Published in final edited form as:

Virus Res. 2011 June ; 158(1-2): 124–129. doi:10.1016/j.virusres.2011.03.022.

Mutations in PA, NP, and HA of a pandemic (H1N1) 2009 influenza virus contribute to its adaptation to mice

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Abstract

In 2009, a swine-origin H1N1 influenza virus caused the first pandemic of the 21st century. To understand the molecular basis of pandemic influenza virus adaptation to new host species, we serially passaged the pandemic (H1N1) 2009 virus strain A/California/04/09 in mouse lungs. After ten passages, the virus became lethal to mice. We found eight amino acid differences between the wild-type and mouse-adapted viruses: one in PB1, three in PA, three in HA, and one in NP. By using reverse genetics to generate mutant viruses, we determined that the amino acid substitutions in PA (at positions 21 and 616), HA (at positions 127 and 222), and NP (at position 375) play independent roles in the increased pathogenicity in mice. Among these five substitutions, an aspartic acid-to-glutamic acid substitution at position 127 in HA contributed to efficient viral replication in mouse lungs. Our results suggest the importance of the viral polymerase complex and of HA in viral adaptation to a new host.

Keywords

pandemic (H1N1) 2009 influenza virus; virulence; new host species; mouse-adaptation

1. Introduction

Influenza viruses in poultry, pigs, humans, and other mammalian species are thought to have originated in migratory free-flying birds (Webster et al., 1992). During repeated infection

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and replication, the viral genomes of these viruses evolve and adapt to their new hosts. In the spring of 2009, a swine-origin H1N1 influenza virus caused an outbreak in Mexico (Perez-Padilla et al., 2009) and rapidly spread worldwide. The pathogenicity in humans of this pandemic (H1N1) 2009 influenza virus was not as severe as that of the 1918 Spanish influenza virus; in most cases, the virus infection was self-limiting, although some patients were hospitalized with pneumonia, respiratory failure, or acute respiratory distress syndrome during the first wave (Dawood et al., 2009; Louie et al., 2009).

To adapt to new hosts, influenza A viruses must acquire amino acid substitutions in their proteins (Brown, 1990; Narasaraaju et al., 2009). Previous studies have shown that repeated passages of influenza viruses from various animal species (e.g., birds, humans, horses) in mice, which are routinely used to evaluate anti-viral agents and vaccine candidates, results in increased virulence with mutations in HA, NP, NA, M, NS, and viral polymerase genes (Brown and Bailly, 1999; Brown et al., 2001; Dankar et al., 2011; Kaverin et al., 1989; Ping et al., 2010; Rudneva et al., 1986; Smeenk and Brown, 1994). Mice infected with pandemic (H1N1) 2009 viruses develop moderate symptoms, although a high dose of some 2009 pandemic strains can cause lethal infection (Itoh et al., 2009; Maines et al., 2009). Recently, a study with mouse-adapted pandemic (H1N1) 2009 viruses, which were established by serial passage in mouse lungs, revealed that optimization of both the receptor specificity of HA and the interaction of the viral polymerase components with cellular factors play key roles in the increased virulence in mice (Ilyushina et al., 2010; Ye et al., 2010). These studies, however, did not address the roles of specific amino acids in these viral proteins in this increased pathogenicity in mice.

Here, to understand the molecular basis for host adaptation of pandemic influenza viruses, we serially passaged a pandemic (H1N1) 2009 virus (A/California/04/09) in mice and identified mutations critical for its adaptation to mice.

2. Materials and methods

2.1. Virus and Cells

A/California/04/09 (H1N1; CA04) was propagated in Madin-Darby canine kidney (MDCK) cells at 35°C. MDCK cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 5% newborn calf serum (Sigma-Aldrich, Inc., St. Louis, MO, USA), and 293T human embryonic kidney cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum; both cell types were cultured at 37°C in 5% CO₂.

2.2 Establishment of mouse-adapted pandemic (H1N1) 2009 virus

Four- to six-week-old female BALB/c mice (Japan SLC, Hamamatsu, Japan) were infected with 10³ plaque-forming units (PFU) of CA04 (50 µl) via the intranasal route. On days 3–5 post-infection (pi), mice were euthanized and their lungs were collected in a 9-fold volume of MEM supplemented with 0.3% bovine serum albumin (BSA: Sigma-Aldrich, Inc., St. Louis, MO, USA). Lung homogenates were then diluted 1,000-fold with MEM supplemented with 0.3% BSA and inoculated (50 µl) into naïve mice via the intranasal route. This procedure was repeated ten times.

2.3. Sequence analysis

Viral RNAs were extracted from viruses in the homogenates of mouse lungs or the supernatants of MDCK cells infected with plaque-purified mouse-adapted CA04 by using a QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) and reverse transcribed with SuperscriptTM III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and an

oligonucleotide complementary to the 12-nucleotide sequence at the 3' end of the viral RNA (Katz et al., 1990). The cDNA products were amplified by using PCR with Phusion High Fidelity DNA polymerase (Finnzymes, Espoo, Finland) and primers specific for each segment of the pandemic (H1N1) 2009 virus. The PCR products were purified by use of a MinElute PCR purification kit (Qiagen, Hilden, Germany) and sequenced with the BigDye terminator kit on an ABI 3130xl (Applied Biosystems, Foster City, CA) by following the manufacturer's instructions.

2.4. Plasmid constructs and reverse genetics

Reverse genetics (rg) systems for CA04 and MA-CA04 viruses were established as described previously (Neumann et al., 1999). Briefly, the cDNAs of the CA04 viral genes were cloned into the pHH21 vector. The characteristic mutations found in MA-CA04 were introduced into the plasmid constructs of CA04 by using site-directed mutagenesis. The eight plasmids for the synthesis of viral RNA and the four expression plasmids for A/WSN/33 (H1N1) virus-derived PB2, PB1, PA, and NP were transfected into 293T cells. Forty-eight hours later, the transfectant viruses were harvested and propagated in MDCK cells to produce stock viruses. Virus titers were determined by using plaque assays in MDCK cells.

2.5. Virulence and replication in mice

Four 6-week-old female BALB/c mice (Japan SLC, Hamamatsu, Japan) per group were intranasally infected with 10^5 or 10^6 PFU (50 μ l) of viruses. Body weight and survival were monitored daily for 14 days. Mice with body weight loss of more than 25% of their pre-infection values were euthanized. For virological analysis, six mice per group were infected with 10^5 PFU of viruses and three mice per group were euthanized on days 3 and 6 pi. Viruses in lungs, nasal turbinates, brains, livers, spleens, kidneys, and colons were titrated by using plaque assays in MDCK cells.

2.6. HA molecule mapping

The sites of the amino acid mutations identified in the HA of MA-CA04 were mapped on the HA crystal structure of A/California/04/2009 (H1N1), which was obtained from the Protein Data Bank (PDB; <http://www.rcsb.org/pdb/home/home.do>, PDB ID: 3LZG) (Xu et al., 2010) by using Pymol software (<http://www.pymol.org/>).

3. Results

3.1. Adaptation of a pandemic (H1N1) 2009 influenza virus to mice

Pandemic (H1N1) 2009 influenza virus A/California/04/09 (CA04) showed mild pathogenicity in mice [MLD₅₀ (dose required to kill 50% of mice): $>10^6$ PFU] (Itoh et al., 2009; Maines et al., 2009). To produce a mouse-adapted pandemic (H1N1) 2009 virus, we passaged CA04 in mice by intranasally infecting mice, making lung homogenates 3–5 days pi, infecting naïve mice with those homogenates, and repeating the process. After ten serial passages, the viruses exhibited high virulence in mice (MLD₅₀: 1.5×10^4 PFU) (Fig. 1A and B).

We then plaque-purified the mouse-passaged virus and compared three of the plaque-picked clones. These three virus clones possessed identical viral genomes (see below) and exhibited comparable pathogenicity in mice (data not shown). We, therefore, selected one of these clones to represent mouse-adapted CA04 (MA-CA04) for subsequent experiments. We then investigated the tissue tropism of the parental and mouse-adapted viruses. The replication of both viruses was restricted to the respiratory tract (lungs and nasal turbinates); no virus was recovered from spleen, kidney, liver, colon, or brain (Fig. 1C).

3.2. Amino acid substitutions in viral proteins during adaptation to mice

To identify amino acid substitutions introduced during virus passaging in mice, the sequences of all of eight segments of MA-CA04 were compared with those of wild-type virus CA04. We found eight amino acid differences between CA04 and MA-CA04 [one in PB1 (a threonine-to-alanine substitution at position 291: PB1-T291A), three in PA (PA-M21I, PA-A70V, and PA-S616P), three in HA (HA-D127E, HA-K142N, and HA-D222G), and one in NP (NP-D375N)] (Table 1). Threonine at position 291 in PB1 (PB1-291T), PA-21M, PA-70A, and PA-616S are highly conserved among human, swine, and avian isolates, although PA616A is conserved in human seasonal H1N1, but not H3N2, viruses. Therefore, the amino acids found at these positions in MA-CA04 were unique to this mouse-adapted virus. Interestingly, about half of the classical swine isolates and one-third of avian-like swine isolates possess HA-127E, HA-142N and HA-222G. By contrast, most pandemic (H1N1) 2009 isolates possess HA-127D, HA-142K, and HA-222D. Most avian H1 HAs possess HA-127E and HA-222G, indicating that during adaptation of CA04 in mice, the virus acquired amino acids commonly found in avian viruses. NP-375D, found in CA04, is observed in most pandemic (H1N1) 2009 virus, most of swine and avian isolates, whereas only a limited number of seasonal isolates (both H1N1 and H3N2) possess D at this position.

3.3. Mutations in PA, HA, and NP contribute to virulence in mice

To identify amino acid substitutions in MA-CA04 responsible for virulence in mice, we used reverse genetics to generate single-gene CA04-MA-CA04 reassortants that possessed the PB1, PA, HA, or NP gene of the MA-CA04 virus and the remaining genes from CA04 (e.g., CA04-MA-PB1 had the PB1 gene from MA-CA04 and its remaining seven genes from CA04), as well as both original viruses, CA04 and MA-CA04. We then infected mice with 10^5 or 10^6 PFU of these viruses and monitored their body weight and survival. None of the mice infected with the reverse genetics-derived CA04 (rgCA04) died and none lost more than 10% of their body weight (Fig. 2). By contrast, mice infected with the reverse genetics-derived MA-CA04 (rgMA-CA04) and all of the single-gene reassortants tested, except for CA04-MAPB1, lost a significant amount of body weight compared to mice infected with rgCA04. CA04-MAPB1 killed one of the four mice infected at 10^6 PFU.

Since the MA-CA04 virus possesses more than one amino acid substitution in PA and HA, we next generated recombinant viruses that possessed a single amino acid substitution in PA or HA in the genetic background of CA04 (e.g., CA04-PA-M21I had an M-to-I substitution at amino acid position 21 in PA of CA04) and examined their virulence in mice as described above. Mice infected with CA04-PA-M21I, CA04-PA-A70V, CA04-HA-D127E, and CA04-HA-E222G lost a significant amount of body weight compared to those infected with rgCA04. These results indicate that the single amino acid substitutions in PA (PA-M21I and PA-S616P), HA (HA-D127E and HA-D222G), and NP (NP-D375N) each contribute independently to the virulence of MA-CA04 in mice. Moreover, the extent to which these mutations contributed to the virulence varied, for example, NP-D375N appeared to have a substantial effect on virulence.

3.4. HA-D127E contributes to efficient and prolonged viral replication in mouse lungs

To evaluate the effect of the mutations found in MA-CA04 on viral replication in mouse lungs, we infected mice with 10^5 PFU of viruses possessing MA-CA04-derived single genes or amino acids in the background of CA04 and determined virus titers in the lungs on days 3 and 6 pi. Although there was a significant difference between rgCA04 and rgMA-CA04 in virus titers, the lung virus titers of all of the mutant viruses except CA04-MA-HA and CA04-HA-D127E were not significantly higher than that of rgCA04 (Table 2). On the other hand, the titers of CA04-MA-HA and CA04-HA-D127E were significantly higher than that

of rgCA04 on day 6 pi, indicating that the D-to-E substitution at position 127 in HA contributes to efficient viral replication in mouse lungs. By contrast, the virus titers in the lungs of mice infected with CA04-HAK142N were significantly lower than those in the lungs of mice infected with rgCA04.

4. Discussion

Here, to gain insight into influenza virus adaptation to new host species, we serially passaged CA04 in mouse lungs and identified eight mutations in PB1, PA, HA, and NP in a mouse-adapted strain (Table 1). Five of these mutations, PA-M21I, PA-S616P, HA-D127E, HA-D222G, and NP-D375N, independently contributed to the pathogenicity and thus adaptation of CA04 to mice (Fig. 2). Further, we found that HA-D127E contributes to efficient viral replication in mouse lung (Table 2). These results suggest that the viral polymerase complex and HA are both important for viral adaptation to a new host species, in agreement with recent studies on the adaptation of pandemic H1N1 viruses to mice (Ilyushina et al., 2010; Ye et al., 2010).

Several amino acid mutations in PA (PA-T20A, K22R, T97I, M155T, D216N, P277S, L315F, P355S and K615N) are associated with virulence in mice (Gabriel et al., 2005; Li et al., 2005; Song et al., 2009). Although, we did not find any of these previously identified mutations, amino acid substitutions at positions 22 and 615 in PA have been reported in mouse-adapted H5N2 and H7N7 virus strains (Gabriel et al., 2005; Song et al., 2009) and these mutations may be involved in the virulence in mice together with other mutations (Gabriel et al., 2005; Song et al., 2009). These findings suggest that PA-M21I and PA-S616P in MA-CA04 may play similar roles to those of the previously identified mutations at positions 22 and 615 in PA.

Three mutations (HA-D127E, HA-K142N, and HA-D222G) were detected in the HA of the mouse-adapted strain. HA binds to cellular receptors and mediates cell entry of the virus (Skehel and Wiley, 2000). Avian and human influenza viruses preferentially bind to α 2,3 and α 2,6 sialic acid-linked receptors, respectively (Rogers and Paulson, 1983; Rogers et al., 1983). All three of the HA mutations are located close to the receptor binding pocket in HA (Fig. 3); suggesting that these mutations may affect receptor binding specificity. In fact, HA-D222G is known to increase the binding specificity for α -2,3 sialic acid-linked 'avian-type' receptors (Takemae et al., 2010; Tumpey et al., 2007). Given that the cells in mouse lungs mainly express avian type receptors (Ning et al., 2009), it is reasonable that an amino acid substitution that increases HA specificity for avian type receptors would be introduced during adaptation to mice. Interestingly, a pandemic (H1N1) 2009 virus possessing HA-D222G was isolated from hospitalized patients, suggesting that this HA substitution may be associated with illness severity (Kilander et al., 2010). Moreover, Ilyushina *et al.* (2010) recently reported on ten amino acid mutations, including HA-D222G, in a mouse-adapted pandemic (H1N1) 2009 virus. HA-D222G was also reported to decrease the transmissibility of the 1918 Spanish influenza virus (Tumpey et al., 2007). These findings suggest that HA-D222G is a critical mutation for adaptation and increased virulence of pandemic influenza virus in mice and possibly humans. The HA-D127E mutation also enhances viral replication and pathogenicity to mice. Although this mutation has not been found in humans yet, it may affect the pathogenicity of pandemic (H1N1) 2009 viruses.

NP-D375N substantially increased the virulence of CA04 compared to the other seven mutations we identified in this study (Fig. 2). NP is implicated in host restriction (Brown, 2000; Scholtissek et al., 1993). Furthermore, NP-375D is conserved in avian, most classical swine viruses, and most pandemic (H1N1) viruses (Dawood et al., 2009; Garten et al., 2009; Smith et al., 2009) (Table 1), whereas most seasonal viruses possesses V or G at this

position. These findings suggest that the amino acid substitution at position 375 in NP may play a role in host-range alteration, especially from avian to mammalian species.

The 2009 pandemic spurred the development of new anti-viral measures, including drugs and vaccines. Our mouse-adapted pandemic (H1N1) 2009 virus strain could be of use in the development and evaluation of novel anti-viral agents and vaccine candidates, since its high virulence in mice would allow clear-cut efficacy assessments.

Acknowledgments

We thank Susan Watson for editing the manuscript. This work was supported by a Grant-in-Aid for Specially Promoted Research, by a contract research fund for the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases from the Ministry of Education, Culture, Sports, Science, and Technology, by grants-in-aid from the Ministry of Health and by ERATO (Japan Science and Technology Agency), by National Institute of Allergy and Infectious Diseases Public Health Service research grants, and by an NIAID-funded Center for Research on Influenza Pathogenesis (CRIP, HHSN266200700010C). Saori Sakabe is supported by a Research Fellowship from the Japan Society for the Promotion of Science for Young Scientists.

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Fig. 1.

Virulence of CA04 and MA-CA04 in mice. Survival rate (A), body weight changes (B), virus titers in respiratory organs (C) of mice infected with either CA04 or MA-CA04. Ten mice per group were infected with 10^5 PFU of CA04 or MA-CA04 and survival (A) and body weight (B) of four mice per group were monitored daily for 14 days. Three mice per group were euthanized and virus titers in the lungs, nasal turbinates, brains, livers, spleens, kidneys, and colons were determined by using plaque assays in MDCK cells (C). The results are expressed as the mean $\pm$ SD. Viruses were not detected in brains, lungs, spleens, kidneys, or colons.

Fig. 2. Pathogenicity of viruses in mice. Four mice per group were infected with 10^5 or 10^6 PFU of the indicated mutant viruses. Body weight and survival were monitored daily for 14 days. * $P < 0.05$: significant difference compared to rgCA04 (Dunnett's multiple comparison method)

Fig. 3.

Position of the amino acid mutations identified in the HA of MA-CA04. Amino acids at position 127 (red), 142 (blue), and 222 (green) in HA were mapped on the CA04 HA crystal structure (Xu et al., 2010). Three components of the HA receptor-binding pocket, the '130 loop' (amino acid positions 131–135), '190 helix' (184–191), and '220 loop' (218–225) (Yang et al., 2010), are shown in pink, light blue, and, yellow, respectively. The region around the receptor-binding pocket is enlarged in the picture on the right.

Table 1

Amino acid differences between CA04 and MA-CA04

Amino acid encoded (Number of strains possessing mutation/total number of strains examined) ^a									
Protein	Amino acid position	CA04	MA-CA04	Human pandemic (H1N1) 2009	Human seasonal H3N2		Swine		Avian
					Human seasonal H1N1	Human seasonal H3N2	Classical	Eurasian avian-like	
PB1	291	T	A	T(397/397)	T(405/409) I(3/409) S(1/409)	T(571/571)	T(89/89)	T(289/293) A(1/293) I(3/293)	T(5584/5593) A(7/5593) V(1/5593) I(1/5593)
PA	21	M	I	M(461/461)	M(341/341)	M(492/494) I(2/494)	M(93/93)	M(266/266)	M(4597/4602) R(3/4602) V(2/4602)
	70	A	V	A(455/461) V(5/461)	A(340/341) V(1/341)	A(521/526) V(4/526) T(1/526)	A(93/93)	A(253/266) V(11/266) S(2/266)	A(4500/4621) V(107/4621) T(11/4621) S(2/4621) M(1/4621)
HA ^b	616	S	P	S(460/461) P(1/461)	F(341/341)	S(544/544)	S(93/93)	S(261/266) T(2/266) P(1/266) L(2/266)	S(4605/4621) T(7/4621) A(4/4621) L(3/4621) P(2/4621)
	127	D	E	D(667/667)	T(825/866) N(38/866) S(2/866) I(1/866)		E(165/317) D(144/317) N(5/317) T(2/317) S(1/317)	D(38/127) E(37/127) T(35/127) S(11/127) N(6/127)	E(169/177) D(7/177) deleted(1/177)
	142	K	N	K(665/667) N(2/667)	S(791/867) N(59/867) R(17/867)		N(214/317) K(78/317) S(18/317) R(3/317) L(2/317) A(1/317) H(1/317)	N(39/127) S(37/127) R(36/127) L(7/127) K(6/127) H(2/127)	S(162/179) N(11/179) K(5/179) R(1/179)
	222	D	G	D(610/667) G(21/667) E(28/667) N(8/667)	D(813/866) N(21/866) G(31/866) E(1/866)		D(170/314) G(130/314) N(8/314) E(2/314) K(2/314) T(1/314) V(1/314)	G(52/126) D(44/126) E(28/126) K(1/126) T(1/126)	G(171/182) D(7/182) N(3/182) E(1/182)

Protein	Amino acid position	Amino acid encoded (Number of strains possessing mutation/total number of strains examined) ^d							
		CA04	MA-CA04	Human pandemic (H1N1) 2009	Human seasonal H1N1	Human seasonal H3N2	Swine		Avian
							Classical	Eurasian avian-like	
NP	375	D	N	D(335/343) N(5/343) B(2/343) G(1/343)	V(186/216) D(14/216) G(7/216) E(9/216)	G(497/512) E(114/512) D(1/512)	D(221/267) E(19/267) G(18/267) N(5/267) V(3/267) Y(1/267)	D(92/113) E(20/113) N(1/113)	D(4495/4608) E(47/4608) N(35/4608) S(19/4608) G(6/4608) V(3/4608) A(1/4608) Q(1/4608) Y(1/4608)

No amino acid differences between the PB2, NA, M, and NS genes of CA04 and MA-CA04 were observed.

^a excludes the completely identical sequences
^b H1 HA (H1 numbering)

Table 2

Virus titers in mouse lungs

Virus	Virus titer (mean log ₁₀ PFU ± SD/g) in mouse lungs	
	Day 3 pi	Day 6 pi
rgCA04	7.8±0.4	6.4±0.1
rgMA-CA04	8.8±0.2*	7.7±0.2*
CA04-MAPB1 (T291A)	7.7±0.4	6.5±0.2
CA04-MAPA	8.4±0.1	6.5±0.1
CA04-MAHA	8.4±0.1	7.9±0.2*
CA04-MANP (D375N)	8.2±0.5	6.4±0.2
CA04-PAM2II	8.3±0.0	6.4±0.1
CA04-PAA70V	7.6±0.2	6.6±0.2
CA04-PAS616P	8.0±0.1	6.6±0.7
CA04-HAD127E	8.0±0.1	7.4±0.1*
CA04-HAK142N	5.0±0.6*	5.3±0.7*
CA04-HAD222G	8.3±0.1	6.7±0.1

Six mice per group were infected with 10⁵ PFU of virus and three mice in each group were euthanized on days 3 and 6 pi, Virus in lungs was titrated by using plaque assays.

* $P < 0.05$: significant difference compared to rgCA04 (Dunnett's multiple comparison method)