Identification of Critical Residues in the Hemagglutinin and Neuraminidase of Influenza Virus H1N1pdm for Vaccine Virus Replication in Embryonated Chicken Eggs

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In 2009, we successfully produced a high-yield live attenuated H1N1pdm A/California/7/2009 vaccine (CA/09 LAIV) by substitution of three residues (K119E, A186D, and D222G) in the hemagglutinin (HA) protein. Since then, we have generated and evaluated additional H1N1pdm vaccine candidates from viruses isolated in 2010 and 2011. The 2010 strains with the new HA substitutions near the HA receptor binding site (N125D and D127E or D127E and K209E) grew well in eggs and formed large plaques in Madin-Darby canine kidney (MDCK) cells. Introduction of these acidic amino acids into the HA of CA/09 also improved vaccine virus growth in eggs to a titer comparable to that of CA/09 LAIV. However, the high growth of A/Gilroy/231/2011 (Gil/11) vaccine virus required modification in both the HA and the NA segments. The residue at position 369 of the NA was found to be critical for virus replication in MDCK cells and eggs. These HA and NA residues had minimal impact on viral entry but greatly improved viral release from infected cells. Our data implied that the HA receptor binding and NA receptor cleaving function of the poor-growth H1N1pdm virus was not well balanced for virus replication in host cells. The high-growth vaccine candidates described in this study maintained vaccine virus antigenicity and induced high levels of neutralizing antibodies in immunized ferrets, making them suitable for vaccine production. The identification of the amino acids and their roles in viral replication should greatly help vaccine manufacturers to produce high-yield reassortant vaccine viruses against the future drifted H1N1pdm viruses.

The 2009 influenza pandemic, caused by swine-origin H1N1 influenza viruses (H1N1pdm), spread to over 215 countries from April 2009 and was responsible for between 151,700 and 575,400 deaths (1, 2). The rapid manufacture of pandemic vaccines is essential in the event of an influenza pandemic. However, human influenza viruses do not normally grow well in embryonated chicken eggs, the substrate for the production of influenza vaccine viruses. Egg adaptation is usually required to improve vaccine virus growth in eggs (3–6). At the onset of the H1N1 pandemic in April 2009, the development of the H1N1pdm vaccine was hampered by poor virus growth (7).

Live attenuated influenza vaccine (LAIV) has been licensed in the United States since 2003 and approved in other countries, including the European Union, more recently (8). Each LAIV virus is a 6:2 reassortant that contains the 6 internal protein gene segments from the master donor virus that confer the temperature-sensitive (*ts*), cold-adapted (*ca*), and attenuation (*att*) phenotypes, which are combined with antigenic hemagglutinin (HA) and neuraminidase (NA) surface glycoprotein gene segments from the wild-type (wt) virus (9). The H1N1pdm 6:2 *ca* reassortant vaccine virus was developed by introduction of three residues (K119E, A186D, and D222G; H1 numbering is used throughout this paper) in the HA protein (10) and was the first H1N1pdm vaccine available in the U.S. market.

The A/California/7/2009 (CA/09)-like H1N1pdm viruses have been circulating since 2009 and replaced seasonal H1N1 viruses. Although the currently circulating H1N1 viruses are still antigenically similar to CA/09, antigenic drift of H1N1 virus is expected and influenza vaccine update will be needed. Thus, it is important to identify genetic signatures that could facilitate virus growth in eggs in order to rapidly generate updated vaccine strains. The HA and NA surface glycoproteins play important roles in virus repli-

cation. HA binds to sialic acid receptors on the cell surface and mediates virus attachment and membrane fusion during virus entry (11). NA catalyzes the removal of terminal sialic acid on the cell surface so that the newly assembled virus particles can be released from the infected cells and spread (12). Both the HA and NA proteins recognize sialosides but with counteracting functions. Therefore, the functional balance between the receptor binding of the HA and the receptor-destroying property of the NA is critical for efficient viral replication (13, 14). We previously reported that replication of influenza A/Fujian/411/2002 (H3N2) virus in eggs and Madin-Darby canine kidney (MDCK) cells could be improved either by changing two HA residues to increase the receptor binding ability of the HA or by changing two NA residues to lower the enzymatic activity of the NA (15). In this report, we identified critical residues in both HA and NA that improve vaccine virus growth in eggs, demonstrating the importance of both proteins in virus replication in host cells. Moreover, it was found that several acidic residues in the HA globular head as well as the NA residues improved virus replication possibly by facilitating virus release and spread in cells. These amino acid substitutions do not affect virus antigenicity and are suitable for vaccine production.

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TABLE 1 HA and NA sequence comparison of recent H1N1pdm strains^a

Virus strain	Hemagglutinin (H1 numbering)																Neuraminidase (N1 numbering)											
	83	97	124	125	127	191	203	205	209	216	222	249	283	300	321	374	11	15	44	106	189	222	241	248	369	419	443	
CA/09	P	D	P	N	D	L	S	R	K	I	D	V	K	I	I	E	G	M	N	V	N	N	V	N	N	R	I	
Bris/10	S			D	E		T								V	K		I		I	S			D		K		
NH/10	S	N	/L		/E	/I	T		/E						V	K	S			I				D				
Gil/11	S	N				/I	T	K		V	/N	L	E	L	V	K			S	I		S	I	D	K		M	

^a The HA and NA sequences of the egg-adapted H1N1pdm viruses A/Brisbane/10/2010 (Bris/10), A/New Hampshire/2/2010 (NH/10), and A/Gilroy/231/2011 (Gil/11) were compared with the wild-type A/California/07/09 (CA/09) reference strain. Only the residues that differ from A/California/07/09 are listed. /X, mixed sequence; X, egg adaptation change.

MATERIALS AND METHODS

Viruses. Egg-grown wild-type H1N1pdm viruses A/Brisbane/10/2010 (Bris/10) and A/New Hampshire/2/2010 (NH/10) were kindly provided by the Centers for Disease Control and Prevention. A/Gilroy/231/2011 (H1N1pdm) (Gil/11) was isolated from the nasal wash of a ferret which contracted human influenza transmitted from a husbandry staff member. The HA and NA sequences were deposited in GenBank with the accession numbers of KC436084 and KC436085, respectively. All the viruses were expanded in both Madin-Darby canine kidney (MDCK) cells (European Collection of Cell Cultures) and embryonated chicken eggs (Charles River Laboratories, Wilmington, MA).

Generation of recombinant viruses by reverse genetics. The HA and NA gene segments of wt H1N1pdm viruses were amplified by reverse transcription-PCR (RT-PCR) and cloned into the pAD3000 vector (16). Site-directed mutagenesis was performed to introduce specific changes into the HA and NA genes using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The 6:2 reassortant vaccine viruses were generated by plasmid rescue as described previously (17). Briefly, the 6:2 reassortant candidate vaccine viruses were generated by cotransfecting eight cDNA plasmids carrying the HA and NA protein gene segments of the H1N1 virus and the six internal protein gene segments of cold-adapted A/Ann Arbor/6/60 (AA *ca*, H2N2) virus into cocultured 293T and MDCK cells. The rescued viruses from the cell supernatants were propagated in the allantoic cavity of 10- to 11-day-old embryonated chicken eggs. The HA and NA sequences of the viruses were verified by sequencing RT-PCR cDNAs amplified from viral RNA (vRNA).

Virus titration. Infectious virus titers were measured by the fluorescence focus assay (FFA) in MDCK cells and expressed as log₁₀ FFU (fluorescent focus units)/ml. Virus plaque morphology was examined by plaque assay as described before (15). The plaque size of the viruses was measured by ImageQuantTL (GE Healthcare Biosciences, Piscataway, NJ). To compare the replication levels of 6:2 reassortant viruses in eggs, eggs were inoculated with 10³ FFU/egg of virus and incubated at 33°C for 3 days. Allantoic fluid was harvested for both FFA and plaque assay.

Virus growth kinetics and virus protein expression. The growth kinetics of recombinant 6:2 reassortants were determined in MDCK cells. MDCK cells were inoculated with the viruses at a multiplicity of infection (MOI) of 5 or 0.005. After 1 h of adsorption, the infected cells were washed with phosphate-buffered saline (PBS), incubated with minimal essential medium (MEM) containing 1 μg/ml *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-trypsin (Sigma-Aldrich, St. Louis, MO), and incubated at 33°C. The cell culture supernatant was collected at different time points, and the virus titer was determined by FFA.

Viral proteins produced in the infected cells and released virions in cell culture supernatants were analyzed by Western blotting. MDCK cells were infected with the viruses at an MOI of 5 as described above. At 8 h and 16 h postinfection (hpi), the cell culture supernatant was collected and cellular debris was removed by centrifugation in a microcentrifuge at 14,000 rpm for 5 min. The infected cells were collected and lysed with RIPA buffer (20 mM Tris Cl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail). Equal

amounts of cell lysate and cell supernatant were electrophoresed on a Novex 12% Tris-glycine gel (Invitrogen, Carlsbad, CA) under the denaturing condition. The proteins were transferred to a nitrocellulose membrane and blotted with influenza virus-specific antibodies.

For immunofluorescence assay, MDCK cells were infected with the viruses at an MOI of 0.005. At 15 h or 48 h postinfection, infected cells were fixed with 10% formalin for 20 min, followed by treatment with ice-cold methanol for 5 min. The cells were then incubated with goat anti-influenza A virus polyclonal antibody (Millipore, Bedford, MA) at a dilution of 1:40 at room temperature for 1 h, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG antibody (Millipore, Bedford, MA) at a dilution of 1:100 for 30 min. The stained cells were examined with a fluorescence microscope (Nikon Eclipse Ti).

Serum antibody detection by HAI assay. Eight- to 10-week-old male and female ferrets ($n = 3/\text{group}$) from Simonsen Laboratories (Gilroy, CA) were inoculated intranasally with 7.0 log₁₀ FFU of virus per 0.2-ml dose. Ferret serum samples were collected 14 days after infection. Hemagglutinin inhibition (HAI) assay was used to determine antibody levels in postinfection ferret sera against homologous and heterologous viruses. Twenty-five microliters of serially diluted serum samples treated with receptor-destroying enzyme (RDE; Denka Seiken Co., Tokyo, Japan) was mixed with 4 HA units of the indicated viruses (25 μl) in 96-well V-bottom microplates. After incubation at room temperature for 30 min, 50 μl of 0.5% chicken erythrocytes (cRBC) was added to each well and incubated for an additional 45 min. The HAI titer was defined as the reciprocal of the highest serum dilution that inhibited virus hemagglutination.

Nucleotide sequence accession numbers. The HA and NA sequences were deposited in GenBank with the accession numbers [KC436084](#) and [KC436085](#), respectively.

RESULTS

H1N1pdm vaccine candidates grew differently in embryonated chicken eggs. Three recent H1N1pdm strains, A/Brisbane/10/2010 (Bris/10), A/New Hampshire/2/2010 (NH/10), and A/Gilroy/231/2011 (Gil/11), exhibited sequence variations in both HA and NA gene segments compared to A/California/7/2009 (CA/09). Mixed sequences at multiple HA positions such as 124, 127, 191, 209, and 222, due to egg adaptation sequence changes, were found in egg-adapted NH/10 and Gil/11 wt viruses (Table 1). To evaluate the growth of LAIV candidates of these viruses, the 6:2 cold-adapted (*ca*) reassortant viruses containing the 6 internal protein gene segments from the master donor virus A/Ann Arbor/6/60 *ca* and the HA and NA genes from the wild-type (wt) H1N1pdm viruses were generated using the eight-plasmid reverse genetics system. The rescued viruses were amplified in embryonated chicken eggs, and infectious virus titers were determined by the fluorescence focus assay (FFA). Plaque morphology was examined by plaque assay in MDCK cells (Fig. 1). The HA gene of egg-derived Bris/10 wt was homogeneous; only one HA variant

FIG 1 The different growth levels of H1N1pdm *ca* viruses in eggs. (A) 6:2 *ca* reassortants with HA and NA gene segments from A/Brisbane/10/2010 (Bris/10), A/New Hampshire/2/2010 (NH/10), or A/Gilroy/231/2011 (Gil/11) were inoculated into eggs, and the infectious titers were determined by FFA. The amino acid changes in the HA protein caused by egg adaptations were indicated. The data represent the averages of three independent experiments with the standard deviations indicated by bars. The limit of detection is 3.2 log₁₀ FFU/ml. *, $P < 0.05$ (one-way analysis of variance). (B) Plaque morphology of Bris/10 *ca* and NH/10 *ca* viruses containing the indicated HA amino acid changes. The plaque assay was performed in MDCK cells, and the plaques were immunostained with polyclonal antiserum against influenza A viruses. The mean plaque size of 10 representative plaques for each virus is indicated.

was cloned (Table 1). In contrast to CA/09, Bris/10 *ca* grew efficiently to a titer of 8.5 log₁₀ FFU/ml and formed large plaques in MDCK cells. Three HA variants with the egg adaptation changes in HA (P124L/L191I, P124L/K209E, and D127E/K209E) were cloned from the egg-adapted NH/10 wt virus. The respective NH/10 *ca* variants grew to different titers in eggs and had distinct plaque morphologies. The P124L/L191I variant had a low titer in eggs and formed pinpoint-sized plaques (approximately 0.3 mm in diameter) in MDCK cells. Both P124L/K209E and D127E/K209E *ca* viruses formed much larger plaques (2.7 to 4.0 mm in diameter), and the D127E/K209E variant reached the titer of 8.2 log₁₀ FFU/ml, indicating that the K209E change mainly contributed to the efficient virus growth. The Gil/11 6:2 *ca* viruses containing the original HA sequence or the HA with an egg adaptation change (D222N) could not be recovered from the plasmid-transfected cells. Correspondingly, Bris/10 wt and the NH/10 wt isolate containing D127E/K209E grew efficiently, while Gil/11 wt grew poorly in both MDCK cells and eggs (data not shown), indicating that the HA and NA genes controlled virus replication. Sequence comparison of these high- and low-growth viruses indicated that the HA residues at positions 125, 127, and 209 may be important for virus growth in eggs.

Identification of the HA residues that support high growth of vaccine viruses. Previously, we have shown that recombinant CA/09 *ca* viruses containing the original HA sequence could not be recovered from plasmid DNA-transfected cells. The HA D222G change in the receptor binding domain enabled virus recovery, but the virus titer was low. The K119E and A186D substi-

FIG 2 HA sequence changes at positions 125, 127, and 209 improve the growth of CA/09 *ca* virus in eggs. (A) CA/09 *ca* reassortants with the indicated amino acid changes in the HA gene were inoculated into eggs, and the infectious titers were determined by FFA. The data represent the averages of three independent experiments with the standard deviations indicated by bars. The limit of detection is 3.2 log₁₀ FFU/ml. *, $P < 0.05$ (one-way analysis of variance). (B) Plaque morphology of the CA/09 *ca* variants containing the indicated HA amino acid changes. The plaque assay was performed in MDCK cells, and the plaques were immunostained with polyclonal antiserum against influenza A viruses. The mean plaque size of 10 representative plaques for each virus is indicated.

tutions in the HA greatly improved virus growth, reaching a titer of approximately 8.5 log₁₀ FFU/ml (10). To confirm that the newly identified amino acid substitutions (N125D, D127E, and K209E) conferred a growth advantage on H1N1pdm vaccine viruses in eggs, each of the identified mutations was introduced into the cDNA of the original CA/09 HA individually or in combination. The 6:2 *ca* reassortant viruses were rescued and examined for their growth in eggs (Fig. 2). All the single mutations (N125D, D127E, or K209E) significantly improved virus growth in eggs. Among them, the virus with N125D formed larger plaques (1.9 mm in diameter) in MDCK cells. The double mutations further improved virus replication, reaching the highest titer at approximately 8.3 log₁₀ FFU/ml, which was comparable to Bris/10 in virus titer and plaque size (Fig. 1). Additional mutations introduced into the double mutants (e.g., the N125D/D127E/K209E triple mutants) did not further improve virus growth (data not shown). Thus, in addition to the K119E and A186D substitutions that we identified previously, the N125D, D127E, and K209E changes in HA also greatly facilitated vaccine virus growth.

Both HA and NA were required for high growth of Gil/11. To determine whether the substitutions at HA positions 125, 127, and 209 could also improve Gil/11 *ca* virus growth in eggs, single or double HA mutations were introduced into the Gil/11 *ca* virus (Fig. 3A, left columns). Although all the HA variants were rescued, they all formed tiny or small plaques (Fig. 3B, upper panel) with low infectious titers of 5.8 to 7.1 log₁₀ FFU/ml. These data suggested that the changes in the HA could not completely improve the growth of Gil/11 *ca* virus.

To assess the possible contribution of the NA protein to virus growth, the NA segment of these Gil/11 *ca* HA variants was replaced with Bris/10 NA by reverse genetics and the recovered

FIG 3 The effect of the NA segment on the Gil/11 *ca* virus growth in eggs. (A) The 6:2 *ca* reassortants containing the Gil/11 HA variants with the indicated amino acid changes and the NA segment from either Gil/11 or Bris/10 were rescued by reverse genetics. The viruses were inoculated into eggs, and the infectious titers were determined by FFA. The data represent the averages of three independent experiments with the standard deviations indicated by bars. The limit of detection is 3.2 log₁₀ FFU/ml. *, $P < 0.05$ (unpaired *t* test) compared to the corresponding virus with Gil/11 NA. (B) Plaque morphology of the viruses described for panel A. The plaque assay was performed in MDCK cells, and the plaques were immunostained with polyclonal antiserum against influenza A viruses. The mean plaque size of 10 representative plaques for each virus is indicated.

Gil/11 *ca* HA variants with the NA segment from Bris/10 were examined for their growth in eggs. As shown in Fig. 3, all the viruses with Bris/10 NA grew to higher titers than did the corresponding viruses with Gil/11 NA. The replacement of Gil/11 NA with CA/09 NA similarly improved virus growth (data not shown). The Gil/11 *ca* variant containing the N125D/D127E double mutation in the HA and the Bris/10 NA grew to the highest titer in eggs (8.2 log₁₀ FFU/ml) and formed large plaques. These data demonstrated that both HA and NA proteins contribute to virus replication in eggs and MDCK cells.

Identification of the NA residues that contribute to efficient growth of Gil/11 *ca* virus in eggs. Sequence comparison showed that Gil/11 had five unique NA residues at positions 44, 222, 241, 369, and 443 (N1 numbering) compared with the NA of CA/09 and Bris/10 (Table 1). To identify if any of these NA amino acid substitutions were responsible for the lower growth of Gil/11 *ca*, S44N, S222N, I241V, K369N, and M443I changes were introduced into the Gil/11 (N125D/D127E in HA) *ca* virus. As shown in Fig. 4, in contrast to the S222N and I241V single mutants that did not significantly improve virus growth, the single mutant K369N increased virus titer by 0.5 log₁₀ FFU/ml and improved virus plaque size. The S44N and M443I changes did not affect virus growth (data not shown). The double mutations had no additional effect on viral growth compared with the single mutations. However, a triple NA mutant with changes at NA residues

FIG 4 The effect of NA residues on the Gil/11 *ca* virus growth in eggs. (A) The Gil/11 *ca* reassortants containing N125D/D127E changes in HA and the indicated amino acid changes in NA were inoculated into eggs, and the infectious titers were determined by FFA. The data represent the averages of three independent experiments with the standard deviations indicated by bars. The limit of detection is 3.2 log₁₀ FFU/ml. *, $P < 0.05$ (one-way analysis of variance). (B) Plaque morphology of the above-described Gil/11 *ca* variants. The plaque assay was performed in MDCK cells, and the plaques were immunostained with polyclonal antiserum against influenza A viruses. The mean plaque size of 10 representative plaques for each virus is indicated.

222, 241, and 369 had the highest virus titer, of 8.3 log₁₀ FFU/ml, and large plaque morphology, comparable to that of the virus with Brisbane virus NA. Thus, not only the HA protein but also the NA accounted for the poor growth of Gil/11 *ca*.

The effect of the HA residues on virus immunogenicity and antigenicity. To assess whether the HA changes in these high-growth *ca* variants affect virus antigenicity and immunogenicity, the Bris/10 *ca* virus, the NH/10 *ca* virus with D127E/K209E in HA (NH/10 v1), and the Gil/11 *ca* virus with N125D/D127E in HA and Bris/10 NA (Gil/11 v1) were examined for their immunogenicity and antigenicity in ferrets. Ferrets were inoculated intranasally with 7.0 log₁₀ FFU of the above-described vaccine candidates, and ferret serum was collected on day 14. The antibody titers against homologous and heterologous H1N1pdm viruses were evaluated by HAI assay (Table 2). All the Bris/10, NH/10, and Gil/11 *ca* viruses were immunogenic and induced high HAI antibody titers (912 to 2,048) against homologous viruses. As expected, all the antisera against the H1N1pdm *ca* viruses did not cross-react to seasonal H1N1 or H3N2 viruses (HAI titer, <8 [data not shown]). Similarly to current CA/09 LAIV, they all cross-reacted well to the H1N1pdm wt viruses and the heterologous viruses (HAI titers were within 4-fold compared to homologous titers), indicating that there were no major antigenic differences between the three strains. Sera from ferrets immunized with viruses containing HA N125D/D127E (Bris/10 and Gil/11) cross-

TABLE 2 Immunogenicity and antigenicity of vaccine variants in ferrets^a

Test virus	HA residue(s)					GMT ^b of HAI of ferret serum immunized with <i>ca</i> virus			
	124	125	127	191	209	CA/09	NH/10	Gil/11	
						LAIV ^c	Bris/10	v1	v1
CA/09 LAIV	P	N	D	L	K	<u>861</u>	724	1,448	724
CA/09 wt				L/I		1,024	1,024	1,448	1,448
Bris/10		D	E			470	<u>912</u>	1,024	724
NH/10 v1			E		E	790	813	<u>2,048</u>	512
NH/10 v2	L			I		362	575	1,024	724
NH/10 wt	L/I		D/E	L/I	K/E	724	1,149	1,024	724
Gil/11 v1		D	E			472	2,048	2,048	<u>1,024</u>
Gil/11 v2			E		E	1,024	512	2,048	1,448
Gil/11 wt				L/I		470	406	1,024	724

^a Groups of ferrets were inoculated intranasally with 7.0 log₁₀ FFU of the indicated H1N1pdm *ca* vaccine viruses. Serum was collected 14 days after immunization, and the antibody titers against different test viruses were determined by the hemagglutination inhibition assay (HAI) using chicken erythrocytes. The HA sequence variations at positions 124, 125, 127, 191, and 209 of the test viruses are indicated. The HAI titers against homologous viruses are underlined.

^b GMT, geometric mean titer.

^c The current LAIV strain contains the changes at the other sites of HA (119, 186, and 222) that improved vaccine virus growth.

reacted well to viruses containing HA D127E/K209E (NH/10 v1 and Gil/11 v2) or HA P124L/L191I (NH/10 v2). Thus, the N125D/D127E or D127E/K209E substitutions could be introduced into H1N1pdm strains to improve vaccine virus growth without altering virus antigenicity.

The HA and NA substitutions improve virus growth by facilitating virus release from infected cells. The identified HA amino acids that improved vaccine virus growth all contained acidic amino acid substitutions (K119E, A186D, N125D, and K209E). To investigate the impact of these residues on virus replication, pairs of viruses with or without the acidic residue changes were compared for their growth kinetics in MDCK cells. The representative data of the low-growth virus CA09-D127E (125N) versus the high-growth virus CA09-N125D/D127E (125D) are shown in Fig. 5A. The 125N virus showed lower replication kinetics than did the 125D virus at both a high MOI and a low MOI, indicating that the multicycle replication of the 125N virus was impaired. The peak titers of the 125D virus at an MOI of 5 (16 hpi) or an MOI of 0.005 (48 hpi) were approximately 2 logs higher than those of the 125N virus.

Viral protein levels in the infected cells and the culture supernatants at different time points with a high-MOI infection were examined by Western blotting (Fig. 5B). The 125N and 125D viruses produced comparable amounts of viral proteins in the infected cells from 8 to 16 h postinfection. However, the amount of viral particles released into the supernatants of cells infected with the 125N virus, as detected by viral HA protein levels, was approximately 38% of the amount for cells infected with the high-growth 125D virus. The data indicated that the low-growth viruses could enter cells and initiate RNA transcription and protein synthesis efficiently, but virus release from infected cells was not efficient, resulting in poor virus spread or multicycle replication. These were reflected in small virus plaques in MDCK cells and lower titers in eggs and MDCK cells.

FIG 5 Replication levels of the 6:2 *ca* reassortants CA/09-D127E and CA/09-N125D/D127E in MDCK cells. (A) MDCK cells were infected with the two viruses at an MOI of 5 or 0.005 and incubated at 33°C. At the indicated time intervals, the culture supernatants were collected and the virus titer was determined by FFA in MDCK cells. The titers are the mean titers of three independent experiments with the standard errors indicated by bars. *, $P < 0.05$ (paired t test). (B) MDCK cells were infected with the two viruses at an MOI of 5 and incubated at 33°C. The infected-cell supernatants and cell lysates were harvested after 8 h or 16 h postinfection and analyzed by Western blotting using a polyclonal antibody against H1N1pdm HA. The relative amounts (%) of total HA proteins of the two viruses were quantified by ImageQuantTL. Numbers at left are molecular weights in thousands. (C) MDCK cells were infected with the two viruses at an MOI of 0.005 and incubated at 33°C. At 15 h or 48 h postinfection, the infected cells were detected by immunofluorescence. The percentage of the infected cells is indicated in each panel.

FIG 6 Viral protein expression and release from infected cells. MDCK cells were infected with Gil/11-N125D/D127E *ca* viruses containing Gil/11 NA or Bris/10 NA at an MOI of 5 and incubated at 33°C. The infected-cell supernatants and cell lysates were harvested after 8 h or 16 h postinfection and analyzed by Western blotting using a polyclonal antibody against H1N1pdm HA. The relative amounts (%) of total HA proteins of the two viruses were quantified by ImageQuantTL. MW, molecular weights in thousands.

The difference of the two viruses in virus spread at a low MOI was confirmed by immunofluorescence (Fig. 5C). At an MOI of 0.005, only a limited number of replication cycles occurred; similarly low percentages of cells (6% versus 10%) were infected at 15 h postinfection for the two viruses. At 48 h postinfection, the majority of the cells were infected by the 125D virus; however, only approximately 10% of the cells were infected with the 125N virus. Similar results were obtained with other pairs of viruses such as CA/09 (D222G) versus CA/09 (D222G)-K119E/A186D, indicating that the acidic residue changes in the HA facilitated virus release from cells.

To investigate the effect of NA on virus replication in MDCK cells, Gil/11 *ca* viruses containing Gil/11 NA or Bris/10 NA were also compared for the viral protein expression in the infected cells (Fig. 6). Similarly, the two viruses showed similar protein expression levels (with a ratio of 102:100) in infected cells. Consistent with virus titers in the cell supernatants, the virus with Gil/11 NA had less viral HA protein detected in the cell supernatants than did the virus with Bris/10 NA (58:100), indicating inefficient virus spread compared to the virus containing Bris/10 NA.

DISCUSSION

The H1N1pdm-like viruses have been circulating in humans since 2009. Although current circulating H1N1pdm strains are still CA/09-like, genetic diversity and subgroups were formed among the new H1N1pdm strains (CDC communication). Data obtained from animal models demonstrated that the emergence of a more virulent H1N1pdm virus was possible through sequence changes or reassortment with other influenza viruses (18–20). It is therefore very important to be able to rapidly produce high-yield vaccine virus in eggs should antigenic drift or shift occur. In this report, we presented data on the production and evaluation of H1N1pdm-like viruses isolated in 2010 and 2011. We identified several novel amino acid residues in both the HA and NA proteins that contributed to virus replication in eggs without affecting virus antigenicity.

We demonstrated that the HA N125D/D127E and D127E/K209E adaptation sites were responsible for the high growth of A/Brisbane/10/2010 and A/New Hampshire/2/1010. Introduction

FIG 7 Locations of the identified HA and NA residues on the HA and NA three-dimensional structures. (A) Locations of the identified HA residues that improve the growth of H1N1pdm viruses on the HA three-dimensional structure (Protein Data Bank code 3LZG; only one monomer shown). (B) Locations of the three identified NA residues on one NA monomer structure (Protein Data Bank code 3NSS). The images were visualized using PyMol software. RBS, receptor binding site; AC, NA activity cavity.

of these substitutions into the heterologous CA/09 *ca* virus could revert its poor growth. The HA residue 125 is located in the antigenic Sa domain and adjacent to the receptor binding site (RBS) (Fig. 7A). A/Brisbane/10/2010-like viruses containing N125D showed high growth in eggs. A/Brisbane/10/2010-like viruses having an H1 HA N125D change were initially detected in late April 2010 in clinical isolates from the Southern Hemisphere. Although these newer strains did not have major antigenicity changes compared to the CA/09 strain, they have been reported to be associated with several vaccine breakthrough infections and were identified in a number of fatal cases (21, 22). The 127 and 209 changes in A/New Hampshire/2/2010 HA resulted from egg adaptation (7, 23). Both residues are located on the surface of the globular head (Fig. 7A). The 127 residue sits outside the antigenic site Sa and the RBS. A mouse-adapted A/CA/04 virus having an HA with D127E was also shown to be associated with a more virulent phenotype in mice (20). The 209 residue is relatively distant from the RBS within the monomer but is close to the RBS in the neighboring monomer in the HA trimer. An alternative K209T change was reported in some high-yield reassortants for inactivated influenza virus vaccines; however, a single K209T change did not greatly improve vaccine yield (7).

In this study, we also identified important genetic signatures in the NA that contributed to vaccine virus growth in eggs. In contrast to Bris/10, the recent 2011 strain A/Gilroy/231/2011 could not replicate well in either MDCK cells or eggs. It could grow well only when both the HA and NA proteins were altered. Further mapping identified that three amino acids at positions 222, 241, and 369 (corresponding to N2 numbering 221, 240, and 372, respectively) were mainly responsible for the poor growth of Gil/11. The three residues are all around the NA catalytic site (Fig. 7B). The 369 residue is close to the conservative catalytic site R371, and both 369 and 222 are on the antigenic surface (12, 24). K369 and I241 in Gil/11 NA are conserved in the previous human seasonal H1N1 strains, and most recent 2011/2012 H1N1pdm strains con-

tain K369 and I241, suggesting that the NA of the recent H1N1pdm strains may have adapted well in humans (25).

The mechanisms by which the identified HA and NA genetic signatures improve virus replication in eggs and MDCK cells are not fully understood. Our previous study (10) and this study demonstrated that the amino acid substitutions D222G, A186D, N125D, D127E, and K209E in HA greatly improved virus growth in eggs or MDCK cells. Most of the changes are acidic residue changes. Egg adaptation changes in HA were believed to increase virus binding to α 2,3-linked sialic acid to improve virus replication in eggs (7, 26, 27). We and others have shown that the D222G change increased virus binding to α 2,3-linked sialic acid (10, 28). However, the receptor binding assay using resialylated red blood cells showed that the viruses with N125D, D127E, or K209E changes remain predominantly bound to α 2,6-linked sialic acid receptors, consistent with other glycan binding reports (23, 29, 30). Possibly, the current *in vitro* methods failed to detect the differences in the receptor binding caused by these changes. Further viral replication studies showed that the acidic residue substitutions greatly improved virus spread in the host cells, and the vaccine viruses reached a high titer that is suitable for vaccine production. These negative charged residues possibly cause the repulsion to the negatively charged sialic acid receptor or cell membrane and increase virus particle release from MDCK cells without affecting viral entry and viral protein synthesis, as demonstrated by Western blotting and immunofluorescence assays. Thus, we hypothesize that the HA residue changes around the receptor binding site favor receptor binding in eggs or MDCK cells and that the acidic surface changes in the HA further help virus release from infected cells to initiate efficient multicycle replication.

The Gil/11 NA could not support virus replication as efficiently as the NA of Bris/10. However, we could not detect significant differences in the NA enzymatic activities using 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUN) substrate (data not shown). The NA enzymatic difference between the NA of Gil/11 and that of Bris/10 might be below the level of detection. We could not exclude the possibility that the interaction of the NA with HA and other viral or host factors could be less optimal during virus assembly, budding, or release (31–33).

Our results underscored the importance of both HA and NA proteins for viral replication in host cells. The poor growth of H1N1pdm may be caused by a nonoptimal balance of HA and NA functions. The HA-NA balance and NA activity were reported to affect H1N1pdm virus transmissibility (34, 35). Recent reports from Xu et al. using glycan binding and NA activity assays showed that the functional balance of the HA and NA activities is important for the emergence of H1N1pdm viruses (36). In addition to the three strains reported here, we also evaluated the growth of other recent H1N1pdm viruses. Except for the A/Brisbane/10/2010-like high-growth viruses, most of the H1N1pdm strains grow poorly in both MDCK cells and eggs. The identification of the genetic signatures in both HA and NA that contribute to antigenic change and virus growth in eggs will guide us to prepare with greater efficiency future vaccine strains that have high yield and authentic antigenicity.

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