Amino acid determinants conferring stable sialidase activity at low pH for H5N1 influenza A virus neuraminidase

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1. Introduction

Since the first isolation of H5N1 highly pathogenic avian influenza A virus (HPAI) from a 3-year-old boy in Hong Kong in 1997, more than 600 confirmed cases of human H5N1 HPAI infection have been reported, mainly from Indonesia, Viet Nam, and Egypt (as of June, 2012). Since 2004, frequent human infections have raised worldwide concerns regarding the pandemic potential of H5N1 HPAI and its high fatality rate (approximately 60% as of 2012 June) [1]. The viral envelope glycoprotein hemagglutinin (HA) has been investigated extensively and is essential to the high virulence and transmission of HPAI to humans, because it possesses receptor binding ability (recognizing both avian- and human-type sialoglycoconjugates). It also has a multi-basic cleavage site that facilitates systemic infection in chicken and mice [2, 3]. Another viral envelope glycoprotein, neuraminidase (NA), has sialidase activity that enhances virus release from the cell surface by removing sialic acid from cellular glycoconjugates and viral glycoproteins. Deletions in the NA stalk and a reduction in the level of sialidase activity of H5N1 HPAI NA per se has not been compared with that of human influenza A virus (IAV) NA or other avian IAV NA [6].

We previously showed that influenza viruses differ in their stability at low pH. All avian IAVs isolated since 2003 had NAs that were unstable at low pH, similar to human epidemic IAVs. These H5N1 viruses are thus already human virus-like and, therefore, have the frequent infections of humans.

Avian influenza A viruses (IAVs) and human 1918, 1957, and 1968 pandemic IAVs all have neuraminidases (NAs) that are stable at low pH sialidase activity, yet most human epidemic IAVs do not. We examined the pH stability of H5N1 highly pathogenic avian IAV (HPAI) NAs and identified amino acids responsible for conferring stability at low pH. We found that, unlike other avian viruses, most H5N1 IAVs isolated since 2003 had NAs that were unstable at low pH, similar to human epidemic IAVs. These H5N1 viruses are thus already human virus-like and, therefore, have the frequent infections of humans.
H3N2 IAV, are also stable at low pH. Viruses possessing a low-pH-stable NA from a pandemic IAV in the background of A/WSN/33 (WSN; H1N1) replicated more efficiently in cell culture and mouse lungs compared with a WSN virus possessing a NA unstable at low pH [8]. This enhanced replicative ability was associated with retention of the sialidase activity under the acidic conditions of the endocytic pathway during virus entry [8]. On the other hand, the NAs of most seasonal human IAVs are unstable at low pH [7,9,10].

Here, we examined the sialidase activity of 42 H5N1 HPAI viruses isolated from humans, chickens, ducks, and other waterfowl and found that most of the NAs, with the exception of three H5N1 viruses, were not stable at low pH. We also identified amino

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**Fig. 1.** The low-pH stabilities of the sialidase activities of H5N1 HPAI NAs, human H1N1 IAV NAs, and non-H5N1 avian IAV NAs. Sialidase activities of NA-expressing cells transfected with each NA gene were measured at pH 4.0 (filled columns), 5.0 (gray columns) and 6.0 (open columns). Sialidase activities are expressed as a percentage of each activity at pH 6.0. The NA genes used were derived from H5N1 HPAIs isolated from humans (A), aquatic birds (B), chickens (C), other avian IAVs (D) and from human H1N1 IAVs (E).
acid determinants that could confer low-pH stability to H5N1 HPAI NAs.

2. Materials and methods

2.1. Cells

Human embryonic kidney 293T cells were maintained in high glucose Dulbecco's modified medium supplemented with 10% fetal bovine serum (FBS).

2.2. NA genes and plasmids

All NA genes inserted into the pCAGGS expression vector and virus abbreviations are listed in Supplementary Table 1. Chimeric NAs between A/duck/Guangdong/1/01 (H5N1) (DKG/1) and A/Hong Kong/213/03 (H5N1) (HK/213) were generated by ligating the MfeI and BamHI sites (at positions 595 and 1145 in the NA gene, respectively) or by ligating two PCR fragments derived from primers with the BsaI site (at position 842 in the NA gene). The Y155H, I289T, and Q313R mutations of DKG/1 NA and the H155Y, T289I, and R313Q mutations of HK/213 NA were introduced by means of PCR.

2.3. Sialidase activity of cell-expressed NA

293T cells (1.5 × 10⁵ cells/well) in a 24-well tissue culture plate were cultured overnight. The 70% confluent cells were transfected with a plasmid (1 μg/well) for NA expression by using TransIT-293 (Mirus, Madison, WI). After a 24-h incubation at 37 °C, the transfected cells were suspended in phosphate-buffered saline (PBS; 1.2 ml/well), and 50 μl of each cell suspension was transferred into microtubes and centrifuged at 100 × g for 10 min. The cell pellets were incubated with 57 μl of 10 mM acetate buffer (pH 4.0, 5.0, or 6.0) at 37 °C for 10 min. Fifty microliters of each suspension was then transferred to a 96-well black plate on ice and reacted with 2.5 μl of 2 mM 2-O-(4-methylumbelliferyl)-N-acetylneuraminic acid (4MU-Neu5Ac; Sigma-Aldrich Corp., St. Louis, MI) at 37 °C for 30 min. The reaction was stopped by the addition of 200 μl of 100 mM sodium carbonate buffer (pH 10.7). The fluorescent intensity (Ex, 355 nm; Em, 460 nm) was measured with an Infinite M1000 microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

The sialidase activities of the cell-expressed NAs were expressed as a percentage of the activity at pH 6.0.

3. Results

3.1. Low-pH stabilities of the sialidase activities of H5N1 HPAI NAs

We tested the low-pH stabilities of the sialidase activity of cells expressing each of the NA genes of 42 H5N1 HPAI viruses isolated from human and avian hosts, as well as 10 other non-H5N1 avian IAVs and eight human H1N1 IAVs. The NAs of all avian IAVs tested were highly stable at low-pH, as described in our previous reports [7,9,10]. Three H5N1 HPAI NAs of A/Hong Kong/483/97 (HK/483), DKG/1, and CKK/3, were also stable at low pH; at pH 4.0, their NAs had more than 30% of the sialidase activity at pH 6.0. Thirteen H5N1 HPAI NAs were also moderately stable at low pH, retaining more than 30% of their sialidase activity at pH 5.0 compared with that at pH 6.0. However, the NAs of the remaining 26 H5N1 HPAIVs, which were isolated since 1997, were not low-pH-stable, like seasonal human IAVs. No distinct host species–specific differences in their low-pH stabilities were apparent among the H5N1 viruses isolated from different hosts such as humans (Fig. 1A), aquatic birds (Fig. 1B), or chickens (Fig. 1C). There were also no differences in the low-pH stabilities of H5N1 HPAI NAs between aquatic and terrestrial birds (e.g., chickens). We also measured the sialidase activities of avian IAV NAs (Fig. 1D) and human H1N1 IAV NAs (Fig. 1E) as controls of avian-like and human-like low-pH stability. All avian IAV NAs tested were highly stable at low pH, whereas most human H1N1 IAV NAs, except Spanish IAV A/Brevig Mission/1/18 (H1N1) NA (BM/1), were not low-pH-stable, consistent with our previous work [7,9,10].

When we measured the sialidase activity after pre-incubating the NAs under acidic conditions, BM/1 NA lost its activity in a time-dependent manner [10], whereas avian IAV NAs retained their sialidase activity even after 60 min of pre-incubation at pH 4.0 [10] (Supplementary Fig. 1G and H). The low-pH-stable NAs of H5N1 HPAI DKG/1 and A/chicken/Kyoto/3/04 (CKK/3) lost their sialidase activities, similarly to BM/1, as a function of increasing pre-incubation time under acidic conditions (Supplementary Fig. 1A and C). The low-pH-unstable H5N1 HK/213, A/whooper swan/Mongolia/6/05 (WSM/6), and A/Viet Nam/1203/04 (VN/1203) NAs also lost their sialidase activity, as did human H1N1
IAV NAs, in this assay (Supplementary Fig. 1B, D, I, and J). These results indicate that most H5N1 HPAIs have low-pH-unstable NAs, like seasonal human IAVs, and that H5N1 HPAIs with low-pH-stable NAs are the exception, like human pandemic Spanish IAVs, and their low-pH stability profiles differ from the highly stable NAs at low pH in more common avian IAVs (Supplementary Fig. 1).

3.2. Identification of amino acid residues responsible for the low-pH stability of an H5N1 HPAI NA

The amino acid sequence of the low-pH-stable DKG/1 NA was most closely related to that of the low-pH-unstable HK/213 NA among the viruses tested here (Supplementary Fig. 2). Therefore, to determine the amino acid residues responsible for the low-pH stability of H5N1 HPAI NAs, we used the DKG/1 NA and the HK/213 NA to generate chimeric NAs. There are eight amino acid differences between the DKG/1 NA and the HK/213 NA in their ectodomains (amino acids 83–468), at positions 155, 224, 257, 267, 289, 313, 341, and 415 (DKG/1 NA numbering) (Table 1). First, we generated eight chimeric NAs (Chimeras 1–8) by using the Mfe I and BamH I sites and PCR primers with the BsaI site (Fig. 2A) and tested their low-pH stability. Based on the properties of chimeras 3 and 4, residues at positions 155, 289, 313, and 341 were identified as candidate determinants of the low-pH stability of DKG/1 NA. Taken together, the residues at positions 155, 289, 313, and 341 appeared to be determinants for the DKG/1 NA's low-pH stability (Fig. 2A, Table 1). We therefore generated an additional eight chimeric NAs in which...
Tyr or His was substituted at position 155 in the different genetic backbones (chimeras 9–16) (Fig. 2B). A striking difference in low-pH stability between chimeras 13 and 14 suggested that the residues at positions 289, 313, and 341 were likely determinants of low-pH stability. Finally, we generated three NAs with substitutions at positions 289, 313, and 341, respectively, in addition to the substitution at position 155 in the DKG/1 NA (Fig. 2C) or the HK/213 NA (Fig. 2D). The dual substitutions at positions 155 and 341 substantially changed the enzymatic stabilities at pH 4.0 and 5.0 (Table 1). We also examined the low-pH stability of the HK/213 NA mutant with substitutions at positions 155 and 341 after it was incubated under acidic conditions in a time-dependent manner. The HK/213 NA with both the H155Y and K341N mutations lost its sialidase activity in a time-dependent manner, like the DKG/1 NA (Supplementary Fig. 1A and F), but it was not as stable as the highly stable NAs at low pH in the more common avian IAVs (Supplementary Fig. 1G and H). The DKG/1 NA with both the Y155H and N341K mutations lost its sialidase activity within five minutes of exposure to pH 4.0 and 5.0, like the HK/213 NA (Supplementary Fig. 1B and E). Therefore, these results show that histidine at position 155 and lysine at position 341 are responsible for the low-pH instability of H5N1 HPAI NAs.

4. Discussion

Here, we showed that most H5N1 HPAI NAs are unstable at low pH, like seasonal human IAV NAs. Thus, the low-pH stability of H5N1 HPAI NAs more closely resembles that of human IAV NAs than that of avian IAV NAs, regardless of the host (i.e., humans and birds) from which the virus is isolated. Although human infections with avian H5N1, H7N3, H7N7, and H9N2 IAVs have occurred, by far the largest number of human infections has been reported for H5N1 HPAI NAs [11]. Our previous research suggested that NA that are unstable at low pH may be more suitable for the adaptation of IAV to humans [9]. The low-pH instability of H5N1 HPAI NAs among avian IAV NAs might contribute to the frequent transmission of H5N1 HPAI to humans.

For the DKG/1 NA and the HK/213 NA, both tyrosine at position 155 and asparagine at position 341 were responsible for the stability at low pH; histidine at position 155 together with lysine at position 341 destabilized NA activity at low pH. In the three-dimensional structure of VN/1203 NA (Fig. 3) [12], the residue at position 155 was near the monomer interface, suggesting that it may be involved in stabilization of the homotetrameric structure. The residue at position 341 was located near the calcium ion-binding site, which is thought to be involved in the conformation of the enzymatic active site [13,14]. We previously identified residues at positions 430, 435, and 454 (BM/1 N1 numbering) as important for the low-pH stability of N1 NAs (Supplementary Fig. 3A) [10] and residues at positions 344 and 466 (N2 numbering) as important for the low-pH stability of N2 NAs (Supplementary Fig. 3B) [15]; these residues are also located near the enzymatic active site, the calcium ion-binding site, and the subunit interfaces.

Of the 42 H5N1 NAs tested, we found that the tyrosine at position 155 that is responsible for the low-pH stability of DKG/1 NA was present in only three NAs [those of viruses HK/483, A/Hong Kong/486/97 (HK/486), and DKG/1], whereas the remaining 39 H5N1 HPAI NAs, including the low-pH-stable CKK/3 NA, had histidine at this position, indicating that the low-pH stability of CKK/3 NA does not require tyrosine at this position. The lysine at position 341 that was responsible for the low-pH instability of HK/213 NA was present only in this NA; the remaining 41 NAs tested here had asparagine at this position. Therefore, these substitutions are not necessarily responsible for the low-pH stabilities of all H5N1 HPAI NAs.

Several moderately stable NAs at low pH were found among the 42 H5N1 HPAI NAs (Supplementary Fig. 2). The amino acid sequences of the low-pH-unstable A/Viet Nam/UT3028II/03 (VN/UT3028II) NA and the moderately stable A/Viet Nam/UT3028/03 (VN/UT3028) NA at low pH differ by only a single substitution at position 344 (DKG/1 NA numbering; cysteine for VN/UT3028II and glycine for VN/UT3028). Also, only two amino acid differences were found between the unstable A/duck/Viet Nam/5001/04 (DKVN/5001) NA and the moderately stable A/Viet Nam/UT30259/04 (VN/UT30259) NA at low pH; the former having lysine and serine and the latter having asparagine and glycine at positions 150 and 249 (DKG/1 NA numbering), respectively. These results indicate that amino acids at positions other than 155 and 341 affect the low-pH stability of H5N1 HPAI NAs.

Among the residues responsible for the low-pH stability of the NAs of H5N1 HPAI viruses, the residues at positions 249 and 344 are located at or near the calcium ion-binding site, whereas the residues at positions 150 and 249 are located near the enzymatic active site. These results are consistent with our previous findings with BM/1 N1 and human N2 [10,15].

Unlike avian IAV NAs, most human IAV NAs are not stable at low pH. Therefore, most H5N1 HPAI NAs isolated since 2003 are more like the NAs of human IAVs in this regard, which might explain their frequent infection of humans. Further studies of the low-pH stability of IAV NAs may enable us to predict low-pH stability on the basis of the amino acid residues located near the active site, the calcium ion-binding site, and the subunit interfaces.
which may, in turn, help us to assess the adaptability of avian IAVs to humans.

Acknowledgments

We thank Susan Watson for scientific editing. This work was supported, in part, by MEXT/JSPS KAKENHI Grant Number (C; 23590549), by a Sasakawa Scientific Research Grant from The Japan Science Society, by a SRI (Shizuoka Research Institute) academic research grant, by the Global COE Program from the Japan Society for the Promotion of Science, by ERATO (Japan Science and Technology Agency), by a grant-in-aid for Specially Promoted Research from the Ministries of Education, Culture, Sports, Science, and Technology, by grants-in-aid from Health, Labor, and Welfare of Japan, by the Japan Initiative for Global Research Network on Infectious Diseases, and by National Institute of Allergy and Infectious Disease Public Health Service research grants.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2012.08.007.

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