

# A molecular mechanism for the low-pH stability of sialidase activity of influenza A virus N2 neuraminidases<sup>1</sup>

Tadanobu Takahashi<sup>2</sup>, Takashi Suzuki<sup>2,\*</sup>, Kazuya I.-P. Jwa Hidari, Daisei Miyamoto, Yasuo Suzuki\*

Department of Biochemistry, University of Shizuoka, School of Pharmaceutical Sciences, CREST, JST, and COE Program in the 21<sup>st</sup> Century, Shizuoka 422-8526, Japan

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**Abstract** Four human pandemic influenza A virus strains isolated in 1957 and 1968, but not most of the epidemic strains isolated after 1968, possess sialidase activity under low-pH conditions. Here, we used cell-expressed neuraminidases (NAs) to determine the region of the N2 NA that is associated with low-pH stability of sialidase activity. We found that consensus amino acid regions responsible for low-pH stability did not exist in pandemic NAs but that two amino acid substitutions in the low-pH-stable A/Hong Kong/1/68 (H3N2) NA and a single substitution in the low-pH-unstable A/Texas/68 (H2N2) NA resulted in significant change in low-pH stability.

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**Key words:** Chimera; Influenza virus; Low-pH stability; Molecular mechanism; Neuraminidase; Sialidase

## 1. Introduction

New pandemic influenza A viruses have been established by genetic reassortment between human and animal influenza A virus genes [1–5]. Pandemic outbreaks of human influenza A viruses with the N2 neuraminidase (NA; EC 3.2.1.18) subtype occurred in 1957 and 1968. Influenza A virus NAs play an important role in the release of new viruses from host cells [6] and are also related to the host range and virulence of influenza A viruses [7–9].

In a previous study, it was found that avian H1N1 influenza A viruses retain their infectivity and sialidase activities after low-pH treatment [10]. We have also found that the 1957 and 1968 human pandemic influenza A viruses as well as duck viruses retained their sialidase activities even at pHs of less than 4.5 but that some of the human H2N2 epidemic strains isolated before 1968 and human epidemic H3N2 strains iso-

lated after 1968 had no sialidase activities under the same conditions [11]. These findings indicate that low-pH stability of influenza A virus sialidase activity may be a critical factor for viral replication in ducks and may be an important point for determining the epidemiology of human influenza A viruses. In this study, we examined the low-pH stability of sialidase activities of cell-expressed N2 NAs by using chimeric NAs and site-specific mutagenesis, and we determined the amino acid residues in the pandemic strain A/Hong Kong/1/68 (H3N2) and the 1968 epidemic strain A/Texas/68 (H2N2) that contribute to the low-pH stability of the sialidase activities of the viruses.

## 2. Materials and methods

### 2.1. Cell cultures

293T human kidney embryonic cells were cultured in high-glucose Dulbecco's modified Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Madin-Darby canine kidney cells were cultured in Eagle's MEM supplemented with 5% heat-inactivated FBS.

### 2.2. Cloning of NA genes

Viral RNA was isolated from virus particles with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and full-length cDNAs of the NA genes were amplified by reverse transcription polymerase chain reaction. The NA genes of A/Japan/305/57 (H2N2) (Jap57), A/Murakami/4/64 (H2N2) (Mur64), A/Texas/68 (H2N2) (Tex68), A/Hong Kong/1/68 (H3N2) (HK68) and A/Memphis/1/71 (H3N2) (Mem71) were subcloned into the *EcoRI* and *XhoI* restriction enzyme sites of the plasmid expression vector pCAGGS/MCS [12] to generate pCAJap57NA, pCAMur64NA, pCATex68NA, pCAHK68NA and pCAMem71NA, respectively.

### 2.3. Construction of chimeric NAs

Utilizing shared restriction enzyme sites for *EcoRI* (at the pCAGGS/MCS multiple cloning site) and *EcoRV* (at N2 gene nucleotide position 869) among pCAJap57NA, pCAMur64NA, pCATex68NA, pCAHK68NA and pCAMem71NA, we generated 12 chimeric constructs (designated 1–12) in all possible pairwise combinations of the low-pH-stable N2 genes (pCAJap57NA and pCAHK68NA) with the low-pH-unstable N2 genes (pCAMur64NA, pCATex68NA and pCAMem71NA). To determine the amino acid residues associated with the low-pH stability of N2, sites for *EcoRV*, *BglII* and *KpnI* (at N2 nucleotide positions 869, 1010 and 1374, respectively) were used to generate eight additional chimeras (designated 13–20) in which portions of the HK68 or Tex68 NA gene had been replaced with corresponding regions of the counterparts (see Fig. 2A). Similarly, sites for *NheI*, *EcoRV*, and *MunI* at N2 nucleotide positions 626, 869, and 1024, respectively, were used to generate four chimeras between pCAJap57NA and pCAMem71NA, (designated 21–24, see Fig. 3A). These constructs were confirmed by automated sequencing using an ABI Prism 373A sequencer (Applied Biosystems, Foster City,

\*Corresponding author. Fax: (81)-54-264-5723.

E-mail address: [suzukit@ys7.u-shizuoka-ken.ac.jp](mailto:suzukit@ys7.u-shizuoka-ken.ac.jp) (T. Suzuki).

<sup>1</sup> Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB101671, AB101672, AB101673, AB101674 and AB101675.

<sup>2</sup> These authors equally contributed to this work.

**Abbreviations:** FBS, fetal bovine serum; MEM, minimum essential medium; 4-MU $\alpha$ -Neu5Ac, 2'-(4-methylumbelliferyl)- $\alpha$ -D-N-acetylneuraminic acid; NA, neuraminidase; PBS, phosphate-buffered saline

CA, USA) or by observation of unique restriction enzyme digestion patterns.

#### 2.4. The low-pH stability of cell-expressed NAs

The pCAGGS/MCS expression plasmid construct for each NA gene or chimeric construct was transfected in 293T cells. Cells were cultured to 70% confluence in 24-well plates and transfected with 0.5 µg of each plasmid per well of the plate by using 25 µl of OPTI-MEM I containing 1 µl of Trans IT-293 (PanVera, Madison, WI, USA). After incubation for 48 h at 37°C, the cells were washed once with phosphate-buffered saline (PBS) (pH 7.2, 131 mM NaCl, 14 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, and 2.7 mM KCl) and suspended in 15 µl of PBS. One microliter of each cell suspension was mixed with 10 µl of 20 mM sodium acetate buffer at pH 4.0 or with 10 µl of the same buffer at pH 5.0 and stored at 37°C for 5 min. Sialidase activities of the mixtures were determined using 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (4-MUα-Neu5Ac) (Sigma-Aldrich, St. Louis, MO, USA) as reported previously [11]. The results were shown as means for duplicate experiments.

### 3. Results

#### 3.1. Low-pH stability of sialidase activity of cell-expressed chimeric NAs

To elucidate the molecular mechanism of the low-pH stability of sialidase activities, we first carried out experiments using cell-expressed NAs to determine whether the N2 NA gene is involved in the pH stability of sialidase activities. Low-pH stability of the sialidase activities of the influenza A viruses tested depended only on NA glycoproteins (unpublished data). We therefore generated a series of chimeric constructs in all possible pairwise combinations of the low-pH-stable N2 genes (pCAJap57NA and pCAHK68NA) with the low-pH-unstable N2 genes (pCAMur64NA, pCATex68NA and pCA-Mem71NA) by utilizing restriction enzyme sites for *EcoRV* (N2 nucleotide position 869) shared with the N2 gene (Fig. 1A). The chimeric NAs were expressed in 293T cells, and sialidase activities of the cell-expressed NAs were examined using the substrate 4-MUα-Neu5Ac. As an indicator of the low-pH stability, the sialidase activity of each cell-expressed NA at pH 4.0 was calculated as a percentage relative to the enzymatic activity of the NA at pH 5.0.

Chimeras 4, 6, and 8 as well as the background pandemic NAs retained their activities at low pH. In contrast, chimeras 3, 5, and 7 showed little sialidase activity at low pH. Chimeras 2 and 12, in which the regions of low-pH-unstable Mem71 and Tex68 NAs had been replaced with the corresponding region of low-pH-stable Jap57 NA as well as that of chimera 8, did not show sialidase activity at low pH. Chimeras 9 and 11, in which the regions of low-pH-stable HK68 and Jap57 NAs had been replaced with the corresponding regions of low-pH-unstable Mur64 and Tex68 NAs, retained about 70–90% of the activities of the background pandemic NAs (Fig. 1B). The results indicate that the amino acid regions responsible for the low-pH stability of sialidase activity in the two pandemic NAs are not the same and that the region of HK68 NA represented by chimeras 4, 6 and 10 may contribute to the low-pH stability of sialidase activity.

#### 3.2. Amino acid residues responsible for low-pH stability

To investigate the molecular mechanism in further detail, we attempted to identify the minimal molecular determinants of the low-pH stability in HK68 NA in comparison with low-pH-unstable Tex68 NA, because they showed the highest homology of the tested N2 NAs (eight amino acid substitutions).

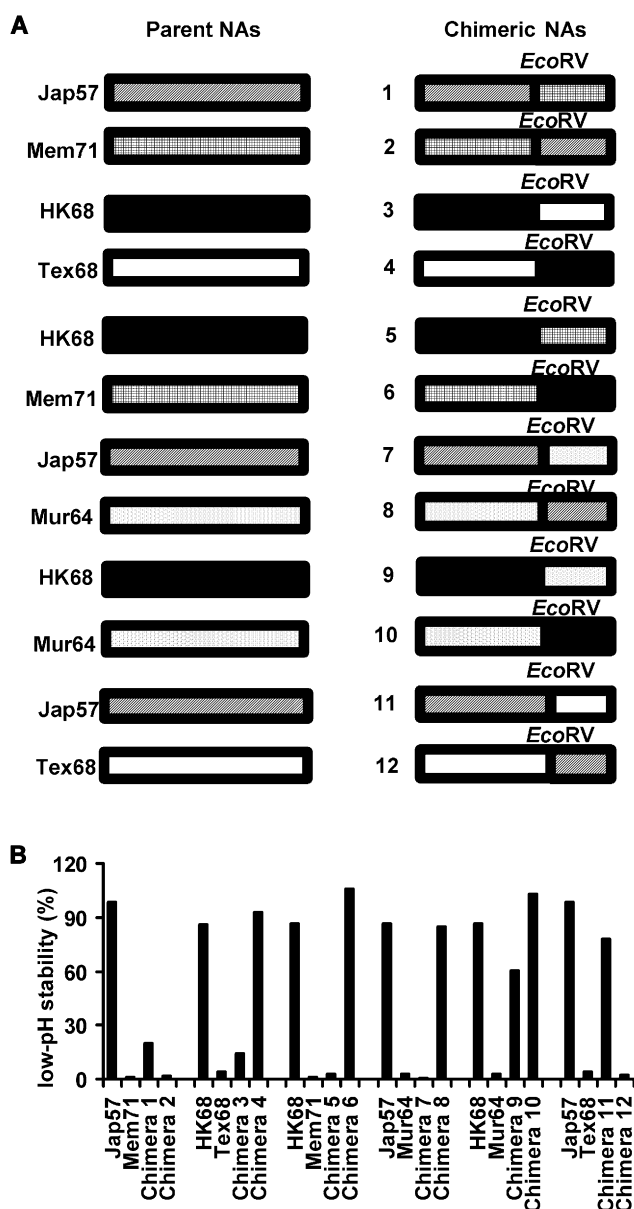


Fig. 1. The low-pH stability of sialidase activities of wild-type NAs and chimera NAs replaced between low-pH-stable and low-pH-unstable NA. Utilizing a shared restriction enzyme site for *EcoRV* (at N2 gene nucleotide position 869) among N2 NA genes, 12 chimeras (designated 1–12) were generated in all possible pairwise combinations of low-pH-stable N2 genes (Jap57 and HK68) with low-pH-unstable N2 genes (Mur64, Tex68 and Mem71). 293T cells were transfected with 0.5 µg of each parental or chimera NA expression vector. Cells expressing parental and chimera NAs were harvested at 48 h post transfection. Sialidase activities of cells expressing NAs were measured using 4-MUα-Neu5Ac. As an indicator of low-pH stability, the sialidase activity at pH 4.0 is shown as a percentage relative to the activity of each cell expressing NA at pH 5.0.

The differences between the low-pH stabilities of chimeras 3 and 4 suggested that the region between amino acid residues 284 and 469 contributes to the low-pH stability of HK68 NA. The regions between amino acid residues 284 and 469 in HK68 NA and Tex68 NA were different only at positions 286, 344 and 466. To elucidate the contribution of three amino acid residues to the low-pH stability, restriction enzyme sites for *EcoRV*, *Bgl*II and *Kpn*I at N2 nucleotide positions

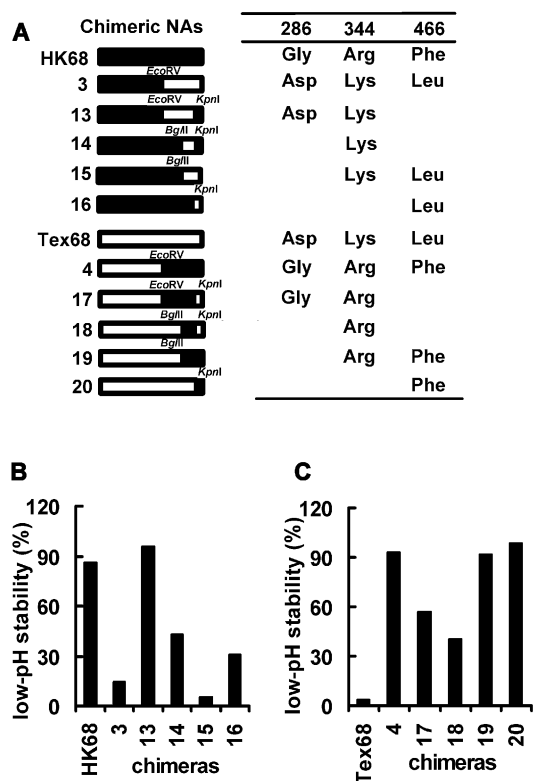


Fig. 2. Determination of amino acid residues responsible for low-pH stability of sialidase activities of HK68 NA and Tex68 NA. A: Chimera NA genes replaced between the HK68 NA (■; pCAH-K68NA) and the Tex68 NA (□; pCATex68NA) by utilizing shared restriction enzyme sites for *EcoRV*, *BglII* and *KpnI* are depicted with only the amino acid residues different from parental NA at positions 286, 344 and 466 in N2 numbering, which are total differences in amino acid residues between HK68 NA and chimera 3 or Tex68 NA and chimera 4. The low-pH stabilities of sialidase activities of (B) chimeric constructs 3, 13–16 derived from parental HK68 NA or (C) chimeric constructs 4, 17–20 derived from parental Tex68 NA were determined.

869, 1010 and 1374, respectively, were used to generate eight additional chimeras in which portions of the HK68 NA gene or the Tex68 NA gene had been replaced with the corresponding regions of the counterparts (Fig. 2A).

The low-pH stability of chimera 13 was almost the same as that of parent HK68 NA. The degree of low-pH stability of sialidase activity of chimera 14, in which position 344 had been changed, and that of chimera 16, in which position 466 had been changed, were only about half of that of the parent HK68 NA. Chimera 15, in which two amino acid residues, at positions 344 and 466, in HK68 NA had been replaced with the corresponding residues from Tex68 NA, did not show the low-pH stability of sialidase activity of parent NA (Fig. 2B). These results indicate that both of the amino acid residues Arg 344 and Phe 466 were indispensable for the property of HK68 NA. In addition, the change in chimera 17 at positions 286 and 344 and the change in chimera 18 at position 344 resulted in almost 50% recovery of low-pH stability of sialidase activity of these chimeras in comparison to no low-pH stability of sialidase activity of parent Tex68 NA. Surprisingly, chimera 20, in which a change in only a single amino acid (Leu to Phe at positions 466) of Tex68 NA (having no low-pH stability of sialidase activity

of almost 0%) had been made, showed low-pH stability of sialidase activity that was almost the same as that of HK68 NA (almost 100%). The almost complete recovery of the low-pH stability of sialidase activity of chimera 19, in which changes of amino acid residues at positions 344 and 466 had been made, also supports the speculation that position 466 contributes significantly to the low-pH stability of sialidase activity. We found that the amino acid substitution of Lys 344 to Arg on Tex68 NA partly contributed to the recovery of low-pH stability of the sialidase activity, but only a single amino acid change at position 466 (Leu to Phe) on Tex68 NA was sufficient for recovery of the low-pH stability of sialidase activity (Fig. 2C).

The low-pH stabilities of four additional chimeric NAs between Jap57 NA and Mem71 NA were also examined (Fig. 3A). Unlike the chimeric NAs between HK68 NA and Tex68 NA, both the region before amino acid position 202 and the region after amino acid position 336 in Jap57 NA were essential for low-pH stability of sialidase activity of Jap57 NA (Fig. 3B). This finding further supports the speculation that Jap57 and HK68 NAs do not possess consensus amino acid regions responsible for their low-pH stability of sialidase activity and suggests that the low-pH stability of sialidase activity is governed by the overall three-dimensional structure of each NA protein.

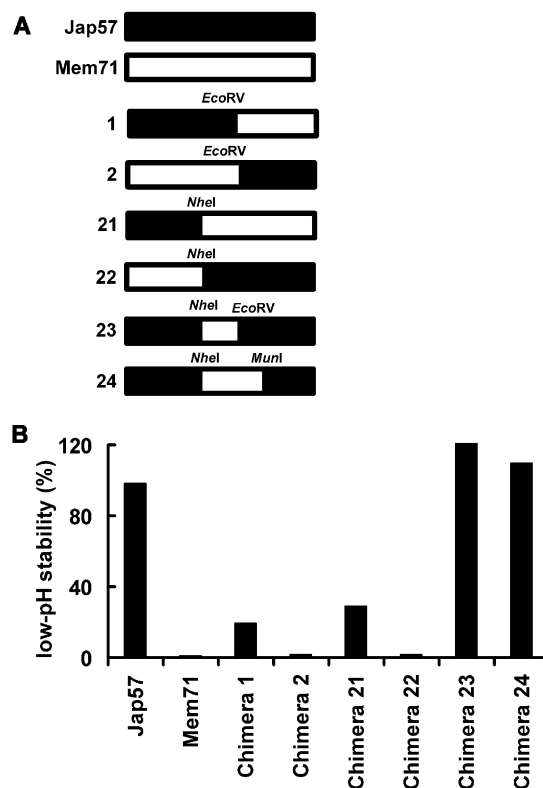


Fig. 3. The low-pH stabilities of sialidase activities of chimeric NAs between the Jap57 NA and the Mem71 NA. A: Chimera NA genes replaced between the Jap57 NA (□; pCAJap57NA) and the Mem71 NA (■; pCAMem71NA) by utilizing shared restriction enzyme sites for *NheI*, *EcoRV*, and *MunI* are depicted. B: The low-pH stabilities of sialidase activities of chimeric constructs were determined as described in the legend of Fig. 1.

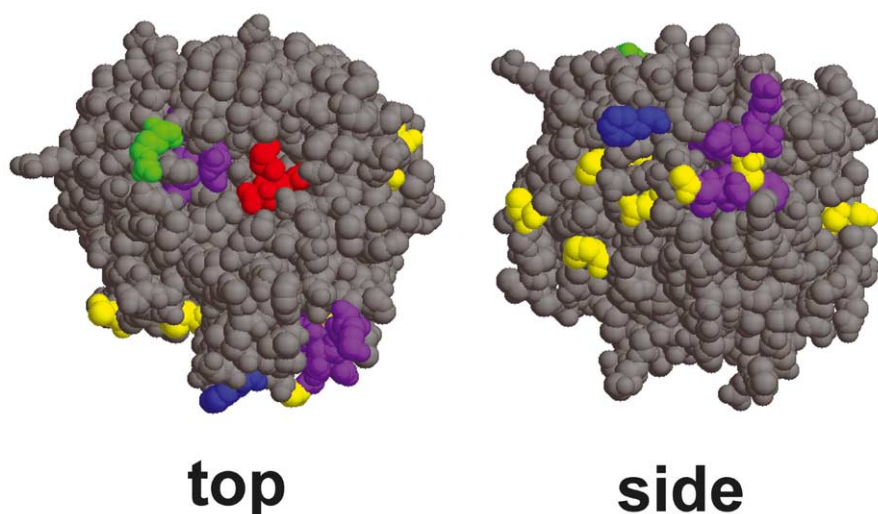


Fig. 4. Location of amino acid residues 344 and 466 in N2 NA protein. The positions of amino acid residues 344 (green) and 466 (blue), the enzymatic active site indicated by the position of the bound sialic acid (red), the calcium ion binding sites (purple), and the subunit interfaces (yellow) are shown on the NA monomer structure of A/Tokyo/3/67 (H2N2) [15].

#### 4. Discussion

In this study, we investigated the molecular mechanism of the low-pH stability of sialidase activities of pandemic influenza A virus NAs by using chimeric NAs replaced between low-pH-stable and low-pH-unstable NAs. The results indicate that consensus amino acid regions responsible for low-pH stability do not exist in Jap57 and HK68 NAs. We therefore examined the minimal molecular determinants of low-pH stability of sialidase activity in the 1968 pandemic HK68 NA. We found that substitutions of both amino acid residues Arg to Lys at position 344 and Phe to Leu at position 466 in HK68 NA reduced the degree of low-pH stability of sialidase activity and that a single amino acid change, Leu to Phe, at position 466 was sufficient to confer the low-pH stability of sialidase activity of HK68 NA to Tex68 NA. Although the amino acid residues at positions 344 and 466 strongly affected the low-pH stability of sialidase activity of HK68 NA, the residues were not located within the active site of N2 NA (Fig. 4). Both of the amino acid residues were located near known calcium ion binding sites in which residues at positions 293, 297, 324, 345, 347, 348, 111–115 and 139–143 are involved. It has been shown that calcium ions are necessary for influenza virus sialidase activity and that the binding of calcium ions to NA plays an important role in stabilizing the protein conformation [13–15]. Substitutions of amino acids around the calcium binding sites in NA would have caused distortion of the overall three-dimensional structure of NA protein. In addition, as the residue at position 466 is located near the subunit interface, in which residues at positions 99, 104, 111, 139, 204, 214, 455, 459 and 460 are involved, its substitution may also have caused distortion of a homotetramer conformation of native NA protein composed of four subunits [16–18] (Fig. 4). The results of our study in which sequences of 12 human, six avian and two swine virus NAs were compared suggest that N2 amino acid residues at positions 153, 253, 307, 329, 344, 347, 356, 368, 390 and 431 are associated with the low-pH stability of sialidase activity of N2 NA [11]. However, none of these positions matches the results of the present study.

A single amino acid alteration in PB2 or NS1 of influenza A virus resulted in an appreciable increase in the rate of virus replication and virulence in mammals [19,20]. We therefore generated mutant influenza A viruses that included N2 NA genes, in which the pH stability of the sialidase activities was changed, by using a plasmid-driven reverse genetics system [21]. The NA mutant viruses showed a significant difference in viral infection (described elsewhere). Further studies are needed to elucidate the role of NA glycoprotein in the appearance of new pandemic viruses and the replication of avian influenza viruses in ducks.

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