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Review Article

Influenza neuraminidase

Gillian M. Air

Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA.
Correspondence: Gillian M. Air, Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, 940 Stanton L. Young Boulevard, Oklahoma City, OK 73104, USA.
Email: Gillian-air@ouhsc.edu

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Influenza neuraminidase is the target of two licensed antivirals that have been very successful, with several more in development. However, neuraminidase has been largely ignored as a vaccine target despite evidence that inclusion of neuraminidase in the subunit vaccine gives increased protection. This article describes current knowledge on the structure, enzyme activity, and antigenic significance of neuraminidase.

Keywords Influenza, NA activity, NA antigenicity, NA inhibitors, neuraminidase.

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Introduction

The influenza neuraminidase (NA) was recognized as a potential drug target in the 1970s. Inhibitors based on the presumed transition state intermediate were synthesized that showed good activity *in vitro*,[1] but bioavailability was found to be a problem and interest lapsed until the crystal structure of NA was solved. The structure showed a large and highly conserved active site that appeared to be quite rigid, making it an ideal target for structure-based drug design. Zanamivir (Relenza™, GlaxoSmithKline, Middlesex, UK) was licensed in 1999, followed by oseltamivir (Tamiflu™, Roche, Basel, Switzerland). Many comprehensive review articles have covered the development and use of these antivirals,[2–6] so this review will focus on recent developments in our understanding of neuraminidase structure and function in relation to its role as a target for antiviral drugs and neutralizing antibodies.

Neuraminidase structural domains

The NA is a tetramer of four identical polypeptides. Each polypeptide contains about 470 amino acids arranged in four domains, an N-terminal cytoplasmic sequence, followed by a membrane-anchoring hydrophobic transmembrane domain and a thin stalk of variable length, ending in a globular "head" domain that carries the enzyme active site. A sequence alignment of the nine NA subtypes indicating the location of key structural elements is shown in Figure S1. Crystal structures of NA encompass the catalytically active heads (Figure 1A), either proteolytically cleaved from the virus[7,8] or engineered as a soluble secreted protein.[9] The intact NA has not been crystallized, but a cryo-electron microscopy study of the X-31 (A/Aichi/68, H3N2) reassortant virus has revealed considerable detail at near atomic resolution.[10] The structure confirms that the N2 NA protrudes slightly further than the hemagglutinin (HA) from the viral membrane, that there are 40–50 NA spikes per virion, and that these occur in clusters amid 300–400 HA spikes on an average sized virion of diameter 120 nm.

There is no posttranslational cleavage of the NA polypeptide. The N-terminal cytoplasmic domain is a short six amino acids (MNPNQK). This sequence is nearly 100% conserved across all influenza A subtypes, yet its function remains unclear. Viruses with mutations in the cytoplasmic tail show reduced budding and changed morphology,[11,12] but interacting partners of the hexapeptide have not yet been identified.

Transmembrane domain

The transmembrane domain that immediately follows the short cytoplasmic sequence is variable in sequence among subtypes, but in all subtypes is predicted to form a transmembrane helix encompassing amino acids 7–29 when analyzed by the highly reliable program TMHMM.[13,14] The transmembrane sequence has a combined signal peptide-anchor function of directing the NA across the endoplasmic reticulum and also retaining it in the membrane.

Stalk domain

In between the transmembrane sequence and the globular head domain is a thin stalk of variable length and unknown structure. All NA subtypes have Cys residues in

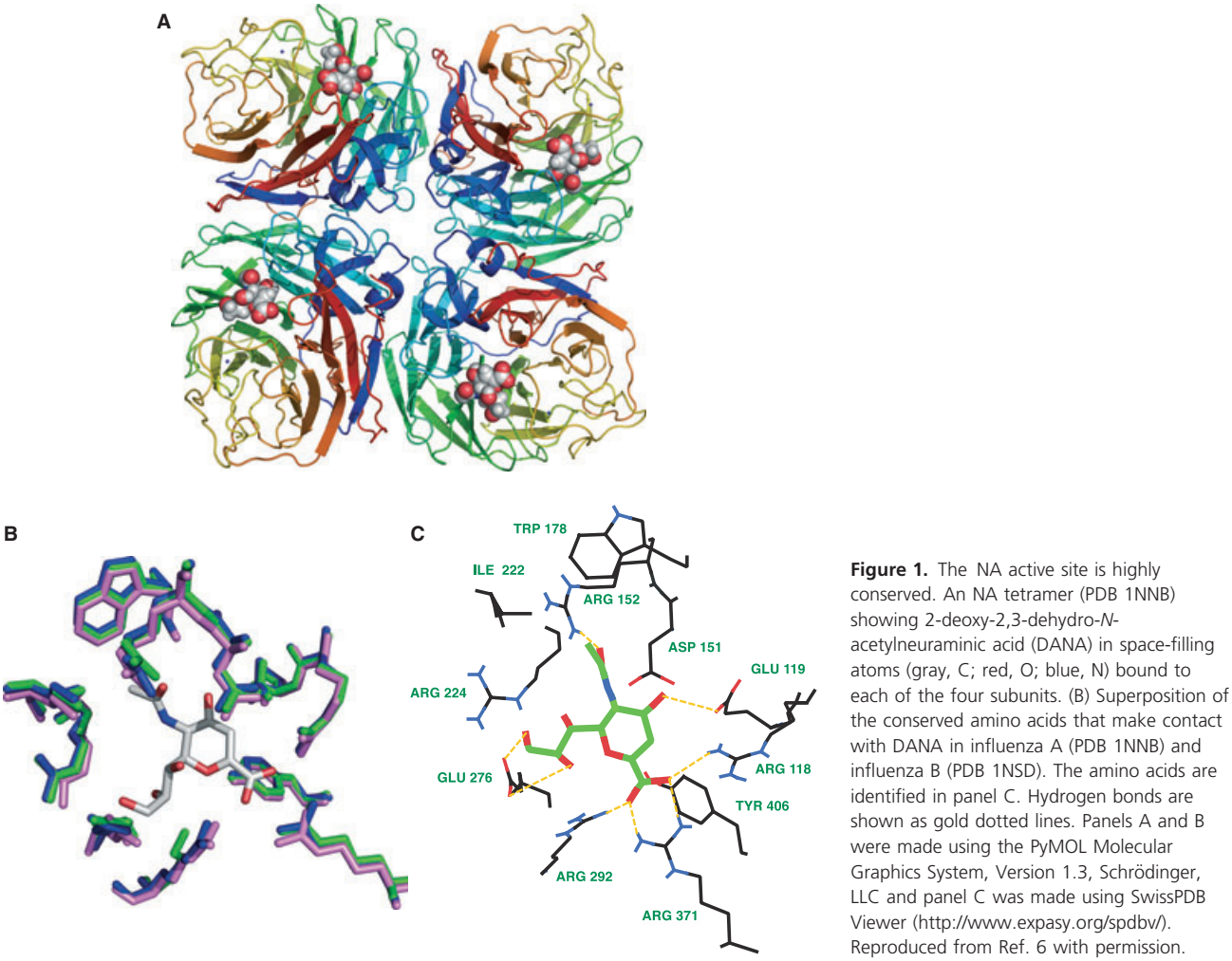

Figure 1. The NA active site is highly conserved. An NA tetramer (PDB 1NNB) showing 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DANA) in space-filling atoms (gray, C; red, O; blue, N) bound to each of the four subunits. (B) Superposition of the conserved amino acids that make contact with DANA in influenza A (PDB 1NNB) and influenza B (PDB 1NSD). The amino acids are identified in panel C. Hydrogen bonds are shown as gold dotted lines. Panels A and B were made using the PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC and panel C was made using SwissPDB Viewer (http://www.expasy.org/spdbv/). Reproduced from Ref. 6 with permission.

the stalk and/or transmembrane domains, and these may assist tetramer formation as a dimer of disulfide-linked dimers. All NA stalks contain predicted sites of N-linked glycosylation, ranging from one in some NAs with short stalks to three or more in full-length stalks (Figure S1). There is no information on whether all these canonical Asn-X-Ser/Thr sites are glycosylated. Most NAs have stalks of approximately 50 amino acids, but deletions of up to 18 amino acids have been found in N1 and N2 NA stalks.[15,16] The cryo-EM study of A/Aichi/68 virions showed a 51-amino acid stalk extending 10 nm from the viral membrane.[10] A helix would extend 7·6 nm, so the stalk structure must be more extended than a helix. Secondary structure programs such as JPred[17] do not predict any helices or coiled coils, but show about 50% beta sheets in the full-length stalks, and measurements of full-length versus deleted stalk also indicated a mixture of extended and folded structure.[16] Several studies have associated deletions in the stalks of N1 NA of avian viruses with transmission from ducks to land-based poultry (an excellent literature review is given by Munier *et al.*[18]), but the mechanism remains obscure. The activity of NA for small substrates is unchanged by the deletion, but activity with complex substrates is reduced,[16,18] suggesting that lower accessibility and hence lower activity are advantages for viruses replicating in chickens compared to those of waterfowl. The full-length NA extends 2 nm beyond the HA from the viral membrane,[10] while the "stubby" variant with a deletion in the stalk was reduced 2·5 nm,[16] putting its sialic acid binding site at the same level or just below the HA binding site. Studies of HA binding specificity and affinity to sialic acids presented on a glycan array have shown that sialic acid binding by the HA is highly dependent on sugar conformation and extension.[19,20] It is tempting to suggest that in poultry the shorter NA allows the HA to engage receptors before they are cleaved by the NA activity, but the well-documented clustering of NA spikes on the viral surface,[10,21] in patches devoid of HA, makes this less likely.

The catalytic "head" domain

Early X-ray crystal structures of NA heads complexed with sialic acid were solved for N2,[22] N9,[23,24] and type B[25] NAs. These showed a conserved six-bladed propeller structure, each blade made up of four antiparallel beta sheets stabilized by disulfide bonds and connected by loops of variable length. The active site is highly conserved in spatial as well as sequence properties and showed little change in conformation on binding a transition state analogue inhibitor [2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DANA)] or the sialic acid product of the reaction. Eleven conserved amino acids make contact with DANA (Figure 1), while another six conserved amino acids form a "second shell"[25] that holds the active site residues in place. The whole appears rather rigid, providing the basis of several drug design approaches (see reviews in Refs. 2,6,26–30).

Recent determinations of N1 NA structures from H5N1, 1918 H1N1, and 2009 swine-origin H1N1 viruses,[7,9,31] and also N4,[7] N6,[32] and N8[7] have greatly expanded our understanding of the dynamics of the head domain. NA sequences fall into two distinct groups[7,33]: Group 1 contains N1, N4, N5, and N8 and Group 2 contains N2, N3, N6, N7, and N9 (Figure S1). The Group 1 structure of N1 NA from A/Vietnam/1203/2004 (H5N1) showed an additional cavity next to the active site that was not present in N2 or N9 structures[7] (Figure 2). The cavity is created by the movement of the "150 loop" (residues 147–152) and was proposed to offer a new target for drug design. When N1, N4, or N8 NAs were complexed with inhibitors, the 150 loop was seen to be in either the open or closed position, depending on the crystal soaking conditions.[7] The 150 loop was seen only in a closed configuration in the structure of N1 NA from the swine-origin virus A/California/04/2009 (H1N1).[31] A molecular dynamics study suggested that the open form is preferred in solution and that the open form may also exist in avian N2 and earliest human N2 structures which lack a salt link from Asp 147 to Lys or His 150 that holds the loop closed in H5N1 NA.[34]

Enzyme activity

The receptor-destroying activity of influenza virus was first observed by Hirst,[35] and the "split product" was identified by Gottschalk[36] as *N*-acetylneuraminic acid, one of the sialic acids, thus establishing the enzyme as a neuraminidase (sialidase). Laver showed that the NA and HA activities resided on different proteins[37] and grew crystals of pronase-released NA heads that launched inhibitor-design studies.[8]

The NA is a tetramer of identical subunits and monomeric forms have no enzyme activity,[38,39] even though the subunit active sites in the crystal structures appear to be quite independent of each other. A drug-resistant mutant E119G was found to have very low activity owing to the dissociation of the tetramer into inactive monomers. The authors suggested that loss of the E119-R156 salt link places the R156 side chain in an orientation that both dis-

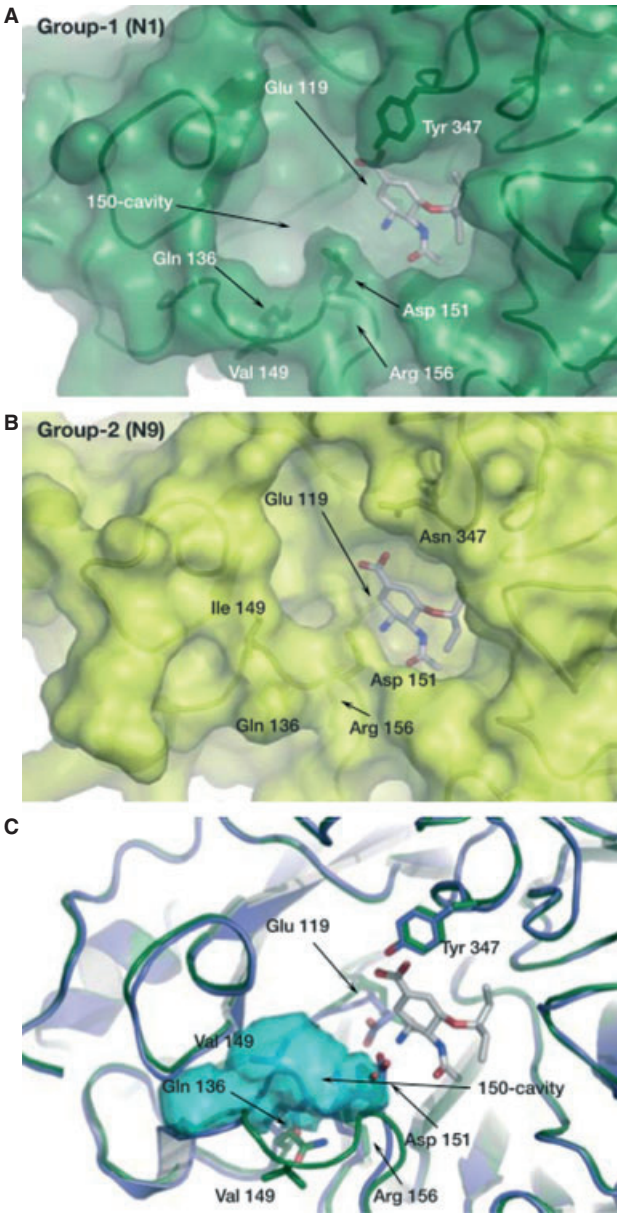

Figure 2. Group 1 NAs such as N1 have an additional cavity in the active site that is not present in Group 2 NAs such as N9. (A, B) Oseltamivir bound to N1 (A, green) and N9 (B, yellow) NAs shown in surface representation with the protein main chain shown in ''worm'' representation. (C) Superposition of the active sites of apo-N1 (green) and N1 complexed with oseltamivir (blue). Electron density from a difference Fourier is shown in blue to indicate the position of the 150-cavity. Reproduced from Russell *et al.*[7] with permission.

rupts the active site and is unfavorable for tetramer formation.[40] However, this does not explain why the wild-type enzyme is only active as a tetramer. Tracking the folding pathway using conformation-specific monoclonal antibodies gave rise to the suggestion that the fifth beta-sheet of the NA propeller structure (amino acids 353–403), which is not stabilized by disulfide bonds, may not form its correct structure unless in the tetramer.[41] However, this region is not involved in the tetramer interface. NA expressed in insect cells as monomers or dimers could not be associated into tetramers, while the tetramer fraction did not dissociate.[42] There were differences in susceptibility to endoglycosidases in that tetramers contained high mannose ($Man_{5-9}GlcNAc_2$) structures, while monomers or dimers contained only complex glycans (truncated to $Man_3GlcNAc_2$ in insect cells but not extended), suggesting that tetramerization only occurs if some mannose structures have not been trimmed, but again this does not explain why only tetramers are active. In looking for a structural explanation, we examined N6 because this is the only NA that crystallized with a complete tetramer in the asymmetric unit.[32] There are many hydrogen bond networks that extend from the active site, through the second shell, and across the subunit interface that potentially provide a connection between tetramer formation and activity, but neither the hydrogen bonds nor the hydrophobic subunit interactions are conserved in other NA subtypes, so there is no obvious common explanation of why NA is only active as a tetramer.

The NA has optimal enzyme activity over the pH range of 5·5–6·5. Ca^{2+} is required for activity and stability,[43,44] and up to five Ca^{2+} ions per tetramer are seen in the crystal structures. One Ca^{2+} sits at the fourfold axis and so may stabilize the tetramer, although it is coordinated via water molecules and its affinity is weak.[25] It is not seen in several structures, owing to either low resolution or low affinity. The N6 structure was determined at the high resolution of 1·85 Å, and there is no Ca^{2+} at the fourfold axis.[32] His119 packs with Y176 in the center of the N6 (and N8) tetramer, perhaps excluding the metal ion (Rudiño-Piñera *et al.*,[32] personal communication from E.F. Garman). The second Ca^{2+} binding site is near the active site of each subunit of the tetramer and may help hold the active site in the appropriate conformation to bind substrate. The Ca^{2+} is coordinated by carbonyl oxygens of D293, G297, G345, and N347 and a carboxyl O of D324 (N2 numbering; see Figure S1). When Ca^{2+} is omitted from the enzyme reaction, or if the protein is dialyzed against EDTA, the extent of loss of activity varies with subtype, probably reflecting how well the Ca^{2+} is buried and inaccessible to solvent. N9 NA that was prepared and crystallized without added Ca^{2+} showed only one Ca^{2+} bound per tetramer. In the three subunits without Ca^{2+}, there is considerable rearrangement of side chains, and Glu276 is moved to a position where it could not play its usual role of binding the glycerol side chain of sialic acid substrate.[45] Thus, the authors hypothesize that this NA would have low activity but did not report that they measured it. A third Ca^{2+} binding site was found in the 1918 N1 and 2009 swine-origin N1 structures. In contrast to the other sites, here the Ca^{2+} is coordinated mostly by side chains (D381, D387, and D379) along with the main chain O of S389 and two water molecules.[9,31] No Ca^{2+} was reported at this site in H5N1 or N4 structures,[7] although the sequences in this region are highly conserved, and higher resolution structures may be needed.

The enzymatic mechanism of NA has been shown to proceed with the retention of configuration.[46,47] The enzyme acts on the α-anomer of sialic acid linked to the next sugar and releases the free sialic acid as the α-anomer that then converts within an hour or so to the β-anomer. The equilibrium mix in aqueous solution is about 95% β- and 5% α-sialic acid. The enzymatic parameters of influenza NA are surprisingly unclear. There are not many studies of the reaction velocity; most work has been done with virus or incompletely purified NA, and comparison of catalytic parameters across different reports shows wide divergence. With the small fluorogenic substrate 4-methylumbelliferyl-*N*-acetylneuraminic acid (MUN), the values of Km range from 6 to 520 μm, while for the trisaccharide NeuAcα2-3Galβ1-4Glc (3′SiaLac) the range is 171–750 μm. Specific activity is rarely reported but for MUN the range is from 100 (N1) to 900 (N9) nmol/second per mg (64–124 for N2 NAs on 3′SiaLac). Unfortunately, when protein was crystallized for structure determination, the enzyme activity of the protein was either not determined or not reported, leaving many unanswered questions, such as whether the Ca^{2+} binding sites are important to activity. It is hoped that more laboratories will follow the example of the McKimm-Breschkin and Wu groups in reporting the activity of their expressed protein.[42,48] A compilation of available data is included as Table S1.

Substrate specificity

Influenza NA cleaves α2-3-linked sialic acid (3'Sia) more efficiently than α2-6 sialic acid (6'Sia). Early studies showed an increase in 6′ activity with time, suggesting that the NA specificity was following the change of HA specificity from avian (3′Sia) to human (6′Sia).[49,50] However, the change was rather minor, from 5:1 to 3:1 preference for 2–3 sialic acid, and in subsequent years, the preference for 3′ stayed the same or increased again.[51] N1 from human viruses gave a 5:1 preference of 3′ over 6′SiaLac, while from pigs and birds, the preference was much higher at 20:1 and 50:1, respectively.[52] N-linked carbohydrates on proteins are typically terminated with the Sia-Galβ1-4GlcNAc (SiaLacNAc)

structure. The NAs of human seasonal H1N1 viruses, but not swine or avian isolates, were found to have lower activity on structures with Sia-Galβ1-3GlcNac (Sia-Le^c) found in O-linked sugars on glycoproteins, and some activities were even lower if the GlcNAc was fucosylated (SiaLe^a).[52] The human viruses cleave 6′ Sia more efficiently than the avian viruses, but in no case does 6′Sia activity exceed the 3′Sia cleavage, in marked contrast to the almost absolute specificity of HA of human viruses for α2-6 sialylated glycans.

Influenza NA shows lower activity on glycoprotein substrates than on small substrates (Table S1). Drzeniek *et al.*[53] found that early H2N2 strains cleaved 3′SiaLac about tenfold faster than fetuin or ovomucoid, and 20 times faster than orosomucoid (α-1 acid glycoprotein), Tamm-Horsfall protein from urine, or porcine submaxillary mucin. A similar gradient from 3′SiaLac to fetuin to bovine mucin was found for A/Aichi/68 (H3N2) NA.[54] The extensive O-acetylation of mucin contributes to its low activity as substrate; 4-*O*-acetylSia is not cleaved at all, while sialic acids with O-acetyl groups at 7, 8, or 9 positions on the glycerol chain are cleaved slowly.[55] In addition, sugars on mucin are O-linked, containing the Sia-Galβ1-3 linkage that is a poor substrate compared to the N-linked structures.[52]

Efforts to show a role of sugar structures downstream of the Sia-Gal in NA specificity have not yielded convincing differences that can be related to structure. The dramatic differences in specificity of binding antigenically variant H3N2 viruses to the Glycan Array (Consortium for Functional Glycomics) are not seen when we use NA to digest the sialic acid from the array. Activity of virus-associated NA is low and specificity is broad for a wide variety of 3′ and 6′ Sia structures [S. Gulati, M. Tappert and G.M. Air, unpublished results at http://www.functionalglycomics.org/glycomics/publicdata/home.jsp (Glycan Array H:1660)]. Thus, the biologically significant substrates of influenza NA remain to be identified.

Some NAs have a second sialic acid binding site

When subtype N9 NA was purified, it was found to strongly bind red blood cells. The NA activity of N9 was inhibited by DANA, but the hemabsorbing activity (HB) was not.[56] Some antibody-selected escape mutants lost HB activity, and the sites of amino acid changes in these mutants mapped the HB site as separate from the NA active site.[57] An HB site was created in the non-hemagglutinating N2 NA by substituting amino acids from two loops of N9 NA into N2 NA.[58] In N9 NA, an X-ray crystal structure (Figure 3) showed a sialic acid molecule bound in the predicted HB site, contacting amino acids Ser367, Ser370, Ser372, Asn400, Trp403, and Lys432 (N2 numbering).[59] This site bears no resemblance to either the NA active site or the HA sialic acid binding site, and indeed, the specificity of binding is unlike that of HA, in that the bound sialic acid is not released by N9 or N2 NA activity but only by bacterial sialidases of very broad specificity.[60] HB activity was also found in the N1 NA of fowl plague virus.[61] Comparisons of sequences in the area of the second binding site suggested that avian viruses have the HB activity while human viruses do not,[59,61,62] but so far a specific function of the HB activity in bird viruses has not been established.

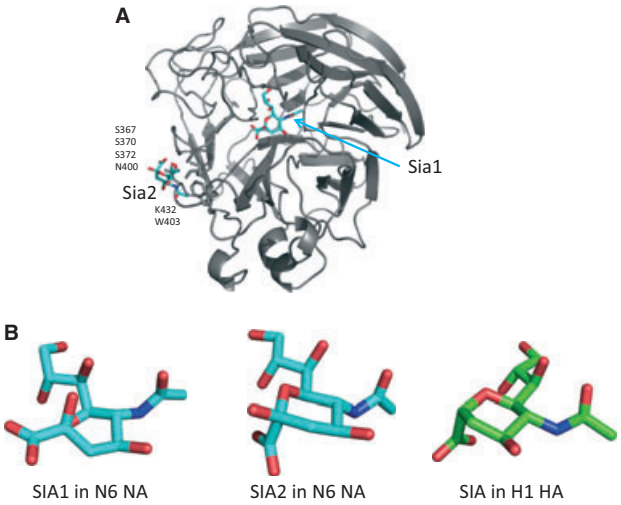

Figure 3. The second sialic acid binding site in N9 and N6 NA. (A) The catalytic site (Sia1) and second sialic acid binding site (Sia2) in N9 NA (PDB 1MWE). (B) Sialic acid bound in the catalytic site of N6 NA is in the boat configuration but in the second site it is in the chair conformation (PDB 1W1X), as in hemagglutinin (1RVX). The figures were made using Pymol.

Recently, the crystal structure of N6 NA from A/duck/England/56 was determined.[32] In contrast to other NA structures, N6 crystallized with the complete tetramer in the asymmetric unit, allowing independent assessment of the four monomers. Under a variety of soaking conditions, sialic acid was seen bound in both the active site and the HB site of all four monomers. In the active site, the sialic acid is in the twisted boat conformation that may result from its tight binding preceding catalysis,[22,63,64] while the sialic acid in the HB site is in the chair conformation as in N9 NB site and in HA.[32,59] Interestingly, although sialic acid in solution exists 95% as the β-anomer, only the α-configuration is seen in the HB binding site (Figure 3), which may explain the difficulty in locating the second sialic acid in N9 NA and the high concentration of sialic acid needed before the sialic acid is clearly seen. We might conclude that the most effective ligand would be a terminal sialic acid α-linked to a glycan, but there are no reports of soaking sialyllactose or longer sugars into the N9 or N6

crystals. Uhlendorf *et al.*[62] noted that HB^+ mutants of A/Singapore/1/57 (H2N2) NA show higher NA activity with multivalent substrates than HB^- NAs, while the monovalent small substrates show no difference in cleavage rate, and the authors suggested that the HB site acts like the lectin domain of some bacterial sialidases, either stabilizing a polyvalent substrate until it is cleaved or ''feeding'' the substrate into the catalytic site. However, the discordance between binding specificity and cleavage specificity of the NA and HB sites in N9 NA [60] does not support this idea, and so the role of the second binding site remains unknown.

NA inhibitors and resistance mutations

The NA is the target of licensed anti-influenza antivirals Relenza™ and Tamiflu™ with several others in clinical trials. The rigid and stable enzyme active site of Group 2 NAs is an excellent target for drug design efforts. The first of these began with a known inhibitor that is a transition state analogue, DANA. In the 1970s, a trifluoroacetyl derivative of DANA was found to be a potent inhibitor of NA *in vitro* and of virus replication in tissue culture, but it failed to protect animal models, apparently due to difficulty in crossing cell membranes coupled with rapid metabolism.[1] Examining how sialic acid sits in the active site, it was predicted and demonstrated that addition of a 4-guanidino group to DANA would improve its binding, and this compound (zanamivir) is now marketed as Relenza™.[65] Scientists at Gilead took a more *a priori* approach, using the crystal structure of the active site to find a backbone that was easier to synthesize than sialic acid and that had better bioavailability, and the result was oseltamivir and its ethyl ester pro-drug marketed as Tamiflu™. By 2008, most of the seasonal H1N1 viruses circulating were resistant to oseltamivir, accelerating the search for new drugs. Peramivir was briefly licensed for emergency use during the swine-origin H1N1 epidemic in an injectable formulation for patients on ventilators and is currently completing clinical trials, and several other backbones as well as further derivatives of zanamivir are being tested. Several recent reviews describe these new developments.[3,5,6,66–69]

For a variable virus such as influenza, drug resistance is an ever-present consideration. Amantadine and its analogue rimantadine are no longer routinely used because resistance develops so quickly. These drugs target the M2 ion channel protein, and because the drug binding site is not at the region critical for the ion channel function, viruses with mutations that confer resistance to amantadine are no less infectious than wild-type viruses. Mutant viruses can be selected in the laboratory to all of the NA inhibitors developed so far, but sometimes only after several passages and in general the resulting virus is less fit. Laboratory-selected resistance is sometimes associated with change in the HA rather than in the NA. The mutant HA has lower affinity for its sialic acid ligands, and the virus can escape from aggregation because of low affinity even though the NA is inactivated by the drug.[70,71] Resistance in natural isolates is associated with mutations in the NA, but mostly these resistant viruses are less fit, only appear sporadically and do not spread.[72] However, seasonal H1N1 viruses with the H275Y (N1 numbering; H274Y in N2) mutation spread throughout the world in 2008, apparently because a compensating mutation had increased their fitness and transmissibility.[73] However, this lineage of H1N1 viruses rapidly disappeared in the face of the swine-origin H1N1 virus that appeared in 2009, so their fitness may have been marginal. More detailed accounts of resistance mechanisms and a tabulation of known NA and HA mutations that lead to resistance are found in recent reviews.[6,74] The swine-origin H1N1 isolates that have replaced the typical human H1N1 viruses since 2009 show as yet a low frequency of oseltamivir resistance that has not been generally transmitted. The sporadic H275Y mutation does not reduce virus replication and transmission in the guinea pig or ferret models but to date has not spread among humans.[75–80]

NA as an antigen

Antibodies against NA do not block the attachment of virus to cells and so are not ''neutralizing'' in the classical sense. This has given rise to a general perception that NA is not an important antigen. NA is less abundant than HA on the virus, and so it is true that HA elicits a higher antibody response, but anti-NA antibodies have been shown to block infection as evidenced by their ability to select escape mutants [57,81–83] and also protect against challenge with a lethal virus is animal models.[84] There is considerable evidence that inclusion of NA in the vaccine would provide additional protection and indeed some cross-protection against avian or swine viruses with N1 NA.[85,86] At this time, licensed subunit influenza vaccines are only required to contain 15 μg of each HA, measured by single radial immunodiffusion, and vaccine efficacy is most commonly measured by hemagglutination inhibition (HAI). The HAI tests became less reliable in the 1990s when human H3N2 and H1N1 isolates lost the ability to agglutinate chicken red blood cells and the avian viruses such as H5N1 also gave erratic results. There is now general agreement that the HAI test is not reliable as laboratories try guinea pig, turkey, horse, or human red cells with varying results owing to low affinity of modern HAs for red blood cells.[87] Recent meetings have discussed the advantages of including NA in the vaccine[85,88]; in current subunit vaccines, NA is present but is not a requirement, and the amount is low and variable.[89] The thought is that NA might provide more

cross-reactive protection because viruses of recent concern (avian H5N1 and human 2009 swine-origin H1N1) both contain the N1 NA. An additional advantage of including NA in the vaccine is that the assays to measure both amount of NA and protective antibodies against NA are straightforward enzyme activity measurements. The neuraminidase-inhibition (NAI) assay does not have the problems associated with changing affinity or specificity that cause variation in the HAI test. The downside of using the NAI assay at present is that there are no suitable fluorogenic or chromogenic substrates of sufficient size to be effectively inhibited by antibodies,[57] and the chemistry of the fetuin assay is more complex than desirable for a standard test. The Warren assay for released sialic acid has been adapted to 96-well plate format,[90,91] and assays that use lectins such as peanut agglutinin to measure newly exposed galactose after cleavage of sialic acid [92,93] are being explored, but substrates that allow direct readout are needed if NA antibodies are to be routinely measured.[88]

As in the case of HA, neutralizing antibodies against NA bind to conformational epitopes. These can be partially mapped by sequence changes in escape mutants selected with monoclonal antibodies. Antibodies can only select escape mutants if they neutralize the parental virus, and for NA, this property correlates with the ability of the antibody to inhibit NA activity in an *in vitro* assay.[83,94,95] The positions of amino acid sequence changes in escape mutants are quite similar in different subtypes of NA, leading to the conclusion that neutralizing anti-NA antibodies bind to epitopes surrounding the enzyme active site. Table 1 shows the positions of escape mutations in N2, N9, and N8 NA. Despite the interest in H5N1 and swine-origin pandemic H1N1 human viruses, there seems to be a dearth of information on the neutralizing epitopes of N1 NA. On the three-dimensional structure of NA, the sites of escape mutations are seen to be in loops surrounding the active site pocket (Figure 4A). However, the epitope bound by an antibody is much larger than the escape mutation sites (Figure 4B).

The only way that complete neutralizing epitopes have been mapped is by obtaining the structure of the NA–antibody Fab complex by X-ray crystallography. The structures of three epitopes on NA are known, two on N9 NA[96,97] and one on N2 NA.[98] By combining the structure with results of escape mutant analysis and mutagenesis experiments, the picture that emerges is that the antibody is in direct contact with 16–20 amino acids of the NA, but that only a small subset of these contacts are so critical to the

Table 1. Sites of escape mutations in NA selected by monoclonal antibodies

Amino acid position (N2 numbering)	N2*			N9**	N8***
	RI/5+/57	Tokyo/67	Memphis/31/98	Tern/Australia/G70c/75	Duck/Ukraine/1/63
150	+				+
198			+		
199			+		+
220			+	+	
221		+	+		
253		+			
284					+
329	+			+	
334	+				
344	+	+			+
346					+
367				+	+
368	+	+		+	
369				+	
370	+			+	
372				+	+
400				+	+
403	+				
432				+	

*N2 variants summarized in Gulati *et al.*[83]
**Webster *et al.*[57]
***Saito *et al.*[81]

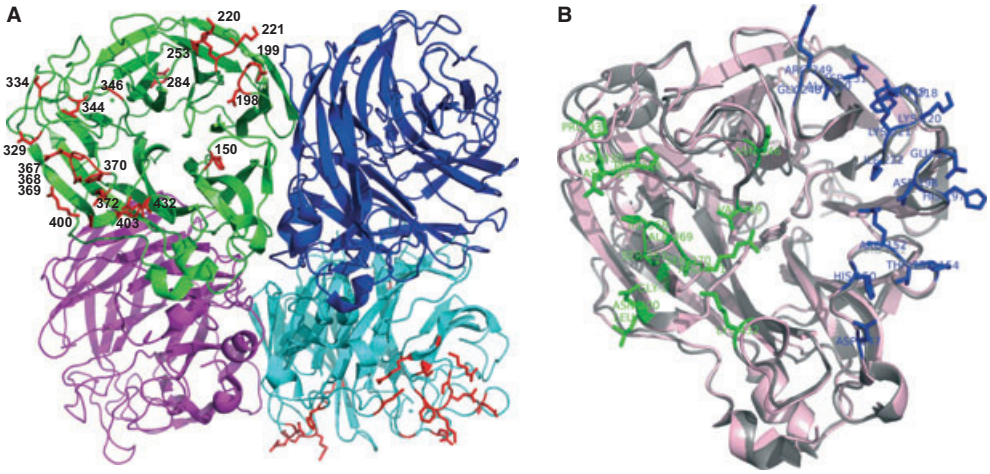

Figure 4. Neutralizing epitopes on NA. (A) Escape mutations in NAs surround the active site pocket. All known escape mutations (Table 1) are shown in the green and cyan subunits. The NA tetramer is tilted so that you are looking straight into the active site of the green subunit, while the cyan subunit shows that escape mutations occur on loops on the top surface. (B) A single subunit of N9 (gray) and N2 (pink) showing the amino acids that make up the epitopes of antibodies NC41 (green) and Mem5 (blue). The orientation is similar to the green subunit in A. An epitope is defined as those amino acids of NA that make direct contact with the antibody heavy and light chains. Each antibody covers about one-third of the top surface of the NA subunit. Images were made using Pymol.

A

AA on NA	147	150	154	197	198	199	220	221	222	249	251
Interaction	s	s	v	v	h,v	s,h	h	h,v	v	v	h
Buried Å^2	47	82	34	64	59	83	75	112	39	122	52
1998	D	H	P	H	D	E	K	K	I	R	D
1999	.	.	.	D	.	.	.	.	.	K	.
2002	.	.	.	D	.	.	.	.	.	K	.
2003	.	.	.	D	.	K	.	.	.	K	.
2004	.	.	.	D	.	K	.	E	.	K	.
2006	.	R	.	D	.	K	.	E	.	K	.
2008	N	R	.	D	.	K	.	E	.	K	.

B

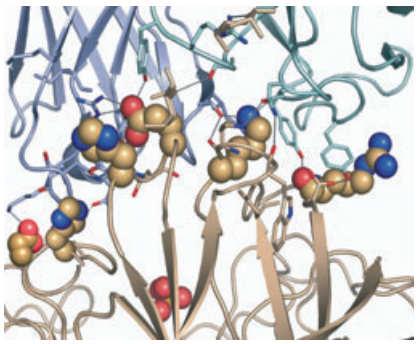

Figure 5. The Mem5 epitope has undergone antigenic drift. (A) Mutations in the Mem5 epitope since 1998. All 11 NA amino acid side chain contacts are shown. Another 6 amino acids make contacts from main chain atoms and these would not be expected to change if the side chain is substituted. (B) The interface between the NA (brown) and antibody heavy (blue) and light (cyan) chains. Amino acids shown in space-filling representation have changed in human isolates since 1998. Adapted from Venkatramani *et al.*[98] with permission.

interaction such that even the most conservative mutations in this subset greatly reduce antibody binding. Mutations in the non-critical contacts may show only a small reduction in binding. Even so-called conservative mutations in these critical contacts are sufficient to abolish the binding of an antibody to NA.[94,98–100]

A question is often asked about the importance of NA in antigenic drift. The crystal structure of Memphis/31/98 N2 NA bound to Fab Mem5 shows 16 amino acids of NA contact the antibody. Five of these are main chain contacts unlikely to be affected by mutation. Of the 11 amino acid side chains on NA that binds antibody, six have changed in human isolates from 1999 to 2008 (Figure 5), suggesting that this epitope defined by a mouse monoclonal antibody is also a dominant epitope in human immune response and that escape from antibodies that bind this epitope has been a driving force in human influenza drift.[98] A simple analysis of amino acid sequence changes in human isolates shows that the extent of change is similar for NA and HA; indeed, the NA has been mutating faster than the HA in recent years (Figure 6). Because HA change is known to be driven by antigenic selection, this analysis suggests that NA is under similar antigenic pressure and provides further evidence that inclusion of NA in the vaccine would be beneficial.

Role of NA

In the absence of NA activity, influenza viruses can undergo a complete cycle of infection and production of progeny virus, but remain aggregated to such an extent that they cannot spread to new host cells to initiate another round of infection. Thus, there is no amplification of virus, and the infection proceeds no further.[101] NA is described

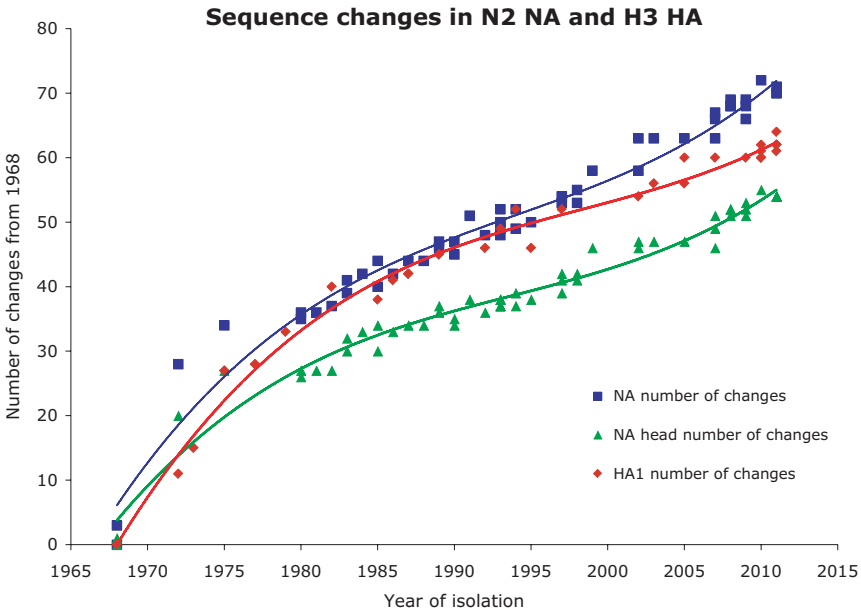

Figure 6. Accumulation of changes in HA1 and NA of representative H3N2 human isolates. The *y*-axis is overall numbers of residues changed from Aichi/1/68, so reversions and multiple substitutions are not counted. The NA changes are given for the whole NA and for the head region only. Since an antibody footprint is fairly constant, the results are given as total numbers of changes rather than percentages.

as a receptor-destroying enzyme, but it has been recognized for many years that some influenza viruses do not elute from agglutinated red blood cells, showing that the NA specificity does not match that of the HA.[51] In recent years, human H3N2 viruses have been isolated with little or no detectable NA activity, owing to large deletions of the coding sequence that remove most or all of the catalytic head domain.[102–104] Many mutations in NA inhibitor-resistant viruses map in the HA rather than the NA, and these have been shown to reduce HA affinity for receptors to the point where NA activity is no longer required for release.[71] The difficulties of the HAI assay since the mid-1990s are apparently a reflection of decreasing sialoglycan affinity of H3N2 and H1N1 viruses. Thus, it is not surprising that influenza viruses are now being isolated with little or no NA activity, because the strict requirement for its activity seems to have been reduced. However, it is not yet clear if the loss of NA occurs in the human host or in the cells used to isolate and grow the viruses. It is possible that NA is still required in host tissues but not in MDCK cells, reflecting the different sialylated glycans that serve as receptors. It may be that the role of NA is not to destroy cellular receptors but to remove sialic acid from the HA (and NA) of progeny virions to avoid aggregation and to facilitate binding to cell receptors. If the HA mutations during antigenic drift also reduce its ability to bind the N-linked sialoglycans on the HA, perhaps while retaining ability to bind receptors on cell surfaces, then the NA may not be required and NA inhibitors will no longer be effective. Most studies have focused on the concept that NA is a receptor-destroying enzyme, but it is quite possible that the NA has additional functions in the infected host, such as reducing sialic acid on cells involved in immune response.

Summary

NA activity is an important component of influenza infection and crystal structures of type A and type B NA allowed structure-based drug design efforts that have been quite successful. Neuraminidase inhibitors are not "magic bullets" that instantly cure the disease, but they do reduce the length and severity of illness. Resistance can be demonstrated in the laboratory but is rare in nature except for the 2008 seasonal H1N1 viruses that were predominantly resistant to oseltamivir. The disappearance of these strains on emergence of the swine-origin H1N1 viruses in 2009 may be due in part to lower fitness that was not apparent until a competing virus appeared that had the advantage of antigenic novelty in the human population. The activity of NA that is inhibited by the drugs is still unclear, and its biological substrates have not been identified. It is likely that the role of NA in infection is more complex than merely destroying viral receptors. As a vaccine, NA alone is less effective than HA, but increased protection has been reported when NA is added to the HA in the vaccine. The manufacturing and regulatory burden if a certain level of NA were to be required in influenza subunit vaccines might be offset by increased and broader protection and a reliable enzymatic assay as a surrogate measure of protection.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Sequence alignment of avian representatives of NA subtypes N1–N9 (N2 numbering).

Table S1. Enzymatic properties of influenza virus neuraminidases.

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