The active1918 pandemic flu viral neuraminidase contains oligomannose N-glycan & has a tryptic resistant stalk region

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ABSTRACT

Neuraminidase activity is essential for the infectious cycle of an influenza virus. The 1918 pandemic flu virus caused one of the most deadly pandemics in human history. To search for unique structural features of the neuraminidase from this virus that might have contributed to its unusual virulence, we expressed this enzyme using a baculovirus system. The purified enzyme appeared as a monomer, a dimer, and a dodecamer, with only the dodecamer showing significant activity. The monomer and dimer could not be oligomerized into the dodecamer in solution, suggesting that some unique structural features were required for oligomerization and activation. These features could be related to oligomannose glycan, since it was found exclusively on the dodecamer. Sequence analysis revealed a cluster of N-glycosylation sites in the stalk region of the enzyme with no accompanying tryptic sites. Indeed, trypsin digestion failed to cleave the stalk region of the dodecamer, which is in sharp contrast to the observation that viral neuraminidases usually can be released from viral particles through tryptic digestion in this region. It might be the combination of a unique glycosylation pattern and the lack of tryptic sites that protected the stalk region from host protease attack and made the virus more robust for infection.

Figure 1: Sulfotransferase assay with oligosaccharides

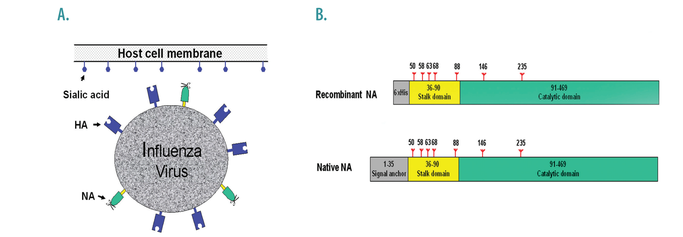

Figure 1: Functions of HA and NA during influenza virus infectious cycle (A) and domain structure of the recombinant 1918 influenza virus (B). HA is a lectin specific for sialic acids on host cell membrane and is responsible for the initial attachment of a virus to a host cell. NA is a neuraminidase that cleaves sialic acids and allows the release of the descendent virus. The native NA contains a signal anchor domain, a stalk domain, and a catalytic head domain. Potential N-glycosylation sites are indicated with red sticks. The signal anchor domain has been replaced with a 6xHis tag in the recombinant protein.

Experiments & Results

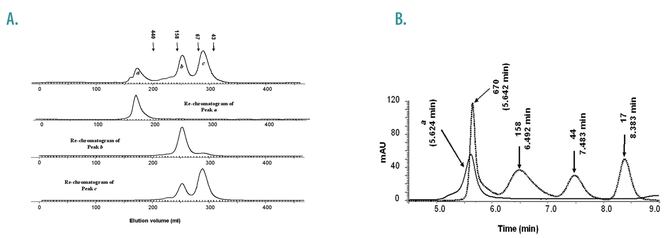

Figure 2: Separation, molecular mass determination, and conversion of the three 1918 NA forms with gel filtration analysis. (A) Separation of nickel affinity-purified recombinant NA on Superdex-200 gel filtration column (top panel) and re-chromatograms of the separated peaks on the same column after two weeks of storage. Peaks a, b, and c correspond to high molecular mass oligomer, dimer, and monomer, respectively. Migrations were based on the molecular standards. **(B)** Chromatogram of peak a in an analytical gel filtration column. The position of the molecule was around 700 kDa, suggesting that it was a dodecamer. The molecular standards (dotted line) used are bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), and horse myoglobin (17 kDa).

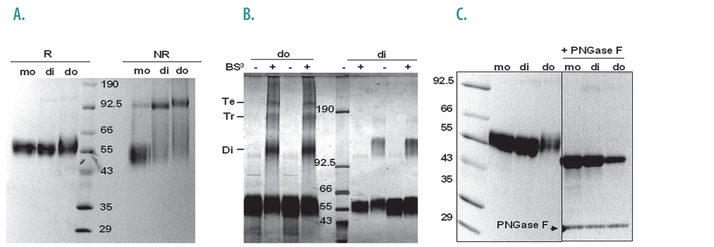

Figure 3: Analysis of the three 1918 NA forms with SDS PAGE. (A) The three forms were separated under reducing (R) and non-reducing (NR) conditions on an SDS gel. Under reducing conditions, both the dimer (di) and the dodecamer (do) were reduced to monomeric form. Under non-reducing conditions, both the dimer and the dodecamer existed in dimeric form. Some dimeric form was also seen in the lane of the monomer (mo) due to disulfide bond formation. **(B)** The dodecamer and the dimer were cross-linked first with BS3 and then separated on a reducing 5-15% gradient SDS gel. The cross-linking products of the dodecamer contained dimer (Di), trimer (Tr) and tetramer (Te). **(C)** The three NA forms were first deglycosylated with PNGase F under denaturing conditions and then separated on 15 % SDS gel. All three forms exhibited a molecular mass of 48 kDa after deglycosylation.

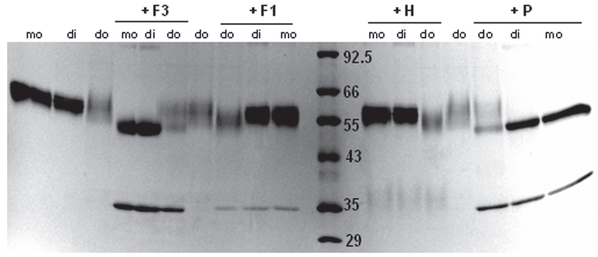

Figure 4: Deglycosylation of the three NA forms with endoglycosidases under native conditions. The dodecamer (do) was partially deglycosylated by all four endoglycosidases, indicating that this form contained both complex and high-mannose types of glycan. The monomer (mo) and dimer (di) could only be deglycosylated with PNGase F and Endo-F3, indicating that these two forms contained only complex N-glycan, but no oligomannose N-glycan. P, PNGase F; H, endoglucosaminidase H; F1, endoglucosaminidase F1; F3, endoglucosaminidase F3. Bands at ~35 kDa corresponded to the deglycosylation enzymes.

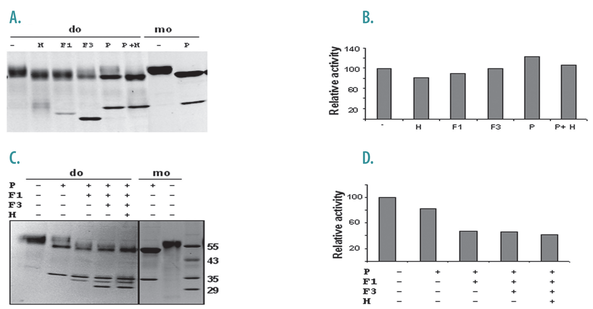

Figure 5: The dodecamer could be deglycosylated under native conditions with a combination of endoglycosidases but the enzyme activity could not be abolished. (A) The dodecamer was treated with endoglycosidases at 37° C for 1 hour and then separated on a reducing SDS gel. **(B)** Relative neuraminidase activities of the samples in A. **(C)** The dodecamer was treated with endoglycosidases at 37° C for 24 hours and then separated on reducing SDS gel. **(D)** Relative neuraminidase activities of the samples in C. In each sample, the initial concentration of the dodecamer was set at 0.1 μg/μL. For each assay, 20 μL sample was used for SDS gel analysis and a 0.5 μL sample was used for activity assay. The activity of the non-treated sample was arbitrarily set to 100%. P, PNGase F; H, endoglucosaminidase H; F1, endoglucosaminidase F1; F3, endoglucosaminidase F3.

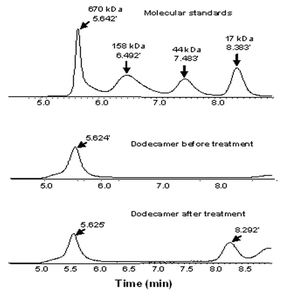

Figure 6: Deglycosylation did not dissemble the dodecamer into dimers under native conditions. (A) Analytical gel filtration chromatogram of molecular standards. (B) Analytical gel filtration chromatogram of the dodecamer (at 5.624 minutes). (C) Analytical gel filtration chromatogram of the PNGase F and Endo F treated dodecamer. Peak eluted at 8.292 min was due to the endoglycosidases.

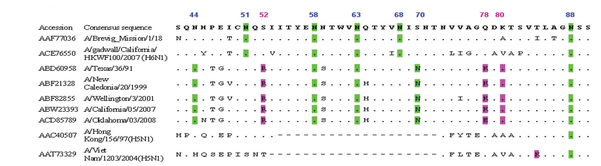

Figure 7: The stalk region of the dodecamer was resistant to trypsin digestion. (A) The stalk region of the 1918 NA (residue #42-90) had a cluster of N-glycosylation sites but no tryptic site. The stalk regions of several influenza viral neuraminidases are aligned. The N-glycosylation sites are highlighted in green and tryptic sites in pink. The positions are numbered according to the protein sequence of 1918 NA (accession AAF77036). Residues identical to the consensus sequence are represented by dots and deletions by hyphens. The consensus sequence was generated by multiple alignment of >30 N1 type viral neuraminidases, only a few of which were shown. (B) Trypsin digestion of monomer (mo), dimer (di), and dodecamer (do). The digestions were separated by SDS-PAGE. Visualizations were done by protein staining of the gel (left panel) and by Western blot of the same gel with anti-His tag antibody (right panel).

CONCLUSIONS

- N-glycans, including both oligomannose and complex types, are needed for NA protein folding.
- Only correctly folded 1918 NA can further oligomerize into a highly active form.
- 1918 NA has a trypsin-resistant stalk region, which could be an important factor contributing to the virulence of this virus.
- This study also suggests that N-glycosylation pathways might be selectively targeted for antiviral drug design.

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