

Surface glycoproteins determine the feature of the 2009 pandemic H1N1 virus

Jin Il Kim^{1,2,#}, Ilseob Lee^{1,2,#}, Sehee Park^{1,2,#} & Man-Seong Park^{1,2,*}

¹Department of Microbiology, ²Center for Medical Science Research, College of Medicine, Hallym University, Chuncheon 200-702, Korea

After the outbreak of the swine-origin influenza A H1N1 virus in April 2009, World Health Organization declared this novel H1N1 virus as the first pandemic influenza virus (2009 pH1N1) of the 21st century. To elucidate the characteristics of 2009 pH1N1, the growth properties of A/Korea/01/09 (K/09) was analyzed in cells. Interestingly, the maximal titer of K/09 was higher than that of a seasonal H1N1 virus isolated in Korea 2008 (S/08) though the RNP complex of K/09 was less competent than that of S/08. In addition, the NS1 protein of K/09 was determined as a weak interferon antagonist as compared to that of S/08. Thus, in order to confine genetic determinants of K/09, activities of two major surface glycoproteins were analyzed. Interestingly, K/09 possesses highly reactive NA proteins and weak HA cell-binding avidity. These findings suggest that the surface glycoproteins might be a key factor in the features of 2009 pH1N1. [BMB Reports 2012; 45(11): 653-658]

INTRODUCTION

Influenza A virus (IAV), a member of the *Orthomyxoviridae*, contains eight segmented RNA genomes within a viral membrane (1, 2). Retrospective studies of the reconstructed 1918 influenza virus revealed that the NS1 (3), hemagglutinin (HA) (4, 5), neuraminidase (NA) (6), PB1-F2 (7), and replication complex (PB2, PB1, PA, and NP) (8) proteins determined the high virulence of the 1918 'Spanish Flu' (H1N1). The well-organized genetic network of 1918 H1N1 allowed this virus to efficiently infect humans, and it claimed an estimated 50 million lives. Two subsequent influenza pandemics, the 1957 'Asian Flu' (H2N2) and 1968 'Hong Kong Flu' (H3N2), resulted from the introduction of avian IAV-derived genes into human IAV (H1N1) (1, 9). When such events occur, humans have no immunity against the novel IAV, and they are vulnerable to infection. Of the two major surface glycoproteins, HA is indispensable for mediating the in-

vasion of host cells, and NA terminates the viral invasion by freeing the progeny virions from the infected cell. HA and NA continuously change their antigenicity through mutations in their amino acid residues or changes in the glycosylation patterns (10-12). Due to this ever-changing antigenicity, new trivalent vaccine viruses against seasonal IAV are selected and recommended by the World Health Organization (WHO) each year.

In April 2009, the first outbreak of the swine-origin influenza A H1N1 virus (S-OIV) was reported in North America (13). Unlike the swine influenza viruses previously characterized in humans, which were poorly transmissible (14, 15), this novel S-OIV produced a number of human infections in many countries and swept through the global community in a very short time. In June 2009, this S-OIV was declared to be the first pandemic influenza A H1N1 virus (2009 pH1N1) of the twenty-first century. Although 2009 pH1N1 virus has an avian-type glutamic acid (E) instead of a human-type lysine (K) at PB2-627, which is disadvantage to be a pandemic virus, 2009 pH1N1 caused high pathogenicity and efficient transmission in animal models (16-18). However, it remains unclear which factors of 2009 pH1N1 contributed to the pandemic characteristics.

In this study, we first compared the growth properties of K/09 to that of control virus S/08 in cells. Interestingly, K/09 had a higher growth than S/08 in three different cell lines. However, K/09 encoded less competent replication complex and non-structural protein 1 (NS1) than did the control virus. To understand the observed efficient growth in cells, the activities of K/09 surface glycoproteins were compared with those of control virus. In a MU-NANA assay, the K/09 NA was found to be highly reactive, and the K/09 HA demonstrated weak cell-binding avidity in an agglutination assay with RDE-treated chicken RBCs. Overall, our findings indicate that the unique properties of 2009 pH1N1 might be attributed to the features of its surface glycoproteins.

RESULTS

Growth properties of the 2009 pH1N1 virus in different cells

To assess the growth properties of the 2009 pH1N1 virus in cell lines, MDCK, LLC-PK1, and A549 cells were inoculated with K/09 at a multiplicity of infection (MOI) of 0.01 and maintained in 0.3% BSA/MEM with or without 1 µg/ml of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin to

*Corresponding author. Tel: +82-33-248-2632; Fax: +82-33-252-2843; E-mail: ms0392@hallym.ac.kr

#These authors contributed equally to this work.
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inhibit contaminating chymotrypsin activity (Fig. 1). Supernatants were collected at 8, 16, 24, 48, and 72 hours post-inoculation (hpi) and titrated using a plaque assay in MDCK cells. S/08 was used as a control virus. A/WSN/33 (WSN), which was used as another control virus, was not discussed here because it exhibited strong growth property in the presence or absence of exogenous trypsin as previously noted (19). In MDCK cells, both K/09 and S/08 grew to more than 10^7 pfu/ml at 24 hpi in the presence of 1 μ g/ml TPCK-treated trypsin (Fig. 1B). At 8 hpi, both viruses had replicated to more than 10^4 pfu/ml. However, this parallel growth curve was not sustained; K/09 managed to replicate up to $10^{5.68}$ pfu/ml without trypsin, while S/08 failed to maintain its growth ($< 10^5$ pfu/ml) (Fig. 1A). Both viruses grew less in A549 cells than in MDCK cells. However, K/09 grew to a higher titer than did S/08 in both the presence and absence of trypsin (Fig. 1C and D). In LLC-PK1 cells, K/09 demonstrated unique growth properties. Without trypsin, K/09 outgrew S/08 by almost two log scales at 16 dpi, and this difference in growth increased more at later time points (Fig. 1E). At 48 hpi, K/09 grew to more than 10^6 pfu/ml when supplemented with trypsin. In contrast, S/08 only yielded 10^4 pfu/ml (Fig. 1F). Taken together, these results indicate that the growth of K/09 is more robust than that of S/08 in MDCK, A549, and LLC-PK1 cells, and K/09 can maintain a robust replication property even in the absence of exogenous trypsin.

Replication properties of the 2009 pH1N1 virus RNP complex

To assess the properties of the K/09 RNP complex, four expression plasmids (pCAGGSII-PB2, -PB1, -PA, and -NP) bearing

S/08, and K/09 RNP components were constructed. pPoll/K09:NA-Gaussia luciferase (pPoll-Gluc) was used as a reporter plasmid (20). In a mini-genome assay, the RNP complex activities of 16 combinations between S/08 and K/09 RNP components were evaluated in 293T and MDCK cells, respectively. To standardize fluorescence expression levels, the fluorescence value of each of the 16 combinations was converted to that of wtS/08 (100%).

In 16 RNP combinations of S/08 and K/09 RNP complex components, the wtS/08 RNP complex had superior activity to the wtK/09 RNP complex. Interestingly, the RNP activities of hybrid combinations were dependent on K/09-PB1. When S/08 RNP components were combined with K/09-PB1 (PB2/PB1/PA/NP = S/K/S/S), the RNP activity was reduced by more than 20% compared to the completely wtS/08 combination (S/S/S/S). In contrast, when K/09 RNP components were combined with S/08-PB1 (K/S/K/K), the activity of the RNP complex increased by more than 20% compared to the completely wtK/09 RNP combination (K/K/K/K) (Fig. 2). Even though a reporter plasmid, pPoll-Gluc, was constructed using K/09-NA non-coding regions, hybrid RNP combinations showed lower activities with K/09-PB1 but retained higher activities with S/08-PB1. These results indicate that the replicative capacity of K/09 is lower than that of S/08.

The NS1 activity of the 2009 pH1N1 virus

It was known that the NS1 protein of influenza virus plays an important role in inhibition of host immune responses (21, 22). Thus, to evaluate the contribution of the NS1 protein of 2009

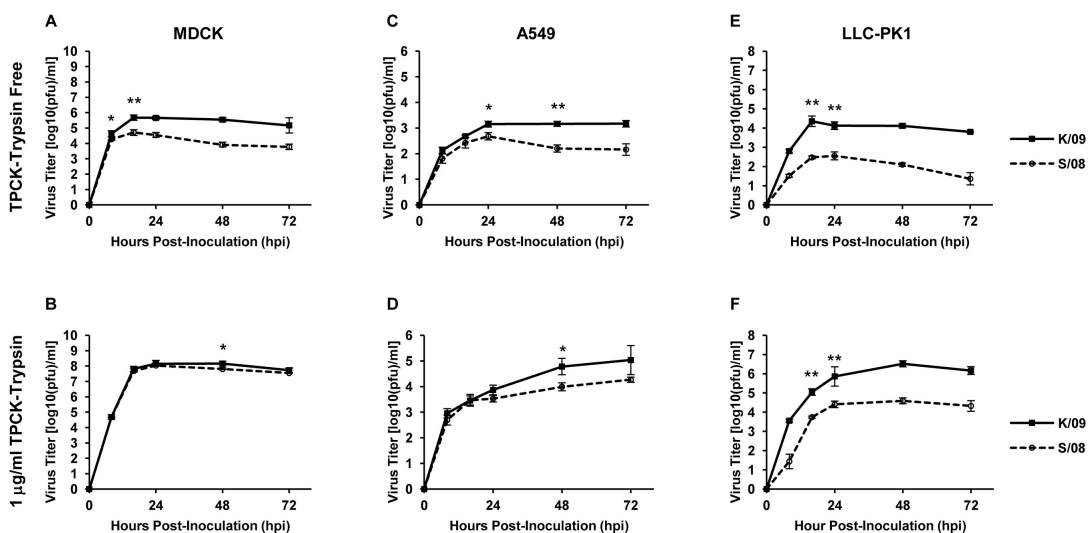


Fig. 1. Growth properties of K/09 and S/08 viruses in cultured cells. Monolayers of MDCK (A, B), A549 (C, D), or LLC-PK1 (E, F) cells were inoculated with K/09 and S/08 viruses at an MOI of 0.01. At one hour after inoculation, the cells were washed five times with PBS and incubated with appropriate media in the absence (A, C, E) or presence (B, D, F) of 1 μ g/ml of TPCK-treated trypsin at 37°C. Supernatants were collected at 8, 16, 24, 48, and 72 hpi, and the viral titer was determined by plaque assay in MDCK cells. The detection limit of viral growth was 10 pfu/ml. The statistical significance of the growth difference between K/09 and S/08 viruses was assessed by the Student's t-test (*P < 0.05; **P < 0.01).

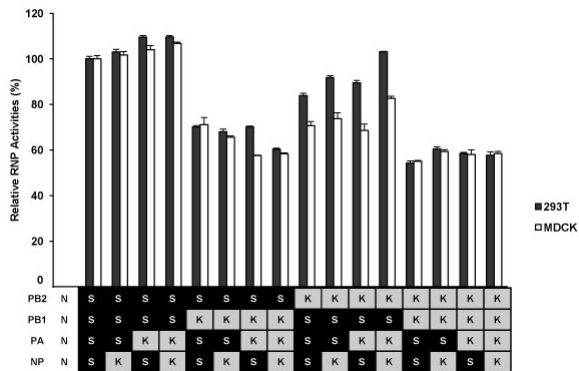


Fig. 2. Relative RNP activities of K/09 and S/08 viral plasmids in a mini-genome assay. The relative activities of the 16 possible RNP combinations (PB2, PB1, PA, and NP) between K/09 and S/08 viral plasmids were measured in 293T and MDCK cells using pPoll/K09:NA-Gussia luciferase. Four RNP plasmids and a reporter plasmid were transfected together into cells. After 24 hours of incubation at 37°C, the relative luciferase activity of each combination was measured and converted to that of the wild-type S/08 RNP complex (S/S/S). The results were expressed as the mean value of two independent experiments, measured in triplicate (N, negative control; S, S/08; K, K/09).

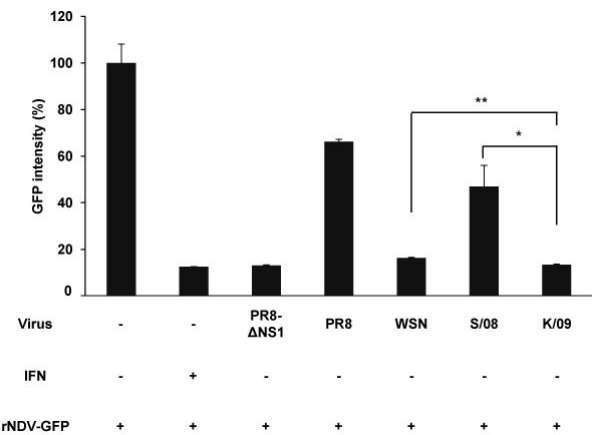


Fig. 3. Analysis of NS1 activity to impede the interferon production. The ability of NS1 to inhibit interferon production was determined by rNDV-GFP bioassay. The K/09 and control viruses were inoculated into monolayers of A549 cells at the same multiplicity of infection (MOI = 1). After one hour, A549 cells were washed three times with PBS and incubated with 0.3% BSA/DMEM. Supernatants were collected at 24 hpi, UV-treated, and incubated with Vero cells for 16 hours. Vero cells were then washed three times with PBS and subsequently inoculated with rNDV-GFP virus at an MOI of 1. GFP expression was measured by a fluorescence reader at 24 hpi. The results represent the mean value of two independent experiments measured in triplicate. The statistical significance of the relative NS1 activity between viruses was assessed by use of the Student's t-test (*P < 0.05; **P < 0.01). IFN, human interferon-β; PR8-ΔNS1, A/Puerto Rico/8/34 virus with truncated NS1; rNDV-GFP, recombinant Newcastle disease virus expressing green fluorescence protein.

pH1N1 to viral pathogenesis, the activities of NS1 from S/08 and K/09 were compared in rNDV-GFP bioassay which was used to measure interferons secreted from cells infected with virus (23). In this assay, confluent monolayers of A549 cells were inoculated with each virus at the same multiplicity of infection (MOI = 1), inducing the expression of interferon. At 24 hpi, supernatants were collected and treated with UV-light to remove all viable viral particles but preserve the interferon that had been secreted from the virus-infected cells. Monolayers of Vero cells were then treated with the interferon-containing supernatants and subsequently inoculated with the rNDV-GFP virus. The resulting levels of GFP expression in each set of Vero cells thus reflected how well the NS1 of the corresponding virus had been able to block the interferon production pathway in the A549 cells. A/Puerto Rico/8/34 (PR8) and PR8-Delta NS1 (PR8 with truncated NS1) viruses were used as controls. Surprisingly, WSN showed a very low NS1 activity, while S/08 had a relatively strong NS1. The

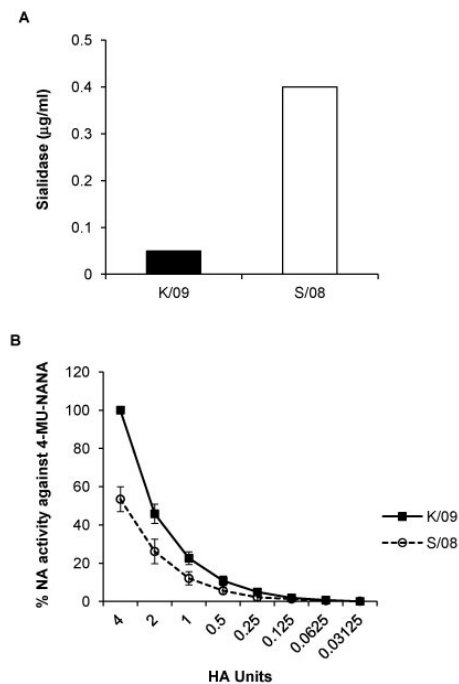


Fig. 4. Activities of surface glycoproteins. (A) Cell binding assay with K/09 and S/08 viruses. The relative binding avidities of K/09 and S/08 HAs to cellular receptors were compared using turkey RBCs. After being treated with different amounts of RDE (0.025-3.2 µg/ml) for one hour at 37°C, 0.5% turkey RBCs were agglutinated with 4 HA units of each virus. The results are expressed as the mean value of the maximal binding avidity measured in two independent experiments. (B) Neuraminidase activities of K/09 and control viruses measured by MU-NANA assay. 4 HA units of each virus were mixed with 100 µM of 2'-O-(4-methylumbelliferyl)-N-acetylneuraminic acid (4-MU-NANA) and incubated for one hour at 37°C. The fluorescence expression was measured with excitation at 365 nm and emission at 460 nm. The results were expressed as the mean value of two independent experiments, measured in triplicate.

2009 pandemic strain K/09 NS1 was determined to be a weak interferon blocker, with activity similar to that of WSN (Fig. 3). This result suggests that the NS1 protein of K/09 may not be the main source for the pathogenesis of 2009 pH1N1.

Membrane glycoproteins of the 2009 pH1N1 virus

To evaluate HA activity, cell binding avidity was examined using chicken red blood cells (RBCs) (24). For the cell binding assay, chicken RBCs were treated with 0.025–3.2 µg/ml of receptor-destroying enzymes (RDE; bacterial neuraminidase of *Vibrio cholerae*) to remove sialic acids from the surface of RBCs. In this assay, S/08 HA formed agglutination with RBC treated with 0.4 µg/ml of RDE, whereas K/09 HA barely produced agglutination with RBC treated with 0.1 µg/ml of RDE (Fig. 4A). This data suggests that S/08 HA is able to bind to RBCs which contain reduced amount of sialic acids. Therefore, receptor binding activity of S/08 HA might be stronger than that of K/09 HA. NA enzymatic activity was also analyzed by a MU-NANA assay in which 50 µl of S/08 or K/09 was subjected to a two-fold serial dilution in reaction buffer and incubated with 50 µl of MU-NANA (2'-O-(4-methylumbelliferyl)-N-acetylneuraminic acid) substrates at 37°C. After one hour, the fluorescence expression in each reaction was measured and compared. The enzymatic activity of K/09 NA was almost two-fold higher than that of the S/08 NA (Fig. 4B). Considered together, our findings demonstrate that K/09 possesses weak HA protein for cell binding and highly reactive NA protein.

DISCUSSION

In contrast to seasonal H1N1 viruses, 2009 pH1N1 caused lower respiratory tract infections in mice, ferrets, and non-human primates (16–18). Certain 2009 pH1N1 isolates killed mice that had not been adapted to the virus (16, 25, 26). However, the genetic features of 2009 pH1N1 that cause its high pathogenicity in animal models and its efficient transmission among humans remain poorly characterized. To clarify the mechanisms underlying the unique properties of K/09, we focused on the capacity of the replicative complex since this complex controls viral replication and transcription (27) and affects viral pathogenesis in the lower respiratory tracts of ferrets (8). However, K/09 was shown to have inefficient replication machinery in a mini-genome assay. Although the virus titer of K/09 attained the same maximum level as S/08 in MDCK cells (Fig. 2A), the RNP complex of K/09 was not a powerful virus manufacturer in the mini-genome assay. These results drove us to investigate surface glycoproteins because HA-NA balance plays an important role in viral life cycle. The viral surface glycoproteins of influenza virus are known to have various roles; HA interacts with sialic acid receptors to infect host cells (12) and NA enzymes release progeny virions to neighboring cells in a short time during infection (28). In this study, by measuring relative degrees of growth properties in cells, and analyzing the relative activities of the replication complex, NS1 protein, and glycoproteins of this virus, we ob-

served that 2009 pH1N1 had a unique genetic composition. Although 2009 pH1N1 had weak HA cell-binding avidity as compared to S/08, the growth kinetics of K/09 showed similar growth kinetics to that of S/08. It might be resulted from the robust enzymatic activity of the K/09 NA protein (Fig. 4B) because strong NA activity is able to easily release progeny virions. Based on these observations, we speculated that 2009 pH1N1 was equipped with balanced active membrane glycoproteins that compensated for the low efficiency of the replicative complex and NS1 protein. Therefore, we suggest that surface glycoproteins might determine the feature of 2009 pH1N1.

MATERIALS AND METHODS

Viruses and cells

Wild-type viruses used in this study were propagated in 10-day-old embryonated chicken eggs. A/Korea/01/09 (K/09) is an isolate of the 2009 pandemic H1N1 strain, A/WSN/33 (WSN) is a mouse-adapted laboratory strain, and seasonal H1N1 influenza (S/08) is a strain that was isolated in Korea in 2008. A recombinant Newcastle disease virus expressing green fluorescence protein (rNDV-GFP) was also propagated in 10-day-old embryonated chicken eggs. Madin-Darby canine kidney (MDCK) cells from ATCC were maintained in EMEM. Human embryonic kidney (293T) cells, human lung epithelial (A549) cells, and African green monkey kidney (Vero) cells from ATCC were maintained in DMEM. Swine kidney epithelial (LLC-PK1) cells from ATCC were maintained in Medium 199.

Plasmids

In the mini-genome assay for replication complex activity, the PB2, PB1, PA, and NP genes of K/09, WSN, and S/08 viruses were cloned into a modified pCAGGSII plasmid (pCAGGSII-PB2, -PB1, -PA, and -NP). For a reporter plasmid, the open-reading frame (ORF) of *Gussia luciferase* was inserted between the 3'- and 5'-non-coding regions of K/09 NA and cloned into a pPoll plasmid (pPoll-Gluc). All plasmid constructs were maxi-prepped, and their sequences were verified before use.

Mini-genome assay

293T or MDCK cells were transfected with each combination of pCAGGS-PB2, -PB1, -PA, and -NP plasmids, along with a pPoll-Gluc reporter plasmid, and maintained in Opti-MEM (Gibco, Invitrogen, CA, USA) 37°C. At 24 hpi, the cells were normalized by being added with WST-1 (Roche, Switzerland) and analyzed for luciferase activity using a Gluc assay kit (NEB, MA, USA). The results were expressed as the mean value of three independent experiments, measured in triplicate.

NS1 bioassay

To evaluate the ability of NS1 to inhibit interferon production, A549 cells were inoculated with each virus at an MOI of 1. After one hour, the A549 cells were washed three times with PBS and incubated with 0.3% BSA/DMEM. The supernatants were col-

lected at 24 hpi, UV treated, and incubated with prepared Vero cells for 16 hours. The Vero cells were then washed three times with PBS and subsequently inoculated with rNDV-GFP virus at an MOI of 1. GFP expression was measured by a SpectraMax M2e multi-mode microplate reader (Molecular Devices, CA, USA) at 24 hpi. The results were expressed as the mean value of two independent experiments, measured in triplicate.

Growth kinetics

For the growth kinetics analysis, monolayers of MDCK, A549, and LLC-PK1 cells were inoculated with each virus at an MOI of 0.01. After one hour, the cells were washed five times with PBS and maintained in the appropriate media supplemented with 0.3% BSA in the presence or absence of TPCK-treated trypsin (1 µg/ml). At the indicated timepoint, the supernatants were collected and titrated for viral presence using a plaque assay in MDCK cells. The results were expressed as the mean titer of each timepoint in three independent experiments.

Cell binding assay

Cell binding assay was previously described by Hensley *et al.* (12). Briefly, chicken red blood cells (RBCs) were treated with 0.025-3.2 µg/ml of RDE (receptor-destroying enzyme, bacterial neuraminidase of *Vibrio cholerae*; Sigma, MO, USA) at 37°C. After one hour, the RDE-treated RBCs were washed once with PBS and diluted to 0.5% RBC solution (v/v), and 50 µl containing 4 HA units of each virus was then allowed to agglutinate with 50 µl of 0.5% RDE-treated RBCs for 30 minutes at room temperature. HA-RBC agglutination was determined in two independent experiments.

MU-NANA assay

To evaluate NA enzymatic activity, 50 µl of 4 HA units of each virus was subjected to two-fold serial dilution in 96-well plates, 50 µl of MU-NANA (2'-O-(4-methylumbelliferyl)-N-acetylneuraminic acid) substrate was added, and the solution was incubated at 37°C. After one hour, the fluorescence expression levels were measured using a SpectraMax M2e multi-mode microplate reader (Molecular Devices, CA, USA). The results were converted to the expression value of K/09 (100%) using the mean values of two independent experiments, measured in triplicate.

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