and establishment of the pandemic (H1N1) 2009 influenza virus in the human population have created a new scenario of possibilities of reassortment with the contemporary, cocirculating viruses, including seasonal H3N2 and H1N1 viruses (3, 12). Therefore, knowledge of the factors limiting reassortment between the pandemic (H1N1) 2009 influenza virus and contemporary human viruses is important for a complete understanding of the factors that will shape the evolution and emergence of future epidemic and pandemic viruses.

In previous studies, when we used reverse genetics (9) to generate a series of single-gene reassortants containing each of the A/California/04/2009 (H1N1) (CA04) genes on an A/Kawasaki/UT-SAI-23/2008 (H1N1) (k23) genetic background, we found that some of the reassortants could not be rescued after three independent attempts; notably, reassortants containing the PB2, PA, or NP genes derived from CA04 on a k23 background could not be generated. As this suggested a limited level of genetic compatibility among ribonucleoprotein (RNP) components between the two viruses, we wished to investigate further this finding, by means of a luciferase-based replicon assay. Plasmid pPolINP(0)luc2(0) (50 ng), which contains the firefly luciferase gene flanked by the noncoding regions of the NP gene derived from A/WSN/33 (WSN), was cotransfected into human embryonic kidney (HEK) 293 cells grown in 48-well plates, along with the protein expression plasmids (pCAGGS/MCS [5, 10]) containing the PB2, PB1, PA, and NP genes derived from k23 and CA04 (50 ng each) in all possible 16 combinations, by using the Trans IT 293 transfection reagent (Mirus, Madison, WI) (3 μl of transfection reagent per microgram of plasmid); 24 h posttransfection, the cells were assayed for luciferase activity by using a Bright-Glo luciferase assay system and a GloMax 96 microplate luminometer (Promega, Madison, WI). We found that the activities of the heterogeneous RNP complexes varied substantially. Although the introduction of CA04 PB1, PA, or NP genes in the k23 RNP background caused some reduction in polymerase activity relative to the all-k23 RNP complex, the introduction of CA04 PB2 caused the greatest reduction in the polymerase activity (Fig. 1A). Interestingly, all mixed RNP complexes that contained a PB2 derived from CA04 in combination with a PB1 derived from k23 showed markedly reduced activity, indicating a possible incompatibility between these two genes.

Next, we wanted to investigate the relevance of this genetic incompatibility in the context of virus growth. However, most of the single gene reassortants containing CA04 RNP compo-
A means that cells. Plaques were measured at 48 h postinfection; values represent remaining genes are derived from WSN. (C) Plaque size in MDCK CA04 are shown in gray-shaded and open boxes, respectively; the measured by plaque assay in MDCK cells. Genes derived from k23 and did not match that of the virus growth of the corresponding

The results from the replicon assay, combined with the differential growth capability of reassortant viruses, point to a limited compatibility of the CA04 PB2 with the remaining polymerase subunits from k23. To determine the region in PB2 responsible for this deficiency, we constructed a series of chimeric PB2 expression plasmids in which different extensions of the genes derived from k23 and CA04 were exchanged (Fig. 2A). The chimeric plasmids, along with expression plasmids for PB1, PA, and NP derived from k23, were used in the replicon assay, as described above. Recombinant RNP complexes containing the chimeric PB2s on a k23 background showed substantial variation in polymerase activity; while substitution for either the N- or C-terminal region of the k23 PB2 by CA04 did not appreciably reduce the activity, constructs containing the central region derived from CA04 PB2 showed a substantial loss of activity of the recombinant RNP complexes (Fig. 2A), suggesting that this region of CA04 PB2 is responsible for its relative inability to function in combination with the remaining RNP components derived from k23.

Reassortant viruses containing the CA04 PB2 with the remaining RNP components derived from k23 showed substantially impaired growth capability, compared with viruses containing either pure k23 or CA04 RNP complexes (Fig. 1B and C). Therefore, we wished to investigate the possibility of adaptation of viruses containing the hybrid RNP complexes. To this end, we subjected the virus CA04PB2-k23RNP-WSN to serial passage in MDCK cells. After five passages, partial restoration of virus growth capability and plaque formation ability was observed (Fig. 2B and C). Sequencing analysis of the adapted viruses showed compensatory mutations in the PB1 gene in three independent experiments (V285I in one experiment and I423T in two experiments) and also a mutation in NP (T423R) in one experiment (Fig. 2B). No mutation was observed in any other genes. To identify the relevance of each of the compensatory mutations for the polymerase activity, we cloned each of the adapted genes into pCAGGS/MCS and used them in combination with the plasmids for the remaining RNP components of CA04PB2-k23RNP-WSN in the replicon assay. As shown in Fig. 2D, only the mutation PB1 I423T corresponded with a significant increase in polymerase activity of the mixed RNP complex. Moreover, a reassortant virus containing the CA04 PB2 on a k23 background containing the PB1 I423T mutation (designated CA04PB2-k23PB1[I423T]-k23) could be generated by reverse genetics. These findings, combined with the severely impaired activity of all mixed RNP complexes containing a CA04 PB2 in combination with a k23 PB1 (Fig. 1A), indicate that the interaction between PB2 and PB1 is an important restricting factor for influenza virus reassortment and that an I423T mutation in PB1 can overcome the restriction of polymerase activity of the hybrid RNP complex. Intriguingly, the mutation PB1 V285I, despite improving the growth of CA04PB2-k23RNP-WSN, did not confer substantial polymerase activity in the replicon assay. The mechanism for

FIG. 1. (A) Polymerase activities of 16 RNP gene combinations measured in a replicon assay. Four expression plasmids (PB2, PB1, PA, and NP) for the 16 RNP gene combinations between k23 and CA04, together with pPolINP(0)luc2(0) for the production of virus-like RNA encoding the reporter luciferase gene, were transfected into HEK 293 cells and assayed for luciferase activity after 24 h of incubation at 37°C. The values shown are means ± standard deviations for three independent experiments and are standardized to the activities of the expression plasmids for the CA04 RNP complex proteins (100%). Genes derived from k23 and CA04 are shown in gray-shaded and open boxes, respectively. (B) Rescue efficiency and titers of stock viruses. Virus derived from k23 and CA04 are shown in gray-shaded and open boxes, respectively. (C) Plaque size in MDCK cells. Plaques were measured at 48 h postinfection; values represent means ± standard deviations (n = 50). The genetic composition of viruses is the same as that indicated in panel B.
the partial restoration of viral growth by this mutation remains unclear.

Inability to form a heterotrimer is one possible mechanism for incompatibility among polymerase subunits between different viruses (7), so we wondered whether this could explain the incompatibility between CA04 PB2 and k23 PB1. To investigate this, we performed immunoprecipitation assays. Lysates prepared from HEK 293 cells transfected with expression plasmids for k23 PB2, k23 PB1-FLAG (a k23 PB1 with a C-terminal FLAG tag), and k23 PA, or CA04 PB2, k23 PB1-FLAG, and k23 PA, were immunoprecipitated by use of an anti-FLAG M2 affinity gel (Sigma, St. Louis, MO). The agarose-bound proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotted with mouse monoclonal anti-PB2 or anti-FLAG antibodies. (A) Cell lysis and immunoprecipitation were carried out in buffer containing 0.15 M NaCl, and 1% NP-40 and 0.1% SDS (a), 2% NP-40 and 0.2% SDS (b), or 5% NP-40 and 0.5% SDS (c), as indicated.

FIG. 3. Immunoprecipitation of polymerase subunits at different salt and detergent concentrations. HEK 293 cells grown in 6-well plates were cotransfected with 750 ng each of expression plasmids for k23 PA, k23 PB1-FLAG, and k23 PB2 or CA04 PB2, as indicated (+). At 24 h posttransfection, cells were lysed and the cleared lysates were immunoprecipitated with anti-FLAG M2 affinity gel (Sigma, St. Louis, MO). The agarose-bound proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotted with mouse monoclonal anti-PB2 or anti-FLAG antibodies. (A) Cell lysis and immunoprecipitation were carried out in buffer containing 0.15 M Tris-HCl, pH 7.8, 1% NP-40, 1 mM EDTA, protease inhibitor cocktail) with different salt concentrations, as indicated. The cell lysates were also directly Western blotted with mouse monoclonal anti-PB2 or anti-FLAG antibodies. (B) Cell lysis and immunoprecipitation were carried out in buffer containing 0.15 M NaCl, and 1% NP-40 and 0.1% SDS (a), 2% NP-40 and 0.2% SDS (b), or 5% NP-40 and 0.5% SDS (c), as indicated.
PB2s pointed to the central region of the gene (Fig. 2A), while adaptation of virus CA04PB2-k23RNP-WSN caused a compensatory mutation in amino acid 423 of PB1 that was able to restore the functionality of the mixed RNP complex to some extent (Fig. 2B, C, and D). However, this position has not been identified as an interaction site between these two proteins in immunoprecipitation experiments, as reported by other groups (reviewed in reference 1), which is in agreement with our finding that CA04 PB2 interacted with k23 PB1 as well as k23 PB2. The reason for the limited activity of the mixed RNP complex therefore remains unclear and is probably due to subtler functional constraints: that is, the level of incompatibility between the two proteins was not explained by the lack of heterotrimer formation, yet the resulting hybrid complex was nonetheless functionally impaired. Isoleucine at position 423 is highly conserved (approximately 99.7%) among influenza A viruses, and although analysis of 3156 unique influenza A virus PB1 sequences, including H1N1, H3N2, and H5N1 subtypes, showed two amino acid substitutions at this position (leucine and valine), threonine was not found. While the significance of this is not clear, the high degree of conservation of isoleucine at this position may indicate that adaptation through an I423T mutation could be restricted due to additional constraints.

The findings reported here contrast with previous observations by our group, in which a high degree of genetic compatibility was found between pandemic (H1N1) 2009 influenza virus and a contemporary H5N1 virus, with all combinations of RNP components between those two viruses showing substantial polymerase activity and producing viruses with high growth capability (13). Further efforts are required for a thorough understanding of the factors governing reassortment in influenza viruses, as this will improve our ability to understand, predict, and control influenza epidemics and pandemics in the future.

We thank Susan Watson for scientific editing.

This work was supported by a contract research fund from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, and in part by Grants-in-Aid for Specially Promoted Research and for Scientific Research, by ERATO (Japan Science and Technology Agency), by the National Institute of Allergy and Infectious Diseases Public Health Service research grants, and by the Center for Research on Influenza Pathogenesis (CRIPT) funded by the National Institute of Allergy and Infectious Diseases (contract HHSN266200700010C).

REFERENCES