# Tuning of influenza A virus neuraminidase activity

*Meiling Dai*

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# Tuning of influenza A virus neuraminidase activity

## Afstemming van influenza A virus neuraminidase activiteit

(met een samenvatting in het Nederlands)

**Proefschrift**

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op vrijdag 22 september 2017 des middags te 12.45 uur

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**Meiling Dai**

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Promotor: Prof.dr. F.J.M. van Kuppeveld

Copromotor: Dr. C.A.M de Haan

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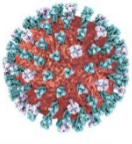
# Contents

# Chapter 1

## General Introduction



Influenza A viruses (IAVs) are zoonotic pathogens that continuously circulate in a wide variety of species, including birds, pigs and humans (1). In humans, IAVs cause seasonal epidemics and occasional pandemics of influenza. Annual epidemics caused by seasonal IAVs can result in millions of human infections and pose a huge health and economic burden (2). Influenza pandemics may be caused by animal viruses that managed to cross the host species barrier and became transmissible among humans (3). IAV pandemics have occurred four times in the 100 years, causing millions of deaths and global devastating effects. The "Spanish" influenza pandemic of 1918–1919, claimed approximately 50 million deaths worldwide and was caused by an H1N1 virus (4). After that, pandemics have been caused by H2N2 virus in 1957 (Asian flu), by H3N2 virus in 1968 (HongKong flu) and more recently by swine-origin, new pandemic H1N1 (H1N1pdm09) in 2009 (5, 6). Currently, H3N2 and H1N1pdm09 viruses are circulating in the human population, giving rise to the seasonal epidemics of IAVs.

As single-stranded, segmented RNA viruses of the Orthomyxoviridae family, influenza viruses are classified into four genera influenza A, B, C and D viruses according to antigenic differences between their nucleoprotein (NP) and matrix 1 (M1) proteins (7-9). While IAVs infect avian, human, swine, and other mammalian animals, influenza B virus (IBV) and influenza C virus (ICV) are reported to principally infect humans, but not other species (10). As a component of seasonal influenza epidemics, IBV infections lead to clinically severe disease (11), while ICV infections typically results in mild respiratory symptoms (12). Recently, several novel influenza D virus (IDV) strains have been isolated from pigs as well as cattle (13, 14). IDV strains are more closely related to ICV than to IAV or IBV (15).

IAVs can be further classified into 18 HA (H1-H18) and 11 NA (N1-N11) subtypes depending on the genetic and antigenic properties of the two major viral envelope glycoproteins haemagglutinin (HA) and neuraminidase (NA) (16, 17). All IAV subtypes are found in wild waterfowl, the IAV natural host reservoir, except that H17-H18 and N10-N11 are only reported in bats (18, 19). Several IAVs have also become endemic in terrestrial poultry in several parts of the world (20). While IAV infection in wild aquatic birds is generally asymptomatic (21), infections of domestic poultry with IAVs may lead to severe disease and large economic losses. This particularly holds true for IAVs that contain H5 or H7 proteins, as these viruses may evolve into highly pathogenic (HP) IAVs, with flock mortality as high as 100% (22, 23). Of the large number of avian IAV subtypes, particularly the highly pathogenic (HP) H5N1 (since 1997) and the novel H7N9 (since 2013) viruses are regarded as pandemic threats to the human population. Both viruses sporadically infect humans thereby causing severe illness, including severe pulmonary disease and acute respiratory distress syndrome, which lead to high mortality rates (up to 35-60%). Fortunately, sustained human-to-human transmission has not been reported yet for these viruses (24-26). In addition to being directly infected by avian influenza virus, humans can also be infected with influenza viruses that have passed through an intermediate host, such as pigs (27). Pigs are widely regarded as “mixing vessel” of IAV, in which reassortment events may occur between avian, swine and human IAV strains resulting in new genetic variants. The most striking example thereof is the H1N1pdm09 virus, which contains a unique gene constellation combining gene segments from human, avian as well as swine influenza viruses (28).

So far, vaccination is the most effective way to prevent infection and severe outcomes caused by influenza viruses. Current seasonal influenza virus vaccines contain antigens from contemporary H1N1pdm09 and H3N2 IAVs and one or two strains of IBV. Although vaccines are an effective countermeasure against infection, they need to be regularly updated because of antigenic drift as they usually induce narrow and strain-specific immunity with limited cross protection (11, 29). Meanwhile, the timely production of novel pandemic virus vaccines remains problematic because of the limitations of current technology. Only few antiviral drugs have been approved for therapeutic use against IAV. Currently, only the NA protein inhibitors zanamivir, oseltamivir and peramivir are being used in the clinic.

## Influenza A virus genome and virion morphology

All influenza viruses contain a segmented negative-sense RNA genome surrounded by a lipid envelope (Figure 1A). Influenza A and B viruses contain two spike glycoproteins, HA and NA, that are, together with the M2 protein, embedded in the envelope that is derived from the host plasma membrane (30). ICV and IDV, by contrast, have only a single membrane glycoprotein, hemagglutinin-esterase-fusion (HEF) (31). The viral envelope and its integral membrane proteins HA, NA, and M2 overlay a matrix of M1 protein, which maintains the virus shape. Within the lipid envelope each of the eight viral RNA segments (vRNAs) is encapsidated by viral nucleoprotein (NP) and bound to a viral polymerase complex (consisting of the polymerase proteins PA, PB1, and PB2) thereby forming the eight viral ribonucleoproteins (vRNPs).

IAV particles contain eight genome segments that can encode up to 17 viral proteins (32-35). The non-structural segment (NS segment) encodes the nuclear export protein (NEP; also known as NS2) and the host antiviral response antagonist non-structural protein 1 (NS1) (36); The matrix protein M1 and the ion channel protein M2 are encoded by the M segment; the haemagglutinin (HA) segment encodes the receptor-binding glycoprotein HA; and the neuraminidase (NA) segment encodes the sialic acid-destroying enzyme NA. Additionally, nucleoprotein (NP) as well as the three components of the RNA-dependent RNA polymerase (RdRp) complex (PB1, PB2 and PA) are encoded by their respective genome segments (30, 37). The PB1 segment also encodes PB1-F2 and PB1-N40 with poorly characterized functions (38). As a pro-apoptotic protein encoded by a second open reading frame (ORF) in the PB1 segment, PB1-F2 is known to localize in mitochondria and to induce apoptosis (39, 40), Additionally, the PA segment also encodes PA-X, which represses cellular gene expression (41) and two N-terminally truncated forms of PA named PA-N155 and PA-N182, which were suggested to possess important functions in the replication cycle of influenza A virus (42).

Influenza virus particles are pleomorphic and known to display a diversity of morphological states, from spherical to filamentous. Spherical virions (80–120 nm in diameter) predominate in extensively-passaged laboratory strains, such as PR8 and WSN (43, 44), whereas clinical isolates that have undergone limited number of passages typically contain filamentous particles (45-47), with lengths exceeding even over 1 μm (48). The M segment of influenza

A virus, which encodes the M1 and M2 proteins, is an important genetic determinant of filamentous morphology, probably as the highly organized layer of the M1 protein beneath the envelope maintains the virus shape (48-50). Furthermore, the virion morphology not only depends on the virus strain but also on the host cell type in which the virus is replicated (51). Several studies have demonstrated that filamentous particles have a polar organization by using cryo electron microscopical techniques (52-54). More specifically, it seems that HA and NA are preferentially distributed to differently curved membranes within particles (55), in which the HA covers most of the virus surface, whereas the NA forms patches at one end of the virus opposite the vRNP-containing tip (52, 55).

## Influenza A virus life cycle

The IAV infection cycle can be divided into several steps, which are shown in Figure 1B (56).

**Virus attachment and entry**

IAV infection is initiated by the attachment of viral HA to host cell glycan receptors that contain terminal sialic acid (SIA) linked to the penultimate galactose by either an α2,6 (α2,6-SIA) or α2,3 linkage (α2,3-SIA) (Figure 2). SIAs are nine-carbon monosaccharides omnipresent on the host cell glycolipids and glycoproteins. Molecular species of sialic acid are largely divided into two moieties: N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) (57). Neu5Gc has been detected in pigs, mice and ducks, but it is not present or only with minor quantities in humans, ferrets, chickens or other poultry (58, 59).

Following receptor binding, IAVs enter cells through receptor-mediated endocytosis (60). IAVs enter either via clathrin-mediated endocytosis (CME), the first endocytic pathway identified as an IAV entry route (61, 62), or by clathrinin-independent uptake routes, including caveolae-dependent endocytosis (63) and macropinocytosis (64). Internalization of IAV via macropinocytosis was shown to be dependent on sialylated N-glycans (65).

**Fusion and uncoating**

After receptor-mediated endocytosis, the low pH of the late endosome triggers conformational changes in the HA that ultimately result in fusion between the viral and the endosomal membranes (66). Prior cleavage of HA by (extra) cellular proteases is required for the exposure of the HA fusion peptide and the subsequent conformational changes that mediate fusion (67). The low pH environment of the endosomes also results in a proton influx via an ion channel formed by the viral M2 protein and in acidification of the interior of the virus particles. This acidification mediates the dissociation of the viral M1 protein from the vRNP complexes (68), allowing the release of vRNPs into the cytoplasm after fusion.

**Virus replication and transcription**

After release of the vRNPs into the cytoplasm, they are transported into the nucleus, through the nuclear pore complex. In the nucleus, primary transcription takes place by the viral polymerase complex formed by PA, PB1 and PB2, which leads to the production of viral mRNAs. Transcription is a primer-dependent process. The primers are obtained via

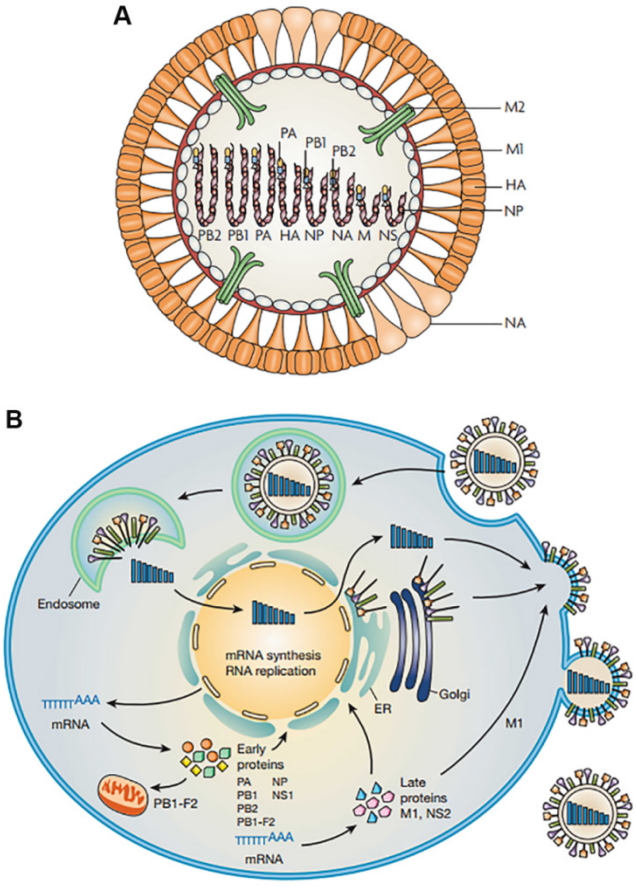



**Figure 1.** (A) **Schematic picture of an IAV particle.** The viral envelope accommodates the two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), together with the M2 ion-channel. Underlying the viral envelope is matrix protein 1 (M1). Inside the viral particle are eight ribonucleoprotein (vRNP) complexes comprising viral genomic RNA (vRNA) segments associated with the nucleoprotein (NP) and three polymerase proteins (PA, PB1, and PB2). The figure was adopted from (228). (B) **Schematic representation of the IAV replication cycle.** The viral HA attaches to host cell receptors that contain terminal α2,6-or α2,3-linked sialic acid (SIA) to initiate influenza A virus infection. Virus particles are internalized by receptor-mediated endocytosis. Following internalization, endosomal acidification induces conformational changes in HA, which lead to fusion between the virion and endosomal membranes and to cytoplasmic release of the vRNP complexes. vRNPs translocate to the nucleus, where the RNA-dependent RNA polymerase transcribes and replicates viral RNA, leading to the production of three types of RNA molecules: viral mRNAs, the complementary RNA (cRNA) and new vRNAs. Viral proteins that are needed in replication and transcription are translocated back to the nucleus. Progeny vRNPs are exported to the cytoplasm for packaging. HA, NA and M2 are inserted into the endoplasmic reticulum (ER) and transported via the secretory pathway to the plasma membrane, where M1 assists in the formation of virus particles. Progeny virions are assembled via budding and released from the host cells, for which the enzymatic activity of NA is essential. The figure was adopted from (6).

'cap-snatching', which involves cleavage of capped RNA fragments from cellular mRNAs by PA (69, 70). The capped RNA fragments are elongated by the viral polymerase complex using vRNA as a template (71). When the 5´ end of the vRNA is reached, the polymerase stutters on a poly-U stretch, resulting in the generation of the poly-A tail (72, 73) . Some mRNAs are spliced, resulting in the generation of M2- and NEP-encoding mRNAs.

The viral mRNAs are exported to the cytoplasm and translated into proteins (74). The PA, PB1, PB2 and NP proteins are imported into the nucleus where they participate in the synthesis of full length complementary copies (cRNAs) of the vRNAs, which in turn are used for the synthesis of nascent vRNAs. The synthesis of cRNA and vRNA is a primer-independent process. Mutations in the polymerase complex, including the well-known K627 mutation in PB2, may increase replication of avian IAVs in mammals. These adaptive mutations are probably required for efficient interaction of the polymerase proteins with mammalian host factors and/or with adaptation of the polymerase complex to the lower temperature in the respiratory tract of mammals (~33°C), compared to the temperature in the avian intestinal tract (~41°C), which is the site of replication of avian IAVs (75-78).

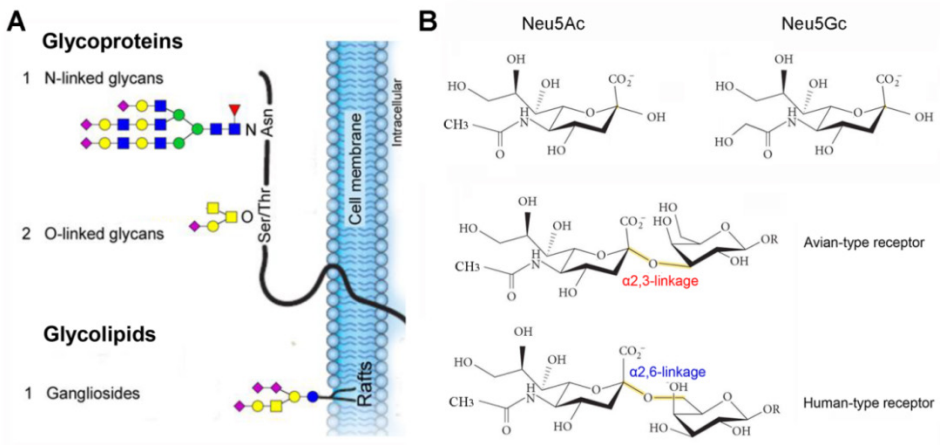



**Figure 2.** (A) Cell surface glycans. Glycans are present on the cell surface either attached to proteins or to glycolipids (gangliosides). Glycan trees are either attached to Asn residues (N-linked glycans) or to Ser or Thr residues (O-linked glycans). SIAs (purple diamonds) are terminal modifications of the glycan trees. The two most common SIAs are N-acetyl neuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) (shown in B). The C5 carbon in Neu5Ac is modified with an N-acetyl group, which can be further hydroxylated to form Neu5Gc. Neu5Ac may be attached to the penultimate galactose (Gal2; yellow circle) via α2,3- or α2,6-linkage (shown in B). Blue and yellow squares indicate N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc), respectively. Green circles and red triangles refer to mannose and fucose moieties, respectively.

### Assembly and release

vRNAs encapsidated by NP and bound to a viral polymerase complex form the eight vRNPs. Progeny vRNPs are exported to the cytoplasm with the assistance of the M1 and NEP proteins, which contain nuclear export signals (79, 80). vRNPs are subsequently transported through the cytoplasm to the plasma membrane on RAB11-positive vesicles (81, 82). RAB11 expression and functional GTPase activity were shown to be crucial for influenza vRNP cytoplasmic transport (82). Incorporation of the eight vRNPs into the virions requires segment-specific packaging signals in the vRNAs (83, 84). The integral membrane proteins HA, NA and M2 are transported to the plasma membrane via the secretory pathway. HA and NA are targeted to lipid rafts that are the site of influenza virus budding (85, 86). Virus budding is mediated by the concerted interactions between the structural proteins HA, NA and M1. While M2 contributes to scission of budding viruses from the plasma membrane (87), the sialidase activity of the viral NA, which cleaves sialic acids from cellular and viral

glycoproteins, allows virus release and prevents virus aggregation (88).

## HA glycoprotein

As the most abundant viral envelope protein present in the virion, HA mediates the attachment of virus binding to host receptors and fusion between the viral and endosomal membranes (66). The HA precursor protein is a type I integral membrane glycoprotein of about 550 amino acids, with a N-terminal signal sequence, a large ectodomain, a transmembrane domain close to the C-terminus and a short cytoplasmic tail. The cleavage of the HA (HA0) by (extra-) cellular proteases generates the HA1 and HA2 subunits, which remain disulfide bonded. The H5 and H7 proteins may contain a multibasic cleavage site between HA1 and HA2, which is cleaved by ubiquitously-expressed subtilisin-like proteases. Viruses containing such cleavage sites are referred to as highly pathogenic (HP) IAVs.

The first crystal structure of influenza A virus HA was described by Wilson *et al.* in 1981 (89). Three protomers of HA1-HA2 form the mature homo-trimeric HA protein. Each HA protomer contains a membrane-distal, globular domain that binds to glycan receptors on host cells via a receptor binding site (RBS) and a membrane-proximal stem domain, which is the main component of the HA fusion machinery (90) (Figure 3A). The RBS comprises a pocket of several highly conserved amino acids, Tyr-98, Trp-153, His-183, and Tyr-195 that form the base element of the RBS and which is edged by three secondary elements: 190-helix, the 130-loop, and the 220-loop (H3 numbering) (91, 92) (Figure 3A). So far, 18 HA (H1-H18) subtypes have been described, which are divided into two groups: group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18) and group 2 (H3, H4, H7, H10, H14, and H15) according to their phylogenetic relationships (93, 94) (Figure 3B).

Specificity of HA for sialylated receptors is a critical determinant of the virus host range (95). Therefore, HA receptor-binding specificity and affinity have been broadly studied by using a variety of methods, including virus hemagglutination of erythrocytes from different species (96), solid-phase microplate binding assay (97, 98), glycan microarray procedures (99, 100), saturation transfer difference nuclear magnetic resonance (STD-NMR) (101, 102) and surface plasmon resonance (SPR) (103-105). In recent years, a novel biophysical approach based on bio-layer interferometry has been used to determine the HA receptor specificity and avidity (106, 107). The main conclusion from these studies is that the HA proteins of human IAVs preferentially bind to α2,6-SIA (108, 109), which is regarded as the human-type receptor and predominantly found in the upper respiratory tract (URT) in humans (110, 111). In contrast, avian IAVs preferentially recognize SIA linked to the terminal oligosaccharide by an α2,3 bond, which is referred to as the avian-type receptor (112-114). This receptor is extensively present on epithelial cells of for example the duck intestine, but is also found in the human lower respiratory tract (LRT) (115, 116). Besides a general preference for the specific linkage between terminal SIA to the penultimate galactose, also terminal sialic acid modifications, internal linkages from the second (Galactose; Gal-2) to the third sugar (N-acetylglucosamine; GlcNAc-3 or N-acetylgalactosamine; GalNAc) as well as modifications of internal sugars (fucosylation or sulphation) can also influence HA binding (117, 118). For example, it has been shown that some influenza viruses interact with greater avidity with

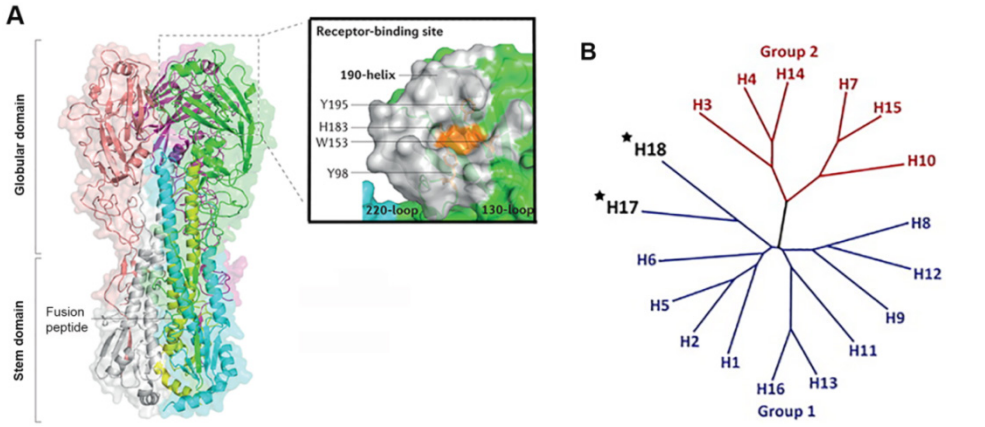



**Figure 3.** (A) Crystal structure of the haemagglutinin (HA) ectodomain. The HA ectodomain contains two domains: the globular domain, which contains the receptor-binding site (RBS), and the stem domain, which contains the elements important for membrane fusion including the fusion peptide. The RBS forms a shallow pocket comprising three secondary elements: the 130-loop, 190-helix and 220-loop. In addition, four highly conserved residues (Y98, W153, H183 and Y195) form the base element of the RBS. The figure was adopted from (35). (B) Phylogenetic tree of the HA gene from each IAV HA subtype. The different HA subtypes can be broadly classified into two groups (group 1 and 2). The newly identified bat-derived H17 and H18 that belong to group 1 are indicated with a star. The figure was adopted from (34).

α2,3-linked glycans that contain sulfated GlcNAc-3 than with glycans that are fucosylated at this site (117). Attachment of influenza virus to sialic acid is also influenced by the length and branching of the sialylated glycan (119). Generally, the interaction of HA with individual sialosides is very weak, with the HA receptor binding affinity to monomeric sugar being in the mM range (120-122). Therefore, the simultaneous interaction of a number of HAs to several receptors is important for attachment of virus to cells during infection. In agreement herewith, greatly increased HA binding avidity was observed with multimeric sialosides when compared with monomeric SIAs (123, 124).

Linear sialylated pentasaccharides LSTc (containing α2,6-SIA) and LSTa (containing α2,3-SIA) or trisaccharides 3' sialyllactosamine (3'-SLN) and 6' sialyllactosamine (6'-SLN) that all contain the three terminal saccharides SIA, Gal-2 and GlcNAc-3 are commonly used as human and avian receptor analogs to study the molecular basis of the HA receptor binding specificity (125-128). In most crystal structures, when bound by HA, the α2,3-linked SIA receptor adopts a *trans* conformation, whereas the human type α2,6-linked SIA receptor adopts a *cis* conformation (Figure 4). As a consequence, a change in HA-binding preference requires amino acid substitutions in the RBS. For example, E190D and G225D substitutions in the 190-helix and 220-loop of H1, including the 1918 and H1N1pdm09 strains, are required for a shift in binding preference from avian- to human-type receptors (109, 129). For the H2 and H3 proteins, Q226L and G228S substitutions are required for such a shift (108, 109). Thus, the specific amino acid substitutions that result in altered receptor-binding specificities may differ between HA subtypes. For HA of airborne transmissible H5N1 influenza viruses, it was reported that amino acid substitutions Q226L and N224K or G228S

result in a switch in receptor specificity (122, 130-132). Particularly the Q226L substitution facilitated binding to α2,6-SIA, while restricting binding to α2,3-SIA (122). Receptor binding may also be affected by HA glycosylation sites that are often removed or introduced to evade host immune responses during the evolution of influenza viruses (133, 134). For example, airborne H5 HAs were found to have lost the glycosylation site (N158D) near the RBS resulting in increased binding avidity (130, 131, 135). The enhanced preference for human-type receptors due to mutation of Asn at position 158 was reported also in other studies (136, 137).

Besides HA receptor specificity, the stability of the HA protein also appears to be important for IAV host tropism. Upon virus attachment and entry into cells, a low-pH-triggered conformational change in HA mediates fusion of the viral membrane with the endosomal membrane to release the viral genome into the cytoplasm.

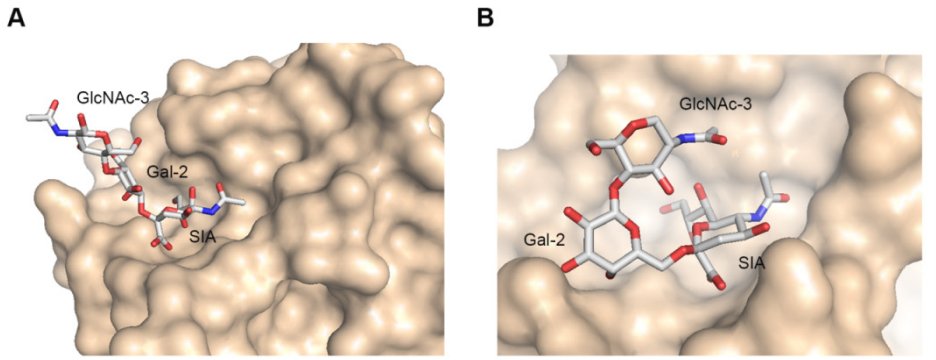



**Figure 4.** Haemagglutinin proteins from the H5 and H1 subtypes in complex with the avian and human receptor analogues. (A) Structure of an avian influenza H5 HA complexed with avian receptor analog LSTa that contains α2,3-linked SIA in a *trans* conformation. The structural figure was created using PyMOL using with the Protein Data Bank (PDB) accession 4K63. The three terminal saccharides SIA, Gal-2 and GlcNAc-3 are shown as sticks (oxygen in red; nitrogen in blue; carbon in white). (B) Crystal structure of H1N1pdm09 HA complexed with human receptor analogue LSTc contaning α2,6-linked SIA in a *cis* conformation. The structural figure was created using PyMOL using with the PDB accession 4JTV. The three terminal saccharides SIA, Gal-2 and GlcNAc-3 are shown as sticks (oxygen in red; nitrogen in blue; carbon in white).

Avian influenza viruses such as H5N1 that replicate in the intestinal tract have been shown to be activated at a relatively high pH (5.6-6.0) (138). In contrast, acid-stabilizing mutations in the H5 HA reducing the pH of fusion enhance the growth of H5N1 influenza virus in the mammalian upper respiratory tract (139, 140). In agreement herewith, efficient transmission between mammals requires a decrease in the pH required for HA (pH 5.0–5.5) (141-143). Thus, lowering the optimal fusion pH of avian influenza viruses is thought to be favorable for enhanced replication in the mammal upper respiratory tract and more efficient transmission.

## Neuraminidase

Neuraminidase (NA) functions as receptor-destroying enzyme. It removes SIA from cell surface receptors during virus infection and thereby facilitate the release of progeny viruses and spread of infection to new cells (144). Moreover, IAV employs NA to cleave sialic acids

from decoy receptors such as mucin glycoproteins during initial infection stages. Cleavage of these mucins is thought to allow IAV to penetrate the mucus layer and to reach and infect epithelial cells (145-147). So far, 11 NA subtypes (N1-N11) have been described (17, 19, 148). Based on phylogenetic analysis all IAV NAs from avian and mammalian species, except bats (N10 and N11), the NA proteins can be divided into two groups, with group 1 containing N1, N4, N5 and N8 and group 2 including N2, N3, N6, N7 and N9 (Figure 5A).

The NA protein is a type II transmembrane glycopro
tein, which forms a homo-tetramer. Each NA subunit contains about 470 amino acids divided over four domains, a short N-terminal cytoplasmic tail, followed by a hydrophobic transmembrane domain (TMD), a thin stalk of variable length, and a globular head domain (Figure 5B). The N-terminal cytoplasmic domain contains six amino acids (MNPNQK) and is nearly 100% conserved in all influenza A subtypes. This cytoplasmic peptide appears to be crucial for efficient virus replication and for virion morphology (149).

**Transmembrane domain**

The NA TMD that immediately follows the cytoplasmic tail is predicted to consist of a helix encompassing amino acids 7–29 (150). The TMD of NA targets NA to the endoplasmic reticulum, facilitates its membrane integration and transport to the plasma membrane. The TMD domain is also important for efficient tetramerization of the NA protein (151, 152). Increased interaction strength between TMDs may compensate for decreased interaction between the NA head domains (152, 153).

**Stalk domain**

The NA protein contains a NA stalk domain of variable length (25-57 aa) between TMD and the globular head domain (154). A minimal stalk length of 10 amino acids is needed for the head domain to fold (151). Cys residues in the stalk domain may assist correct folding and di/tetramerization of the NA protein via the formation of intermolecular disulfide bonds (155). Glycosylation sites in the stalk domain were shown to modulate NA enzymatic activity and IAV replication (156). In avian IAVs, NA stalk deletions are considered a hallmark of adaptation to terrestrial poultry (157, 158). These deletions, which generally range from 15 up to 27 amino acids (159-161) are associated with decreased enzymatic activity in the context of the virus particle resulting from decreased access to sialosides (162, 163). Such stalk deletions are associated with enhanced replication and virulence/pathogenicity in mice and chickens (160, 161).

**NA head domain and active site**

Crystal structures of NA head domains complexed with SIA have been solved for all IAV NA subtypes (164-170) and B (171) viruses. These IAV NA structures revealed that the NA head domain forms a conserved 6-bladed propeller structure. Each blade is made up of four antiparallel beta sheets stabilized by disulfide bonds and connected by loops of variable length (168, 171) (Figure 5C). The NA active site located in the head domain comprises 8 highly conserved functional residues that directly contact the SIA substrate: R118, D151, R152, R224, E276, R292, R371 and Y406 (N2 numbering) and 11 framework residues that hold the catalytic residues in place, including E119, R156, W178, S179, D198, I222, E227,

H274, E277, N294 and E425 (171, 172) (Figure 5D). Superposition of the structures of NAs from phylogenetic groups 1 and 2 shows that the positions of the active site residues are virtually identical among all the NAs (164). However, there are notable differences between the group 1 and group 2 structures in the conformation of the 150-loop (formed by amino acids 147–152, N2 numbering) that is adjacent to the active site. Only group 1 NAs (but not the H1N1pdm09 N1) possess an open conformation that forms an additional 150-cavity, while no 150-cavity has been observed in group 2 NAs (173). The 150-cavity has been proposed to be a novel target for group 1 specific influenza NA inhibitors (174). Notably, monomeric NAs display no enzyme activity (175, 176). Tetramerization of the NA protein is essential for the formation of the active site and for enzymatic activity (177).

The receptor-destroying activity of NA was first reported by Hirst (178). The ‘‘split product’’ was later identified as N-acetylneuraminic acid (Neu5Ac) (179). The enzymatic mechanism of NA can be divided into four major steps (180-183). The first step is binding of NA to the SIA substrate. This step involves charge interactions between arginine residues at positions 118, 292, and 371 of NA with the SIA carboxylate group and steric constraints from residue Y406 at the base of the active site. During the second step of the catalytic reaction, active site residues D151, R152 and E277 are engaged to stabilize the transition state intermediate (180), which involves proton donation from the solvent. The third stage of the reaction involves the transition state intermediate covalently being bound to the hydroxyl group of Y406 and then being hydroxylated with the solvent. The final step is the formation of Neu5Ac and its release from the enzyme active site.

**Calcium binding sites**

Calcium binding by influenza NA has been shown to be critical for thermostability and enzymatic activity (184). Up to three $Ca^{2+}$ ions have been observed in each NA protomer (165, 185). A conserved calcium binding site present in each NA protomer of all known type A and B NAs (186) is formed by the four backbone carbonyl oxygens of N293, D297, G345, and G347, a carboxyl oxygen from D324, as well as a water molecule, and this conserved site was proposed to be important in stabilizing otherwise flexible loops in a reactive conformation of the active site (187). Additionally, NA of IBV, IAV N3 and H1N1pdm09 have been reported to contain a $Ca^{2+}$ binding site in the center of tetramer, which may stabilize the tetrameric conformation (171). Interestingly, NA of the 1918 pandemic virus and of H1N1pdm09 contain an additional calcium ion in each protomer. The main residues that make up this calcium binding site are D379, N381, and D387 (165).

**NA enzymatic activity and substrate specificity**

In comparison with the well-studied HA receptor specificity, information on the NA enzymatic activity and substrate specificity of IAVs is limited. One of the most commonly used substrates to determine the NA enzymatic activity is small fluorogenic substrate 4-methylumbelliferyl-N-acetylneuraminic acid (MUNANA) (195-198). The cleavage of this small molecule by NA leads to the production of fluorescent 4-methylumbelliferone (4-MU) that can be easily detected. The main disadvantage of this assay is that this monovalent substrate is not representative of the multivalent display of sialosides *in vivo*, while in addition it is not informative about the ability of NA to cleave α2,3 linked or α2,6 linked SIA.

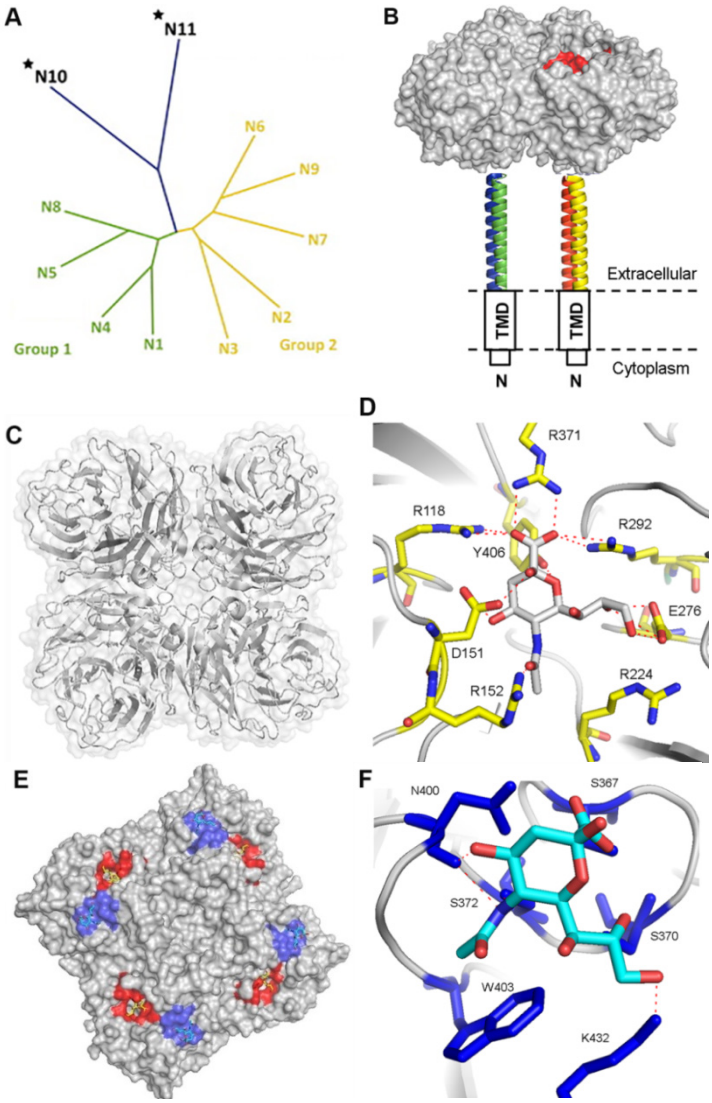



**Figure 5.** (A) Phylogenetic tree of the NA gene of each IAV NA subtype, adopted from (34). All the known NAs can be grouped into two groups (group 1 and 2), except that the bat-derived N10 and N11 (marked with a star) do not belong to either group. N10 and N11 do not possess sialidase activity and are referred as NA-like molecules. (B) Schematic representation of neuraminidase structure. NA is a homo-tetramer with each NA subunit containing a cytoplasmic tail, transmembrane domain (TMD), stalk domain and head domain. (C) Crystal structure of the head domain of the NA from A/Vietnam/1203/04 (H5N1) (PDB ID: 2HTY) (164), shown as a tetramer. The NA head domain is composed by conserved 6-bladed propeller structure with each blade contains four anti-parallel beta sheets stabilized by disulfide bonds and connected by loops of varying length. (D-F) Structure of A/tern/Australia/G70C/75 N9 NA complexed with sialic acid (PDB code: 1MWE). (D) Highly conserved NA active site residues and Neu5Ac are shown as sticks. For the active site residues oxygen is shown in red; nitrogen in blue; carbon in yellow. For Neu5Ac oxygen is colored red; nitrogen blue; carbon white. Hydrogen bonds are shown as dashed red lines. (E) Surface representation of the N9 structure with active (red area) and 2$^{nd}$ SIA-binding sites (periwinkle blue) indicated. Neu5Ac moieties in these sites are shown as sticks (F) 2$^{nd}$ SIA-binding site of NA in complex with Neu5Ac. NA and Neu5Ac are shown as sticks (oxygen in red; nitrogen in blue; carbon in cyan for Neu5Ac; 2$^{nd}$ SIA-binding site residues that directly contact with

Neu5Ac are shown in blue and their numbering is indicated, hydrogen bonds are shown as dashed red lines. The figures were made using PyMOL.

So far, a variety of assays has been used to investigate NA substrate specificity, including glycan array-based neuraminidase assay (199, 200), STD-NMR (201), enzyme linked lectin assay (ELLA) (202-204), and assays using BODIPY-labeled synthetic oligosaccharides (205-207). Overall, these studies suggest that most if not all influenza NA proteins prefer cleavage of α-2,3 (avian-type receptor) over α-2,6 (human-type receptor) sialylosides, although the human viruses appear to cleave α-2,6 SIA relatively more efficiently than the avian viruses (208, 209). The increased cleavage of human-type receptors by the NA proteins of human viruses is probably correlated with the altered binding properties of the corresponding HA proteins and with the importance of a well-tuned HA-NA functional balance as is detailed below. The N2 proteins of human H2N2 and H3N2 viruses isolated between 1957 and 1987 displayed an increase in their ability to cleave α2,6 linked SIAs with time (210, 211). Substitution I275V was identified as being important for the enhanced specificity for α2,6 linked sialylosides of human N2 viruses, and valine at position 275 was maintained in all later human viruses (211). Furthermore, the amino acid at position 431 was found to influence the ability of N2 protein to cleave Neu5Gc, which is found in swine but not humans. Lysine at this position conferred high Neu5Gc specificity while lower Neu5Gc specificity was observed with the presence of proline, glutamine or glutamic acid.

## HA-NA balance

While HA functions in the attachment of the virus particles to the SIA-containing host cell receptors, NA facilitates the release of virus particles from cells and decoy receptors by cleavage of SIA. Intuitively it makes sense that the activities of the HA and NA proteins need to be balanced. For example, low binding avidity of HA to receptors in combination with high NA activity is likely to disturb efficient binding of viruses to the host cells, whereas low NA activity combined with high HA receptor-binding avidity may lead to insufficient SIA cleavage and subsequent aggregation of virus particles (212). The balance between the HA and NA activities is thought to be critical for influenza virus replication as well as transmission (212, 213), however only little is known about the importance of HA-NA balance for the escape of decoy receptors and the attachment to host cells at the molecular level.

The balance between HA and NA may need to be readjusted when IAV crosses the host species barrier. The switch in HA receptor-binding preference during the adaptation of avian IAVs to mammals may drive subsequent adaptation of the NA activity to maintain an optimal balance between attachment to and cleavage of SIA (212-214). For instance, previous analysis of evolution of the N2 enzymatic activity in human H2N2 and H3N2 IAVs demonstrated that the NA cleavage capacity of α2,6-linked SIAs was gradually upregulated in the direction of the preferred H2 and H3 receptor determinant (210, 211). Analysis of recombinant HA and NA proteins suggested a different HA-NA balance for human H1N1 viruses compared to swine viruses (200), which may be related to different SIA receptor profiles in these host species (215).

HA-NA balance may also be affected when the activity of the HA or NA proteins is altered

for example by selection of mutations that either alter the antigenicity of these proteins or confer resistance to antiviral drugs. For example, NAI-resistance mutations in NA, which generally negatively affect NA activity, may be compensated by changes in HA to restore viral fitness (216). Such mutations in HA may confer lower affinity for SIA receptors, as such viruses are less dependent on NA activity (217). Also antibody-driven antigenic variation in the H1 may lead to compensatory mutations in NA that result in NA antigenic variation and acquisition of drug resistance (218).

## IAV antiviral drugs

Neuraminidase inhibitors (NAIs), such as oseltamivir (trade name: Tamiflu) and zanamivir (trade name: Relenza) are transition state analogues designed to target conserved residues at the NA enzyme active site in influenza A and B viruses. However, treatment of IAV-infected individuals with an NAI can drive emergence of resistance within treated individuals (219-221). Resistance to oseltamivir most commonly results from mutations which directly or indirectly alter the shape of the NA catalytic site, thus reducing the inhibitor binding ability. The two phylogenetic groups of NAs (N1 and N2) differ in sensitivity to NAIs and distinct resistance mutations are found. Generally, H275Y (N1 numbering) is often observed in N1, while R292K and E119V are most common in N2 (222). While clinical use of NAIs in IAV-infected individuals can generate resistant viruses, attention should be also paid to the emergence and circulation of NAI-resistant human IAV strains in the absence of selective drug pressure. By 2008, most of the seasonal H1N1 viruses circulating were resistant to oseltamivir in the absence of oseltamivir selection pressure, indicating that the fitness of these seasonal viruses was no longer compromised by the resistance mutation. This was explained by the presence of several “permissive” mutations (196, 223) that increase NA cell surface expression and enzymatic activity. There is concern that also the H1N1pdm09 viruses may similarly acquire oseltamivir resistance as permissive substitutions that increase expression and activity of cell-associated NA proteins have already been found in currently circulating viruses (224-226).

## Aim and outline of the thesis

As indicated above, a functional balance between the HA and NA proteins is likely of high importance for maintaining optimal virus replication as well as transmission. While HA receptor-binding avidity and specificity have been studied in detail, much less is known about the molecular determinants that mediate (changes in) the specificity and activity of IAV NA proteins. The overall aim of this thesis was to unravel to what extent and how IAVs modulate the activity of their NA proteins.

Recombinant soluble NA proteins have been used for structure determination, enzymatic activity and immunogenicity analyses. Once the NA sequence is known, the recombinant proteins can be produced within a relatively short time and can be easily purified, which facilitates their use in various downstream assays. Moreover, there is no need to cultivate potentially dangerous viruses. As we wanted to study a large number of NA proteins, we first

aimed to establish an optimal recombinant soluble NA protein to study the (enzymatic) characteristics of these proteins. To this end, we compared and analyzed different recombinant soluble expression approaches for several HP H5N1 NA proteins **(Chapter 2)**. By doing so, we not only identified an optimal recombinant soluble NA expression approach, but also identified residues that affected NA folding and/or enzymatic activity.

Previously, we compared the HA receptor-binding properties of the novel emerging H7N9 virus (from 2013) that frequently infects humans with a closely related avian H7N9 virus from 2008. The H7 of the novel H7N9 virus displayed a modest increase in binding to α2,6 sialosides and reduced, but still dominant, binding to α2,3 linked SIAs compared to the H7N9 virus from 2008. In view of the presumed importance of the HA-NA balance, the (enzymatic) properties of the corresponding N9 proteins were studied in **Chapter 3** using the optimized recombinant soluble NA expression approach developed in Chapter 2. In agreement with the reduced receptor-binding avidity of the novel H7 proteins, the N9 proteins displayed reduced cleavage of and binding to sialosides resulting from a single mutation in the 2$^{nd}$ SIA-binding site.

The H1N1pdm09 virus is the first human pandemic IAV to have emerged in the genomics era. Sequencing of numerous IAV isolates has provided us with a wealth of sequence information. In **Chapter 4**, we used phylogenetic analysis in combination with the recombinant NA expression approach to analyze the evolution of the H1N1pdm09 NA protein upon the introduction of the virus in the human population in detail. More specifically, the effects of NA substitutions on enzymatic activity, substrate specificity, expression and antigenicity were investigated. Our results indicate that the NA proteins evolve within a certain bandwidth of these different phenotypic properties and suggest that evolution of the NA protein is a continuous trade-off between these different aspects.

In the **Chapter 5** we focused in more detail on to what extent differences in the enzymatic activity of different NA proteins derived from avian and human viruses can be attributed to differences in their 2$^{nd}$ SIA-binding site. The results indicate that mutation of the 2$^{nd}$ SIA binding site provides viruses with an additional mechanism to manipulate the enzymatic activity of their NA proteins without having to mutate their active site residues directly. Finally, a summarizing discussion is provided as the last chapter (**Chapter 6**), in which the main results and conclusions of the research in this thesis are analyzed and discussed in a broader perspective.

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# Chapter 2

## Identification of residues that affect oligomerization and/or enzymatic activity of influenza virus H5N1 neuraminidase proteins

Meiling Dai[1], Hongbo Guo[1], Jos Dortmans[1], Jojanneke Dekkers[1], Johan Nordholm[2], Robert Daniels[2], Frank J.M. van Kuppeveld[1], Erik de Vries[1] and Cornelis A.M. de Haan[1]*

1. Virology Division, Department of Infectious Diseases & Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands
2. Department of Biochemistry and Biophysics, Center for Biomembrane Research, Stockholm University, SE-106 91 Stockholm, Sweden

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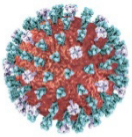

## Abstract

Influenza A virus attachment to and release from sialoside receptors is determined by the balance between hemagglutinin and neuraminidase (NA). The molecular determinants that mediate the specificity and activity of NA are still poorly understood. In this study, we aimed to design the optimal recombinant soluble NA protein to identify residues that affect NA enzymatic activity. To this end, recombinant soluble versions of four different NA proteins from H5N1 viruses were compared with their full-length counterparts. The soluble NA ectodomains were fused to three commonly used tetramerization domains. Our results indicate that the particular oligomerization domain used does not affect the Km value, but may affect the specific enzymatic activity. This particularly holds true when the stalk domain is included and for NA ectodomains that display a low intrinsic ability to oligomerize. NA ectodomains extended with a Tetrabrachion domain, which forms a nearly parallel four-helix bundle, better mimicked the enzymatic properties of full-length proteins than when other coiled coil tetramerization domains were used, which probably distort the stalk domain. Comparison of different NA proteins and mutagenic analysis of recombinant soluble versions thereof resulted in the identification of several residues that affected oligomerization of the NA head domain (position 95), and therefore the specific activity, or sialic acid binding affinity (Km value; positions 252 and 347). This study demonstrates the potential of using recombinant soluble NA proteins to reveal determinants of NA assembly and enzymatic activity.

## Introduction

Virus particles contain dedicated proteins that recognize cell-surface molecules. While some viruses evolved to bind specific protein receptors, others bind carbohydrate moieties, such as sialic acids (SIAs) that are omnipresent on the cell surface as well as in the mucus. Several of these latter viruses, including influenza A virus (IAV), carry receptor destroying activities in addition to receptor binding proteins or domains. The receptor destroying activity plays an important role in viral fitness, pathogenicity and host tropism as it is important for release of (newly assembled) particles from host cells and from non-functional (decoy) receptors.

IAVs are important pathogens of animals and humans (1). They are enveloped, segmented negative-strand RNA viruses belonging to the *Orthomyxoviridae* family (2). Their virus particles contain two main surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). IAVs are classified based on the subtypes of both HA and NA. So far, 18 HA (H1–H18) and 11 NA (N1–N11) subtypes have been identified, almost all of which have been found in aquatic birds (3-6). In humans, IAVs cause seasonal epidemics and occasional pandemics. The pandemics resulted from animal viruses that managed to cross the host species barrier and gained the ability to transmit among humans (7). Highly pathogenic avian H5N1 IAVs are regarded as a pandemic threat because of their high virulence and fatality rate, global prevalence and wide diversity of avian hosts (8-10).

The HA and NA proteins have a critical role in determining IAV pathogenicity and host tropism. The HA protein is responsible for virus-cell attachment via binding to sialylated receptors at the cell surface, and it also induces virus-cell fusion after endocytic uptake of the virus particles (11, 12). The NA protein is the receptor-destroying enzyme and responsible for removing sialic acid (SIA) from host glycoproteins as well as glycolipids, thereby allowing release of progeny virions from cells and decoy receptors and preventing virus self-aggregation. Several studies indicate that a functional balance between the HA and NA proteins is of importance for maintaining optimal virus replication as well as transmission across different host species (13-15). However, while numerous studies describe HA receptor-binding avidity and specificity in detail, much less is known about the enzymatic properties and substrate specificities of different NA proteins.

NA is a homo-tetrameric type II transmembrane glycoprotein, with each NA monomer containing a globular head domain with the enzyme active site, a thin stalk of variable length, a hydrophobic transmembrane domain (TMD) and a short N-terminal cytoplasmic tail. The NA head domain forms a conserved 6-bladed propeller structure. Each blade is formed by four anti-parallel beta sheets, which are stabilized by disulfide bonds and connected by loops of variable length. The active site of NA is composed of highly conserved catalytic and structural residues that either directly contact the SIA substrate or hold the catalytic residues in place (16-18). Tetramerization of the NA protein is important for the formation of the active site and synthesis of active enzymes (19). Recent studies indicate that the neuraminidase TMD facilitates oligomerization of NA in coordination with the head domain to reach optimal assembly (20, 21).

Previously, we and others have generated recombinant soluble NA proteins for structure determination, enzymatic activity and immunogenicity analyses (22-30). The use of recombinant soluble glycoproteins provides several advantages. Importantly, there is no need to cultivate potentially dangerous viruses. Once the sequence is known, the recombinant proteins can be produced within a relatively short time and, compared to their membrane-anchored counterparts, the soluble glycoproteins can be easily purified, which facilitates their use in various downstream assays. In view of the importance of NA oligomerization for enzymatic activity, N-terminal tetramerization domains are generally fused to the recombinant soluble NA proteins. These tetramerization domains may be artificial like GCN4-pLI (22, 23) or derived from bacterial or mammalian proteins such as the *Staphylothermus marinus* Tetrabrachion protein (27, 28) or human vasodilator-stimulated phosphoprotein (VASP) (24, 29, 30). Although the recombinant soluble NA proteins have been used for different analyses, it is not known how the different N-terminal oligomerization domains affect the NA protein assembly and enzymatic properties.

In this study, we aimed to design the optimal recombinant soluble NA protein to identify residues that affect NA enzymatic activity. To this end, recombinant soluble versions of four different NA proteins from H5N1 viruses were compared with their full-length counterparts. The soluble NA ectodomains were fused to three commonly used tetramerization domains. The results indicate that the design of the recombinant soluble NA protein including the particular tetramerization domain is an important determinant for maintaining the enzymatic properties within the head domain. Comparison of different NA proteins and mutagenic analysis of recombinant soluble versions thereof resulted in the identification of several residues that are important for the enzymatic activity of the N1 protein by affecting oligomerization of the NA head domain (position 95), and therefore the specific activity, or sialic acid affinity (Km value; positions 252 and 347).

## Materials and Methods

### NA genes preparation

Human codon optimized NA ectodomain (head plus stalk domain, a.a.62–469; N2 numbering) encoding cDNAs (Genscript, USA) of A/duck/Hunan/795/2002 (GenBank accession no. BAM85820.1, referred to as HN), A/Vietnam/1194/04 (GenBank accession no. AAT73327, referred to as VN), A/turkey/Turkey/1/2005 (GenBank accession no. ABQ58915.1, referred to as TK) and A/Hubei/1/2010 (GenBank accession no. AEO89183.1, referred to as HB) were cloned into a pFRT expression plasmid (Thermo Fisher Scientific). The soluble NA-encoding sequences were preceded by sequences coding for an N-terminal signal sequence derived from *Gaussia* luciferase (Gluc), a double Strep-tag for affinity purification (One-STrEP; IBA GmbH), either a GCN4-pLI (GCN4) (31), human VASP (32) or *Staphylothermus marinus* Tetrabrachion (33) tetramerization domain and a two amino acid (GT) linker. When indicated, the two amino acid linker was replaced with an extended linker (GSGGT). The corresponding FL-NA protein-coding plasmids were generated by replacement of the non-NA coding sequences by sequences encoding the NA transmembrane domain and cytoplasmic tail. Mutations of interest were introduced into the

corresponding NA genes by using the Q5 Site-Directed Mutagenesis Kit (NEB) and confirmed by sequencing.

**Protein expression**

HEK293T cells were transfected with the appropriate NA expression plasmids using polyethyleneimine (PEI) in a 1:10 ratio (g DNA to g PEI). After an overnight incubation period, the transfection medium was replaced by 293 SFM II expression medium (Invitrogen) for the soluble NA constructs or by DMEM containing 2% FCS for the full-length NA constructs. 5 days post transfection, cell culture media containing soluble NA were harvested, cleared by centrifugation and the protein expression levels of the soluble NA proteins were analyzed by SDS-PAGE analysis followed by Western blotting with HRP-conjugated Strep-tag II-specific monoclonal antibody (IBA). The NA protein concentrations in the cell culture media were determined by extrapolation using a standard consisting of different amounts of purified NA proteins. The purified NA proteins were quantified by quantitative densitometry of coomassie blue stained proteins using a BSA standard. The blot signals were imaged and analyzed with an Odyssey imaging system (LI-COR). Oligomerization of the proteins was determined by blue-native polyacrylamide gel electrophoresis (BN-PAGE) similarly as described previously (34). Cells expressing full-length NA proteins were harvested at 36 h to 48 h post transfection. Cells were lysed with PBS containing 0.05% Triton X-100 on ice for half an hour. Cell lysates were cleared by centrifugation at 12.000 rpm for 2 minutes and protease inhibitor cocktail (Roche) was added to prevent protein degradation. Quantification of the protein expression levels was performed by Western blot, similarly as described above, using a polyclonal goat antiserum raised against N1 proteins from A/Vietnam/1203/2004 (H5N1) and A/Hong Kong/483/1997 (H5N1) (NR-9598; BEI Resources). The different N1 proteins used in this study were recognized to approximately the same extent by this polyclonal antiserum (data not shown). Rabbit anti-goat immunoglobulins/HRP (DAKO) was used as secondary antibody. All results were confirmed 2-4 times using independently generated protein preparations.

**Neuraminidase enzyme activity and kinetics**

The activity of recombinant soluble and full-length NAs was determined by using a fluorometric assay similarly as published before (35). In this assay, the substrate 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA; Sigma-Aldrich) is hydrolyzed by neuraminidase resulting in fluorescent 4-methylumbelliferone (4-MU). To this end, NA preparations were subjected to 2-fold serial dilutions in reaction buffer (50mM Tris/HCl, 4mM $CaCl_2$, PH 6.0) in a flat-bottom 96-well black plate (Greiner Bio-One). Subsequently, a similar volume of reaction buffer containing 200μM MUNANA was added to each well, mixed well, and incubated at 37℃ for 60min. The reaction was terminated with the stop solution (0.1M Glycine, 25% ethanol, PH 10.7). The fluorescence of the 4-MU reaction product was immediately determined in relative fluorescence units (RFUs) using a Fluostar OPTIMA plate reader (BMG Labtech, Mornington, Australia) with excitation and emission wavelengths at 340 and 490 nm, respectively. The specific activity (activity/ng) of the different NA proteins was determined from the linear parts of the resulting curves and graphed relative to a NA reference sample. Kinetic analysis was performed to determine the Km values of the different NA proteins. NA samples, resulting in approximately 20% of the

maximum RFU level after 1 h incubation period with 100 μM MUNANA, were incubated with different concentrations of MUNANA ranging from 3.9 to 500 μM (using two-fold serial dilutions) in a total volume of 100μL. The fluorescence of 4-MU was measured at 37°C every 5min for 40 min. The data were fitted to the Michaelis-Menten equation by non-linear regression using the Prism 6.05 software (GraphPad) and the Km value was determined.

**GALLEX TMD interaction system**

The NA TMD interactions from the HN and VN strains were determined using the *E. coli* GALLEX assay (36). The coding sequences for residues 2-35 and 2-42 from each NA were inserted into the pBLM plasmid between the LexA DNA binding domain and the MBP gene. These regions encompass the TMD (residues 7-34), the conserved N-terminal amino acids (residues 2-6), and either 1 or 8 juxtamembrane amino acids on the C-terminal side. SU101 cells, which contains a lacZ gene regulated by the LexA operator, were first freshly transformed with the resulting pBLM expression plasmids and grown overnight in LB containing ampicillin (100 μg/ml). The cultures were then diluted in LB until $A_{600}$=0.1, induced with 0.05 mM IPTG for 2.5 h at 37°C until $A_{600}$≈0.6. The measurement of the β-galactosidase activity and the relative TMD interactions were calculated as previously described (37).

**Statistical analysis**

All statistical analyses were performed either by two-tailed t-test or one-way ANOVA analysis of variance in combination with Bonferroni's multiple comparisons test using Prism 6.05 software. The data shown are the means of three independent experiments. Error bards indicate standard deviations. The level of significance was determined as *$P$<0.05, ** $P$<0.01, *** $P$<0.001, **** $P$<0.0001.

## Results

**Construction and expression of recombinant NA proteins**

As we aim to study the enzymatic activity for a large panel of recombinant NAs, we first analyzed the extent of which the specific recombinant protein expression approach affected the activity of the resulting NA proteins. We started our analysis by comparing the enzymatic activity of N1 proteins derived from different H5N1 viruses that were expressed either as full-length proteins (FL-NA) or recombinant soluble ectodomains fused to an artificial GCN4-pLI tetramerization domain (GCN4-NA). A schematic representation of the recombinant proteins is shown in Fig. 1A. The FL-NA proteins contain a N-terminal cytoplasmic tail, transmembrane domain (TMD), stalk, and head domain. The GCN4-NA proteins contain a cleavable signal sequence, GCN4-pLI leucine zipper tetramerization domain, NA stalk and head domain. Expression of the NA proteins was achieved by transient transfection of the appropriate plasmid into HEK 293T cells. Four N1 proteins (designated HN, VN, TK, and HB after their geographic origin) derived from viruses belonging to different H5N1 clades were expressed. Expression of the NA proteins was confirmed by gel electrophoresis of cell lysates for the FL-NA or of the cell culture supernatants for the GCN4-NA proteins followed by Western blotting. For all proteins, their electrophoretic mobilities were in agreement with the expected molecular weights (Fig. 1B).

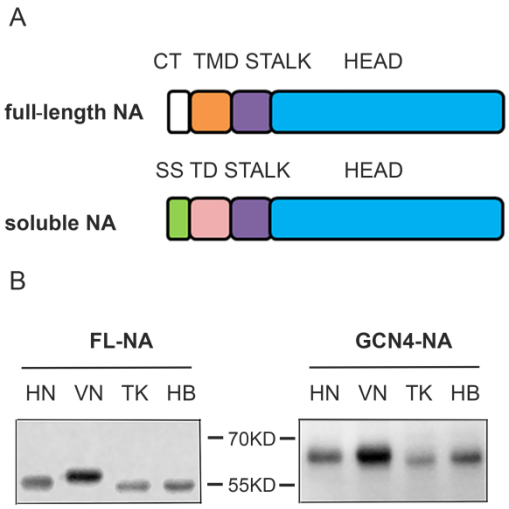


**Figure 1. Expression of recombinant NAs**. (A) Schematic representation of the recombinant full length NA (FL-NA) and soluble NA proteins. The FL-NA protein contains the NA head, stalk, transmembrane domain (TMD) and cytoplasmic tail (CT). The soluble NA protein contains the head and stalk regions fused with a tetramerization domain (TD) and a cleavable signal sequence (SS). (B) Recombinant full-length NA (FL-NA) and soluble GCN4-containing NA (GCN4-NA) proteins HN, VN, TK and HB were expressed in HEK293T cells and their expression levels were analyzed by SDS-PAGE followed by western blotting as described in the Materials and Methods section. The position on the gel of the relevant molecular weight marker proteins is shown.

**Enzymatic activity of the recombinant NA proteins**

The enzymatic activity of the different NA proteins was studied using the MUNANA fluorometric assay. To this end, serial dilutions of preparations containing defined amounts of FL-NA and GCN4-NA of HN, VN, TK and HB were analyzed (Fig. 2A and B). Based on these results, and as detailed in the Materials and Methods section, the specific activities of the different NA proteins were calculated. As shown in Fig. 2C, the full-length proteins (HN, TK, HB) were approximately 4~5 fold more active than their soluble counterparts containing the GCN4-pLI tetramerization domain. Furthermore, it is clear that the specific activities of the VN proteins are lower than of the other NA proteins. However, the difference is much bigger between the soluble proteins (approximately 12 fold) compared to the full-length protein (approximately 1.5 fold). To exclude that the large difference between the VN and HN proteins somehow results from using a short linker (GT) between the GCN4-pLI and NA sequences, the effect of using an extended linker (GSGGT) was analyzed (Fig. 2D). Similar results were obtained with the short and extended linkers. In addition, we determined the Km values of the different NA proteins. The Km value is the substrate concentration at which the reaction rate is half of the maximum and is indicative of the affinity of NA for its substrate. The results indicated that the FL-NA proteins of HN, TK and HB had significantly lower Km values than FL-NA VN, indicating that the VN protein has a lower binding affinity for sialic acid (Fig. 3A). Similar differences in the Km values were also observed for the GCN4-NA proteins (Fig. 3B). Our results indicate that although the FL-NA and GCN4-NA proteins differ in their specific activities, they display similar Km values, which are significantly higher for the VN proteins than for the other proteins.

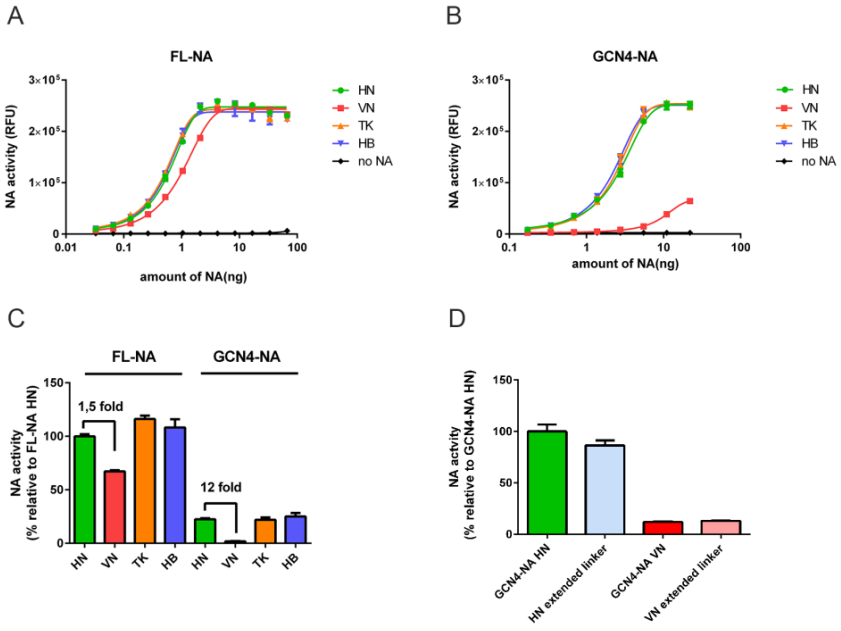


**Figure 2. Activity of recombinant NA proteins derived from different H5N1 viruses**. The NA enzymatic activity of preparations containing different amounts of the FL-NA (A) or GCN4-NA (B) proteins HN, VN, TK and HB was determined using the MUNANA fluorometric assay (Relative fluorescent units; RFU). (C) Specific activity (RFU/ng) of the different NA proteins was determined from the linear parts of the curves shown in (A) and (B) and normalized to the specific activity of the FL-NA HN protein. The fold difference between the specific activities of the FL- and GCN4-NA HN and VN proteins is indicated. (D) Specific activity of GCN4-NA HN and VN as well as of their counterparts with extended linkers is shown normalized to that of GCN4-NA HN. All graphs represent the mean of three independent experiments. Error bars indicate standard deviations.

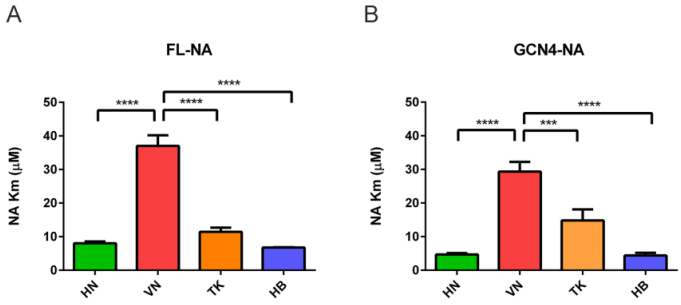


**Figure 3. Km values of FL- and GCN4-NA proteins**. (A) Km values of FL-NA proteins. (B) Km values of soluble GCN4-NA proteins. All graphs represent the mean of three independent experiments. Error bars indicate standard deviations. Significant differences by one-way ANOVA in combination with Bonferroni's multiple comparisons test relative to NA VN are indicated (*P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001).

## Differences between GCN4-NA proteins are not explained by changes in oligomeric status or thermostability

These results prompted us to elucidate the reason for the observed differences in the enzymatic activity of the different GCN4-NA proteins. As a first step, we compared the oligomeric status of the GCN4-NA HN and VN proteins as it is known that oligomerization of

the NA proteins is essential for their enzymatic activity. To this end, the two proteins were subjected to BN-PAGE followed by Western blotting. When the proteins were not heat-denatured or only for a very short time (5 seconds), they both ran at a high position in the gel in agreement with their tetrameric configuration (Fig. 4A). Only after prolonged heating of the samples, a minor protein species could be detected at a lower position in the gel that likely corresponds with NA monomers. These results indicate that the enzymatic difference between the two recombinant soluble NA proteins is not likely associated with differences in their oligomerization state *per se*. Next, the thermostability of the two proteins were compared by heating them to 50°C for different time periods and monitoring their enzymatic activity (Fig. 4B). The activity of both enzymes decreased prolonged exposure to 50°C. The enzymatic activity of the VN protein appeared somewhat more thermostable than that of the HN protein. This result indicates that the differences in the specific activity between the GCN4-NA HN and VN proteins are not explained by the VN protein being less thermostable.

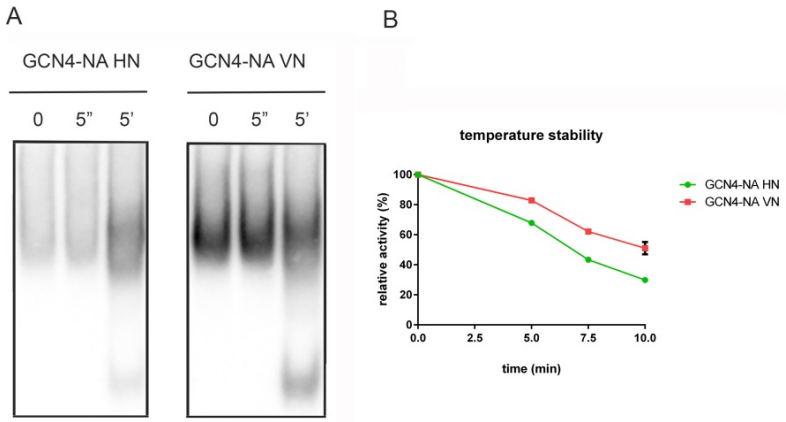


**Figure 4. Stability of GCN4-NA HN and VN proteins**. (A) BN-PAGE analysis of the GCN4-NA HN and VN proteins. NA samples were subjected to gel electrophoresis without boiling (0”) or after boiling for 5” or 5’. The positions of the presumed monomeric and tetrameric forms of NA in the gel are indicated. (B) Analysis of the enzymatic activity of GCN4-NA HN and VN samples heated at 50°C for 5, 7.5 and 10 minutes relative to their unheated controls. A representative experiment performed in triplicate is shown.

### Sequence comparison of HN and VN proteins

Sequence comparison of the VN and HN proteins revealed 10 residues that differ between the HN and VN proteins that are located either in their stalk (S46A and L54F) or head domain (K84T, N95R, Y100H, H252Y, S343P, G347Y, E385G and G454S; N2 numbering) (Fig. 5). Although the GCN4-extended HN and VN proteins differed dramatically in their Km values and in their specific activity, none of these residues belong to the active site or framework residues (16-18) (Fig. 5 and 6). The residue at position 347, which is located close to the active site, forms a calcium ion binding site together with other amino acids (38). Calcium binding is important for thermostability and enzyme activity (39, 40) and differences at this position may therefore affect the enzymatic properties of NA. The other residues are located further away from the active site and are expected to affect NA enzyme activity indirectly. Two residues, at positions 95 and 454, are located close to the NA head domain interface, and might affect oligomerization of the NA head domains.

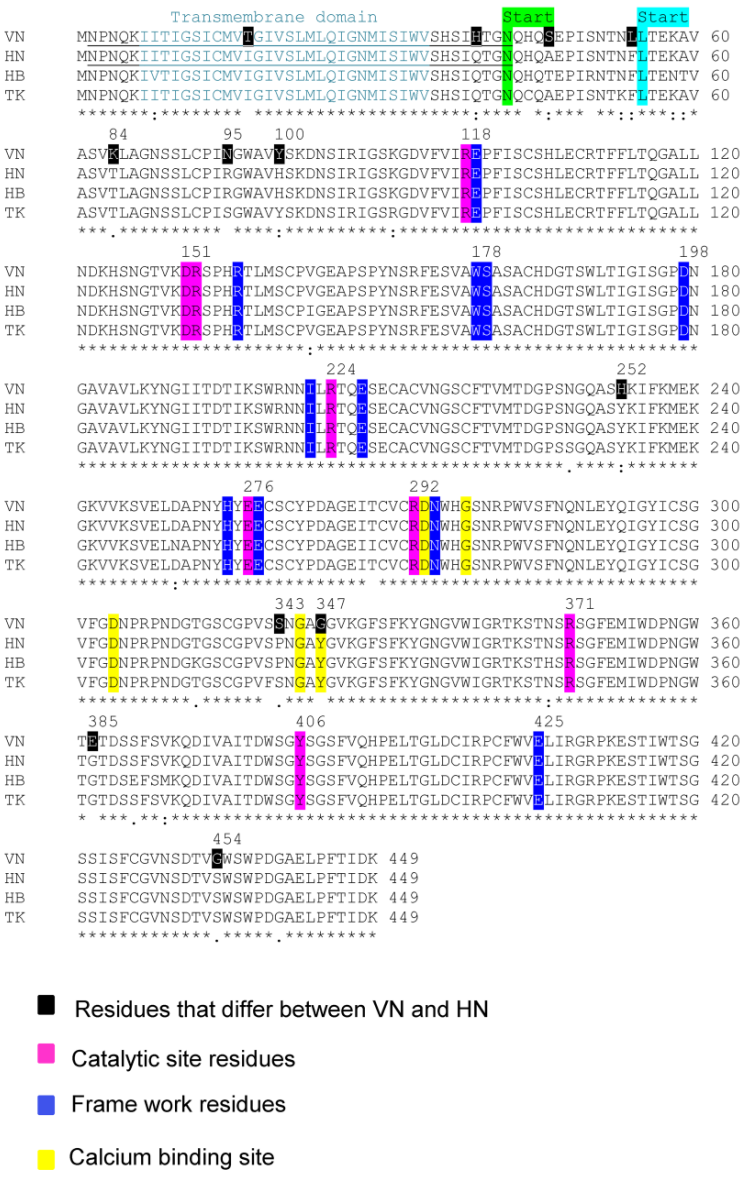


**Figure 5. Alignment of the N1 proteins used in this study.** Clustal Omega multiple sequence alignment of the N1 proteins used in this study is shown. Residues that differ between the VN and the HN proteins are indicated by the black boxes and their numbering is indicated (N2 numbering is used from residue 84 onwards). NA starting residues of the recombinant soluble NA and NA head proteins are also indicated by the green and cyan boxes, respectively. The transmembrane domains are indicated with the blue letters, while the extended versions thereof used in the GALLEX system (36) are underlined. Residues within the active site that have direct interaction with the substrate at the catalytic site and the framework residues that stabilize the catalytic site are indicated by purple and blue boxes, respectively (16-18). The residues that form a $Ca^{2+}$ binding site are indicated by the yellow boxes.

**Identification of residues in NA that affect Km**

To identify the residues that are responsible for the different Km values of the HN and VN proteins, we performed a mutagenic analysis. The residues that differ between the two proteins in the stalk and in the head domain were mutagenized one by one in the background of the GCN4-NA VN protein. All mutant VN proteins were expressed and their Km values were determined (Fig. 7A). Of the 10 mutant NA proteins tested, only two (H252Y and G347Y) displayed Km values that were significantly lower than that of the parental VN protein. Similar results were obtained when these substitutions were introduced in the FL-VN proteins (Fig. 7B).

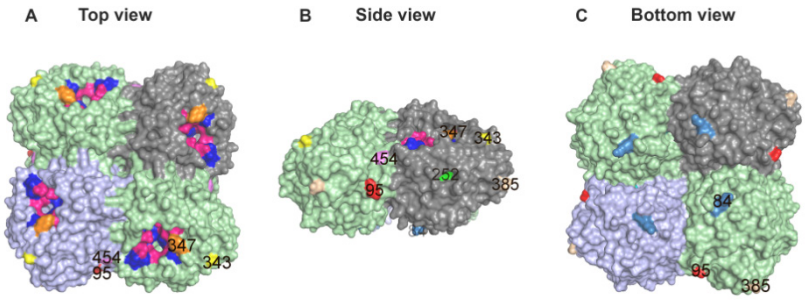


**Figure 6. Structure of the H5N1 NA protein.** Top (A), side (B) and bottom (C) views of the structure of NA protein of A/Vietnam/1203/04 generated with PyMol software are shown (PDB 2HTY) (52). This protein differs from the VN protein used in this study at only two positions (283N and 347Y). The residue at position 283 (not indicated) does not differ between the HN and VN N1 proteins The NA catalytic site and the frame work residues (as indicated in Fig. 5) are colored pink and blue, respectively. These residues do not overlap with the residues that differ between the HN and VN ectodomains and that are labeled with different colors (84 sky blue, 95 red, 100 purple, 252 green, 343 yellow, 347 orange, 385 wheat, 454 violet). The residue at position 100 is not visible in the structure, while the residues that differ in the stalk region between the VN and the HN proteins are not indicated as the structure thereof is not solved.

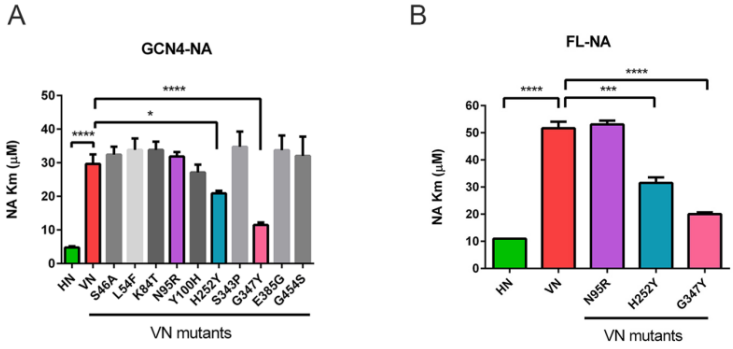


**Figure 7. Km values of mutant NA VN proteins.** The indicated single amino acid changes were introduced into the background of the NA VN protein and the Km values of the resulting GCN4-NA (A) and FL-NA (B) proteins were determined. Wildtype VN and HN GCN4- and FL-NA were used as a reference. All graphs represent the mean of three independent experiments. Error bars indicate standard deviations. Significant differences by one-way ANOVA in combination with Bonferroni's multiple comparisons test relative to NA VN are indicated (*P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001).

In agreement with the TK and HB proteins also having a low Km value, the residues at these two positions were identical to those in the HN protein. From these results we conclude that the identity of the residues at position 252 and 347, the latter of which is part

of a calcium binding site, can influence the sialic acid binding affinity of the NA protein likely by causing structural perturbations that influence the active site conformation.

**Identification of residues that affect specific activity of NA**

We next analyzed the specific activity of the set of GCN4-NA VN mutant proteins. Of the 10 substitutions tested, only one (N95R) resulted in a significantly increased specific activity to a level approximately half of that of the HN protein (Fig. 8A-B). The two substitutions that altered the Km value, also slightly increased the specific activity, but to a lower extent that was not as significant as N95R. Substitution of the residue at position 84 resulted in a lower specific activity. While the VN and HN proteins contain an N or R residue at position 95, respectively, the TK protein contains an S at this position. To get more insight into the importance of the residue at position 95 for the NA specific activity, recombinant proteins were made in the background of both HN and VN that contain either a R, N, or S residue at this site. Regardless of the NA protein background used, the NAs had the lowest activity when an N was present at position 95, an intermediate activity with an S at this position, and the highest activity with an R at this position (Fig.8C-D). However, additional residues also contribute to the differences between the HN and VN proteins as changes at position 95 alone do not completely rescue the enzymatic activity.

**Identity of the residue at position 95 is of importance for NA head domain oligomerization**

We hypothesized that the residue at position 95 may alter the oligomerization of the head domain, as its substitution had a large effect on the NA specific activity, but not on the Km value (Fig. 7 and data not shown). Furthermore, it is located close to the interface between NA monomers, and oligomerization is required for NA enzymatic activity (19). To test this hypothesis, we expressed the HN and VN head domains in the absence of the artificial tetramerization domain. In addition, the stalk domain was deleted as it was previously shown that removal of the NA stalk domain restores NA activity in the absence of the TMD and an artificial tetramerization domain (20). Removal of the stalk domain from the GCN4-pLI extended NA proteins (GCN4-$NA_{head}$) had a small negative effect for the HN protein, but clearly increased the activity of the VN protein (Fig. 9). Additional deletion of the tetramerization domain resulted in undetectable enzymatic activity for the VN protein ($NA_{head}$ VN), while the activity of the HN protein ($NA_{head}$ HN) was much less affected. Introduction of N at position 95 of the HN head domain ($NA_{head}$ HN R95N) abolished its activity, while introduction of R at the same position of the VN protein ($NA_{head}$ VN N95R) resulted in detectable activity. These results indicate that in contrast to the head domain of the HN protein, the head domain of the VN protein is not able to form bioactive molecules in the absence of an artificial oligomerization domain, a characteristic for which the identity of the residue at position 95 appears to play an essential role.

In view of the importance of oligomerization for NA protein activity (41, 42), we next analyzed the oligomerization state of the NA head domains by using BN-PAGE. In the presence of GCN4-pLI, the majority of the NA head domains of HN and VN migrated at a high position in the gel in accordance with these proteins being a tetramer (Fig. 10). Heating of the samples resulted in the appearance of NA monomers. In the absence of the

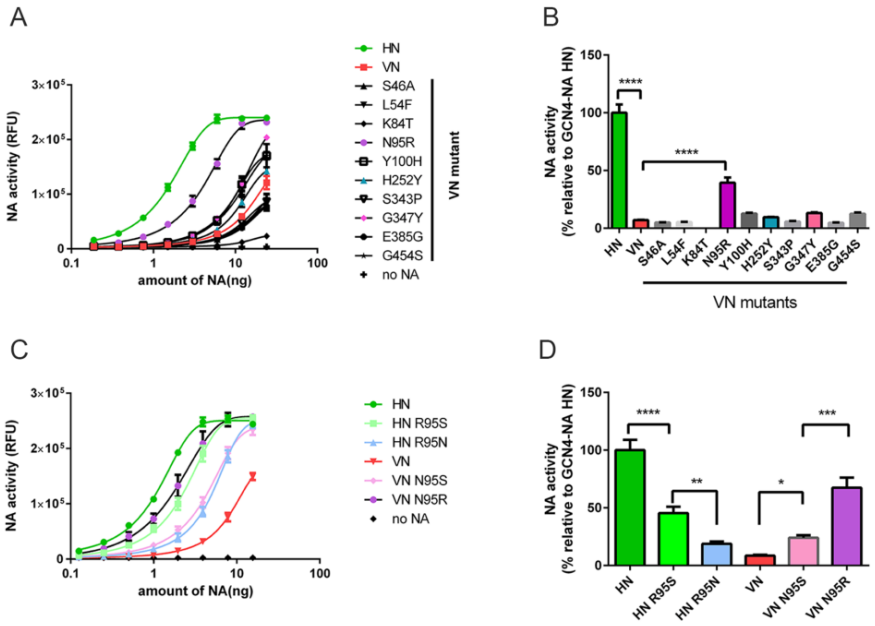


**Figure 8. Enzymatic activity of mutant GCN4-NA VN and HN proteins.** (A) Single amino acid changes were introduced into the background of the GCN4-NA VN protein and preparations containing different amounts of these proteins were analyzed for their enzymatic activity as described in the legend to Fig. 2. Wildtype VN and HN GCN4-NA were taken along as a reference. (B) The specific activity of the different NA proteins shown in (A) is graphed relative to the specific activity of the GCN4-NA HN protein. (C) The residue at position 95 in the GCN4-NA VN and HN proteins was substituted with N, S or R as indicated. Preparations containing different amounts of these proteins were analyzed for their enzymatic activity as described in the legend to Fig. 2. Wildtype VN and HN GCN4-NA were taken along as a reference. (D) The specific activity of the different NA proteins shown in (C) is graphed relative to the specific activity of the GCN4-NA HN protein. All graphs represent the mean of three independent experiments. Error bars indicate standard deviations. Significant differences by one-way ANOVA in combination with Bonferroni's multiple comparisons test relative to GCN4-NA VN (B), or VN N95S or HN R95S (D) are indicated (*P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001).

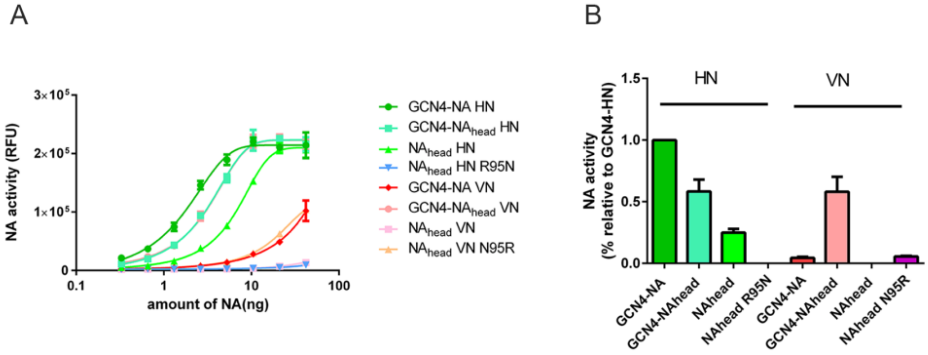


**Figure 9. Enzymatic activity of NA proteins in the absence of the stalk and tetramerization domain.** (A) Enzymatic activity of preparations containing different amounts of HN and VN GCN4-NA, GCN4-NA proteins lacking the stalk (GCN4-$NA_{head}$), NA head domains lacking the GCN4 domain ($NA_{head}$) and NA head domains with R95N or N95R substitutions. (B) Specific activity of the different NA proteins shown in (A) is graphed relative to the specific activity of the GCN4-NA HN protein. All graphs represent the mean of three independent experiments. Error bars indicate standard deviations.

tetramerization domain, the majority of the NA head domains of HN still appeared to migrate as a tetramer, in agreement with this protein displaying enzymatic activity, while monomers were observed after heating of the samples. In contrast, the majority of the enzymatically inactive VN head domains appeared to migrate as monomers, even without heating of the samples. Substitution of the residue at position 95 in the head domain of HN, which resulted in an enzymatically inactive protein, gave rise to the increased presence of monomers, even in the absence of heating, although higher order structures could still be observed. Introduction of the reciprocal mutation in the VN head domain also affected the gel electrophoretic behavior of the resulting protein to some extent, but did not appear to result in a clear appearance of NA tetramers, which might be expected in view of the low enzymatic activity of this preparation. From these results we conclude that, in the absence of the GCN4 domain, the VN and HN head domains differ in their oligomerization in agreement with their observed difference in NA enzymatic activity. Mutation of the residue at position 95 affected the migration of the resulting proteins to some extent, in agreement with these mutations affecting the activity of the NA head domain. The results also indicate that other residues, besides the residue at position 95, must be of importance for the difference in enzymatic activity and oligomerization of the NA head domains of VN and HN.

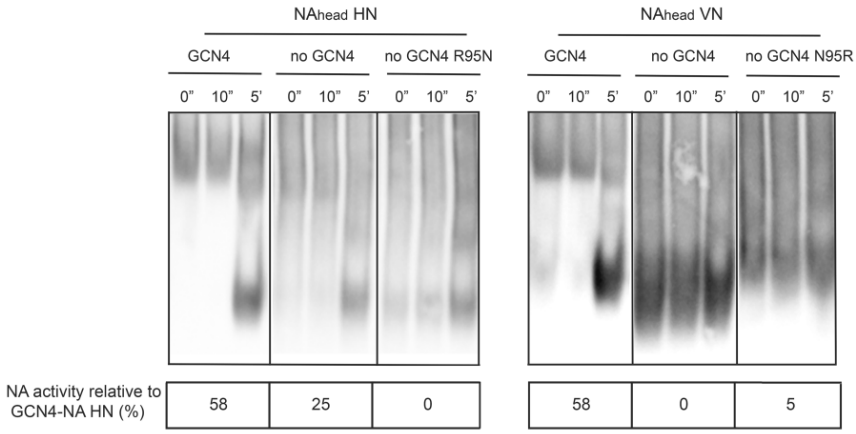


**Figure 10. BN-PAGE analysis of HN and VN NA head domains.** BN-PAGE analysis of the HN and VN NA head domains in the presence or absence of GCN4 domain, with and without the substitution at position 95. NA samples were subjected to gel electrophoresis without boiling (0") or after boiling for 10'' or 5'. The relative specific activity of each NA protein preparation normalized to GCN4-NA HN (based on the results shown in Fig. 9) is indicated.

**Importance of the residue at position 95 in full-length NA**

As shown in Fig. 2, the specific activity of HN and VN NA proteins differed considerably for the recombinant soluble proteins, but much less so for the full-length proteins. As the large difference between the soluble proteins is partly explained by the identity of the residue at position 95, we wondered to what extent this residue is responsible for the minor activity difference between FL-NA proteins. Substitution of the R residue at position 95 in the HN protein to N had a minor effect on the activity of the resulting protein. The reciprocal substitution in the VN protein resulted in increased activity (Fig. 11A and B). So, also in the

full-length protein, the identity of the residue at position 95 may affect the activity of the resulting protein, although it appears to depend on the background of the NA protein.

The small difference between the full-length HN and VN proteins may be explained by the TMD being able to compensate for the lower oligomerization capability of the VN protein head domain. Recent studies indicate that NA TMDs form an amphipathic tetramer that stabilizes the stalk and allows the head domain to fold (21, 37). As it appears that the TMD and head domains of NA have coevolved, it may be that the TMD domains of HN and VN also differ in their ability to interact. To test this hypothesis, the interaction strengths of the TMDs were measured in the GALLEX system (36), which is a two-hybrid system with which the oligomerization of membrane proteins can be measured. The interaction between the TMDs (residues 7-34) were determined in the presence of the 5 highly conserved N-terminal residues and either 1 (construct containing NA residues 2-35) or 8 (construct containing NA residues 2-42) C-terminal juxtamembrane residues. These regions of the VN NA protein differ from the HN protein by containing a polar substitution at position 17 (I17T) and a Gln to His substitution at position 39 (Fig. 5). The results indicated that the relative interaction strength of the VN TMD was slightly higher than the HN TMD, but when larger portion of the C-terminal juxtamembrane residues were present, both constructs showed an equally strong association (Fig. 11C). In agreement with the TMD domains not differing significantly in their interactions, substituting the residues at position 17 and 39 in the full-length VN protein did not affect the specific activity (Fig. 11D). A negative effect was observed, however, when the reciprocal mutations were made in the HN protein. Thus, just as for the residue at position 95, we observe a background-dependent effect for the residues at position 17 and 39 in the full-length proteins. The results indicate that the TMD compensates for the lower oligomerization capacity of the VN head domain, regardless whether it is derived from the VN or the HN protein. This is possibly achieved by the TMD promoting the proper assembly of the stalk region, which has the potential to influence the structure of the first few NA head amino acids that include residue 95.

**Effect of tetramerization domain on NA activity**

Based on the proposal that TMD could influence the structure of the stalk region and ultimately the head domain, we examined whether other tetramerization domains are more suitable than the GCN4-pLI domain to oligomerize the NA ectodomain. To this end, we exchanged the left-handed coiled coil GCN4-pLI domain with other tetramerization domains that were used previously for the synthesis of recombinant soluble NA proteins (24, 27). The Tetrabrachion domain (33) forms a parallel tetramer, while the VASP domain (32) forms a right-handed coiled coil (Fig.12A). The specific activity of the resulting NA proteins is shown in Fig. 12B. The VASP domain-extended protein displayed similar specific activities as the GCN4-pLI-extended proteins, with the VN protein being much less active that the HN protein. In contrast, the Tetrabrachion-extended NA proteins were more active than the other soluble NA proteins and the difference between the VN and HN proteins was smaller. The Km values of the soluble HN and VN proteins were not affected by the tetramerization domains (Fig. 12C). From these results we conclude that the structure at the N-terminus of NA can influence the specific activity of NA proteins. The enzymatic properties of NA proteins fused to the parallel Tetrabrachion oligomerization domain better mimic the enzymatic properties

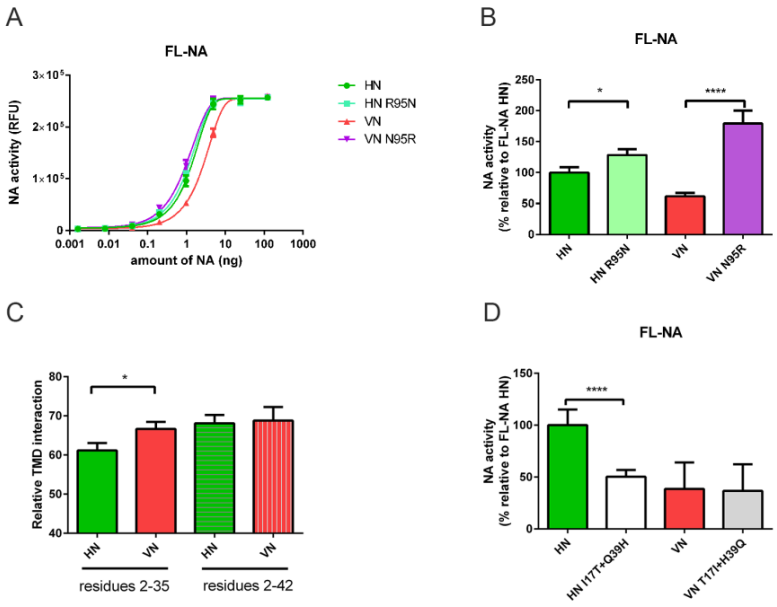


**Figure 11. Substitution of residues located in the TMD and at position 95 in the FL-NA proteins.** (A) Enzymatic activity of preparations containing different amounts of FL-NA HN and VN proteins with or without the indicated substitution of the residue at position 95. (B) The specific activity of the FL-NA proteins based on the results shown in (A) relative to the specific activity of the FL-NA HN protein. (C) The relative strength of the HN and VN TMD interactions was determined using the GALLEX system (36). The interaction strengths between the two TMDs were compared in the presence of either 1 (residues 2-42) or 8 (residues 2-42) juxtamembrane C-terminal amino acids (see Fig. 5 for their sequences). (D) The specific activity of the FL-NA HN and VN as well as of their TMD mutants is shown relative to the specific activity of FL-NA HN protein. All graphs show the mean of three independent experiments. Error bars indicate standard deviations. Significant differences between the specific activity of the wildtype FL-NA proteins and the mutants thereof (B and D) and the VN and HN TMD interaction strengths (C) were determined using the two-tailed t-test (*P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001).

of FL-NA proteins compared to the other coiled-coil tetramerization domains.

Finally, we studied the effect of substituting the residues at positions 95, 252 and 347 in the background of the TE-NA HN and VN proteins. As shown in Fig. 13, substitution of the residues at position 252 and 347 in the background of the VN protein did not affect the specific activity of the resulting proteins, which is in agreement with the results obtained with the GCN4-pLI-extended NA proteins (Fig. 8). Substitution of the residues at these positions in the HN protein had a small, but significant, positive effect on the specific activity. Mutation of the residue at position 95 in the HN or VN protein had a clear negative or positive effect, respectively. Substitution of the indicated residues in the VN protein affected the Km to a similar extent as was observed for the GCN4-NA and FL-NA proteins (compare Fig. 13 with Fig. 7). Introduction of the reciprocal mutations in the HN protein, did not affect the Km value for the residues at position 95 and 252, while the Km value was increased by mutation of the residue at position 347. The results obtained with the TE-NA proteins mimic the results obtained with the full-length NA proteins, with the exception of R95N mutation in the HN protein (compare Fig. 11B and 13A). The results furthermore confirm the importance of the

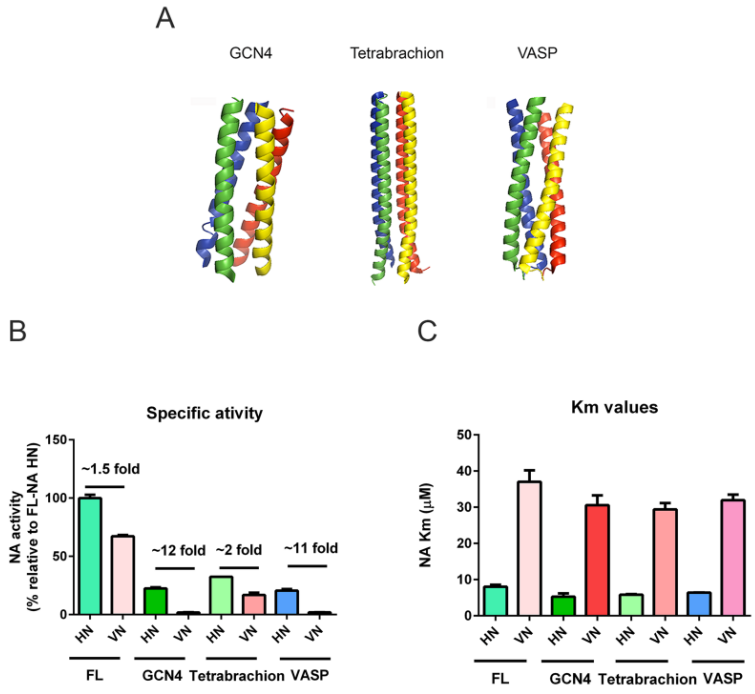


**Figure 12. Enzymatic activity of HN and VN proteins extended with different tetramerization domains**. (A) Structure of GCN4, Tetrabrachion and VASP tetramerization domains generated with PyMol software are shown (PDB ID code 1GCL, 1FE6 and 1USE) (31-33). (B) Specific activity of FL-NA proteins and of soluble version thereof fused to different tetramerization domains relative to that of the FL-NA HN protein. (C) Km values of HN and VN FL-NA and soluble NA proteins fused to different tetramerization domains. All graphs show the means of three independent experiments. Error bars indicate standard deviations.

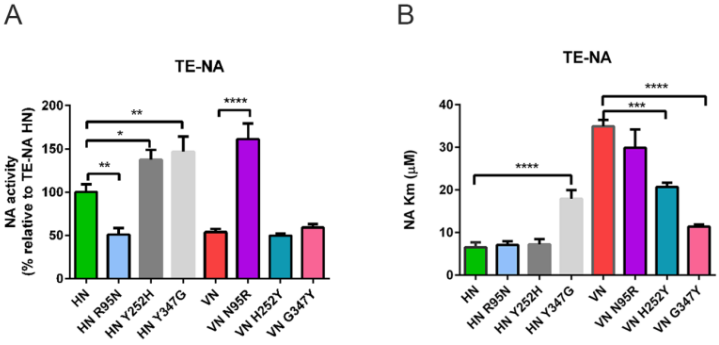


**Figure 13. Specific activity and Km values of mutant TE-NA proteins.** (A) Specific activity of TE-NA HN and VN as well as the indicated mutant proteins is shown relative to the specific activity of TE-NA HN. (B) Km values of the indicated wildtype and mutant TE-NA HN and VN proteins is shown. All graphs represent the mean of three independent experiments. Error bars indicate standard deviations. Significant differences by one-way ANOVA in combination with Bonferroni's multiple comparisons test relative to the wildtype TE-NA HN and VN is indicated (*P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001).

residue at position 347 for the Km value. For the residue at position 252 we observe a background-dependent effect on the Km value. The importance of this residue for the Km value in the VN protein is observed, however, for the FL-, GCN4- and TE-NA protein.

## Discussion

Here, we compared the enzymatic activities of four NA proteins derived from different H5N1 viruses were expressed either as soluble proteins fused to different commonly used tetramerization domains or as full-length versions. The NA proteins differed in their Km values, which depended on specific mutations in their head domain at position 252 and 347, but their Km values were not influenced by the N-terminal extensions used. However, the N-terminal extension/tetramerization domain affected the specific activity of the resulting NA proteins. While relatively small differences were observed for the different full-length proteins or for ectodomains extended with a Tetrabrachion tetramerization domain, these differences were much larger when GCN4 or VASP tetramerization domains were used. Differences in specific activities of the NA proteins were explained by different intrinsic capacities of the NA ectodomains to oligomerize, which could be largely attributed to a residue close to the NA head domain interface at position 95. Our results suggest that the Tetrabrachion domain, which forms a parallel four-helix bundle, probably causes less distortion of the stalk domain compared to the other oligomerization domains, which is particularly apparent for NA ectodomains with a low intrinsic capacity to oligomerize.

The VN N1 protein was shown to display a much higher Km value and thus a lower sialic acid binding affinity than the other N1 proteins analyzed. This difference is largely explained by the proteins differing at residues 252 and 347. Substitutions H252Y and G347Y in the background of the VN protein decreased the Km values, while the opposite effect was observed for the Y347G substitution in the HN protein (Fig. 7 and 13). In agreement with our results, others have shown that introduction of a Y at position 347 resulted in lower Km values in different NA proteins (43). The effect on the Km value may be explained by the residue at position 347 forming together with other amino acids a calcium ion binding site (38). Binding of calcium ions has previously been shown to be important for NA activity (39, 40). The residue at position 252 is located more distantly from the enzyme active site (Fig. 6). This residue may indirectly affect the enzyme active site via hydrogen bonding with the residue at position 274 (44), which may also explain the importance of this residue in sensitivity of the NA protein towards oseltamivir carboxylate (44-46). H5N1 viruses with an H at position 252 of the NA protein, were shown to be more susceptible to oseltamivir carboxylate than viruses with an Y at this position (47-50). Similar results were obtained when our recombinant N1 proteins were analyzed for oseltamivir sensitivity (data not shown). In the background of the HN protein, the identity of the residue at position 252 did not affect, however, the Km value (Fig. 13).

The N1 protein of VN consistently displayed a lower specific activity than the other N1 proteins tested. Substitution of the residue at position 95 affected the specific activity, but not the Km value. Furthermore, as this residue is located close to the interface between the

NA monomers (Fig. 6) and oligomerization is a prerequisite for NA enzyme activity (42), we hypothesized that the residue at position 95 may indirectly affect the NA enzyme activity by affecting oligomerization of the NA head domain, which is more apparent in the (GCN4-extended) soluble NA protein than in its full-length form. In agreement herewith, the VN and HN head domains clearly differed in their ability to form oligomeric complexes and in their enzymatic activity when expressed without oligomerization domains. Mutation of the residue at position 95 in the VN (N95R) and HN (R95N) head domains was shown to increase and decrease the enzymatic activity, respectively, and to affect the electrophoretic behavior of the resulting head domains to some extent as analyzed by BN-PAGE. However, our results indicate that other residues must also be of importance for their oligomerization and enzymatic activity. Indeed substitution of several residues in the VN protein was shown to decrease (K84T) or increase (e.g. Y100H, G347Y and G454S) the specific activity (Fig. 8A and B), although the effects of the latter mutations were much smaller than observed after substitution of the residue at position 95. Of note, the residue at position 95 was recently shown to constitute, together with other adjacent residues, a novel epitope on the N1 protein of the new pandemic H1N1 virus (51). Mutation of the residue at this position was shown to reduce binding of monoclonal antibodies that are able to protect against an otherwise lethal IAV challenge. The variation that is observed at this position in N1 proteins of different H5N1 viruses may therefore result from/contribute to NA antigenic drift.

The N-terminal oligomerization domain, to which the NA ectodomain was fused, clearly affected the specific activity, but not the Km value, of the resulting NA proteins. The full-length proteins displayed higher specific activities than the recombinant soluble NA proteins which carried non-native oligomerization domains. Relatively small differences that were observed between full-length NA proteins, appeared much larger when recombinant soluble NA proteins extended with GCN4 or VASP domains were analyzed. These domains adopt left-handed or right-handed coiled coils (31, 32) (Fig. 12). They might therefore distort the adjacent stalk domain and thereby the folding and oligomerization of the head domain as the large difference between the HN and VN protein was no longer observed when the stalk domain was absent (Fig. 9). The distorting effect particularly became apparent when the NA head domain of VN was used, which has a low intrinsic capacity to oligomerize. We do not know why deletion of the stalk had a negative effect on the activity of the HN protein. Possibly, the stalk domain may also contribute to folding of the NA head domain. The difference in specific activity between the HN and VN proteins was smaller when the Tetrabrachion oligomerization domain was used. This domain forms a nearly parallel four-helix bundle which probably better mimics oligomerization driven by the TMD, and which suffices to overcome the oligomerization defect in the VN head domain. These results are in agreement with previous studies that showed the importance of the TMD for folding and assembly of the NA head domain (20, 21).

The use of recombinant soluble NA proteins is an attractive approach to study NA protein activity and to perform structure analyses. Recombinant soluble NA proteins are easily purified from cell culture media and applied in downstream antigenicity and activity assays. It is Important, however, to keep in mind that the design of the recombinant soluble NA protein and the particular oligomerization domain used may affect the specific activity of the

resulting protein, especially when using NA head domains with a relatively low intrinsic capacity to oligomerize. In this respect, the Tetrabrachion tetramerization domain seems the better choice of the tetramerization domains tested, although also the Tetrabrachion-extended proteins do not perfectly mirror the full-length proteins in all cases. In addition, this study demonstrates the potential of using recombinant soluble NA proteins to reveal determinants of NA assembly and enzymatic activity.

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# Chapter 3

## Mutation of the second sialic acid-binding site, resulting in reduced neuraminidase activity, preceded emergence of H7N9 influenza A virus

Meiling Dai[a], Ryan McBride[b], Jos C.F.M. Dortmans[a#], Wenjie Peng[b], Mark J.G. Bakkers[a$], Raoul J. de Groot[a], Frank J.M. van Kuppeveld[a], James C. Paulson[b], Erik de Vries[a] and Cornelis A.M. de Haan[a]*

[a] Virology Division, Department of Infectious Diseases & Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

[b] Departments of Cell and Molecular Biology, Chemical Physiology, and Immunology and Microbial Science, The Scripps Research Institute, La Jolla, California, USA

# Current address: GD Animal Health, Deventer, the Netherlands

$ Current address: Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, USA

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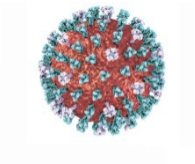

## Abstract

The emergence of novel influenza A virus (IAV) H7N9 since 2013 has caused concerns about the ability of this virus to spread between humans. Analysis of the receptor-binding properties of the H7 protein of a human isolate revealed modestly increased binding to α2,6 sialosides and reduced, but still dominant, binding to α2,3-linked sialic acids (SIAs) compared to a closely related avian H7N9 virus from 2008. Here we show that the corresponding N9 neuraminidases display equal enzymatic activity on a soluble monovalent substrate and similar substrate specificity on a glycan array. In contrast, solid phase activity and binding assays demonstrated reduced specific activity and decreased binding of the novel N9 protein. Mutational analysis showed these differences to result from substitution T401A in the 2$^{nd}$ SIA-binding site, indicating that substrate binding via this site enhances NA catalytic activity. Substitution T401A in the novel N9 protein appears to functionally mimic the substitutions that are found in the 2$^{nd}$ SIA-binding site of NA proteins of avian-derived IAVs that became human pandemic viruses. Our phylogenetic analyses show that substitution T401A occurred prior to substitutions in HA causing the altered receptor-binding properties mentioned above. Hence, in contrast to the widespread assumption that such changes in NA are only obtained after acquisition of functional changes in HA, our data indicate that mutations in the 2$^{nd}$ SIA binding site may have enabled and even driven the acquisition of altered HA receptor-binding properties, and contributed to the spread of the novel H7N9 viruses.

## Introduction

Influenza A viruses (IAVs) cause seasonal epidemics and occasional pandemics of influenza, and therefore pose a significant threat to the public health and economy worldwide. Pandemics are caused by animal IAVs that managed to cross the host species barrier and gained the ability to transmit among humans (1). Since 2013, human infections caused by avian-origin H7N9 IAVs have frequently been reported in China, raising concerns about the pandemic threat of these viruses. Although sustained human-to-human transmission has not been reported yet, the novel H7N9 viruses acquired amino acid changes associated with adaptation to human receptor binding and transmission in prior pandemics (2, 3) and appear to transmit from birds to humans more readily than other avian IAVs. In addition, human infections with H7N9 IAVs have a high mortality rate (up to 35%), in part because protective antibodies against these viruses in humans are lacking (4, 5).

IAVs are enveloped, negative-strand RNA viruses that contain two main surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) (6). HA binds to sialic acid (SIA)-containing receptors on the host cell and triggers fusion of the viral envelope with the endosomal membrane. The NA protein has receptor-destroying activity and by cleavage of SIAs from glycans contributes to the release of (progeny) viruses from the host cell surface as well as from non-functional decoy receptors (7, 8). Based on antigenic and genetic properties of these two surface glycoproteins, IAVs are divided into different subtypes. So far, 16 HA (H1-16) and 9 NA (N1-9) subtypes have been found in wild aquatic birds, which are regarded as the natural reservoir of IAVs (9).

The receptor-binding specificity of the HA protein is a major determinant of IAV host range. While avian IAVs generally prefer binding to α2,3-linked sialosides, human viruses preferentially bind to glycans containing α2,6-linked SIAs (10). Analysis of the receptor-binding properties of the novel H7N9 viruses indicated a dual receptor specificity when complete viruses were used (2, 5, 11, 12). The use of recombinant soluble HA proteins indicated that the novel H7 protein prefers binding to avian-type receptors (α2,3-linked sialosides), while also displaying weak binding to human-type receptors (α2,6-linked sialosides) (2, 13-15). The binding to avian-type receptors was reduced, however, in comparison to ancestral avian H7 viruses (15). Mutational analysis showed that substitutions at position 186 and 226 were largely responsible for the observed altered receptor-binding properties of the novel H7 protein (15).

The balance between the HA and NA activities is considered critical for IAV replication and transmissibility. Changes in the receptor-binding properties of the HA protein are often accompanied with changes in the enzymatic properties of NA (16, 17). The type II transmembrane NA protein forms a homo-tetramer with each NA monomer consisting of a globular head domain, a thin stalk of variable length, a hydrophobic transmembrane domain (TMD) and a short N-terminal cytoplasmic tail. The NA head domain contains the active site composed of highly conserved catalytic and structural residues that either directly contact the SIA or stabilize the catalytic residues (6, 18, 19). In contrast to the detailed knowledge on receptor-binding specificity of the HA protein, little is known about the cleavage specificity

of the NA protein compared to receptor-binding properties of the HA protein. In general, it seems that NA proteins from both avian and human viruses prefer cleavage of α2,3 over α2,6 SIAs, although this difference appears smaller for the NA of human IAVs (20-22). However, in most studies cleavage of only a very limited number of synthetic glycans was analyzed.

The NA proteins of several avian IAVs (N1, N2 and N9) have been shown to display hemadsorption activity (23-26), which was attributed to the presence of a 2nd SIA-binding site. For N9, X-ray crystallography (27) revealed that the 2nd SIA-binding site is a shallow pocket composed of three surface peptide loops, located adjacent to the catalytic site. The presence of a 2nd SIA-binding site in N1 was also demonstrated using saturation-transfer difference (STD) NMR spectroscopy (28). Both by hemadsorption assays and STD NMR it was shown that N1 and N2 proteins of swine and human IAVs display severely reduced binding compared to their avian counterparts (26, 28). Sequence analysis indicates that five out of six SIA contact residues in the N9 2nd SIA-binding site are highly conserved across all avian NA genotypes. This conservation is lost in human N1 and N2 genotypes, in agreement with the >100-fold reduced hemadsorption activity reported for their NAs (17, 26, 29). The biological significance of the 2nd SIA-binding site in avian NAs remains elusive and essentially nothing is known about its SIA-binding specificity. It has been suggested that this site may serve to enhance the catalytic efficiency of NA as changes in hemadsorption activity of N2 correlated with changes in the ability of N2 to cleave SIA from multivalent, but not monovalent substrates (26).

Previously, we compared the HA receptor binding properties of a human isolate of the novel H7N9 virus isolated in 2013 with a closely related avian H7N9 virus from 2008. In line with the results obtained by others (13, 14), the H7 of the novel H7N9 virus displayed modestly increased binding to α2,6 sialosides and reduced, but still dominant, binding to α2,3-linked sialic acids (SIAs) compared to the H7N9 virus from 2008 (15). In the present study, we analyzed the enzymatic activities and binding properties of the corresponding N9 proteins of these H7N9 viruses. After optimizing the recombinant protein expression approach for NA, we analyzed the enzymatic activities of the N9 proteins using monovalent and multivalent substrates. The results indicate that the two N9 proteins display equal specific activity on the monovalent substrate MUNANA and similar substrate specificity as revealed by glycan array analysis. In addition, glycan array analysis was for the first time used to determine the binding specificity of the 2nd SIA-binding site. Binding and substrate specificity, as revealed by glycan array analyses, were shown to correlate well for the avian N9 protein from 2008. The novel N9 protein displayed reduced binding to and enzymatic activity against multivalent substrates. Mutational analysis, involving loss and gain of function NA phenotypes, attributed these difference to a T401A substitution in the 2nd SIA-binding site. Phylogenetic analysis indicated this substitution to be unique to the novel H7N9 viruses. Remarkably, the T401A mutation in NA preceded the mutations found in H7 at position 186 and 226 that are essential for the altered receptor-binding properties of this virus, indicating that mutations in the 2nd SIA-binding site may have enabled the acquisition of altered receptor-binding properties of HA.

## Materials and Methods

### Genes, expression vectors, protein expression and purification

Expression constructs of the recombinant soluble H7 and N9 proteins of A/Anhui/1/2013 (GISAID Isolate EPI439507, referred to as H7 Anhui and N9 Anhui) and of A/Anas crecca/Spain/1460/2008 (GenBank accession no. CAY39406 and HQ244409.1, referred to as H7 Spain and N9 Spain) have been described before (15). In these N9 expression constructs, sequences encoding the NA head domain (a.a.76–470) of
ence, a double Strep-tag for purification (One-STrEP; IBA GmbH), and a GCN4-pLI (47) tetramerization domain (referred to as GCN4-$NA_{head}$). The NA ectodomain-encoding sequences in this construct were extended with sequences encoding the stalk domain (starting at residue 42) by insertion of a primer-dimer and cloned into a pFRT expression plasmid (Thermo Fisher Scientific) downstream sequences encoding the signal peptide of Gaussia luciferase and GCN4-pLI (referred to as GCN4-NA) (31). Finally the GCN4-pLI tetramerization domain was exchanged with the *Staphylothermus marinus* Tetrabrachion tetramerization domain (31, 48) (referred to as TE-NA). Mutations of interest were introduced into the corresponding NA genes by using the Q5 Site-Directed Mutagenesis Kit (NEB) and confirmed by sequencing. HA and NA expression plasmids were transfected into HEK293T cells (ATCC), and recombinant soluble NA or HA proteins were purified from the cell culture supernatants using Strep-tactin beads (IBA) as described previously (40). Quantification of the purified proteins was performed by comparative coommassie gel staining using standard BSA samples (Sigma-Aldrich) with known concentrations as a reference.

### NA enzymatic assays

Purified N9 proteins were assayed for their ability to cleave different substrates. The activity of N9 proteins towards the synthetic monovalent substrate 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA; Sigma-Aldrich) was determined by using a fluorometric assay similarly as described before (31) using a Fluostar OPTIMA plate reader (BMG Labtech, Mornington, Australia) with excitation and emission wavelengths at 340 and 490 nm, respectively. Activity of the NA proteins towards multivalent glycoprotein substrates was analyzed using a previously described enzyme-linked lectin assay (ELLA) (49) with some modifications. In brief, 2.5 µg/ml fetuin- or 25 µg/ml transferrin-coated (both from Sigma-Aldrich) 96-well Nunc MaxiSorp plates were incubated with serial dilutions of recombinant soluble NA proteins in reaction buffer (50mM Tris/HCl, 4mM $CaCl_2$, pH 6.0). After overnight incubation at 37°C, plates were washed and incubated with either biotinylated Erythrina Cristagalli Lectin (ECA, 1.25 µg/ml; Vector Laboratories), biotinylated peanut agglutinin (PNA, 2.5 µg/ml; Galab Technologies), biotinylated Sambucus Nigra Lectin (SNA, 1.25 µg/ml; Vector Laboratories) or biotinylated Maackia Amurensis Lectin I (MAL I, 2.5 µg/ml; Vector Laboratories). The binding of ECA, PNA, SNA and MAL I was detected using horseradish peroxidase (HRP)-conjugated streptavidin (Thermo Fisher Scientific) and tetramethylbenzidine substrate (TMB, BioFX) in an ELISA reader EL-808 (BioTEK) reading the OD at 450 nm. Both for the MUNANA assay and the ELLA, the data were fitted by non-linear regression using the Prism 6.05 software (GraphPad). The resulting curves were used to determine the amount of NA protein corresponding to half maximum lectin binding. The

inverse of this amount is a measure of specific activity (activity per amount of protein) and was graphed relative to other NA proteins or substrate-lectin combinations. In similar way, the specific activity for MUNANA was determined. The mean values of 2-4 experiments, with independently generated protein preparations, performed in triplicate are graphed. Analysis of the enzymatic specificity of the NA proteins using glycan array analysis was performed as described previously (50). In short, glycan arrays were treated with 20 µg/ml NA protein for 2 h at 37°C, after which the arrays were analyzed for binding with biotinylated ECA (10 µg/ml; VectorLabs). Binding of ECA was detected using Alexa Fluor 555-labeled streptavidin (2 µg/ml; Invitrogen). The values shown are the means of the mean intensities of 6 glycan spots.

**HA and NA binding assays**

Binding ability of HA and N9 proteins was analyzed using fetuin solid-phase binding and hemagglutination assays similarly as described previously (40, 51). In brief, 4µg of purified, soluble trimeric H7 and tetrameric N9 proteins were precomplexed with HRP-conjugated anti-Strep tag mouse antibody (IBA) and with HRP-linked anti-mouse IgG (DAKO) in 4:2:1 molar ratio on ice for half an hour prior to incubation of limiting dilutions on the fetuin (50 µg/ml) or aisalofetuin (Sigma-Aldrich) (50 µg/ml) coated 96-well Nunc MaxiSorp plates at 4 °C for 2 hours. 1µM Oseltamivir carboxylate (OsC; kind gift of Roche) was added to the N9 protein mixtures in order to inhibit NA enzymatic activity. HA and N9 binding was detected using tetramethylbenzidine substrate (TMB, BioFX) in an ELISA reader EL-808 (BioTEK) reading the OD at 450 nm. The hemagglutination assay was performed as described before (40). In brief, 2-fold dilutions of the HA or NA antibody complexes described above were incubated with 0.5% chicken erythrocytes for 2 hours at 4°C in the presence or absence of 1µM OsC, with a starting concentration of 40 µg/ml. The glycoprotein binding and hemagglutination assays were performed at least twice in duplicate. The mean values of these experiments are graphed. Binding of the NA proteins to the glycan arrays was performed similarly as described previously for HA proteins (51). N9 proteins (50 µg/ml) were precomplexed with anti-Strep tag mouse antibody (25 µg/mL; IBA) and Alexa Fluor 647-linked anti-mouse IgG (12.5 µg/ml; Invitrogen), similarly as described above, before their application to the array. The values shown are the means of the mean intensities of 6 glycan spots.

**Statistical analysis**

All statistical analyses were performed by two-tailed t-test using Prism 6.05 software. Error bars indicate standard deviations. The level of significance was expressed as $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$.

**Phylogenetic analysis**

All full-length and unique H7 and N9 protein sequences of avian viruses and of the novel H7N9 viruses were downloaded from the Genbank and GISAID databases. H7 and N9 protein trees were constructed by using the PHYLIP Neighbor Joining algorithm using the mPAM distance matrix. This tree was used as a guide-tree to select H7 and N9 sequences representing all main branches of the tree. The selected H7 and N9 proteins were used to

construct a summary-tree of similar topology as the guide-tree. The H7 and N9 protein trees are rooted by the A/Anas crecca/Spain/1460/2008 isolate.

## Results

**Comparison of enzymatic activity of different recombinant soluble N9 constructs**

Previously, we compared the HA receptor-binding properties of a novel H7N9 virus isolated from a human patient in 2013 (A/Anhui/1/2013, referred to as "Anhui") with a closely related avian H7N9 virus from 2008 (A/Anas crecca/Spain/1460/2008, referred to as "Spain") (15). In the same study we also made a preliminary comparison of the enzymatic properties of the corresponding N9 proteins. Detailed analysis of the N9 proteins was precluded, however, by the low expression level and activity of the recombinant soluble N9 proteins. These proteins consisted of the NA head domain (residue 76-469 of the N9 protein) N-terminally extended with a GCN4-pLI tetramerization domain (15) (referred to in this study as GCN4-$N9_{head;}$ Fig 1A). However, the NA stalk domain may contribute to correct folding (30) and affect enzymatic activity (31, 32). Therefore, we constructed novel expression vectors encoding N9 protein head domains extended with their stalk sequences (Fig 1A; starting with residue 42, referred to as GCN4-N9). The GCN4-N9 proteins migrated, as expected, at a higher position in the gel than the GCN4-$N9_{head}$ proteins lacking the stalk domain (Fig 1B) and displayed 2- to 3-fold higher expression level. Importantly, in the MUNANA assay the GCN4-N9 proteins displayed a much higher specific activity than the GCN4-$N9_{head}$ proteins (Fig 1C). We previously showed that replacing GCN4-pLI by a tetrabrachion tetramerization domain also resulted in NA proteins with a higher specific activity (31) and therefore made N9 constructs with such a tetrabrachion domain. The resulting proteins (referred to as TE-N9) displayed the expected electrophoretic mobility (Fig 1B) and a higher specific activity than the GCN4-N9 proteins (Fig 1C). Specific activity for MUNANA was similar for the N9 Anhui and Spain proteins. TE-N9 proteins, displaying the highest specific activity, were used in all subsequent analyses.

**Enzymatic activity of the TE-NA N9 proteins with multivalent substrates**

Next, we analyzed the enzymatic activities of the TE-N9 Anhui and Spain proteins on multivalent, surface-coated, fetuin and transferrin glycoprotein substrates, which better resemble the multivalent in vivo substrates of NA than the soluble monovalent MUNANA substrate. Fetuin contains mono-, bi-, and triantennary glycans with α2,3- and α2,6-linked SIAs in 2:1 ratio (33). Transferrin contains two biantennary N-linked glycan chains with only α2,6-linked SIAs (34, 35) as confirmed by linkage-type specific lectin-binding assays (data not shown). Fetuin and transferrin, coated to 96-well plates, were incubated with serially diluted TE-NA proteins, after which the resulting cleavage of SIAs was quantified by probing the 96-well plates with lectins with different binding specificities, namely enzyme-linked lectin assay (ELLA). ECA specifically recognizes glycans containing terminal Galβ1-4GlcNAc, which generally correspond to desialylated N-linked sugars (36), while PNA binds to terminal Galβ1-3GalNAc corresponding with desialylated O-linked sugars (37). MAL I and SNA specifically bind α2,3- or α2,6-linked SIAs, respectively (38, 39). From the resulting curves, the specific activities (activity per amount of protein) of the TE-N9 proteins were determined for each glycoprotein-lectin combination, as indicated in Materials and Methods,

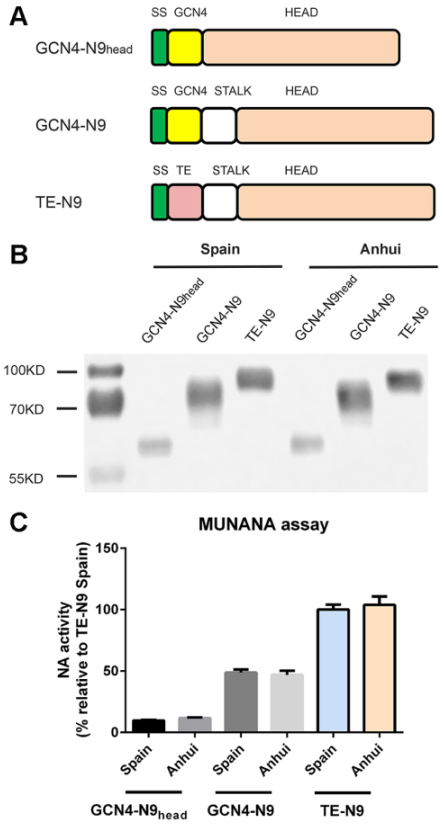


**Fig 1. Optimization of recombinant soluble N9 expression approach.** (A) Schematic representation of the recombinant soluble N9 proteins. The GCN4-N9$_{head}$ contains the NA head, GCN4 tetramerization domain (GCN4) and a cleavable signal sequence (SS). The GCN4-N9 construct contains the head and stalk regions fused with GCN4, and a signal sequence (SS). TE-N9 proteins have a similar schematic structure as GCN4-N9, but carry a Tetrabrachion (TE) instead of the GCN4 domain. (B) Expression of the different versions of the N9 Spain and Anhui proteins in HEK293T was analyzed by SDS-PAGE followed by western blotting. The position on the gel of the relevant molecular weight marker proteins is shown. (C) Specific activity of the different N9 Spain and Anhui proteins using the MUNANA substrate is shown normalized to that of TE-N9 Spain. Means of three independent experiments are graphed. Error bars indicate standard deviations.

and plotted relative to the specific activity determined for fetuin-ECA. Both TE-N9 proteins displayed a higher specific activity when fetuin was used as a substrate compared to transferrin (Fig 2A and B). This substrate preference corresponds with both proteins preferring cleavage of α2,3- (determined with fetuin-MAL I) over α2,6- (determined with fetuin-SNA and transferrin-SNA) linked SIAs (Fig 2A and B). Potential fine-specificity was investigated in detail by assessing cleavage of SIA from a large number of sialylated glycans on a glycan array. Cleavage of SIAs was quantified by binding of fluorescently-labeled ECA. The results (Fig 2C and D; see Table S1 in the supplemental material) indicate that both TE-N9 proteins prefer cleavage of α2,3- over α2,6-linked sialosides. Bi- and tri-antennary glycans containing multiple LacNAc repeats were most efficiently cleaved, although this observation may partly reflect the high affinity of ECA for such glycans (36). The TE-N9 Anhui was less active than the TE-N9 Spain protein in the glycan array analysis. Direct comparison of the specific activities on fetuin and transferrin revealed that the TE-N9 Spain protein indeed had higher specific activity than the TE-N9 Anhui protein (Fig 2E and F). In summary, we conclude that the N9 Spain and Anhui proteins display similar cleavage specificity. Furthermore, although the two proteins do not differ in their specific activity when using a monovalent soluble MUNANA substrate, the N9 Spain protein has a higher specific activity than the N9 Anhui protein when substrates are presented in a multivalent manner.

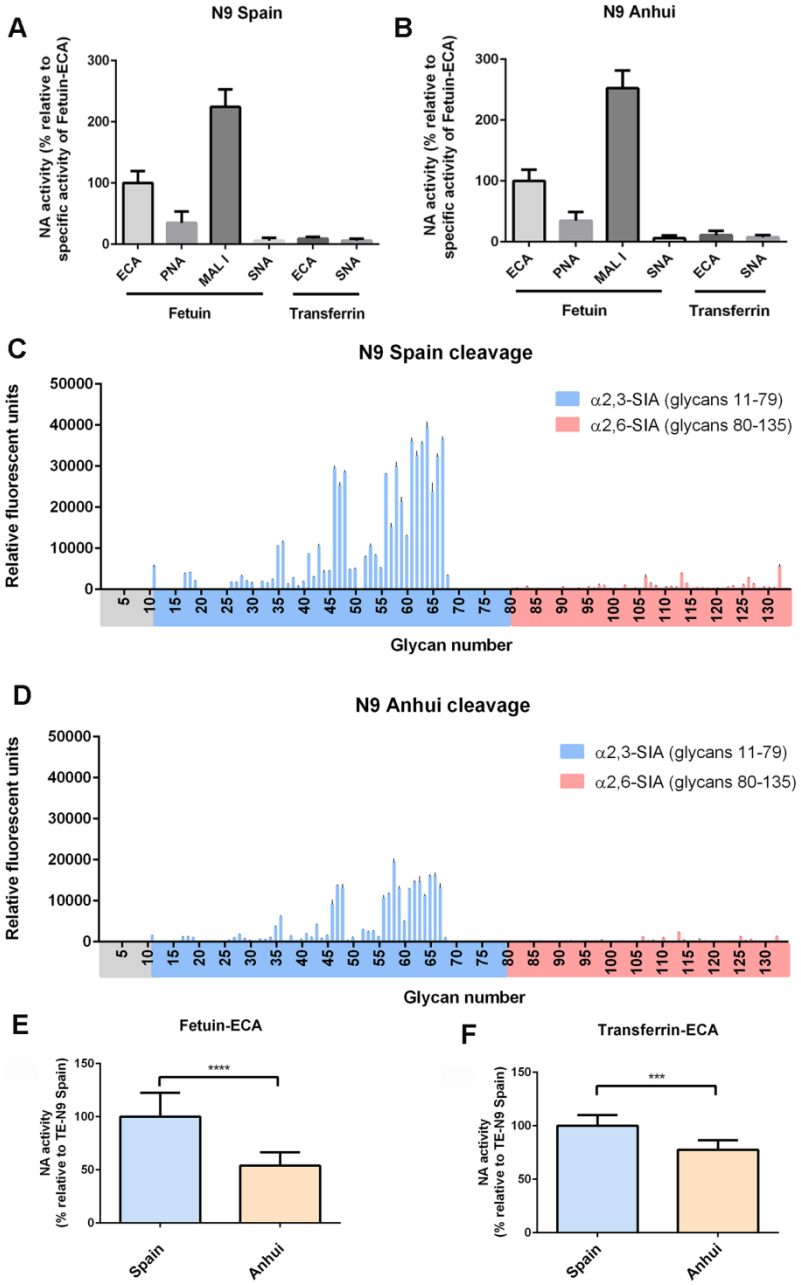


**Fig 2. Specific activity and cleavage specificity of the N9 proteins.** (A) Specific activity of TE-N9 Spain as determined by ELLA using different glycoprotein-lectin combinations is graphed normalized to that of fetuin-ECA. (B) Specific activity of TE-N9 Anhui as determined by ELLA using different glycoprotein-lectin combinations is normalized to that of fetuin-ECA. (C and D) Glycan array analysis of the cleavage specificity of N9 Spain (C) and Anhui (D). Desialylation of the glycans was detected by ECA binding. Colors of the bars correspond to the type of SIA (blue bars: α2,3-SIA, glycans 11 to 79; salmon bars: α2,6-SIA, glycans 80 to 135). Numbers correspond to the numbers indicated in Table S1 in the supplemental material. Glycans that were bound by ECA without NA treatment were excluded from the analysis (non-sialylated glycans 1-10). Desialylated glycan structures that cannot be bound by ECA were also

excluded from the analysis. These latter glycans contain upon desialylation terminal Galβ1-3GlcNAc (glycans 15, 21-23 and 51), Galβ1-3GalNAc (glycans 24, 25 and 31), Galβ1-4Glc (glycans 16, 70, 71 and 82), GalNAcβ1-4Gal (glycans 20 and 86), 6S-Galβ1-4GlcNAc (glycans 13 and 14) or Galβ1-4GlcNAc with GlcNAc being fucosylated (glycans 12, 14 and 72-79). The glycan array values shown are the means of the mean intensities of 6 glycan spots. (E and F) Specific activity of the TE-N9 Spain and Anhui as determined by ELLA using fetuin (E) or transferrin (F) in combination with ECA is graphed normalized to that of TE-N9 Spain. Means of three to four independent experiments are shown for the ELLA assay. Standard deviations are indicated. Significant differences were determined using the Student t test (E and F), significance is as follows: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

### Binding of N9 proteins to sialosides

Mutations in the 2nd SIA binding site of N2, which resulted in severely reduced hemadsorption activity, were previously shown to affect catalytic efficiency of NA against multivalent, but not monovalent substrates (26). Differences in the specific activity for multivalent substrates of the two N9 proteins (that have similar specific activity for monovalent MUNANA), may therefore be caused by differences in their ability to bind to multivalent substrates. To test this hypothesis, the TE-N9 proteins were subjected to different glycoprotein binding assays, similarly as has been performed previously for different HA proteins (15, 40). The results show that the TE-N9 proteins indeed differ in their ability to bind fetuin, with the Spain and Anhui proteins displaying efficient and negligible binding, respectively (Fig 3A and B) in the presence the NA inhibitor Oseltamivir Carboxylate (OsC). OsC occupies the NA active site, inhibits NA activity and prevents binding of substrates via the active site. No binding was observed without OsC or when desialylated fetuin was used (data not shown). As positive controls, the corresponding H7 proteins were taken along. In agreement with previous results (15), the H7 Anhui protein bound fetuin with lower avidity than the H7 Spain protein. Similar differences between the H7 and N9 proteins were observed in a hemagglutination assay (Fig 3C). Binding specificity of the N9 proteins was studied in more detail using glycan array analysis. The N9 Spain protein displayed efficient binding to a large number of α2,3-linked sialosides and weak or no binding to α2,6-linked sialosides. Glycan structures lacking SIAs did not bind (Fig 4A; see Table S1 in the supplemental material). Glycans that were efficiently bound by N9 were also efficiently cleaved (Fig 4C). In agreement with the fetuin-binding and hemagglutination assay, little binding if any was observed for the N9 Anhui protein (Fig 4B; see Table S1 in the supplemental material).

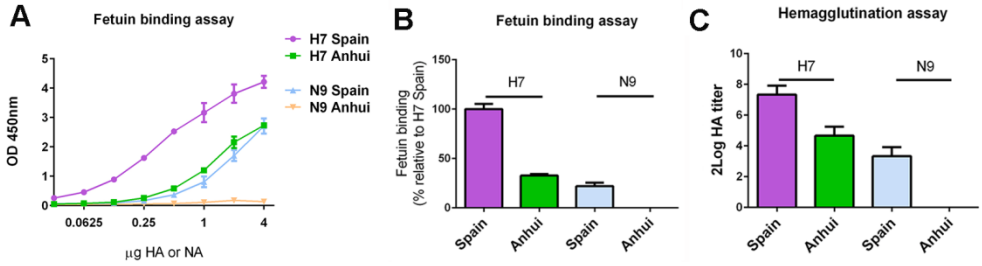


**Fig 3. Binding properties of recombinant soluble N9 proteins.** (A) Limiting dilutions of soluble H7 or N9 proteins, complexed with HRP-conjugated antibodies, were applied in the fetuin-binding assay. 1μM OsC was added to the NA samples to inhibit NA catalytic activity. The optical density at 450 nm (OD 450nm) corresponds with binding of the indicated proteins to fetuin. A representative experiment is shown. (B) Similar curves as shown in (A) of three independent experiments were used to determine the amount of HA or NA proteins corresponding to half maximum binding. The inverse of this amount is a measure for the relative binding avidity and is graphed normalized to that of H7 Spain. (C) Hemagglutination titers of antibody-complexed recombinant soluble H7 and N9 proteins. The means of three independent experiments performed in duplicate are shown. Standard deviations are indicated.

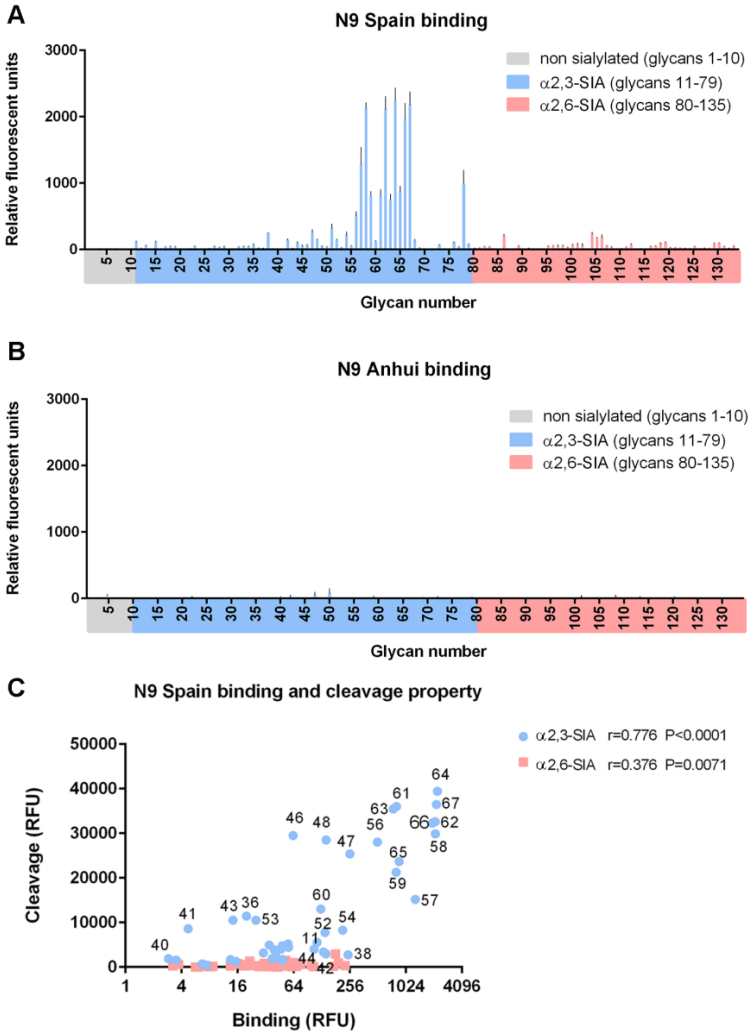


**Fig 4. N9 binding specificity determined by glycan array assay.** (A and B) Glycan array analysis of the binding specificity of N9 Spain (A) and Anhui (B). Colors of the bars correspond to the description given in the legend to Fig. 2. In addition, binding to non-sialylated glycans was evaluated (glycans 1-10; gray bars).(C) Scatterplot of binding to and cleavage of glycans on the array by N9 Spain. Pearson correlation coefficients r and the corresponding P values are indicated for each of the different sialoside types. The numbers correspond to those in Table S1 in the supplemental material.

### Sequence and structural analysis of N9 proteins

Protein sequence alignment showed that the Anhui and Spain N9 proteins differ at only 8 amino acid positions, in addition to a small deletion of 5 amino acids in the stalk (Fig 5A). None of these changes were located in or close to the enzyme active site (Fig 5B). However, Thr401 (N2 numbering) is part of a loop (residues 399-403, in green) that forms an H-bond via Asn400 with SIA bound to the 2$^{nd}$ SIA-binding site (Fig 5B to D) and provides a crucial stacking interaction between the Sia 5-N-methyl group and the aromatic side chain of Trp403. Thr401 is not in direct contact with the bound SIA but substitution of Thr401 by

Ala401 in N9 Anhui results in loss of a water-mediated hydrogen bond to Asp402. Loss of this interaction may increase the flexibility and possibly affect local folding of the 2nd SIA-binding site and thereby reduce binding to SIA.

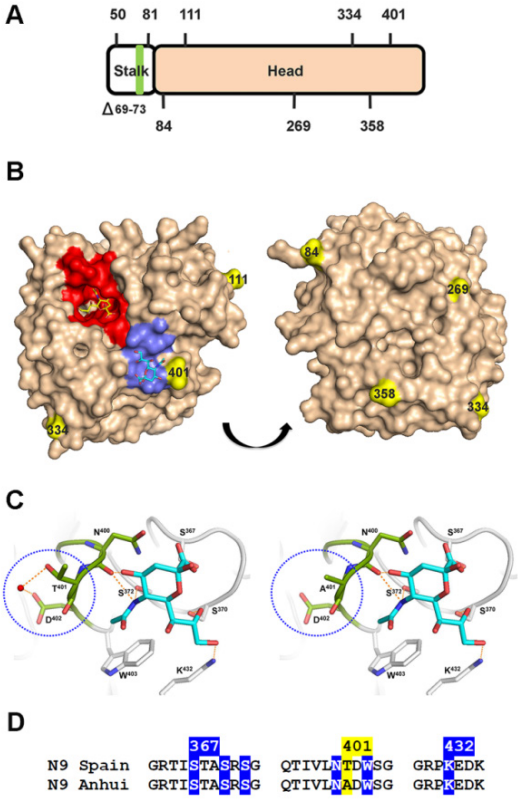


**Fig 5. Sequence and structural analysis of the N9 proteins.** (A) Schematic representation of the residues that differ between the recombinant soluble N9 Spain and Anhui proteins. Two residues are located in the NA stalk domain, while another six residues are located in the NA head domain. The 5 amino acid NA stalk deletion observed in N9 Anhui is indicated by the green rectangle. (B) Crystal structure of N9 from A/tern/Australia/G70C/75 in complex with Neu5Ac (Protein Data Bank [PDB] accession no. 1MWE) (27) depicted using PyMol software. Top and bottom views are shown. The NA active site (6) and the residues in the 2nd SIA-binding site that have direct interaction with SIA (27) are colored red and blue, respectively. The Neu5Ac moieties in these sites are shown as sticks. Residues that differ between N9 Spain and Anhui in the NA head region are colored yellow. The residues that differ in the stalk region are not indicated, as the structure of this part of NA is not solved. (C) Crystal structure of the secondary receptor-binding site of NA in complex with Neu5Ac (PDB accession no. 1MWE). NA and Neu5Ac are shown as sticks (oxygen in red, nitrogen in blue, and carbon in gray or green [for NA] or cyan [for Neu5Ac]), and water molecules are shown as red spheres and hydrogen bonds as dashed orange lines. The image was made using PyMOL. (D) Sequence alignment of the three loops that form the 2nd SIA-binding site and surrounding residues of the Anhui and Spain N9 proteins. Residues in the 2nd SIA-binding site that have direct interaction with SIA are shaded in blue. The residue at position 401 that differs between the Spain and Anhui N9 proteins is shaded in yellow. The N2 numbering of some residues is indicated.

### Residue at position 401 is largely responsible for the different N9 properties

To determine whether Thr401 in the 2nd SIA-binding site is important for binding to and cleavage of multivalent substrates, the T401A and the A401T mutations were introduced in the Spain and Anhui N9 proteins, respectively. The wild type and mutant proteins were

subsequently subjected to the different binding assays. Mutation of the residue at position 401 either abolished (Spain-T401A) or established (Anhui-A401T) binding of the N9 proteins to fetuin (Fig 6A) and to red blood cells (Fig 6B). Similarly, the T401A substitution in the Spain N9 protein decreased binding as determined by glycan array analysis, while the reciprocal effect was observed for the A401T mutation in the Anhui protein (Fig 6C and D; see Table S1 in the supplemental material). Next, we analyzed the enzymatic activities of the mutant N9 proteins. Mutation of the residue at position 401 had no effect on the specific activity using the monovalent MUNANA substrate (Fig 6E) nor did it change cleavage specificity (see Table S1 in the supplemental material). This is in agreement with the Anhui and Spain proteins displaying similar substrate specificity and specific activity using MUNANA. However, in agreement with the binding assay results, the introduction of substitution T401A in the Spain N9 protein decreased the specific activity when using the multivalent fetuin substrate. The reciprocal effect was observed after introduction of the A401T substitution in the Anhui protein (Fig 6F-H). Thus, the ability of N9 proteins to cleave SIAs from fetuin corresponds with their binding avidity to this glycoprotein. These differences could be largely attributed to the residue at position 401 in the 2$^{nd}$ SIA binding site.

**Phylogenetic analysis of N9 proteins**

To get more insight into the evolutionary history of the N9 and H7 proteins of the novel H7N9 viruses, phylogenetic analyses were performed (Fig 7 and 8 and Table 1). The results indicate that the T401A mutation in N9 is unique for the novel H7N9 viruses and occurs at the root of the novel H7N9 clade, after which it is strictly conserved (Fig 7). The T401A mutation preceded a small 5 amino acid deletion in the stalk, which has been described previously as being characteristic for the novel H7N9 viruses (41) (Table 1). The phylogenetic analyses furthermore indicate that only after the acquisition of these NA mutations, the substitutions in H7 at position 186 and 226, which are associated with the altered receptor-binding properties of these viruses (15), are observed (Fig 8 and Table 1).

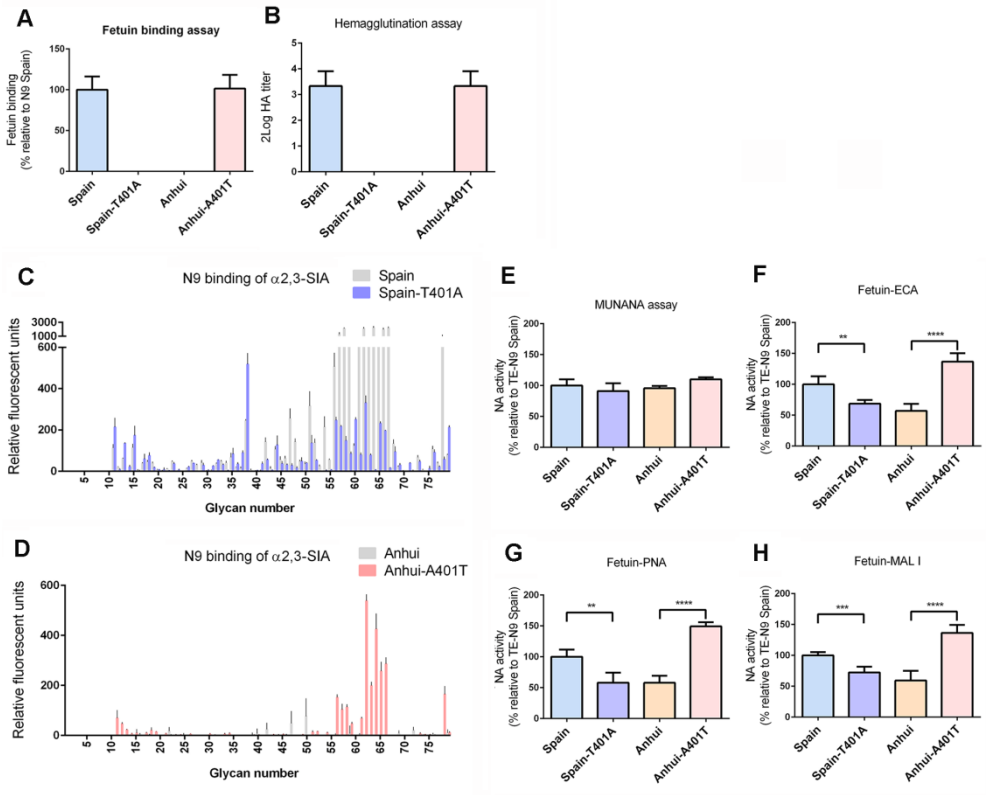


**Fig 6. Effect of substitutions at position 401 on N9 binding and enzymatic activity.** Binding properties of wild type N9 proteins as well as their counterparts with substitution at position 401 determined by fetuin-binding (A), hemagglutination (B) and glycan array (C and D) assays in the presence of 1μM OsC similarly as described in the legends to Fig. 3 and 4. Only the binding to non-sialylated (glycans 1-10) and α2,3-SIA glycans (glycans11-79) is shown. The glycan array values shown are the means of the mean intensities of 6 glycan spots. (E-H) Specific activity of N9 and their mutant proteins determined by MUNANA assay (E) or by ELLA using fetuin in combination with different lectins; ECA (F) , PNA (G) , MAL I (H) were graphed normalized to that of N9 Spain. Means of three to four independent experiments performed in duplicate/triplicate are shown for the fetuin binding, hemagglutination and ELLA assays. Standard deviations are indicated. Significant differences between wild type proteins and their mutants as determined by Student t test are indicated as follows: *P < 0.05; **P < 0.01; ***P <0.001; ****P < 0.0001.

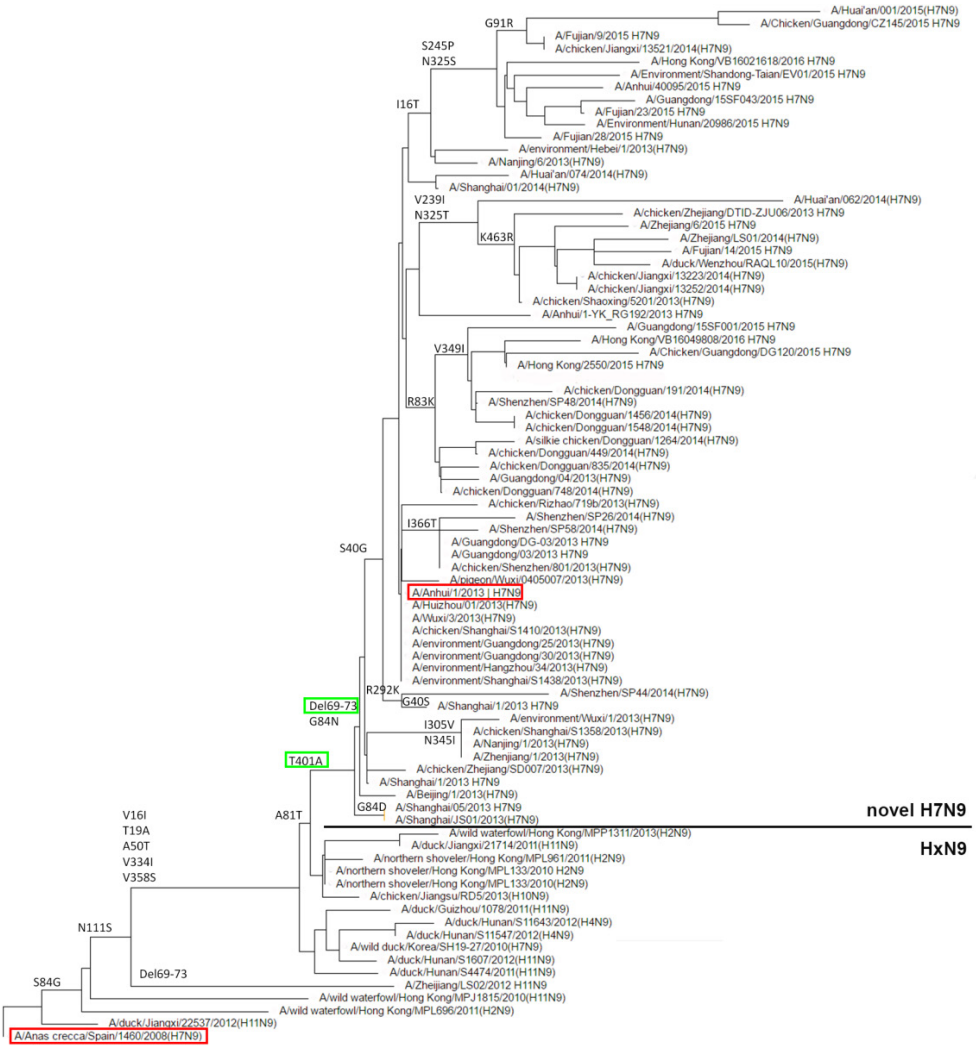


**Fig 7. Phylogenetic analysis of N9 proteins derived from HxN9 and novel H7N9 viruses.** All full-length and unique N9 protein sequences of avian IAVs and of the novel H7N9 viruses were downloaded from the Genbank and GISAID databases. An N9 protein tree was constructed as described in Materials and Methods. Key residues that differ between different branches are indicated. The N9 protein tree is rooted by the A/Anas crecca/Spain/1460/2008 isolate (N9 Spain). The two N9 proteins (N9 Anhui and Spain) that were compared in the present study are indicated by red rectangles. Green rectangles indicate the mutation at position 401 and the deletion in the stalk.

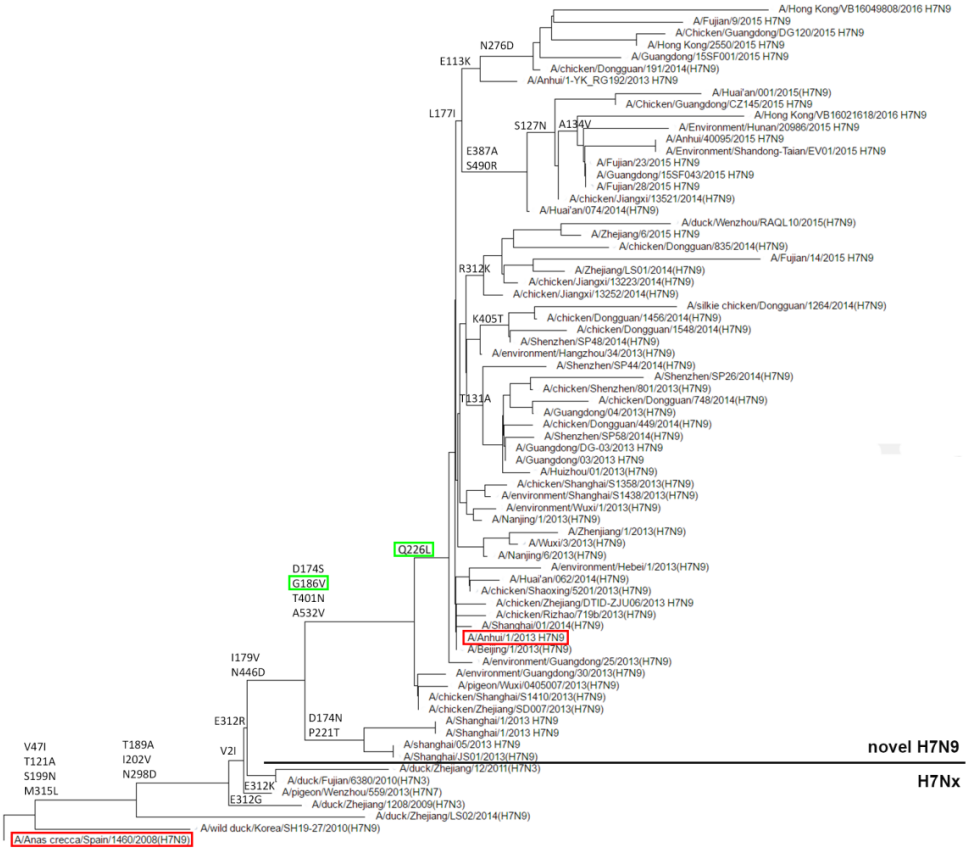


**Fig 8. Phylogenetic analysis of HA proteins derived from H7Nx and novel H7N9 viruses.** All full-length and unique H7 protein sequences of avian IAVs and of the novel H7N9 viruses were downloaded from the Genbank and GISAID databases. An H7 protein tree was constructed as described in Materials and Methods. Key residues that differ between different branches are indicated. The H7 protein tree is rooted by the A/Anas crecca/Spain/1460/2008 isolate (H7 Spain). The two H7 proteins (H7 Anhui and Spain) corresponding to the N9 proteins that were compared in the present study are indicated by red rectangles. Green rectangles indicate the mutations at position 186 and 226 that are responsible for the altered receptor binding properties of the H7 Anhui protein.

**Table 1. Occurrence of key residues in H7 and N9 proteins**

| Influenza A virus | N9 protein | | H7 protein | |
|---|---|---|---|---|
| | 401 | Δ69-73[a] | 186 | 226 |
| H7Nx and HxN9 prior to 2013[b] | T | - | G | Q |
| A/Anas crecca/Spain/1460/2008 | T | - | G | Q |
| A/Shanghai/05/2013 | A | - | G | Q |
| A/Shanghai/JS01/2013 | A | - | G | Q |
| A/Shanghai/1/2013 | A | + | G | Q |
| A/Shanghai/1b/2013 | A | + | G | Q |
| A/chicken/Zheijang/SD007/2013 | A | + | V | Q |
| A/chicken/Shanghai/S1410/2013 | A | + | V | Q |
| A/pigeon/Wuxi/0405007/2013 | A | + | V | Q |
| A/environment/Guangdong/30/2013 | A | + | V | Q |
| A/Bejing/1/2013 | A | + | V | L |
| A/Nanjing/1/2013 | A | + | V | L |
| A/environment/Guangdong/25/2013 | A | + | V | L |
| A/Anhui/1/2013 | A | + | V | L |
| A/chicken/Dongguan/748/2014 | A | + | V | L |
| A/Fujian/9/2015 | A | + | V | L |
| A/Jong Kong/VB16021618/2016 | A | + | V | L |

[a] Δ69-73 indicates a deletion of 5 amino acids in the NA stalk.

[b] The only exception is the N9 protein of A/Zheijang/LS02/2012 H11N9, which contains a similar 5 amino acid deletion in the NA stalk.

## Discussion

Compared to its well-studied functional counterpart HA, much less is known about the binding and cleavage properties of the IAV NA protein. In the present study, the detailed binding properties of the 2nd SIA-binding site of IAV NA were analyzed in correlation with NA cleavage properties, for the first time, using glycan array analyses. Our study establishes the functional significance of the 2nd SIA-binding site, which has long remained underappreciated. Analysis of N9 proteins of the novel H7N9 IAVs that have been infecting humans since 2013 allowed us to directly correlate NA cleavage with NA binding via the 2nd SIA-binding site. Substrate binding via this site clearly enhances NA catalytic efficiency against the same substrate. A single T401A mutation in the NA 2nd SIA-binding site of novel H7N9 viruses, which reduced binding to and cleavage of multivalent substrates, appears to functionally mimic the substitutions that are found in the 2nd SIA-binding of NA proteins of IAVs that managed to cross the host species to become human viruses (20, 26, 42).

However, in contrast to the widespread assumption that such changes in NA are only obtained after acquisition of functional changes in HA, our phylogenetic analyses indicate that the T401A substitution in N9 occurred prior to substitutions in HA causing the altered receptor-binding properties of novel H7N9 viruses. Possibly, the initial functional changes in the N9 protein have subsequently enabled the acquisition of altered receptor-binding properties of the H7 protein and may contribute, besides the mutations found in HA, to the spread and to the increased ability of this virus to infect humans in comparison to other avian viruses.

The presence of a 2nd SIA binding site in NA was already demonstrated in the 1980s using hemadsorption assays (23-25). Direct experimental evidence for the presence of this site was obtained decades later by X-ray crystallography (27) and STD-NMR (28), but detailed analysis of its binding specificity was lacking so far. By using glycan array analysis, we now show that the N9 protein of an avian IAV prefers binding to α2,3-linked sialosides, particularly the bi- and tri- antennary glycans with multiple LacNAc repeats. No binding was observed to α2,6-linked sialosides. In contrast, the 2nd SIA binding-site of a cell-surface expressed N2 protein was shown to enable binding to red blood cells that had been resialylated using either α2,3- or α2,6-sialyltransferases with approximately equal efficiency (26). Whether this difference reflects a different binding specificity for the N2 and N9 proteins or resulted from the different experimental set ups remains to be determined.

The biological function of the NA 2nd SIA binding-site has long remained elusive. Only a single study showed that the ability of NA to bind red blood cells corresponded with cleavage efficiency of other multivalent substrates (26). We now directly show that substrate binding via the 2nd SIA-binding site enhances NA catalytic efficiency against the same substrate. The 2nd SIA-binding site, which is adjacent to the actual NA active site, may enhance the catalytic efficiency of NA by recruiting and keeping multivalent sialosides close to the active site (26, 43). Enhancement of catalytic efficiency resulting from catalytic and carbohydrate-binding domains interacting simultaneously with a polyvalent substrate appears to be a general feature for most glycoside hydrolases, as most of these enzymes have been shown to contain lectin domains, including eukaryotic and bacterial NA proteins (44) as well as other viral receptor-destroying enzymes (45, 46).

The presence of a 2nd SIA binding site provides IAV with an additional, potentially more subtle, option to modulate the catalytic efficacy of its NA protein by circumventing the necessity of mutation of the highly conserved key residues in the NA catalytic site. This mechanism may allow IAV to readily achieve an optimal HA-NA balance when adapting to a novel receptor repertoire upon infection of a novel host species and/or when coping with HAs with altered receptor-binding properties. For example, the emergence of pandemic human IAVs was not only accompanied with the well-known changes in the receptor-binding properties of the HA proteins, but also with changes in the NA 2nd SIA-binding site, which decrease hemadsorption activity (17). We now show that modulation of the NA catalytic activity via mutation of the 2nd SIA binding site may also be observed for avian IAVs. The decreased ability of the N9 protein of novel H7N9 IAV to bind and cleave sialosides correlates well with the low receptor-binding avidity of the corresponding HA protein (15).

Moreover, the substitution at position 401 shows that substrate binding via the 2nd SIA-binding site may be modulated by mutation of other residues in the 2nd SIA-binding site than those that directly contact the SIA moiety. It will be of interest to study the effect of other substitutions in NA proteins of avian viruses at positions neighboring the highly conserved SIA-contact residues in the 2nd SIA-binding site.

Decreased receptor-binding of the N9 protein of the novel H7N9 viruses is another property in which this virus appears to resemble (early) human viruses, besides its decreased and modestly increased HA binding to avian- and human-type receptors, respectively. However, in contrast to the widespread assumption that such changes in NA are only obtained after acquisition of functional changes in HA, our phylogenetic analyses indicate that the T401A substitution in the 2nd SIA-binding site was obtained prior to the acquisition of mutations in H7 (at position 186 and 226) that are responsible for the altered receptor-binding properties (2, 15). The T401A mutation in NA, possibly in combination with the small stalk deletion, may therefore have allowed and even have driven these altered HA properties to restore the balance between HA and NA. Although it is not clear why and in which animal species these mutations in NA and HA were selected, it seems plausible that they have contributed to the wide spread of the novel H7N9 viruses and to the relatively high ability of these avian viruses to infect humans. In view of our results, geno- and phenotypic screening of animal IAVs to monitor their potential to cross the host species barrier should not only focus on the HA protein, but also on the catalytic properties of NA using the appropriate multivalent substrates.

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**Supplemental material**

Table S1 can be found at: http://jvi.asm.org/content/91/9/e00049-17/suppl/DCSupplemental.

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# Chapter 4

## Phenotypic drift of the neuraminidase protein of the new pandemic influenza A(H1N1) virus after its introduction in the human population

Meiling Dai[1], Erik de Vries[1], Tim Leenders[2], Tom Wennekes[2], Berend Jan Bosch[1], Guus Rimmelzwaan[3], Frank J.M. van Kuppeveld[1], Cornelis A.M. de Haan[1]*

1. Virology Division, Department of Infectious Diseases & Immunology, Faculty of Veterinary Science, Utrecht University, Yalelaan 1, 3584CL Utrecht, the Netherlands.
2. Department of Chemical Biology and Drug Discovery, Faculty of Science, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, the Netherlands
3. Department of Viroscience, Erasmus MC, Wytemaweg 80, 3015 CN Rotterdam, the Netherlands

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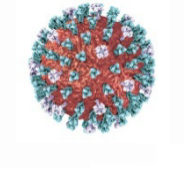


## Abstract

The influenza A virus (IAV) neuraminidase (NA) protein is essential for virion release from cells and decoy receptors. It is an important target for antiviral drugs, while antibodies targeting NA contribute to protection. The emergence of the new pandemic IAV H1N1 in 2009 (H1N1pdm09), the first pandemic IAV of the genomics era, allowed us to analyze the genetic and phenotypic drift of such a virus in great detail. This drift probably entails the selection of NA mutations resulting from adaptation to the new host as well as from selective pressure by the host immune system. Phylogenetic analysis showed that several mutations became fixed in NA after the emergence of IAV H1N1pdm09. The accumulative effect of these mutations, in the order in which they appeared, on different phenotypic properties of NA was analyzed. Within the first two years after its emergence, the H1N1pdm09 virus acquired mutations in NA that resulted in increased enzymatic activity (N369K) and protein expression (V241I). These mutations were previously identified to be permissive for obtaining NA-inhibitor resistance mutations. In subsequent years, mutations were obtained that decreased NA enzymatic activity (K432E) and protein expression (N270K) again, and which therefore may be regarded as non-permissive for the acquisition of NA-inhibitor resistance mutations. These latter two substitutions were accompanied either with an increased ability of NA to cleave substrates at low pH (K432E) or with altered NA antigenicity (N270K), which may explain their selection. Residues at position 369 and 432 are both located in the 2$^{nd}$ sialic acid-binding site, indicating that human viruses can modify the enzymatic activity of their NA protein via (further) mutation of this site. Our results also indicate that the enzymatic activity of H1N1pdm09 NA is maintained within a certain bandwidth, rather than to evolve to maximal sialidase activity. In addition, different NA phenotypes were shown to be intertwined as several mutations affected more than one phenotypic property. This intertwinement of NA phenotypic properties probably is an important characteristic of the evolution of NA.

## Introduction

Influenza viruses (IAVs) are important human respiratory pathogens that cause seasonal epidemics and occasional pandemics (1). Influenza pandemics occur when a for humans novel animal IAV manages to cross the host species barrier and is efficiently spread between humans (2). In subsequent years, these once pandemic viruses may give rise to seasonal epidemics, which also result in millions of human infections with a substantial economic burden (3). Three pandemics were recorded during the 20th century: the 1918 H1N1 Spanish flu, the 1957 H2N2 Asian flu and the 1968 H3N2 Hong Kong flu (4). The first IAV pandemic of the 21$^{st}$ century started in April 2009 and was caused by a novel swine-origin H1N1 IAV (H1N1pdm09) (5). This so far only pandemic in the genomics era allows us for the first time in history to follow the evolution of a pandemic IAV spreading in the human population in great detail. This evolution probably entails the selection of mutations in viral proteins resulting from adaptation to the new host as well as from selective pressure by the host immune system.

IAV particles contain 2 glycoproteins, the hemagglutinin (HA) receptor-binding and fusion protein and the neuraminidase (NA) protein, which has receptor-destroying activity. Both proteins are important determinants of host tropism, pathogenesis and transmission and prime targets of the host immune system. HAs of avian viruses preferentially bind to sialic acids (SIAs) linked to the proximal galactose by an α2,3 bond (avian-type receptor) (6, 7). Human IAVs, including the H1N1pdm09 virus, preferentially bind α2,6-linked SIAs (human-type receptor), which are abundantly present on epithelial cells of the human upper respiratory tract (8, 9). The IAV neuraminidase (NA) protein cleaves SIAs from glycoproteins as well as glycolipids. The NA protein thereby facilitates release of progeny virions from infected cells and decoy receptors (e.g. in mucus) and prevents virus self-aggregation. The NA protein is an important target for antiviral drugs, while antibodies targeting NA contribute to protection against influenza (10, 11). Several studies indicate that a functional balance between HA binding and NA cleavage is of importance for maintaining optimal virus replication as well as transmission across different host species (12-16). For example, respiratory-droplet transmissibility between ferrets of a swine IAV was much enhanced by the introduction of the N1 gene from H1N1pdm09 into the swine IAV (13). However, the molecular details of the HA/NA balance are poorly understood.

Analysis of the receptor-binding properties of IAV H1N1pdm09 showed that its HA protein exhibited much lower binding avidity for human-type receptors than a swine progenitor HA, which resulting from amino acid substitutions near the receptor binding site (14, 17). While IAV H1N1pdm09 displayed limited binding to linear and short sialoglycans, more efficient binding to extended, branched N-glycans was observed (18). During the first three years of its emergence, IAV H1N1pdm09 was furthermore shown to obtain mutations in HA that increased binding avidity, which were subsequently compensated for by the acquisition of other mutations in HA (19). Shortly after its emergence, the H1N1pdm09 HA protein also acquired a stabilizing mutation in its stem region, which lowered its activation pH (20) and may have contributed to its pandemic spread (21). Only more recently, accumulation of mutations in H1 modified its antigenicity to an appreciable extent. This resulted in

replacement of an A/California/7/2009 H1N1pdm09-like virus with an A/Michigan/45/2015 H1N1pdm09-like virus in the vaccine for the 2017 southern and 2017-2018 northern hemisphere (22)

The NA protein is a type II glycoprotein that forms homotetramers. The NA ectodomain contains a thin stalk and a globular head domain containing a six-bladed beta-propeller structure. SIA cleavage is mediated by the active site located in NA head domain (23). The NA active site is composed of several highly conserved catalytic residues that directly contact the SIA substrate as well as structural residues that stabilize catalytic residues in place (24, 25). The NA protein of H1N1pdm09 was shown to preferentially cleave α2,3- over α2,6-linked SIAs (14, 26) with an optimal pH range of 5.5–6.5 (25, 27). $Ca^{2+}$ is required for NA catalytic activity and thermostability (25) and three $Ca^{2+}$-binding sites have been identified for the NA protein of H1N1pdm09 (28). NA catalytic activity for multivalent substrates may be enhanced by the presence of a functional 2$^{nd}$ SIA binding site (29, 30), a shallow pocket composed of three surface loops located adjacent to the active site (31). The N9 protein was recently shown to prefer binding to α2,3-linked sialosides via its 2$^{nd}$ SIA-binding site (30). Five of the six SIA-contact residues identified by structural analysis of the N9 protein (31) are highly conserved among the NAs of avian IAVs (31, 32), but this conservation is lost in the N1 and N2 proteins of human seasonal IAVs (29, 32), including the H1N1pdm09 virus. In agreement herewith, the N1 proteins of H1N1pdm09 and seasonal human H1N1 IAVs display severely reduced, but still detectable binding to α2,3-sialyllactose compared to their avian counterparts as determined by saturation-transfer difference (STD) NMR (33).

In the current study we aim to understand to what extent and how the phenotypic properties of the NA proteins (expression, enzymatic activity and antigenicity) have evolved after the emergence of the H1N1pdm09 virus in 2009 and how these different properties are intertwined and shape NA evolution. To this end, we performed phylogenetic analysis of H1N1pdm09 NA sequences deposited between 2009 and 2016 and analyzed the accumulative effect of those mutations that became fixed in the virus population, in the order in which they appeared, on the phenotypic characteristics of NA. Recombinant soluble tetrameric NA proteins were produced and analyzed for the different phenotypic properties. The expression of recombinant soluble NA proteins allows the rapid production of NAs encoded by primary isolates and circumvents the need for producing and culturing complete viruses that may incorporate additional changes during propagation in eggs or MDCK cells. Within the first two years after its emergence, the H1N1pdm09 virus acquired mutations in NA that resulted in increased expression and activity. In subsequent years, these traits were lost again by other substitutions in NA, which in addition increased low pH activity and altered NA antigenicity. This latter feature was particularly affected by a single mutation that also decreased protein expression. Besides by mutations that affected protein expression, H1N1pdm09 NA enzymatic activity was modulated by mutations that are located in the 2$^{nd}$ SIA-binding site. Furthermore, NA activity was shown to evolve within a certain phenotypic bandwidth, rather than to obtain and maintain maximal sialidase activity. Our results also indicate that different NA phenotypic properties are intertwined, with several mutations affecting more than one property.

## Materials and Methods

### NA genes preparation, protein expression and purification

Human codon-optimized NA ectodomain (amino acids.42–469; N1 numbering)-encoding cDNAs (Genscript, USA) of A/California/04/2009(H1N1) (GenBank accession no. ACP41107.1, referred to as CA/09) and A/Wisconsin/09/2013(H1N1) (GenBank accession no. AGV29183.1, referred to as WI/13) were cloned into a pFRT expression plasmid (Thermo Fisher Scientific). The soluble NA-encoding sequences were preceded by sequences coding for an N-terminal signal sequence derived from *Gaussia* luciferase, a double Strep-tag for affinity purification (One-STrEP; IBA GmbH), and a *Staphylothermus marinus* Tetrabrachion tetramerization domain (34), similarly as described previously (30, 35). Mutations of interest were introduced into the corresponding NA genes by using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) and confirmed by sequencing (Table 1). NA proteins were expressed by transfection of HEK293T cells (ATCC) with equal quantities of the NA gene-containing plasmids, similarly as described previously (36). For determination of the NA protein expression levels, cell culture media were harvested at 72h post transfection and cleared by centrifugation. Anti-Strep tag antibody conjugated to horseradish peroxidase (IBA) was used to detect the recombinant proteins in the cell culture supernatant after Western blotting. The blot signals were visualized and analyzed using the Odyssey imaging system (LI-COR). Recombinant soluble NA proteins were purified from the cell culture supernatants using Strep-tactin beads (IBA) as described previously (36). Quantification of the purified proteins was performed by comparative coommassie gel staining using standard BSA samples (Sigma-Aldrich) with known concentrations as a reference. These purified proteins were used for the enzymatic and antigenic analyses of the NA proteins and as a standard in the analysis of the protein expression levels.

### Phylogenetic analysis

All full-length and unique NA sequences of A(H1N1)pdm09 viruses in the NCBI database were downloaded. NA gene trees were constructed by using the PHYLIP neighbor-joining algorithm with the F84 distance matrix. This tree was used as a guide-tree to select NA sequences representing all main branches of the tree. The selected NA genes were used to construct a summary-tree of similar topology as the guide-tree. The N1 tree was rooted by the A/California/04/2009 isolate.

### NA enzymatic assays and kinetic analysis

Unless indicated otherwise, purified NA proteins were assayed for their ability to cleave different substrates. The activity of serially-diluted NA proteins against monovalent substrates was determined by using a fluorometric assay similarly as described before (35) using either 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA; Sigma-Aldrich) or 2'-(4-methylumbelliferyl)-α-D-N-glycolylneuraminic acid (MUNGNA; synthesis described below) as substrate. When indicated NA samples were incubated at 50°C in the absence or presence of 1mM Ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich) prior to incubation with MUNANA to determine NA thermostability. The MUNANA assay was also performed in 0.1 M citrate reaction buffer (at pH 4.0, 4.6, 5.0 or 6.0). NA kinetic analysis was performed

to determine the Km values (substrate binding affinity) of the NA proteins. NA samples, resulting in approximately 20% of the maximum relative fluorescence units level after 1 h incubation with 100 µM MUNANA, were incubated with different concentrations of MUNANA ranging from 3.9 to 500 µM (two-fold serial dilutions) in a total volume of 100µL. The generated fluorescence was measured at 37°C every 5min for 30 min. The data were fitted to the Michaelis-Menten equation by non-linear regression using the Prism 6.05 software (GraphPad) and the Km values were determined.

Activity of the NA proteins towards multivalent glycoprotein substrates was analyzed using a previously described enzyme-linked lectin assay (ELLA) (30, 37). Briefly, 2.5 µg/ml fetuin- or 25 µg/ml transferrin-coated (both from Sigma-Aldrich) 96-well Nunc MaxiSorp plates were incubated with serial dilutions of recombinant soluble NA proteins in reaction buffer (50mM Tris/HCl, 4mM $CaCl_2$, pH 6.0). After overnight incubation at 37°C, plates were washed and incubated with either biotinylated Erythrina Cristagalli Lectin (ECA, 1.25 µg/ml; Vector Laboratories), biotinylated peanut agglutinin (PNA, 2.5 µg/ml; Galab Technologies), biotinylated Sambucus Nigra Lectin (SNA, 1.25 µg/ml; Vector Laboratories) or biotinylated Maackia Amurensis Lectin I (MAL I, 2.5 µg/ml; Vector Laboratories). The binding of ECA, PNA, SNA and MAL I was detected using horseradish peroxidase (HRP)-conjugated streptavidin (Thermo Fisher Scientific) and tetramethylbenzidine substrate (TMB, BioFX) in an ELISA reader EL-808 (BioTEK) reading the OD at 450 nm. Both for the fluorometric assay and the ELLA, the data were fitted by non-linear regression using the Prism 6.05 software (Graph Pad). The resulting curves were used to determine the amount of NA protein corresponding to half maximum activity or lectin binding. The inverse of this amount is a measure for specific activity (activity/amount of protein) and is graphed relative to other NA proteins or substrate-lectin combinations.

**Synthesis of MUNGNA**

MUNGNA was chemically synthesized according to previously published procedures (38, 39) with some modifications. Starting with commercially available neuraminic acid (Sigma Aldrich), a Boc-protecting group strategy on the C5-NHAc position to selectively deprotect and introduce the N-glycolyl group was used. Chlorination of the anomeric position with hydrochloric acid gas in acetyl chloride and subsequent glycosylation with the sodium salt of 4-methylumbelliferone provided the protected MUNGNA. Deprotection and reversed-phase silica gel purification provided MUNGNA.

**Hemagglutination assay**

The SIA-binding ability of N1 proteins was analyzed using hemagglutination assays. Purified NA protein (4 µg) was precomplexed with anti-Strep tag mouse antibody (IBA; 2 µg) and with lumazine synthase nanoparticles genetically fused to domain B of protein A (0.25 µg; Bosch and coworkers, submitted for publication) on ice for half an hour prior to incubation of limiting dilutions of these complexes with 0.5% human erythrocytes for 2 hours at 4°C in the presence of 5 µM Zanamivir (GlaxoSmithKline). N9 proteins with different binding properties, as shown in a previous study (30), were taken along in the hemagglutination assay as controls. The hemagglutination assays were performed twice in duplicate. The mean values of these experiments are graphed.

**Enzyme-linked immunosorbent assay (ELISA) and NA inhibition ELLA.**

ELISAs and NA inhibition assays were performed using ferret sera raised either against a classical swine H1N1 strain (A/NL/386/86), H1N1pdm09 vaccine strain (A/California/007/09) or several other H1N1pdm09 viruses isolated in the Netherlands (Table 2 and 3). For the ELISA, 96-well plates coated with 100ng of the different purified NA proteins were incubated with two-fold serially-diluted ferret sera at room temperature (RT) for 2 h. After extensive washing, peroxidase-conjugated goat anti-ferret IgG(H+L) (Immune systems) was added to each well. After incubation at RT for 1 h, and washing of the plates, antibody binding was determined by using TMB substrate as described above. Inhibition of NA activity by the ferret sera was measured by ELLA as described previously with modifications (30, 40). Briefly, serial dilutions of ferret sera were mixed with a purified NA protein diluted in reaction buffer (50mM Tris/HCl, 4mM $CaCl_2$, PH 6.0). The mixture was transferred to 96-well plates coated with 2.5 µg/ml fetuin (Sigma-Aldrich) and incubated at 37°C for 2 h. After extensive washing, the plates were incubated with biotinylated ECA (1.25 µg/ml; Vector Laboratories) at RT for 1 h. The amount of ECA binding was determined as described above. The anti-NA ELISA and ELLA titers of the sera correspond to the dilutions at which 50% of maximal binding or NA enzyme activity was achieved as determined by nonlinear regression analysis (Graph Pad Prism 6.05).

**Antigenic cartography**

Antigenic cartography was performed with AntigenMap 2D (41) and AntigenMap 3D (42) using the http://sysbio.cvm.msstate.edu/welcome website and the titers generated in the ELISA and ELLA. Antigenic maps shown in the manuscript were generated with the AntigenMap 2D software, while the antigenic distances between the different NA proteins were determined using the AntigenMap 3D software.

**Statistical analysis**

The mean values of 2-6 experiments performed in duplicate/triplicate with independently generated protein preparations are graphed. All statistical analyses were performed by one-way analysis of variance (ANOVA) using Tukey's multiple comparisons test (Graph Pad Prism 6.05). The level of significance was expressed as *$P$<0.05, ** $P$<0.01, *** $P$<0.001.

## Results

**Evolution of the NA protein of H1N1pdm09 from 2009 until 2015**

To get more insight into the evolution of the NA protein of H1N1pdm09 virus, we first reconstructed the evolutionary path of the NA protein starting with the emergence of the H1N1pdm09 virus in April 2009 and determined which substitutions in NA became fixed in the virus population and the order thereof. To this end, we constructed a NA protein phylogenetic tree (Fig. 1). Careful analysis of the different NA gene sequences revealed that several amino acid substitutions (N248D, V106I, N369K, V241I, N44S, I106V, N200S, I321V, I34V/K432E, N386K, L40I, I314M, and V13I/V264I/N270K) were successively acquired since the emergence of the H1N1pdm09 virus (Fig. 1 and Table1). For some substitutions (I34V/K432E and V13I/V264I/N270K) the order in which they were acquired

could not be resolved. The substitutions were also analyzed with respect to their location in the NA crystal structure (Fig. 2), with the exception of the substitutions located in the transmembrane domain or the stalk region (V13I, I34V, L40I and N44S) as the structure of this part of the NA protein is not resolved. Although some substitutions are located relatively

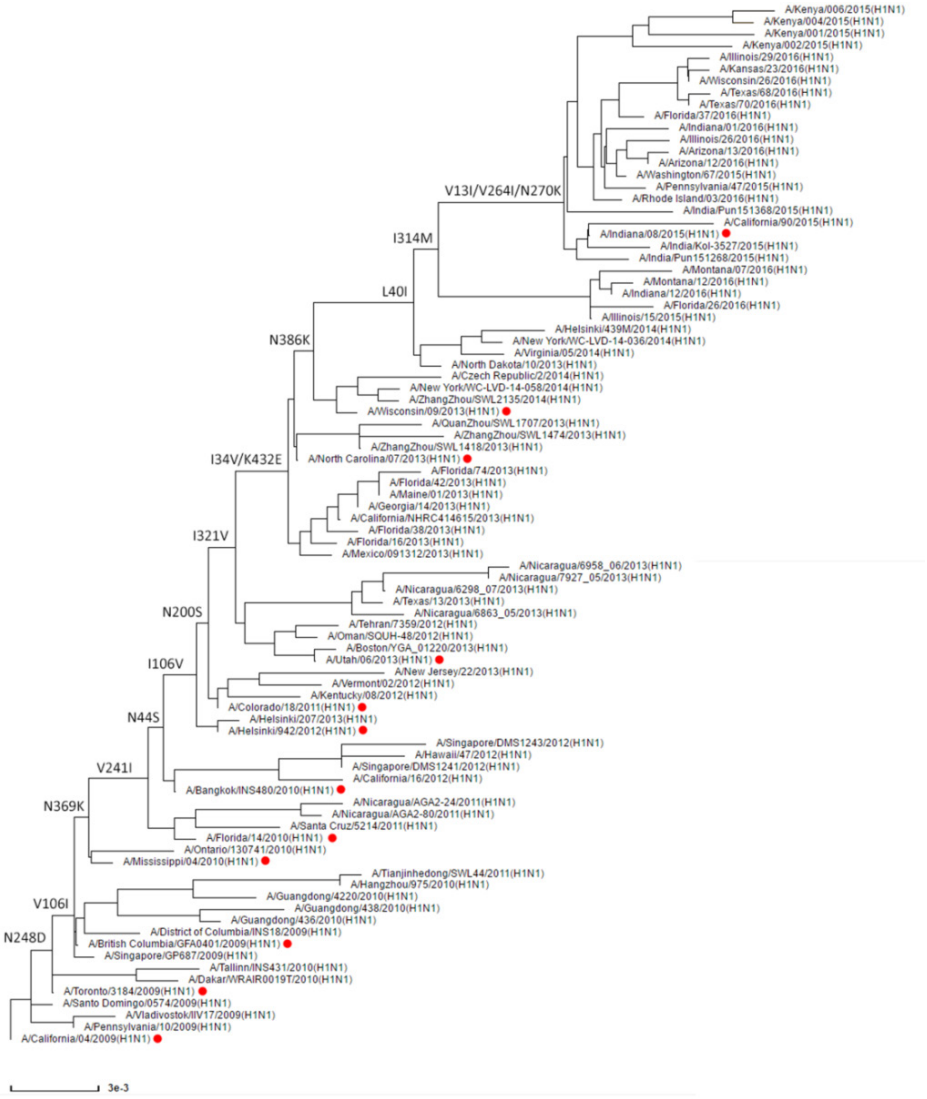



**Fig 1. Phylogenetic analysis of H1N1pdm09 N1 proteins from 2009 until 2015.** All full-length and unique N1 protein sequences of H1N1pdm09 were downloaded from the NCBI database and used to construct a N1 gene guide-tree, which was used to select N1 sequences representing all main branches of the tree. The selected N1 genes were used to construct a summary-tree of similar topology as the guide-tree. The N1 tree is rooted by the A/California/04/2009 isolate. Substitutions specified on the tree backbone indicate specific protein mutations that became fixed in the virus population. Red dots indicate virus isolates, the NA proteins of which only contain the indicated substitutions and were expressed in this study. An alignment of these proteins is shown in Fig. S1, while the mutations are also indicated in Table 1.

close to the active site (N200S, N248D, V241I, and K432E), none of them involves catalytic or framework residues (24, 25) (Fig. 2). Substitutions N369K and K432E are located in the 2nd SIA-binding site at positions known to interact with SIA according to the N9 crystal structure (31). The other residues, with the exception of the residue at position 106, are scattered across the surface of the NA head domain. Of note, N386K and N44S result in the disruption and generation of a N-glycosylation consensus sequence, respectively. The residue at position 386 is part of a $Ca^{2+}$-binding site, while the residue at position 106 is located close to the subunit interfaces and to the $Ca^{2+}$-binding site located at the 4-fold axis (27, 28). A sequence alignment of the NA proteins of the viruses indicated with the red dot in Fig 1. is shown Fig S1.

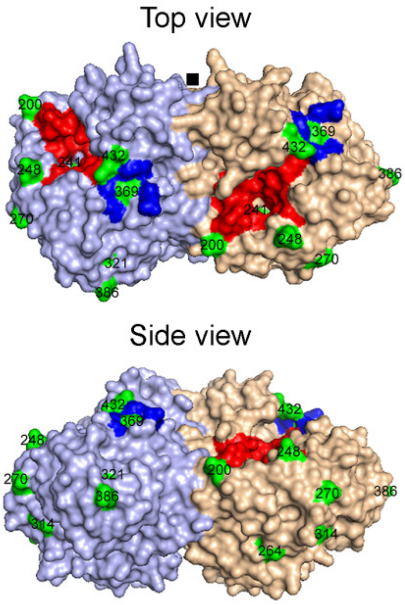



**Fig 2. Structural analysis of the N1 proteins.** Top (A) and side (B) views of the structure of the NA protein from A/California/04/2009 (PDB 3NSS) generated with PyMol software (69). The NA active site and the 2nd SIA-binding site are colored red and blue. The amino acids in the NA head domain, substitution of which is indicated in Fig. 1 and which became fixed in the population, are colored green and their numbering is indicated. V241I is located under the active site but doesn't belong to the catalytic or framework residues. K432E and N369K are in the 2nd SIA-binding site. The residue at position 106 is not visible in this surface representation of the structure, while the substitution located in the stalk region (N44S) is not indicated as the structure thereof is not solved. The substitutions are also indicated in the alignment shown in Fig. S1 and in Table 1.

**Expression of the recombinant NA proteins**

The NA protein sequence changes detected with our phylogenetic analysis prompted us to evaluate the possible accompanying phenotypic changes in NA functionality. To this end, these mutations were introduced into recombinant soluble tetrameric versions of the NA protein of H1N1pdm09 virus (starting at position 42) in the order in which they appeared according to the phylogenetic analysis. As for some substitutions the order in which they appeared could not be resolved, these mutations were combined (V264I+N270K). Initially, we made all NA proteins, for which corresponding viruses could be identified (indicated by the red dots in Fig. 1). These NA proteins are abbreviated with two letters that refer to the location and two digits that refer to the year of isolation (Table 1). Thus, CA/09 refers to the NA protein of the A/California/04/2009 isolate from the beginning of the pandemic. In addition, we made a recombinant NA protein containing the I314M substitution in the background of the WI/13 NA protein. As this mutation is only found in combination with other (non-fixed) mutations, this NA protein is referred to as WI/13-314.

Table 1. NA proteins analyzed

| Abbrevia-tion | Isolate[a] | Mutation(s) introduced[b] | All mutations relative to A/California/04/2009 |
|---|---|---|---|
| CA/09 | A/California/04/2009 | | |
| TO/09 | A/Toronto/3184/2009 | N248D | N248D |
| BC/09 | A/British Columbia/GFA0401/2009 | V106I | N248D+V106I |
| MS/10 | A/Mississippi/04/2010 | N369K | N248D+V106I+N369K |
| FL/10 | A/Florida/14/2010 | V241I | N248D+V106I+N369K+V241I |
| BK/10 | A/Bangkok/INS480/2010 | N44S | N248D+V106I+N369K+N44S+V241I |
| HS/12 | A/Helsinki/942/2012 | I106V | N248D+V106I+N369K+N44S+V241I+I106V |
| CO/11 | A/Colorado/18/2011 | N200S | N248D+V106I+N369K+N44S+V241I+I106V +N200S |
| UT/13 | A/Utah/06/2013 | I321V | N248D+V106I+N369K+N44S+V241I+I106V +N200S+I321V |
| NC/13 | A/North Carolina/07/2013 | K432E | N248D+V106I+N369K+N44S+V241I+I106V +N200S+I321V+K432E |
| WI/13 | A/Wisconsin/09/2013 | N386K | N248D+V106I+N369K+N44S+V241I+I106V +N200S+I321V+K432E+N386K |
| WI/13-314 | | I314M | N248D+V106I+N369K+V241I+N44S+I106V +N200S+I321V+K432E+N386K+I314M |
| WI/13-(314+264) | | V264I | N248D+V106I+N369K+V241I+N44S+I106V +N200S+I321V+K432E+N386K+I314M+V264I |
| WI/13-(314+270) | | N270K | N248D+V106I+N369K+V241I+N44S+I106V +N200S+I321V+K432E+N386K+I314M+N270K |
| IN/15 | A/Indiana/08/2015 | V264I+ N270K | N248D+V106I+N369K+V241I+N44S+I106V +N200S+I321V+K432E+N386K+I314M +V264I+N270K |

**a** Virus isolates carrying NA proteins identical to those analyzed in this study are indicated. These isolates correspond to the isolates indicated with the red dots in Fig. 1.
**b** Mutation(s) introduced relative to the precursor virus are indicated.

Upon the expression of the different NA proteins, we noticed remarkable differences in their expression levels. Therefore, we first performed a quantitative evaluation of these expression levels by Western blot analysis of the cell culture supernatants using an antibody against the Strep tag. The expression levels of the different NA proteins were normalized to that of the CA/09 protein. The results indicate that the expression of the NA proteins of viruses isolated in 2009 and 2010 gradually increased (Fig. 3A). Although each of these NA proteins did not significantly differ compared to its precursor protein, the successive

introduction of mutations resulted in significantly increased expression levels compared to CA/09 from the FL/10, which additionally contains the V241I mutation, until the WI/13 protein. Of note, the positive effect of the V241I substitution on NA protein expression was previously also observed in the context of the full length NA protein (43). Additional introduction of the mutation at position 314 (WI/13-314) reduced the expression level again, but this difference was not statistically significant compared to the WI/13. Finally, introduction of mutations at position 264 and 270 (IN/15) significantly decreased the protein expression level.

As a large difference in the protein expression levels was observed after the introduction of two mutations (V264I+N270K; IN/15), the effect of single substitutions was also analyzed. Introduction of either the V264I or the N270K substitution in the background of the WI/13-314 protein [resulting in WI/13-(314+264) or WI/13-(314+270), respectively] both decreased the expression level, with the mutation at position 270 having the largest effect (Fig. 3B). All NA proteins displayed a similar, high thermal stability (>85% compared to non-heated samples), as evaluated by analysis of NA enzymatic activity in the cell culture supernatants after a 5 min incubation at 50°C using the MUNANA fluorometric assay, indicating that differences in protein expression levels did not result from differences in (thermo)stability

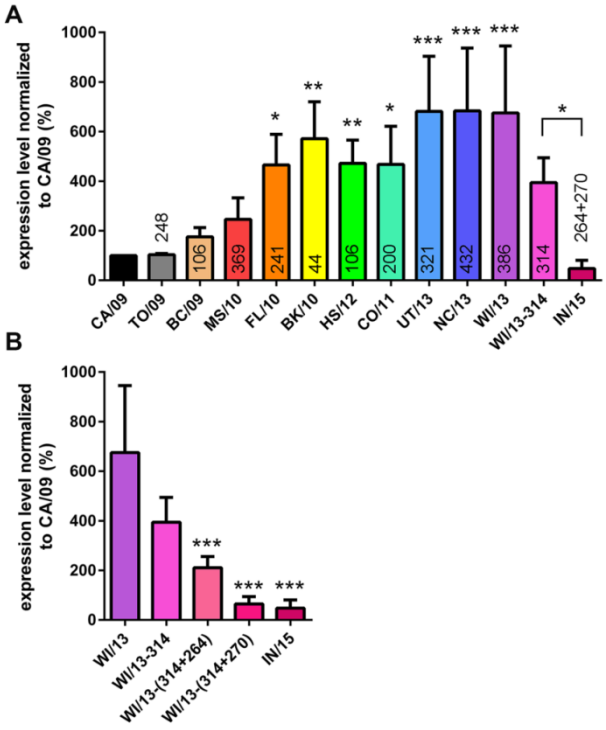



**Figure 3. NA protein expression levels.** (A-B) Expression levels of indicated recombinant soluble H1N1pdm09 NA proteins were determined by Western blot and graphed normalized to NA of CA/09. Values are the mean of two independent experiments, each analyzed in duplicate. Error bars indicate standard deviations. For each NA protein, significant differences relative to the CA/09 NA protein and to its precursor NA (indicated by brackets) (A), or to the WI/13 NA protein (B) are indicated (*P<0.05, ** P<0.01, *** P<0.001).

(data not shown). In summary, the successive introduction of mutations in the N1 protein resulted in increased followed by decreased expression levels, with the expression of the IN/15 protein finally being approximately similar to that of the CA/09 protein. The largest positive and negative effects were observed after introduction of the V241I and N270K substitutions, respectively.

**Cleavage of monovalent substrates**

Substitutions in NA may result from adaptation of the H1N1pdm09 virus to the new SIA-receptor repertoire of the human host. Therefore, the enzymatic properties of the NA proteins were analyzed. NA enzymatic activity was first investigated with monovalent substrates that either contain N-Acetylneuraminic acid (Neu5Ac; MUNANA) or N-Glycolylneuraminic acid (Neu5Gc; MUNGNA). While humans only synthesize Neu5Ac, swine express both SIA forms. The specific activity (activity/amount of protein) of the NA proteins against the Neu5Ac-containing substrate was enhanced by the sequential addition of several NA substitutions, but finally returned for the IN/15 protein, to similar levels as the CA/09 protein (Fig. 4A and Fig. S2). For none of the NA proteins, the substitutions resulted in significantly altered specific activity compared to its precursor protein. Most substitutions also did not affect the MUNANA substrate affinity of the NA proteins (Fig. 4B). An increased Km value (decreased substrate affinity) was only observed after introduction of the K432E mutation (NC/13) (Fig. 4B). This higher Km value was maintained in all other proteins containing this substitution (WI/13, WI/13-314 and IN/15). Analysis of the specific activity of the NA proteins using the Neu5Gc-containing substrate MUNGNA revealed that most proteins cleaved this substrate to a same extent relative to their ability to cleave MUNANA as the CA/09 protein, with the exception again of the NA proteins containing the K432E mutation (NC/13, WI/13, WI/13-314 and IN/15), which were consistently decreased in their ability to cleave MUNGNA (Fig. 4C). In summary, most substitutions in NA had a limited effect on the cleavage of soluble monovalent substrates, only mutation of the residue at position 432, which is located next to the active site, decreased the affinity of NA for MUNANA and the relative specific activity against MUNGNA. The specific activity of NA against MUNANA was not affected, however, by this mutation.

**Cleavage of multivalent substrates**

The enzymatic activities of the different NA proteins were also analyzed with the glycoproteins fetuin and transferrin using ELLAs described previously (30). The multivalent presentation of different glycans on the surface-coated fetuin and transferrin better resembles the *in vivo* substrates of NA than the soluble monovalent substrates used above. Fetuin contains mono-, bi-, and triantennary glycans with α2,3- and α2,6-linked SIAs in 2:1 ratio (44). Transferrin contains two biantennary N-linked glycan chains with only α2,6-linked SIAs (45, 46). Cleavage of SIAs from fetuin and transferrin by serially diluted NA proteins was quantified by analyzing the binding of lectins with different binding specificities. ECA specifically recognizes glycans containing terminal Galβ1-4GlcNAc, which generally correspond to desialylated N-linked sugars (47), while PNA binds to terminal Galβ1-3GalNAc corresponding with desialylated O-linked glycans (48). MALI and SNA specifically bind α2,3- or α2,6-linked SIAs, respectively (49, 50). First, the specific activities of the CA/09 protein for each glycoprotein-lectin combination were determined and plotted relative to the

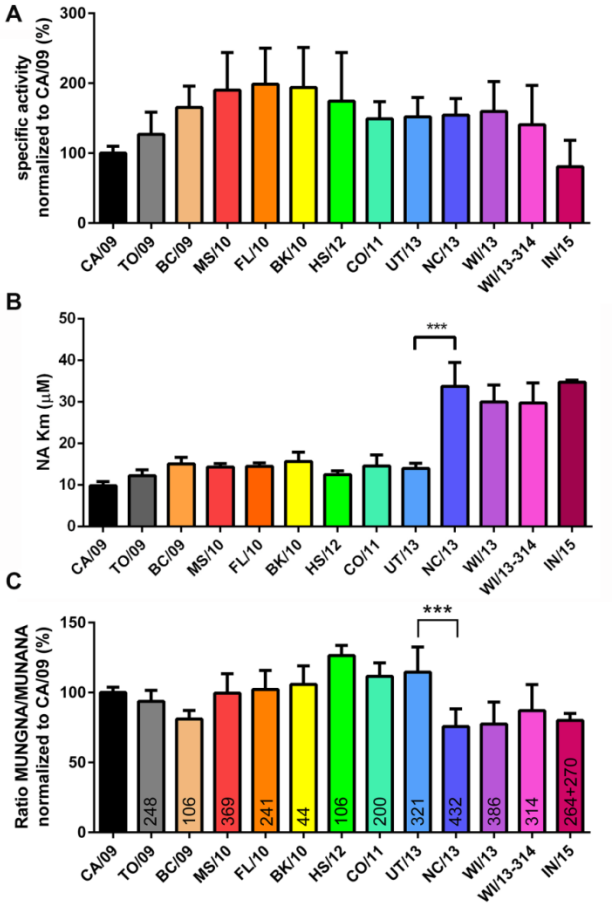
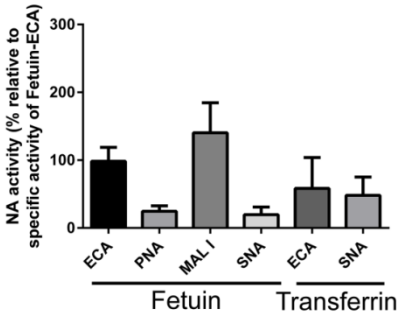



**Figure 4. Activity of H1N1pdm09 NA proteins using monovalent substrates.** (A) Specific activity of indicated NA proteins using the substrate MUNANA is graphed normalized to the specific activity of CA/09 NA. (B) Km values of the indicated NA proteins for MUNANA. (C) Ratio of the specific activity of the indicated NA proteins using the Neu5Gc-containing MUNGNA and the Neu5Ac-containing MUNANA (MUNGNA/MUNANA) graphed normalized to that of CA/09 NA. The graphs represent the mean of 2-6 independent experiments performed in triplicate. Error bars indicate standard deviations. (A-C) For each NA protein, significant differences relative to its precursor NA are indicated (*P<0.05, ** P<0.01, *** P<0.001).

**Fig 5. Specific activity of CA/09 NA using multivalent substrates.** Specific activity of CA/09 NA was determined by ELLA using different glycoprotein-lectin combinations and graphed normalized to the specific activity as determined by fetuin-ECA. Values are the mean of at least three independent experiments performed in triplicate. Error bars indicate standard deviations.



specific activity determined for fetuin-ECA (Fig. 5). The results indicate that CA/09 NA prefers cleavage of α2,3- (determined with fetuin-MALI) over α2,6- (determined with fetuin-SNA) linked SIAs. In agreement herewith, the cleavage of SIAs from fetuin was more efficient than from transferrin when determined with ECA. The preferred cleavage of α2,3-linked SIAs by this N1 protein appeared, however, less pronounced than that of the N9 proteins of avian H7N9 viruses (30).

Next, the specific activities of the different NA proteins were determined and plotted relative to the specific activity of the CA/09 protein for each glycoprotein-lectin combination (Fig. 6). Although small differences can be observed for the different fetuin-lectin combinations (Fig. 6A-D), the results consistently show a large positive effect of the N369K substitution (compare BC/09 and MS/10) and a large negative effect of the K432E mutation (compare UT/13 and NC/13) on the cleavage of SIA from fetuin. The other consistent effect, statistically significant when SNA or MALI were used, was the increased specific activity resulting from the N386K substitution (compare NC/13 and WI/13). No positive effect of the N369K substitution was observed when transferrin was used as substrate (Fig. 6E-F), while the negative effect of the K432E substitution was much smaller and not significant. Strikingly, also when transferrin was used, the positive effect of the N386K mutation, which results in the loss of a N-linked glycosylation site, could be observed, which was significant in combination with ECA.

Previously, we showed that increased cleavage of multivalent substrates correlated with increased binding of NA to glycans via the 2$^{nd}$ SIA-binding site (30). As the substitutions at position 369 and 432 are located in the 2$^{nd}$ SIA binding site of the N1 protein and affect the specific activity when the multivalent substrate fetuin (Fig. 6A-D), but not or much less when soluble, monovalent MUNANA (Fig. 4A) or transferrin, which only contains α2,6-linked SIAs (Fig. 6E-F) was used. We analyzed the ability of several NA proteins, complexed to lumazine synthese nanoparticles to increase the valency, to hemagglutinate red blood cells. Of note, the N9 protein of a recent H7N9 virus (N9 Anhui), which was not able to hemagglutinate red blood cells when complexed with antibodies alone (30), gave positive results in this more sensitive nanoparticle assay. However, none of the N1 proteins tested displayed any hemagglutinating activity (Fig. S3), indicating a very low avidity for the interaction of the N1 proteins of H1N1pdm09 with sialosides via the 2$^{nd}$ SIA-binding site.

**Low pH activity**

The ability of NA to cleave sialosides at low pH during virus entry has been shown to enhance virus replication in vitro (51, 52) and was suggested to contribute to the spread of pandemic viruses (27, 52). Therefore we also analyzed the enzymatic activity of the NA proteins at different pH compared to the activity at the routinely used pH 6.0 using the MUNANA assay. All proteins displayed a similar specific activity at pH 5.0 and pH 6.0, while their activity was completely abrogated at pH 4.0 (data not shown). At pH 4.6, the different NA proteins showed differential amounts of remaining activity, which was relatively low for the proteins with K432 (CA/09-UT/13) and high for the proteins containing the K432E substitution (NC/13-IN/15) (Fig. 7A). This result indicates that the K432E substitution has a positive effect on the cleavage of substrates at low pH.

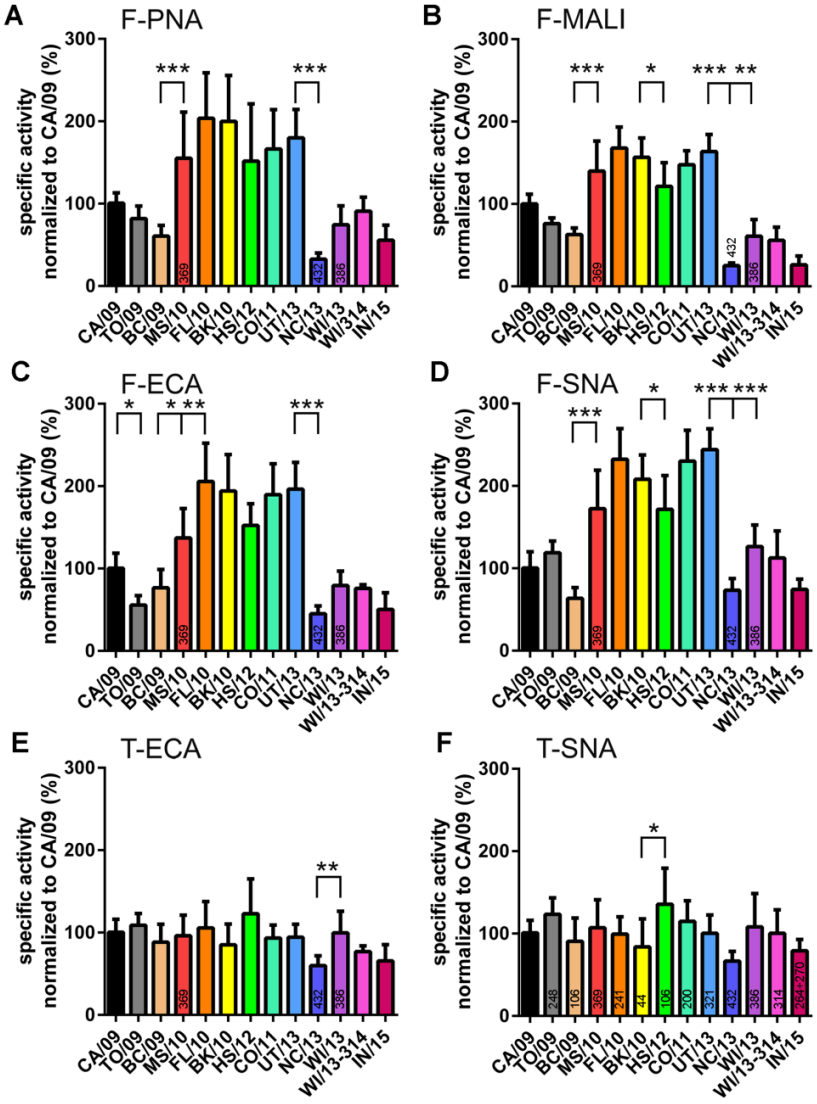



**Fig 6. Specific activity of H1N1pdm09 NA proteins with multivalent substrates.** Specific activity of indicated H1N1pdm09 NA proteins was determined by ELLA using different glycoprotein-lectin combinations: (A) fetuin-PNA (F-PNA) (B), fetuin-MAL I (F-MALI) (C), fetuin-ECA (F-ECA), (D) fetuin-SNA (F-SNA), (E) transferrin-ECA (T-ECA), and (F) transferrin-SNA (T-SNA) and graphed normalized to that of NA CA/09. Means of at least three independent experiments performed in duplicate/triplicate are shown. Standard deviations are indicated. For each NA protein, significant differences relative to its precursor protein are indicated (*P < 0.05; **P < 0.01; ***P <0.001).

## Thermostability

As several substitutions are located at or close to $Ca^{2+}$-binding sites and $Ca^{2+}$-binding is known to be important for thermal stability (53-55), we also analyzed the thermostability of NA activity by incubating the NA proteins at 50°C for 5 min using the MUNANA assay. As indicated above, NA proteins in cell culture supernatants all displayed a high thermal

stability (>85%) after a 5 min incubation at 50°C (data not shown). However, in the presence of EDTA, remarkable differences were observed in the thermostability of purified proteins as shown by graphing the residual activity of the proteins relative to a non-heated sample (Fig. 7B). The CA/09 and TO/09 proteins were completely inactivated by the incubation at 50°C in the presence of EDTA. Introduction of the V106I substitution (BC/09) increased the thermal stability to some extent, which was subsequently greatly enhanced by the N369K mutation (MS/10). Thermal stability was severely decreased again by the I106V mutation (HS/12), after which it remained low or non-detectable. Our results indicate that the residues at position 106 and 369 are important determinants of $Ca^{2+}$-dependent thermostability, probably by affecting the affinity of NA for $Ca^{2+}$. Mutations that affected $Ca^{2+}$-dependent thermostability did not affect the activity of the NA proteins at low pH and vice versa.

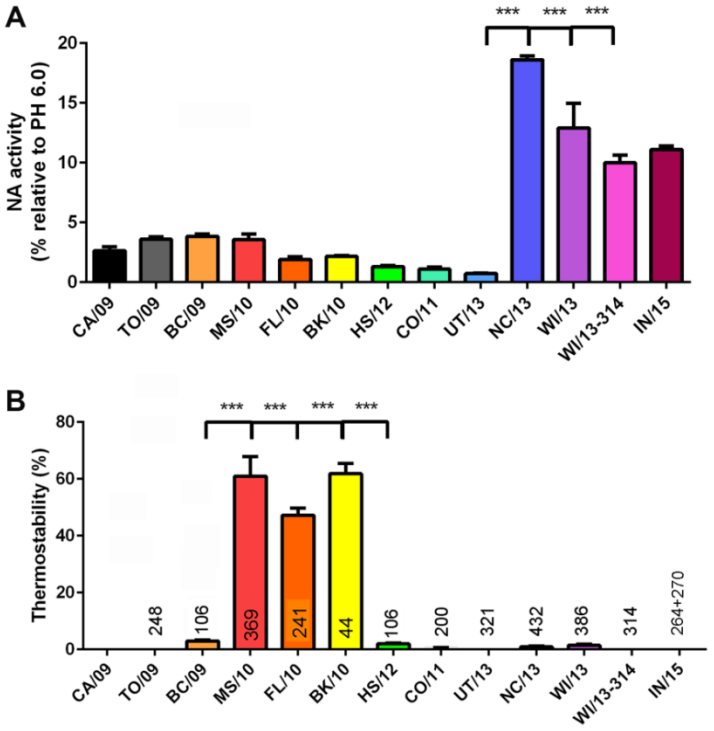



**Figure 7. Low pH activity and $Ca^{2+}$-dependent thermostability of H1N1pdm09 NA proteins**. (A) The activity of the different NA proteins was determined at pH 4.6 using the MUNANA assay and graphed relative to the activity at pH 6.0. (B) $Ca^{2+}$-dependent thermostability was analyzed by determining the NA activity using the MUNANA assay after heating NA samples for 5 min at 50°C in buffer lacking $Ca^{2+}$ and containing EDTA. Values are graphed relative to unheated controls. (A-B) Representative experiments performed in triplicate are shown. For each NA protein, significant differences relative to its precursor protein are indicated (*P < 0.05; **P < 0.01; ***P <0.001).

### Analysis of antigenicity

Substitutions in NA may affect the enzymatic activity, but they may also be selected under immune pressure when they change the antigenicity of NA. The antigenic properties of the different NA proteins were first tested in ELISA by determining anti-NA IgG titers of a panel

of ferret antisera raised against different H1N1 viruses including a classical swine H1N1 virus (A/NL/386/86), the H1N1pdm09 vaccine strain (A/CA/007/09) and several H1N1pdm09 viruses isolated in the Netherlands from 2009 to 2015 (Table 2). Generally, the lowest titers were observed for the ferret antisera raised against classical swine H1N1 virus. When CA/09 or TO/09 proteins were used as antigens, titers were lower when the ferret serum raised against the virus from 2015 was used. The reciprocal effect could be observed for the IN/15 and WI/13-(314+270) proteins; lowest and highest titers were observed when antisera raised against viruses from 2009 and 2015 were used, respectively. While the IN/15 and WI/13-(314+270) proteins in contrast to the other NA proteins both carry the 270 substitution, the H1N1pdm09 virus from 2015, against which the ferret serum was raised that displayed the highest titer against these proteins, had not yet obtained this mutation (data not shown). NA-specific titers were also determined by performing NA inhibition ELLAs (Table 3). Titers and differences between titers were lower with the NA inhibition assay than with the NA ELISA.

Based on the results shown in Table 2 and 3, two dimensional antigenic maps were generated (Fig. 8 and 9). According to the antigenic map based on the ELISA data, NAs appeared to group into three clusters (Fig. 8A). CA/09 and TO/09 NAs were closely positioned to each other, just as WI/13-(314+270) and IN/15. All other NA proteins also clustered together. The largest antigenic difference appeared to be induced by the N270K substitution present in WI/13-(314+270) and IN/15. This was confirmed when the antigenic distance between the NA proteins was determined based on a three dimensional antigenic map (Fig. 8B). The largest antigenic distance between each NA protein and its precursor and between each NA protein and the CA/09 protein was observed after introduction of the N270K substitution. A similar clustering of NA proteins was observed when the antigenic map was based on the NA inhibition ELLA (Fig. 9A). Antigenic distances between the three clusters were however smaller compared to those based on the ELISA data (Fig. 8B and 9B).

**Table 2. NA ELISA titer**

| Antigen[a] | Ferret sera raised against[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A/NL /386/ 86 | A/CA/007 /09 | A/NL/602 /09 | A/NL/007 /10 | A/NL/195 /12 | A/NL/529 /12 | A/NL/016 /14 | A/NL/148 /15 |
| CA/09 | 9,43[c] | 11,89 | 11,27 | 11,63 | 12,09 | 13,32 | 12,14 | 9,23 |
| TO/09 | 8,91 | 11,58 | 10,73 | 11,80 | 11,93 | 13,16 | 11,87 | 9,06 |
| BC/09 | 9,44 | 11,99 | 11,24 | 12,36 | 12,43 | 13,78 | 12,42 | 10,98 |
| MS/10 | 9,00 | 11,88 | 11,33 | 12,72 | 12,87 | 13,80 | 12,24 | 12,09 |
| FL/10 | 8,99 | 11,89 | 11,08 | 12,48 | 12,78 | 13,63 | 12,24 | 12,11 |
| BK/10 | 8,89 | 11,74 | 11,05 | 12,46 | 12,94 | 13,81 | 12,20 | 12,04 |
| HS/12 | 8,89 | 11,56 | 10,81 | 12,25 | 12,53 | 13,50 | 11,98 | 11,85 |
| CO/11 | 9,15 | 12,02 | 11,11 | 12,60 | 12,75 | 13,93 | 12,32 | 12,13 |
| UT/13 | 9,43 | 11,84 | 11,21 | 12,41 | 12,51 | 13,72 | 12,30 | 10,97 |
| NC/13 | 9,20 | 11,77 | 11,01 | 12,84 | 12,41 | 13,94 | 12,27 | 12,29 |
| WI/13 | 9,10 | 11,67 | 10,96 | 12,47 | 12,23 | 13,66 | 12,26 | 12,30 |
| WI/13-314 | 9,02 | 11,46 | 10,69 | 12,44 | 12,26 | 13,39 | 12,15 | 12,42 |
| WI/13-(314+264) | 9,00 | 11,47 | 10,68 | 12,34 | 12,12 | 13,47 | 12,19 | 12,54 |
| WI/13-(314+270) | 7,84 | 7,89 | 7,76 | 12,14 | 10,65 | 11,13 | 10,58 | 12,72 |
| IN/15 | 7,51 | 7,43 | 7,42 | 11,71 | 10,27 | 10,67 | 10,52 | 12,74 |

[a] NA proteins used in the ELISA assay are indicated

[b] viruses used to raise the ferret sera are indicated

[c] Mean titers (Log2) of 2-3 experiments performed in duplicate/triplicate are shown.

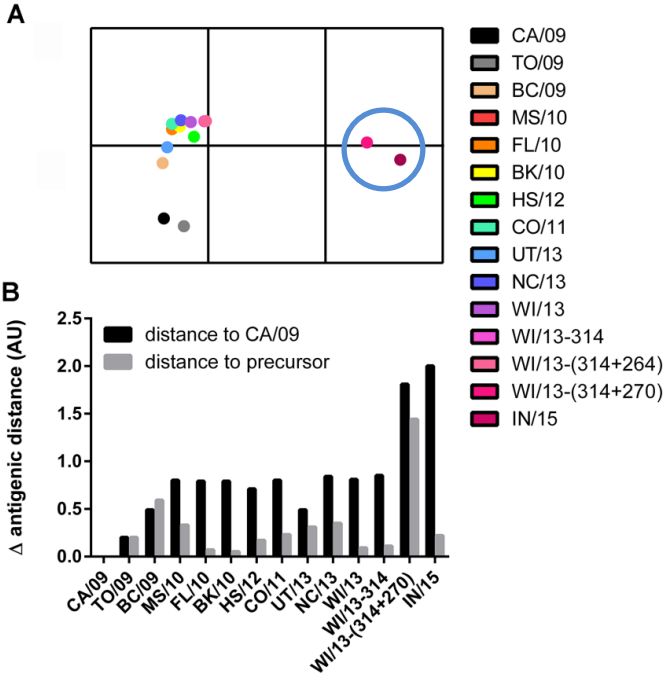



**Figure 8. H1N1pdm09 NA antigenicity determined by ELISA.** (A) Antigenic map showing antigenic differences between the H1N1pdm09 NA proteins was constructed using the ELISA data shown in Table 2 and AntigenMap 2D (41). The spacing between the grid lines is one unit of antigenic distance, which corresponds to a twofold dilution of ferret antisera in the ELISA. The orientation of the map within vertical and horizontal axes is arbitrary. The circle indicates the two NA proteins containing the N270K mutation. (B) For each NA protein, the antigenic distances relative to NA CA/09 (black bars) or its precursor NA (gray bar) based on the ELISA data shown in Table 2 were determined using AntigenMap 3D (42).

**Table 3. NA inhibition titer**

| Antigen[a] | Ferret sera raised against[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A/NL/386/86 | A/CA/007/09 | A/NL/602/09 | A/NL/007/10 | A/NL/195/12 | A/NL/529/12 | A/NL/016/14 | A/NL/148/15 |
| CA/09 | 7,78[c] | 8,44 | 8,00 | 8,00 | 8,90 | 8,69 | 8,92 | 7,08 |
| TO/09 | 7,55 | 8,12 | 7,97 | 7,64 | 9,19 | 9,66 | 8,81 | 7,22 |
| BC/09 | 7,27 | 7,85 | 7,12 | 7,69 | 8,18 | 8,58 | 7,90 | 6,78 |
| MS/10 | 7,76 | 7,35 | 7,13 | 7,41 | 8,79 | 8,86 | 7,38 | 7,14 |
| FL/10 | 7,07 | 7,14 | 7,00 | 7,24 | 8,73 | 8,87 | 7,08 | 6,77 |
| BK/10 | 6,60 | 6,90 | 6,52 | 6,85 | 8,48 | 8,71 | 6,84 | 6,61 |
| HS/12 | 6,67 | 7,13 | 6,88 | 7,17 | 9,24 | 9,38 | 7,13 | 6,98 |
| CO/11 | 7,04 | 7,13 | 6,78 | 7,35 | 8,55 | 8,71 | 7,08 | 7,72 |
| UT/13 | 6,86 | 7,07 | 6,84 | 7,16 | 8,58 | 8,86 | 7,21 | 7,66 |
| NC/13 | 7,09 | 7,09 | 7,04 | 7,36 | 7,12 | 7,52 | 7,18 | 7,12 |
| WI/13 | 6,66 | 6,86 | 6,71 | 6,96 | 7,25 | 7,65 | 7,25 | 7,33 |
| WI/13-314 | 6,39 | 7,25 | 6,52 | 7,42 | 8,03 | 8,62 | 7,64 | 8,19 |
| WI/13-(314+264) | 6,84 | 7,29 | 6,83 | 7,45 | 8,11 | 9,04 | 7,97 | 8,37 |
| WI/13-(314+270) | 6,15 | 6,63 | 6,26 | 8,92 | 8,11 | 9,38 | 8,03 | 9,86 |
| IN/15 | 6,18 | 6,71 | 6,54 | 8,53 | 8,11 | 9,14 | 7,78 | 9,63 |

a NA proteins used in the ELLA are indicated

b viruses used to raise the ferret sera are indicated

c Mean titers (Log2) of 2-3 experiments performed in duplicate/triplicate are shown.

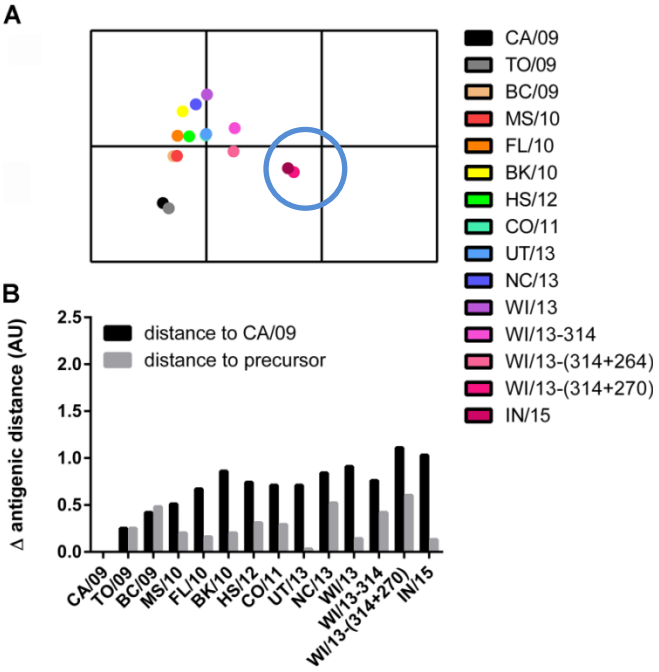



**Figure 9. H1N1pdm09 NA antigenicity determined by NA inhibition ELLA.** (A) Antigenic map showing antigenic differences between the H1N1pdm09 NA proteins was constructed using the NA inhibition ELLA data shown in Table 3 and AntigenMap 2D (41). The spacing between the grid lines is one unit of antigenic distance, which corresponds to a twofold dilution of ferret antisera in the NA inhibition ELLA. The orientation of the map within vertical and horizontal axes is arbitrary. The circle indicates the two NA proteins containing the N270K mutation. (B) For each NA protein, the antigenic distances relative to NA CA/09 (black bars) or its precursor NA (gray bar) based on the NA inhibition ELLA data shown in Table 3 were determined using AntigenMap 3D (42).

## Discussion

In this study, we analyzed the evolution of the phenotypic properties of the NA protein of the H1N1pdm09 virus starting with its emergence in 2009 until 2015. Significant changes in several of these properties, including expression level, cleavage of sialosides, low pH activity and antigenicity were observed (summarized in Fig. 10). In 2009 and 2010, the NA protein acquired several mutations (at positions 106, 241 and 369) that increased expression, activity against multivalent substrates, and $Ca^{2+}$-dependent thermostability. Only a limited effect on antigenicity as determined with polyclonal ferret sera was observed. In the following years, first the thermostability of the NA protein decreased followed by decreased cleavage of multivalent substrates resulting from mutations at position 106 and 432, respectively. The substitution at position 432 was accompanied with increased cleavage ability at low pH. Finally, also the NA expression levels decreased by substitutions at position 314 and particularly 270. Currently, it is not clear whether the mutations in NA are adaptive mutations of NA for the sialoside receptor repertoire of the new host, and/or are selected to compensate for changes in the functionality of HA. Concomitant with the decrease in expression, resulting from the substitution at position 270, also the antigenicity of the NA protein changed, which may explain why this mutation was selected. Strikingly, the phenotypic properties of the IN/15 protein, with the exception of its antigenicity, are rather similar to those of the CA/09 protein, suggesting that the CA/09 protein was already well adapted to the human host and that the NA protein does not evolve to obtain and maintain maximal sialidase activity.

Shortly after the emergence of the H1N1pdm09 virus, substitutions appeared in NA that increased protein expression levels (V241I) and cleavage of multivalent substrates (N369K). Previously, the positive effect of these mutations on expression and activity was also observed using full-length NA proteins (43), thereby indirectly validating the use of recombinant soluble NA proteins to elucidate such phenotypic changes. The V241I and N369K mutations were identified as being permissive for oseltamivir-resistance as they compensate for the negative effect of the H275Y oseltamivir-resistance mutation on cell surface expression and NA activity (43). For the seasonal H1N1 viruses, such permissive mutations in NA were shown to facilitate the emergence and spread of oseltamivir-resistant viruses in the absence of oseltamivir selection pressure (56-58). Nevertheless, prevalence of oseltamivir-resistant H1N1pdm09 viruses so far remained relatively low (1-2%) (59-61). This may in part be related to the emergence of substitutions in NA that have a negative effect on enzymatic activity (K432E) or protein expression (N270K). We speculate that these latter substitutions may be regarded as non-permissive and decrease the chances of H1N1pdm09 viruses to acquire NA inhibitor-resistance mutations and to outcompete non-resistant viruses in the absence of oseltamivir selection. The presence of the K432E non-permissive mutation may for example explain why a large cluster of oseltamivir-resistant H1N1pdm09 viruses found in Japan during the 2013-2014 influenza season was rapidly outcompeted once the wild-type virus began to circulate in the community (62). An important role for the N386K mutation was proposed in this process, however, according to our analyses, this mutation did not negatively affect NA stability as suggested (62), but rather had a positive effect on NA activity (Fig. 10).

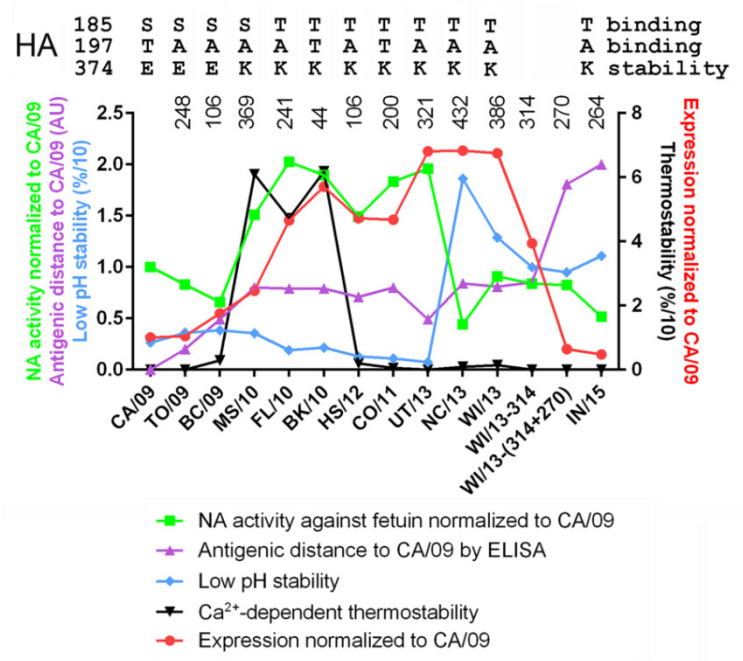



**Figure 10. Evolution of phenotypic properties of H1N1pdm09 NA proteins.** Different phenotypic properties of H1N1pdm09 NA proteins are graphed. These properties include specific activity using fetuin (green; mean of all fetuin-lectin combinations) as substrate normalized to that of CA/09 NA, antigenic distance to CA/09 NA (purple; based on ELISA), expression level normalized to CA/09 NA (red), Low pH activity relative to activity at pH 6.0 (light blue), and $Ca^{2+}$-dependent thermostability of NA proteins relative to unheated controls (black). Letters shown on top indicate amino acid residues found in the corresponding HA proteins at positions that are known to affect binding (185 and 197) or pH stability/activation (374).

The sequence signature composing the 2nd SIA binding site is highly conserved in avian, but not in human, IAV NA proteins (31, 32). For example, one of the SIA-contact residues (S at position 369) is mutated in NA of H1N1pdm09. However, low level binding of H1N1pdm09 NA to sialosides via this site could be demonstrated using STD-NMR (33). Previously, we showed for the N9 protein of avian IAV that binding via the 2nd SIA binding site positively affects the catalytic activity against multivalent substrates (30), probably by keeping them close to the active site. Although we were not able to directly demonstrate binding of the H1N1pdm09 N1 proteins to sialosides, even when coupled to nanoparticles to increase the valency, several of our results indicate that also for the human N1 protein this binding contributes to cleavage of sialosides. First, NA cleavage of fetuin-linked sialosides was shown to be positively (N369K) or negatively (K432E) affected by substitutions localized in the 2nd SIA-binding site. The N369K mutation probably restores interaction with SIA to some extent, while the 2nd SIA binding site is disturbed by mutation of the K432 contact residue. Second, although the K432E substitution increased the Km value for the MUNANA substrate, both the N369K and the K432E mutations did not affect the specific activity of NA for monovalent MUNANA. Third, effects of the N369K and K432E mutations in N1 are much larger for fetuin, containing both α2,3- and α2,6-SIAs, than for transferrin, containing only α2,6-SIA. These results suggest that the N1 protein prefers binding to α2,3-SIAs, just as the

N9 protein (30). Of note, the N386K substitution located outside of the 2nd SIA binding site increased NA activity equally for transferrin and fetuin, while having no effect on the cleavage of monovalent substrates. This mutation abolishes an N-glycosylation site and is therefore proposed to facilitate the accessibility of the active site for bulky substrates. In conclusion, our results show for the first time that also in human IAVs carrying a mutated 2nd SIA binding site, NA activity may be modulated by further mutation of this site.

Mutations in NA result either from stochastic genetic drift or from positive selection. Selected mutations may for example better equip the NA proteins to cleave the novel sialoside receptor repertoire of the new human host. Substitutions selected relatively early after the emergence of H1N1pdm09 virus that increase NA expression level (V241I) or enzymatic activity against multivalent substrates (N369K) might be regarded as such adaptive mutations. During evolution in a new host, phenotypic characteristics of lesser/no importance may also get lost. For example, humans do not express Neu5Gc in contrast to swine. Mutation of the residue at position 432 in the 2nd SIA binding site resulted in a significantly decreased cleavage of the Neu5Gc form of MUNANA (MUNGNA). Why the K432E mutation affects the cleavage of MUNGNA is not known. Possibly this mutation affects the conformation of the active site to some extent as it also increased the Km value for MUNANA and ability of NA to cleave at low pH. Of note, mutation of a neighboring residue (at position 431) in the N2 protein of human viruses was previously shown to also negatively affect cleavage of Neu5Gc sialosides (63).

Mutations in NA may also be selected to tune the balance between HA and NA, and therefore may reflect (or alternatively induce) changes in the functionality of HA. These changes in HA can involve the interaction with sialosides, but also other characteristics such as (low pH) stability. In 2009 and 2010, the HA protein of H1N1pdm09 acquired mutations that increased the receptor binding (T197A; (17)) and low pH stability of HA (E374K) (20, 64) (Fig. 10 and Fig. S4). These mutations in HA may be balanced by mutations in NA, which increase NA protein expression (V241I) and enzymatic activity (N369K). Residues at positions 248 and 106 were previously reported to be important for low pH activity of NA (27). In our assays, however, substitutions at these positions had only minor effects on low pH activity. The substitutions at position 106 and 369 did have large effects on the $Ca^{2+}$-dependent thermostability, indicating that they affect the affinity of NA for $Ca^{2+}$ in view of the known importance of $Ca^{2+}$ binding for NA thermostability (25). The relevance of this latter phenotype for virus replication *in vivo* is not clear, however. In contrast, the ability of NA to cleave sialosides at low pH enhances virus replication (51). This may explain the selection of the K432E mutation, which resulted in increased cleavage at low pH, even though it had a negative effect on the cleavage of fetuin-linked sialoglycans. The K432E mutation in NA may balance the E374K mutation resulting in low pH stability of HA (20,64). We cannot exclude, however, that substitutions in HA, which affect receptor binding (S185T, A197T and T197A) (19) (Fig. 10 and Fig. S4), (also) contributed to the selection of the K432E mutation in NA.

Most substitutions observed in H1N1pdm09 NA are located at the cell surface and several of them in or close to previously identified antigenic sites (e.g. positions 248, 270, and 369)

(65-67). Substitutions can therefore also, or even primarily, be selected for their effect on NA antigenicity. For example, the N369K mutation was reported to abolish the binding of monoclonal antibody HF5 to CA/09 NA, suggesting that this epitope is probably targeted by human NA-specific antibodies (67). Changes in NA functionality resulting from such antigenic changes (e.g. increased cleavage of multivalent substrates and $Ca^{2+}$-dependent thermostability resulting from N369K in NA) may subsequently drive compensatory mutations in HA and/or NA. Nevertheless, most of the mutations appear to have only limited or no effect on the antigenicity of NA when measured with polyclonal antisera in binding or inhibition assays. Only the substitution at position 270 significantly affected NA antigenicity, particularly in the ELISA assay. This antigenic change came at a cost, however, as it had a dramatic effect on the NA protein expression level. Also for seasonal H1N1 NA, a single mutation was previously shown to be largely responsible for the observed antigenic drift analyzed with polyclonal sera (68).

In our study we reconstructed the evolutionary path taken by the NA protein of the H1N1pdm09 virus. Several mutations were identified that affect one or more functional characteristics of NA. The results indicate that within a certain bandwidth the NA protein may differ significantly with respect to expression, stability and activity and that the NA protein not necessarily evolves to obtain and maintain maximal activity. The results furthermore indicate the different NA phenotypic properties to be intertwined, with several substitutions affecting more than one characteristic, including NA antigenicity. Most likely, the phenotypic changes of NA are also linked to the properties of the HA protein and corresponding HA-NA balance. It will be of interest to further study how all these checks and balances affect the evolution of IAVs. As a first step to do so, we aim to study the effect of the different mutations in NA in the context of virus particles using different particle binding, release and infection models.

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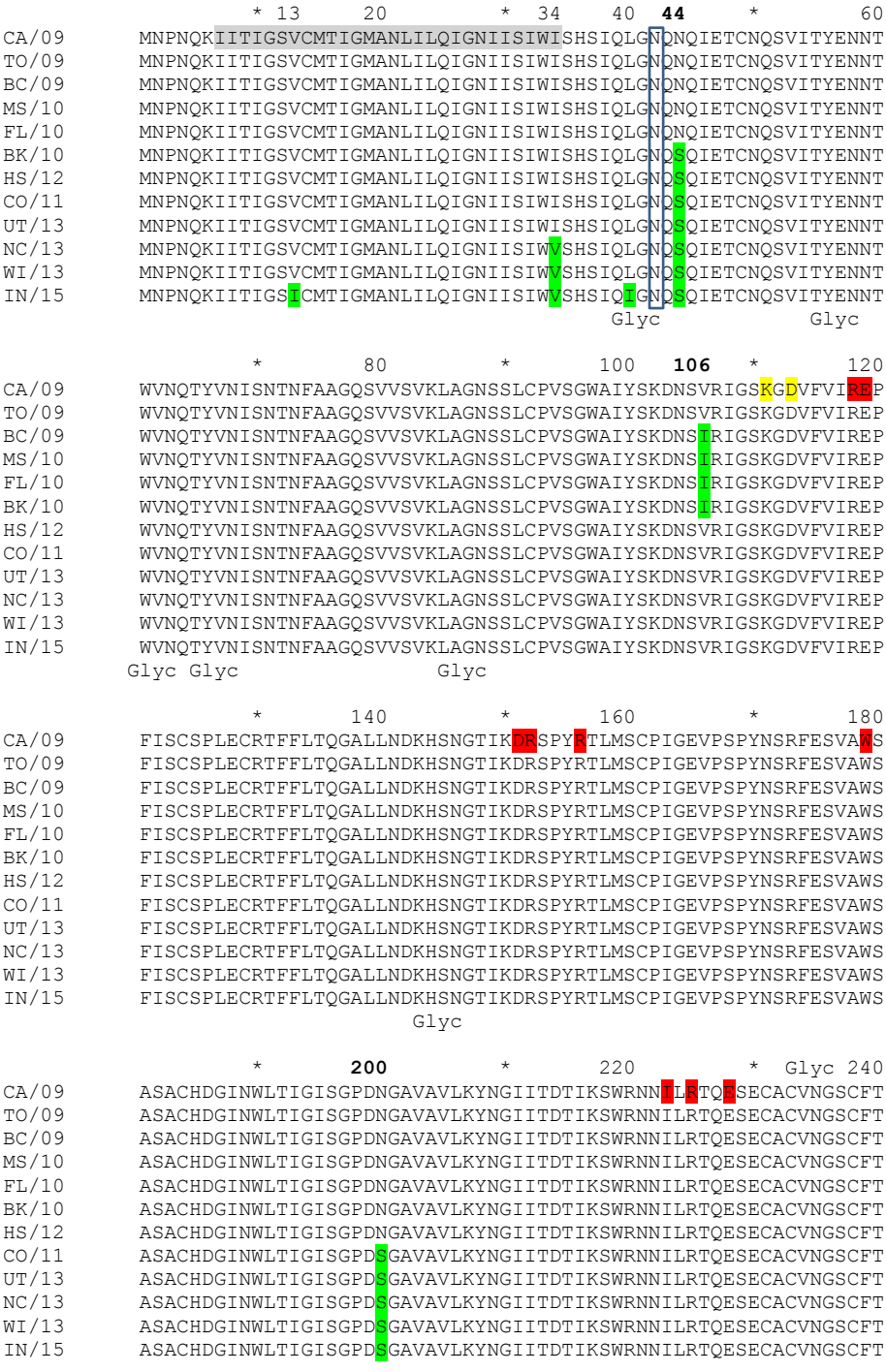
## Supplementary figures



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CO/11      IMTDGPSDGQASYKIFRIEKGKIVKSVEMNAPNYHYEECSCYPDSSEITCVCRDNWHGSN
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NC/13      IMTDGPSDGQASYKIFRIEKGKIVKSVEMNAPNYHYEECSCYPDSSEITCVCRDNWHGSN
WI/13      IMTDGPSDGQASYKIFRIEKGKIVKSVEMNAPNYHYEECSCYPDSSEITCVCRDNWHGSN
IN/15      IMTDGPSDGQASYKIFRIEKGKIIKSVEMKAPNYHYEECSCYPDSSEITCVCRDNWHGSN

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NC/13      RPWVSFNQNLEYQIGYICSGVFGDNPRPNDKTGSCGPVSSNGANGVKGFSFKYGNGVWIG
WI/13      RPWVSFNQNLEYQIGYICSGVFGDNPRPNDKTGSCGPVSSNGANGVKGFSFKYGNGVWIG
IN/15      RPWVSFNQNLEYQMGYICSGVFGDNPRPNDKTGSCGPVSSNGANGVKGFSFKYGNGVWIG

                  369          380    386   *        400         *        420
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TO/09      RTKSISSRNGFEMIWDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRP
BC/09      RTKSISSRNGFEMIWDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRP
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HS/12      RTKSISSRKGFEMIWDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRP
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NC/13      RTKSISSRKGFEMIWDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRP
WI/13      RTKSISSRKGFEMIWDPNGWTGTDNKFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRP
IN/15      RTKSISSRKGFEMIWDPNGWTGTDNKFSIKQDIVGINEWSGYSGSFVQHPELTGLDCIRP
                                 Glyc

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FL/10      CFWVELIRGRPKENTIWTSGSSISFCGVNSDTVGWSWPDGAELPFTIDK
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HS/12      CFWVELIRGRPKENTIWTSGSSISFCGVNSDTVGWSWPDGAELPFTIDK
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WI/13      CFWVELIRGRPEENTIWTSGSSISFCGVNSDTVGWSWPDGAELPFTIDK
IN/15      CFWVELIRGRPEENTIWTSGSSISFCGVNSDTVGWSWPDGAELPFTIDK
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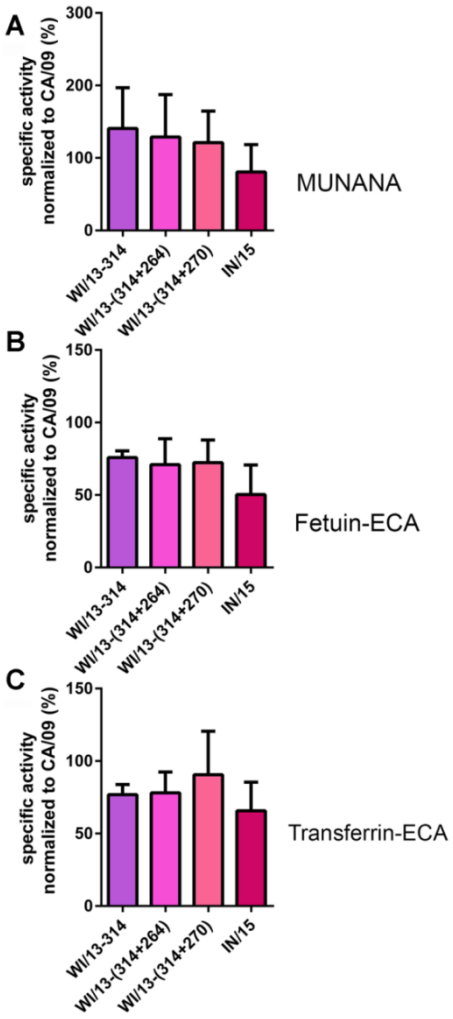
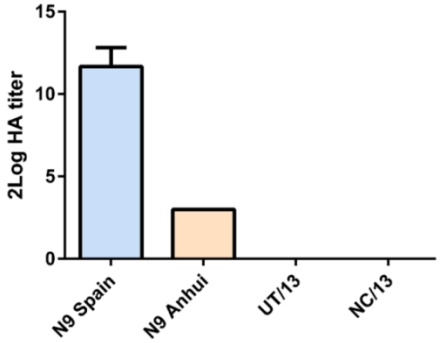
**Supplementary Fig. S1. Alignment of NA of npH1N1.** Alignment of the N1 proteins analyzed in this study. Differences between the different proteins are highlighted in green. Active site residues (including both residues that have direct interaction with the substrate and framework residues that stabilize the catalytic site) are indicated in red

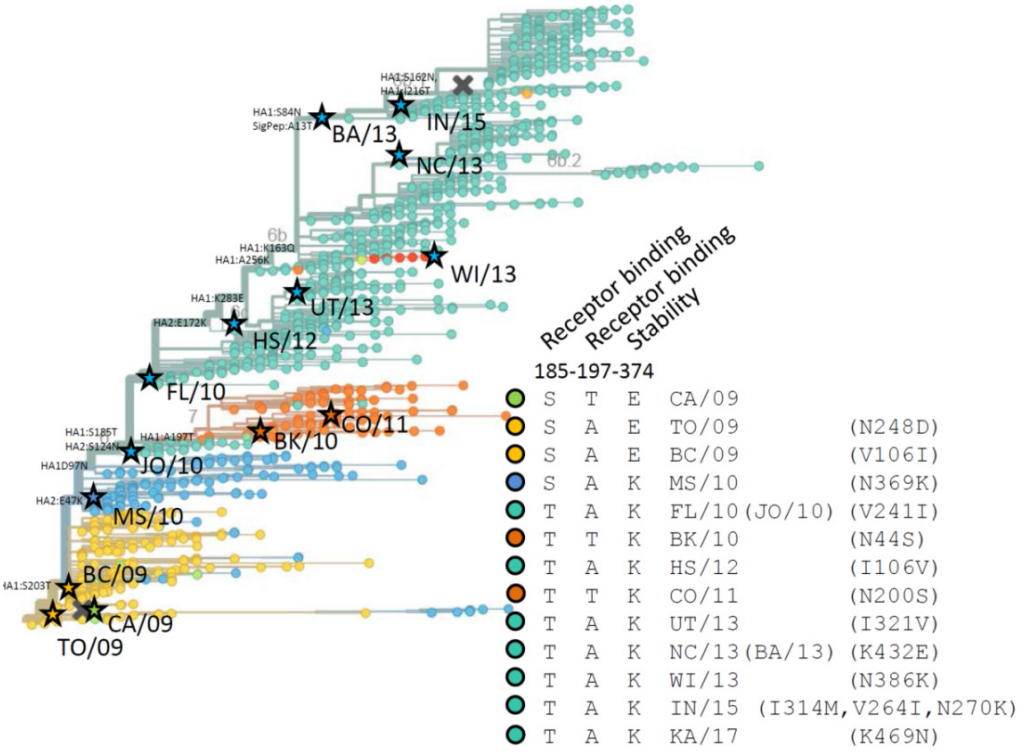
(1). Residues that form the $Ca^{2+}$ binding sites (site 1: positions 111 and 113, site 2: positions 293, 297, 324, 345 and 347, site 3: positions 376, 379, 384, and 386) are indicated in yellow (2). Residues corresponding to residues in the N9 protein that have direct interaction with SIA in the 2nd SIA-binding site are in highlighted in blue (3). The transmembrane domain is indicated in grey, while N-glycosylation sites are indicated by "Glyc". NA starting residue of the recombinant soluble NA proteins is indicated by the blue rectangle (N42).




**Supplementary Fig. S2. Specific activity of H1N1pdm09 NA proteins.** Specific activity of indicated H1N1pdm09 NA proteins was determined by MUNANA assay and by ELLA similarly as indicated in the legends to Fig. 4 and 6.




**Supplementary Fig. S3.** Hemagglutination titers of recombinant soluble NA proteins. UT/13 and NC/13 NA proteins and N9 proteins with different binding properties (4) were complexed to lumazine synthase nanoparticles displaying domain B of protein A using anti-Strep tag monoclonal antibodies. Limiting dilutions of these complexes were incubated with red blood cells in the presence of Zanamivir. The means of two independent experiments performed in duplicate are shown. Standard deviations are indicated.




**Supplementary Fig. S4. Position of H1N1pdm09 viruses analyzed in this study in HA gene phylogenetic tree.** The HA genes corresponding to the NA proteins analyzed in this study (or closest relatives) were mapped in the HA gene phylogenetic tree available via the website http://nextflu.org/h1n1pdm/12y/ (5). The tree is colored according to the identity of residues in HA at positions known to affect receptor binding (185 and 197) or pH stability/activation (374) (6, 7). Amino acid substitutions in HA along the trunk of the tree are indicated. For the FL/10 and NC/13 viruses, the position of the HA genes of a virus with identical NA protein are also indicated (JO/10 and BA/13, respectively). JO/10 and BA/13 refer to A/Johannesburg/119/2010 and A/Bangladesh/3003/2013, respectively.

## Supplemental References

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# Chapter 5

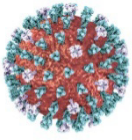
## Important role of the 2nd sialic acid-binding site for influenza A virus N1 neuraminidase activity

Meiling Dai, Erik de Vries, Frank J.M. van Kuppeveld,

Cornelis A.M. de Haan

Virology Division, Department of Infectious Diseases & Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584CL Utrecht, the Netherlands

*Manuscript in preparation*



## Abstract

The influenza A virus (IAV) neuraminidase (NA) protein plays an essential role in the release of virus particles from cells and decoy receptors. IAVs need to tune their NA enzymatic activity to match the host receptor repertoire and the activity of their hemagglutinin attachment proteins. In this study, we analyzed to what extent differences in the enzymatic activities of N1 NA proteins derived from avian, human and laboratory-adapted IAVs can be attributed to changes in the 2$^{nd}$ sialic acid (SIA)-binding site. To this end, enzymatic activities of recombinant soluble NA proteins were analyzed in detail using different monovalent and polyvalent substrates. Our results indicate that substitutions in the 2$^{nd}$ SIA binding site, that are observed in avian, human and laboratory-adapted IAVs affected cleavage of sialosides linked to glycoprotein substrates in solid-phase cleavage assays. These mutations included both SIA-contact residues and residues that do not directly interact with SIA in all three loops of the 2$^{nd}$ SIA-binding site. Generally no or much smaller effects were observed when a monovalent soluble substrate was used or when mutations were introduced at different locations in NA. The lack of detectable substrate binding via the 2$^{nd}$ SIA-binding site of N1 indicates these interactions to be of very low avidity. Our results indicate that IAVs can modify the enzymatic activity of their N1 proteins by mutation of residues at different positions in the 2$^{nd}$ SIA-binding site. Low avidity binding of N1 proteins to substrates via the 2$^{nd}$ SIA-binding site probably explains, at least in part, the observed effects.

## Introduction

Influenza A virus (IAV) particles contain a hemagglutinin (HA) protein that binds sialic acid (SIA)-containing receptors and a neuraminidase (NA) protein that cleaves SIA from sialosides. The NA protein is essential for release of virus particles from infected cells and decoy receptors (e.g. in mucus) and for preventing virion aggregation. The NA protein is also an important target for the immune response and for antiviral drugs (1-3). How IAVs tune the enzymatic activity and specificity of their NA proteins is poorly understood.

The IAV NA protein is a tetrameric, type II transmembrane protein, of which 9 subtypes are known (N1-N9). Its globular head domain is linked to the transmembrane domain via a thin stalk. Tetramerization of NA is required for enzymatic activity (4). The head domain contains the active site, which is composed of several highly conserved catalytic residues that contact SIA and structural residues that keep the catalytic residues in place (5, 6). In addition, the NA proteins of some IAVs have been shown to bind sialosides via a 2$^{nd}$ SIA binding-site that is located next to the catalytic site (7). Binding of substrates via the 2$^{nd}$ SIA-binding site enhances the catalytic activity of NA against these substrates (8, 9), although this has so far only been studied for N2 and N9 proteins. In general, most residues that contact SIA in the 2$^{nd}$ SIA-binding site are highly conserved in avian IAVs, but much less so in human IAVs (8, 10).

A functional balance between the IAVs HA and NA proteins is thought to be important for efficient replication and transmission (11-13), although it is not clear what this functional balance entails at the molecular level. The optimal balance between the HA and NA proteins is probably adapted to the receptor repertoire of a specific host (12) and needs tuning when IAVs encounter the SIA repertoire of a new host or when IAVs acquire altered HA receptor-binding properties. The HA proteins of avian and human viruses generally prefer binding to α2,3- and α2,6-linked sialosides, respectively. While NA proteins of human viruses generally prefer cleavage of α2,3-linked SIAs just as their avian counterparts, they appear relatively better in cleaving α2,6-linked SIA (14-16). In most of these studies, however, monovalent soluble substrates are used, which are not representative of the multivalent substrates found in mucus or on host cells.

The enzymatic activity of the NA protein may be modified via mutation of the catalytic site residues, although these residues are generally highly conserved (5, 6). In addition, residues have been identified that affect the enzymatic activity of NA via long range interactions (17, 18). The overall NA enzymatic activity can also be adapted via modification of NA expression levels or NA folding and oligomerization (17). Alternatively, the NA enzymatic activity may be decreased by shortening the stalk domain, thereby reducing the substrate accessibility of the NA proteins in the context of virus particles (19, 20). More recently, it was shown for N2 and N9 that the enzymatic activity against multivalent substrates may also be adapted by manipulating the binding of these substrates to NA, via the 2$^{nd}$ SIA-binding site (8, 9). N2 proteins of human pandemic viruses were shown to display decreased SIA binding resulting from mutation of a SIA-contact residue in the 2$^{nd}$ SIA binding site (8). For the N9 protein, which preferentially binds α2,3-linked sialosides, it

was shown that substitution of a non-SIA-contact residue could also affect binding and cleavage of substrates (9). To what extent other substitutions in the 2nd SIA-binding site and the 2nd SIA binding site in other NA subtypes affect/contribute to binding and cleavage of multivalent substrates is not known.

Previously, it was shown that also N1 proteins bind SIA, presumably via their 2nd SIA-binding site, using saturation-transfer difference (STD)-NMR (21). N1 proteins of two human viruses displayed much lower affinity than N1 of an avian virus. The functional consequences of these differences in SIA binding are, however, not known. Furthermore, it is not known which substitutions in the 2nd SIA-binding site are responsible for the observed differences. In the present study, we made a comparative analysis of the enzymatic activity of NA proteins derived from highly pathogenic avian IAV H5N1, new pandemic human IAV H1N1 (H1N1pdm09), and a laboratory-adapted human IAV H1N1 (WSN) by using a recombinant soluble expression approach in combination with different mono- and multivalent substrates. The avian and human N1 proteins were shown to differ significantly in their activity and specificity, with the N1 proteins derived from human viruses being relatively better in cleavage of α2,6-linked sialosides. Mutations were identified in the 2nd SIA-binding site, involving both SIA-contact and non-SIA-contact residues, that affected activity and specificity of the N1 proteins. The results indicate that IAVs can modify the enzymatic activity and/or specificity of their N1 proteins by mutation of residues at different positions in the 2nd SIA-binding site, and that binding of substrates via the 2nd SIA-binding site is likely to play an important role in N1 activity and specificity.

## Materials and Methods

### Protein expression and purification

Expression plasmids encoding the N1 ectodmain (head plus stalk domain) of A/duck/Hunan/795/2002(H5N1) (GenBank accession no. BAM85820.1; referred to as HN), A/Vietnam/1194/04(H5N1) (GenBank accession no. AAT73327; referred to as VN), A/turkey/Turkey/1/2005(H5N1) (GenBank accession no. ABQ58915.1; referred to as TK), A/Hubei/1/2010(H5N1) (GenBank accession no. AEO89183.1; referred to as HB), A/California/04/2009(H1N1) (GenBank accession no. ACP41107.1, referred to as CA/09), fused to a *Staphylothermus marinus* tetrabrachion tetramerization domain and a double Strep-tag have been described previously (9). A similar expression plasmid was constructed for the N1 protein of A/WSN/1933(H1N1) (Genbank accession no. AAA91328.1, referred to as WSN). Mutations of interest were introduced into the corresponding NA genes by using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) and confirmed by sequencing. NA proteins were expressed by transfection of HEK293T cells (ATCC) with the NA gene-containing plasmids and the proteins were purified from the cell culture supernatants, similarly as described previously (22). Quantification of the purified proteins was performed by comparative coommassie gel staining using standard BSA samples (Sigma-Aldrich) with known concentrations as a reference.

**Enzyme activity assays**

The activity of N1 proteins toward the synthetic monovalent substrate MUNANA (Sigma-Aldrich) was determined by using a fluorometric assay similarly to what was described previously (17). The activities of the NA proteins toward multivalent glycoprotein substrates fetuin and transferrin were analyzed by enzyme-linked lectin assay (ELLA) similarly as described previously (9). For both the MUNANA assay and the ELLA, the data were fitted by nonlinear regression using Prism 6.05 software (GraphPad) and the resulting curves were used to determine the specific activity (activity per amount of protein) as described previously (9, 17).

**Statistical analysis**

The mean values of at least 2 experiments performed in duplicate/triplicate with independently generated protein preparations are graphed. All statistical analyses were performed by one-way analysis of variance (ANOVA) using Tukey's multiple comparisons test (Graph Pad Prism 6.05). The level of significance was expressed as *P<0.05, ** P<0.01, *** P<0.001.

## Results and Discussion

We compared the enzymatic activity of the N1 proteins derived from different highly pathogenic H5N1 viruses (HN, VN, TK and HB). Previously, we expressed recombinant soluble tetrameric versions of these proteins and analysed their enzymatic activities using the monovalent substrate MUNANA (17). Differences in the enzymatic activities between the VN and the HN proteins could be attributed to mutations in the VN head domain that affected sialidase activity either via long range effects (position 252), via modification of a $Ca^{2+}$-binding site (position 347) or by influencing the oligomerization of the head domain (position 95). In the current study we analysed the enzymatic activity of these proteins using the glycoproteins fetuin and transferrin in addition to the synthetic substrate MUNANA. Multivalent sialylated glycoproteins better mimic the substrates that NA proteins encounter *in vivo* than the monovalent MUNANA. Furthermore, the contribution of SIA binding to NA catalytic activity cannot be observed with MUNANA, but only with multivalent substrates. Fetuin contains mono-, bi-, and tri-antennary glycans with α2,3- and α2,6-linked SIAs in 2:1 ratio (23), while transferrin contains two bi-antennary N-linked glycan chains with only α2,6-linked SIAs (24, 25). Cleavage of SIAs from fetuin and transferrin by serially diluted NA proteins was quantified by analysing the binding of lectins with different binding specificities. ECA specifically recognizes glycans containing terminal Galβ1-4GlcNAc, which generally correspond to desialylated N-linked sugars (26), while PNA binds to terminal Galβ1-3GalNAc corresponding with desialylated O-linked glycans (27). MALI and SNA specifically bind α2,3- or α2,6-linked SIAs, respectively (28, 29).

Analysis of the specific activities of the different proteins when using the MUNANA substrate indicated that the HN, TK and HB proteins cleave this substrate 2- to 3-fold more efficiently than the VN protein (Fig. 1A), which confirms and extents previously reported results (17). A remarkably different result was obtained when the specific activity was determined using the

fetuin-ECA combination (Fig. 1B). While the specific activities of the HN, VN and TK proteins reflected the specific activity as determined with MUNANA, the specific activity of the HB protein was much higher when fetuin was used as substrate. This increase was smaller with transferrin, which only contains α2,6-linked sialosides (Fig. 1C), while similarly increased specific activities for the HB protein were obtained when other lectins in combination with fetuin were used (Fig. 1D). All proteins preferred cleavage of α2,3- (determined with fetuin-MALI) over α2,6-(determined with fetuin-SNA) linked SIAs (Fig. 1D). In agreement herewith, the specific activities were higher when determined with fetuin-ECA than with transferrin-ECA. Increased cleavage of sialosides present on fetuin, but not of MUNANA, by HB is indicative of increased binding of this NA protein to fetuin via its 2nd SIA-binding site.

Sequence analysis of the H5N1 NA proteins showed that the HB protein differs from the others by mutation N369H in the 370 loop of the 2nd SIA binding site (Table 1). Although this substitution does not involve a SIA-contact residue according to available crystal structures (7, 30) (Fig. 2A and B), we analysed whether the observed increased cleavage of fetuin by the HB protein could be attributed to this mutation. To this end, we introduced the N369H

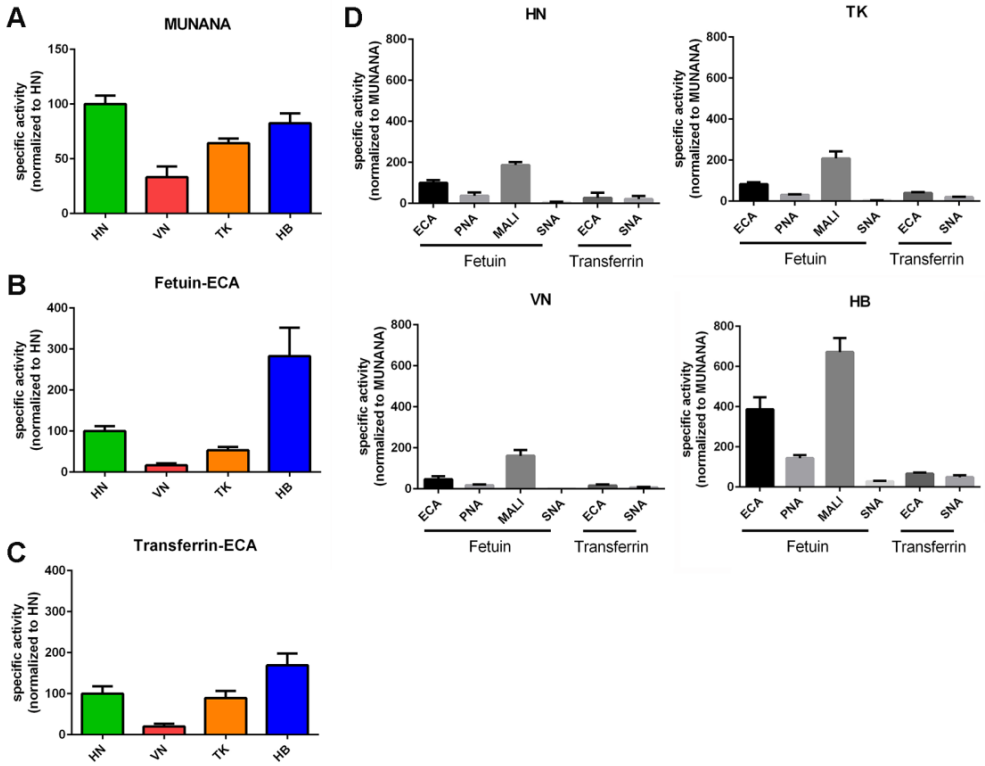



**Figure 1. Specific activity of H5N1 NA proteins using monovalent and multivalent substrates.** (A) Specific activity of indicated H5N1 NA proteins using the substrate MUNANA is graphed normalized to the specific activity of NA HN. (B-C) Specific activity of indicated NA proteins was determined by ELLA using different glycoprotein-lectin combinations, (B) Fetuin-ECA and (C) Transferrin-ECA, and graphed normalized to that of NA HN. (D) The specific activities of the H5N1 NA proteins were determined by ELLA using different glycoprotein-lectin combinations and graphed normalized to the specific activity determined with MUNANA. Means of 2-3 independent experiments performed in triplicate are shown. Standard deviations are indicated.

**Table 1. Sequence alignment of 2nd SIA-binding site of N1 proteins used in this study**

| N1 protein | 370 loop[a] | 400 loop | 430 loop |
|---|---|---|---|
| HN/TK/VN | KSTNSRS | AITDWS | RPKES |
| HB | KSTHSRS | AITDWS | RPKES |
| WSN | KSDSSRH | AMTDRS | LPEED |
| CA/09 | KSISSRN | GINEWS | RPKEN |
| Recent H1N1pdm09 | KSISSRK | GINEWS | RPEEN |
| HN N369H | KSTHSRS | AITDWS | RPKES |
| HN TN-IS | KSISSRS | AITDWS | RPKES |
| HN TN-DS | KSDSSRS | AITDWS | RPKES |
| HN S372H | KSTNSRH | AITDWS | RPKES |
| HN S372N | KSTNSRN | AITDWS | RPKES |
| HN S372K | KSTNSRK | AITDWS | RPKES |
| HN I400M | KSTNSRS | AMTDWS | RPKES |
| HN W403R | KSTNSRS | AITDRS | RPKES |
| HN K432E | KSTNSRS | AITDWS | RPEES |
| HN S434D | KSTNSRS | AITDWS | RPKED |
| Other HN mutants | KSTNSRS | AITDWS | RPKES |

[a] Positions of the residues in the 2nd SIA-binding site that have direct interaction with SIA in the N9 protein (see Fig. 2) are coloured blue. Residues that differ between the HN and other NA proteins in the 2nd SIA-binding site are coloured purple.

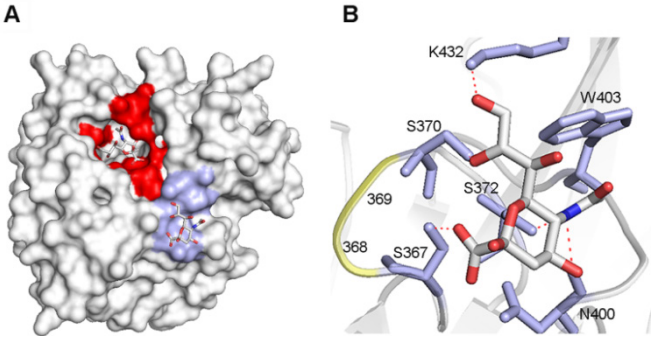



**Figure 2. Structure of the N9 2nd SIA-binding site.** (A-B) Crystal structure of N9 from A/tern/Australia/G70C/75 in complex with SIA Neu5Ac (PDB 1MWE)(7). (A) Surface representation. The NA active site and the 2nd SIA-binding site (SIA-contact residues) are coloured red and light blue, respectively. The Neu5Ac moieties in these sites are shown as sticks (oxygen in red; nitrogen in blue; carbon in gray). (B) Structure of the 2nd SIA-binding site. Neu5Ac is shown as sticks (oxygen in red; nitrogen in blue; carbon in gray). Residues in the 2nd SIA-binding site that directly contact Neu5Ac (S367, S370, S372, N400, W403 and K432) are shown in stick representation (light blue), amino acids at positions 368 and 369 that differ between HN and WSN, and CA/09, are shown in cartoon representation (yellow). Hydrogen bonds between Neu5Ac and residues in the 2nd SIA-binding site are shown as dashed red lines. Figures were made using PyMOL.

mutation in the HN protein. As expected, this mutation did not appreciably affect the ability of the resulting protein (HN-N369H) to cleave MUNANA or transferrin (Fig. 3A and C). In contrast, this mutation resulted in a higher specific activity, which was similar as that of the HB protein, when fetuin was used as substrate (Fig. 3B).

These results may be explained by the mutant HN and the HB proteins displaying increased binding to fetuin via their 2nd SIA-binding site. We were not able, however, to demonstrate binding of the N1 proteins to fetuin in a solid phase-binding assay or to chicken or human erythrocytes in a hemagglutination assay when the N1 proteins were complexed using antibodies (data not shown). The N1 proteins were also negative in the hemagglutination assays when the antibody-NA complexes were linked to lumazine synthase nanoparticles (data not shown). In this latter, more sensitive assay, the N9 protein of A/Anhui01/2013 (H7N9), previously found to be binding-negative (9), was shown to be hemagglutination positive (Chapter4/manuscript in preparation). We conclude that the increased specific activity of the HB protein against fetuin can be attributed to a single mutation in the 2nd SIA-binding site. This mutation does not involve a SIA-contact residue, but is presumed to increase the SIA-binding by this site. The smaller effect of this mutation on the cleavage of sialosides linked to transferrin suggests that it particularly enhances binding to α2,3-linked SIAs. The binding avidity of the N1 proteins was, however, below the limit of detection.

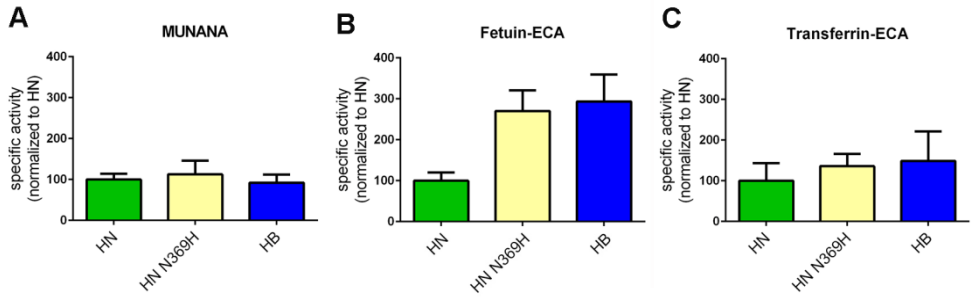



**Figure 3. Substitution N369H affects H5N1 NA enzymatic activity.** Specific activities of the indicated (mutant) H5N1 NA proteins determined by MUNANA assay (A) or by ELLA using fetuin (B) or transferrin (C) in combination with lectin ECA were graphed normalized to that of N1 HN. Means of at least two independent experiments performed in triplicate are shown for the MUNANA and ELLA assays. Standard deviations are indicated.

Next we compared the enzymatic activity and specificity of the HN protein with the N1 proteins of the H1N1pdm09 virus (CA/09) and the laboratory-adapted WSN virus (WSN) (Fig. 4A). The CA/09 and the WSN proteins displayed 2- to 3-fold lower specific activity than the HN protein when MUNANA was used. A similar (CA/09) or even larger (WSN) decrease in specific activity was observed with fetuin-ECA (Fig. 4B). The specific activities of CA/09 and WSN NA proteins were relatively higher with transferrin as substrate (Fig. 4C-D), with the CA/09 protein even displaying a similar specific activity as the HN protein. These results may reflect decreased binding to and/or cleavage of α2,3-linked SIAs that are present on fetuin, but not transferrin, while the WSN and CA/09 proteins may also be relatively better in binding and/or cleavage of sialosides linked to transferrin. When the different specific activities are graphed relative to the MUNANA specific activity (Fig. 4D), it is clear that particularly the WSN protein displays a decreased ability to cleave α2,3-linked SIAs (determined with fetuin-MALI). The CA/09 protein was relatively better in cleavage of α2,6-linked SIAs than the HN protein, which was most apparent when transferrin was used as substrate.

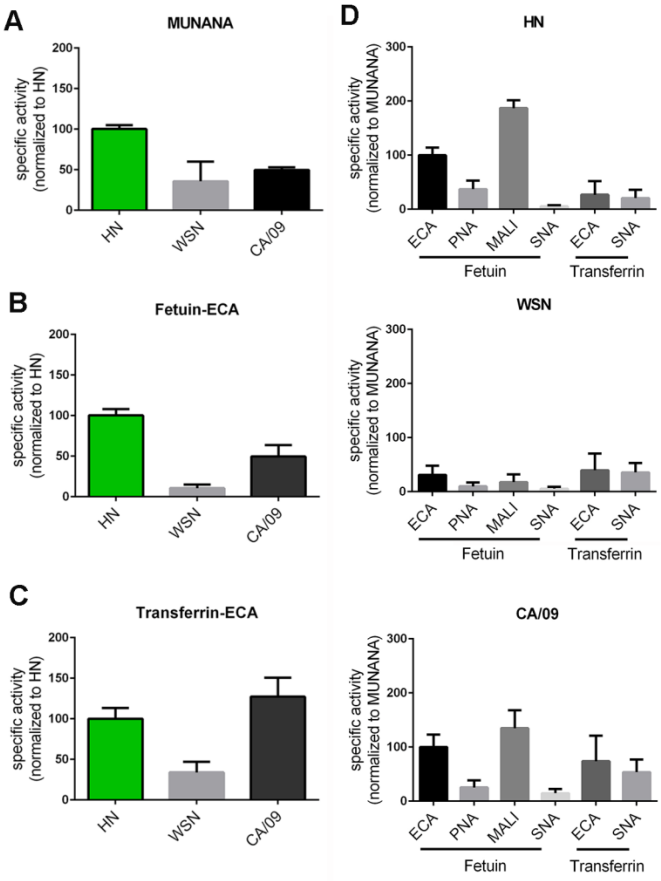



**Figure 4. Enzymatic activity of HN, WSN and CA/09 NA proteins using monovalent and multivalent substrates.** Specific activities of indicated N1 proteins determined by MUNANA assay (A) or by ELLA using fetuin (B) and transferrin (C) in combination with lectin ECA were graphed normalized to that of N1 HN. (D) Substrate specificities of the indicated N1 proteins were determined by ELLA using different glycoprotein-lectin combinations and graphed normalized to the specific activity determined with MUNANA. Means of at least two independent experiments performed in triplicate are shown for the MUNANA and ELLA assays. Standard deviations are indicated.

In the 370 loop, HN NA differs from WSN NA at positions 368/369 (TN vs DS), while again other residues (IS) are found in the CA/09 protein at these positions (Table 1). In addition, S372, which is in contrast to residues at position 368/369 a SIA-contact residue (Fig. 2), is replaced with H in the WSN and with N in CA/09 protein. In more recent H1N1pdm09 viruses a K is present at this position. In the 400 and 430 loops, positions that are SIA-contact residues in N9 (Fig. 2) are substituted. In the 400 loop, residues I400 and W403 of HN are replaced in WSN NA with M and R, respectively. In the 430 loop, the K432E substitution is observed in WSN NA and in the NA proteins of recent H1N1pdm09 viruses.

All the substitutions mentioned above were introduced in the background of the HN protein (Table 1) and the enzymatic activity of the resulting proteins was investigated using MUNANA, fetuin and transferrin as substrates. Mutation of the TN residues at position 368 and 369 to IS or DS, had only limited effect on the cleavage of MUNANA (Fig. 5A). While

the TN to IS substitutions had a small positive effect on the cleavage of both fetuin and transferrin, the TN to DS substitutions had a large negative effect on the cleavage of sialosides linked to fetuin. This negative effect appeared smaller when transferrin was used (Fig. 5B and C). As a result, the HN TN-DS protein was relatively better in cleavage of α2,6-linked SIAs linked to transferrin (transferrin-ECA/SNA) than of α2,3-linked SIAs linked to fetuin (fetuin-ECA/MALI) compared to the wild type HN and the HN TN-IS proteins (Fig. 6). These results indicate that, similar to the N369H mutation, substitutions of residues in the 2nd SIA-binding site not directly contacting SIA can affect cleavage of glycoprotein-linked sialosides. Most likely, this is not explained by these mutations affecting cleavage *per se* (as determined with MUNANA), but rather because they affect the interaction of the NA protein with multivalent substrates via the 2nd SIA-binding site.

Mutation of the SIA-contact residue at position 372 into N, K or H (found in the H1N1pdm09 or WSN N1 proteins) had no significant negative effects on the cleavage of MUNANA (Fig. 5A), while cleavage of both glycoproteins was consistently negatively affected (Fig. 5B and C). The S372H mutation present in WSN NA had the largest negative effect. Previously, we

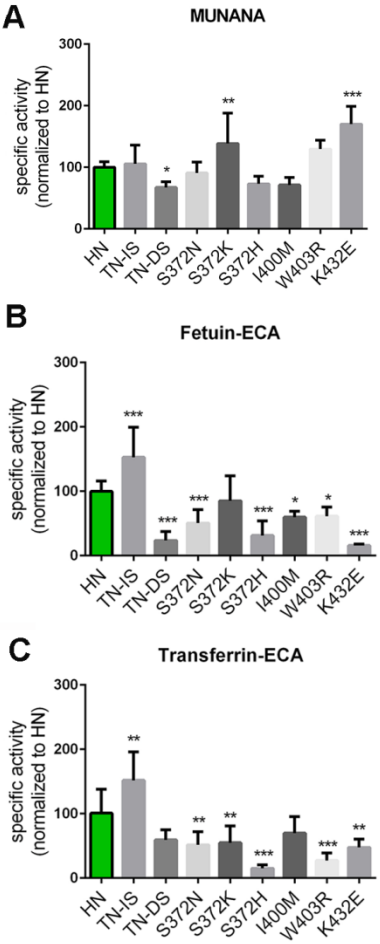



**Figure 5. Effect of substitutions in the 2nd SIA-binding site on NA enzymatic activity.** Specific activities of wild type and mutant HN determined by MUNANA assay (A) or by ELLA using fetuin (B) and transferrin (C) in combination with lectin ECA were graphed normalized to that of wild type HN. Means of at least two independent experiments performed in triplicate are shown. Standard deviations are indicated. The level of significance was expressed as *P<0.05, ** P<0.01, *** P<0.001.

found that a N to K mutation at this position in a H1N1pdm09 NA protein had a positive effect on the cleavage of fetuin-linked glycans, but not of MUNANA or transferrin-linked glycans (chapter4/manuscript in preparation). In the background of the HN protein, however, the presence of a N or K at this position did not appear to result in significant differences. Apparently, the effect of mutations in the 2nd SIA-binding site may be background dependent.

Substitution of residues in the 400-loop either had a small negative effect on cleavage of all substrates including MUNANA (I400M) or specifically had a negative effect on the cleavage of sialosides linked to fetuin or transferrin (W403R) (Fig. 5A and B). While a N at position 400 was also shown to be a SIA-contact residue in the N9 protein (Fig. 2C), this interaction is probably already lost by the presence of an I in the HN protein, thereby explaining why the I400M mutation did not result in a specific negative affect on the cleavage of glycoprotein-linked glycans. The negative effect of the substitution at position 403 is in agreement with the W being a SIA-contact residue (Fig. 2C). Interestingly, the negative effect of this latter substitution on the cleavage of transferrin-linked sialosides appeared larger than that of fetuin-linked sialosides, suggesting that N1 proteins may also bind to some extent to α2,6-linked SIAs and that mutations in the 2nd SIA-binding site may differentially affect binding to α2,3- or α2,6-linked SIAs.

The largest negative effect on the cleavage of fetuin-linked sialosides was observed when the SIA-contact residue in the 430-loop (i.e. K432) was substituted by E as observed in WSN NA and in more recent H1N1pdm09 viruses (Fig. 5B). This substitution, which even resulted in increased cleavage of MUNANA (Fig. 5A), also decreased cleavage of transferrin-linked glycans (Fig. 5C), but to a smaller extent than the cleavage of fetuin-linked sialosides. In agreement herewith, the HN K432E NA protein displayed a much less pronounced preference for the cleavage of fetuin- over transferrin-linked sialosides (Fig. 6). The large negative effect of the K432E mutation particularly on the cleavage of fetuin-linked glycans was previously also observed in the background of the npH1N1 NA protein (Chapter4/manuscript in preparation).

To determine how specific the observed effects are of the substitutions in the 2$^{nd}$ SIA-binding site, several other mutations found in WSN NA compared to the HN protein, at positions that are also substituted in the CA/09 protein, were introduced in the HN protein and the specific activities of the resulting proteins were determined using MUNANA as well as fetuin and transferrin. Almost none of these single and double substitutions (V99I, H155Y, TS186/187MG, M257I, A284T, Y347N, S389R and S434D; N2 numbering) appreciably affected the specific activities of the mutant HN proteins regardless of the substrates used, with the exception of the H155Y and the S434D mutations (Fig. 7). The H155Y substitution resulted in reduced specific activity against all substrates (Fig. 7A-C). This substitution was previously linked to increased low pH stability of NA and was suggested to be involved in stabilization of the homotetrameric structure as it is located near the protomer interface (31). In contrast, the S434D mutation reduced the cleavage of sialosides linked to fetuin and transferrin (Fig. 7B and C), while the specific activity against MUNANA was not affected (Fig.

7A). Of note, the S434D mutation is located only two residues downstream of the K432 SIA-contact residue (Table 1) and probably affects substrate binding of the 2nd SIA-binding site.

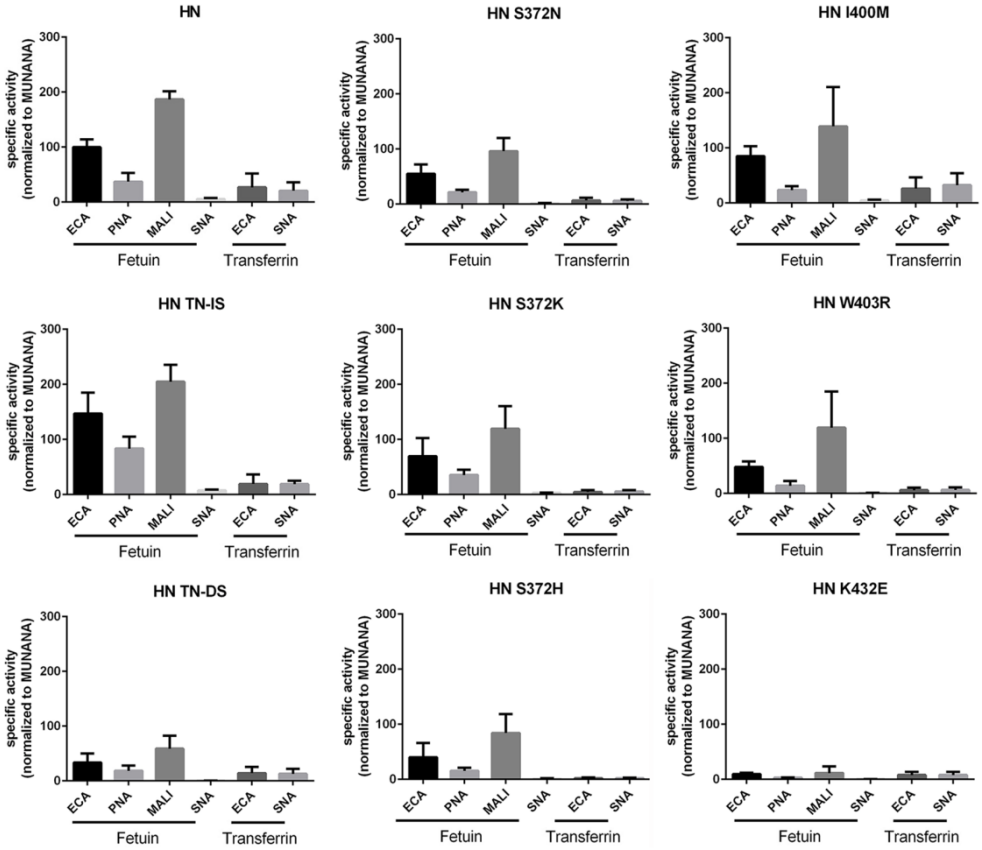



**Figure 6. Substrate specificity of mutant HN NA proteins.** Substrate specificities of indicated mutant HN proteins (same as shown in Fig. 5) were determined by ELLA using different glycoprotein-lectin combinations and graphed normalized to the specific activity determined with MUNANA. Means of at least two independent experiments performed in triplicate are shown. Standard deviations are indicated.

Our results indicate that IAVs can modulate the enzymatic activity of their NA proteins by mutation of the 2nd SIA-binding site in these proteins. Most of these mutations only had a limited effect on NA activity *per se* as measured with the monovalent MUNANA substrate. The effects were often much larger, however, when analysed with multivalent glycoproteins in solid phase cleavage assays, which probably much better mimic the substrates found in mucus or on the cell surface. Although none of the N1 proteins analysed here was positive in SIA-binding assays, our results suggests that low avidity binding of N1 to the glycoprotein substrates explains, at least in part, the observed effects. The observation that also cleavage of sialosides linked to transferrin can be dramatically affected by mutation of the 2nd SIA-binding site suggests that the N1 proteins may also be able to bind to α2,6-linked

sialosides via this site. Previously, we showed that N9 proteins only bind to α2,3-linked sialosides via their 2nd SIA-binding site (9). In contrast, the N2 protein was shown to hemadsorb erythrocytes containing either type of sialosides (8). Altogether, these results indicate that NA proteins of different subtypes differ in their SIA-binding specificities, although a head-to-head comparison is needed to support this hypothesis.

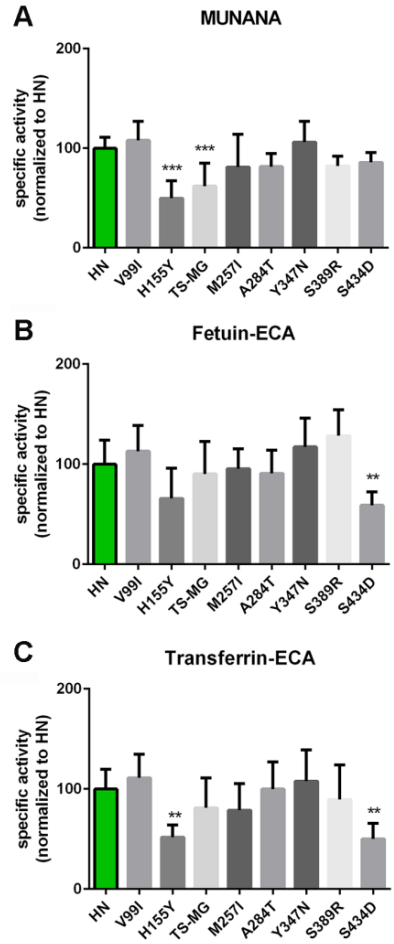



**Figure 7. Effect of substitutions outside the 2nd SIA-binding site on NA enzymatic activity.** Specific activities of wild type and mutant HN determined by MUNANA assay (A) or by ELLA using fetuin (B) or transferrin (C) in combination with lectin ECA were graphed normalized to that of wild type HN. Means of at least two independent experiments performed in triplicate are shown. Standard deviations are indicated. The level of significance was expressed as *P<0.05, ** P<0.01, *** P<0.001.

Different mutations in the 2nd SIA-binding site were shown to different effects on NA cleavage activity and specificity. While some mutations particularly affected cleavage of fetuin-linked glycans (N369H and TN368/369DS in the 370 loop, and K432E in the 430 loop), other mutations appeared to similarly affect cleavage of fetuin- and transferrin-linked sialosides (S372N/K/H and S434D) or even to have a larger effect on the cleavage of sialosides linked to transferrin (W403R). Furthermore, NA activity can not only be modified by mutation of SIA-contact residues in the 370 and 400 loops (8) (this study), but also by substitution of non-SIA-contact residues in the 370 loop (this study), the 400 loop (9) and the 430 loop (this study). Surprisingly, substitution of non-SIA-contact residues in the 370

loop (position 368/369) even resulted in larger effects than mutation of a contact residue (position 372) in this loop. The most dramatic effects, however, were observed after substitution of the K432 residue in the 430 loop. Strikingly, while the residue at position 432 appears to be very important for binding and enzymatic activity, it is less conserved than the other SIA-contact residues in the 2nd SIA-binding site of the NA proteins of avian IAVs (30). Collectively, these results demonstrate the large arsenal available to IAVs to modulate the activity of their NA proteins via the 2nd SIA-binding site, which is a thus far underappreciated feature.

Substitutions in the 2nd SIA-binding site, which are observed in all pandemic human viruses (8, 10), may be selected as they tune the balance between the (novel) receptor repertoire of humans, the (altered) receptor binding-properties of HA and the enzymatic activity of NA. Alternatively, these mutations may be selected as a result of immune pressure on the surface-exposed 2nd SIA-binding site. The selective advantage of being able to bind to avian-type receptors via the 2nd SIA-binding site may be lost for human viruses carrying HA proteins that only bind to human-type receptors. The high conservation of the SIA-contact residues in the 2nd SIA-binding site of NA proteins of avian viruses suggests an important role for this binding site for virus replication and/or transmission of viruses carrying HA proteins preferring binding to avian-type receptors. However, the importance of the 2nd SIA-binding site for replication and transmission *in vivo* remains to be established. Mutation of the 2nd SIA-binding site in a N2 protein, which reduced NA hemadsorption activity, resulted in 10-fold less efficient replication in chicken embryo fibroblasts, but not in ducks compared to the control virus (32). Other studies on the importance of substrate binding via the 2nd SIA-binding for virus replication and transmission are lacking. In future studies, we aim to address the links between substrate binding of the 2nd SIA-binding site, for example as determined with STD-NMR (21), NA enzymatic activity, and virus replication *in vitro* and *in vivo*.

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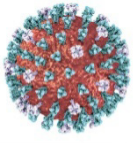
# Chapter 6

## Summarizing discussion



Influenza A virus (IAV) infections constantly pose great threats to public health and cause large economic burdens. Seasonal influenza viruses circulate and cause influenza epidemics in humans every year. Currently, influenza A(H1N1) and A(H3N2) are the circulating seasonal influenza A virus subtypes. The A(H1N1) virus is derived from virus that caused the 2009 influenza pandemic and referred to as H1N1pdm09. Additionally, viruses derived from two lineages of influenza B viruses, namely Victoria lineage and Yamagata lineage, are also circulating as seasonal influenza viruses.

There are two important glycoproteins in the IAV virus membrane, hemagglutinin (HA) and neuraminidase (NA), both of which recognize sialic acids (SIAs). Initiation of virus infection involves binding of multiple HAs to SIAs on carbohydrate side chains of cell surface glycoproteins and glycolipids (1-3). As the second most abundant viral glycoprotein, NA is a receptor-destroying enzyme that removes SIAs from infected cell surfaces upon virus replication so that newly synthesized viruses are released from these cells and can infect new cells (4). The sialidase activity also prevents aggregation of newly synthesized virions by removing SIAs from their own glycoproteins. Although IAVs can infect and complete one replication cycle in the absence of NA activity, progeny viruses will remain aggregated on the host cell surface and fail to spread (4). The HA protein also binds to SIAs on decoy receptors, that are for example abundantly present on mucins. Irreversible binding of influenza virions to mucus will prevent them from accessing the underlying epithelium (5). Therefore, the enzymatic activity of NA is also thought to facilitate virus infection by enabling penetration of the respiratory tract mucus and by removing decoy receptors from the glycocalyx of epithelial cells during the early stage of viral infection (5, 6). The importance of NA enzymatic activity for replication and transmissibility in vivo is demonstrated by numerous studies in which NA inhibitors are used or antibodies that inhibit NA activity (7-10). The important role of NA in transmission is also demonstrated by studies with the H1N1pdm09 virus, showing that increased NA activity contributed to the release of virus particles into the air and aerosol transmission (11, 12). After the discontinuation of the use of M2 channel inhibitors as antiviral drugs, inhibitors targeting the NA protein are the sole IAV antiviral drugs currently used in the clinic. Inclusion of NA protein in vaccine preparations has been shown to result in increased protection against influenza (13, 14). Nevertheless, current inactivated vaccines on the market only monitor and focus on the amounts of HA present (15-17).

Despite the important role of NA in virus infection, replication and transmission, it appears that this protein is relatively understudied compared to its HA counterpart. While HA receptor-binding avidity and specificity have been studied in detail, much less is known about the molecular determinants that mediate the specificity and activity of IAV NA proteins. The overall aim of this thesis was to unravel to what extent and how IAV NA protein activity is modulated during virus evolution. The preceding chapters of this thesis described our investigations in the NA functionality, mainly enzymatic activity, by using a recombinant soluble protein approach. In this last chapter, the main findings will be summarized and discussed in a broader perspective. This chapter is divided over three main topics involving 1) investigation of NA enzyme activity and substrate specificity, 2) mechanisms of tuning IAV NA activity, and 3) HA/NA balance and virus evolution.

## 1) Investigation of NA enzyme activity and substrate specificity

The receptor-destroying activity of influenza virus was first observed in the 1940s by Hirst (18), who found that IAV was able to hemagglutinate red blood cells (RBC) at 4℃, but this hemagglutination was reversible by heating up to 37℃, indicating that some enzyme was involved for this process. The substrate of this enzyme was identified as N-acetylneuraminic acid by Gottschalk, thus establishing this enzyme as a neuraminidase (sialidase) (19). The first NA crystal structures were successfully solved for NA protein head domains released and purified from virus preparations (20, 21). The X-ray structural studies identified the catalytic and framework residues within the active site, which are highly conserved across all influenza A and B viruses. NA structural information and understanding of NA catalytic mechanism provided valuable opportunities for the discovery and development of NA inhibitors, such as zanamivir (22-25) and oseltamivir (26, 27). Although the enzymatic mechanism of NA has been studied in detail using synthetic glycans and for example been shown to react with retention of configuration (27, 28), enzymatic parameters of NA still remain largely unknown, especially when using more natural substrates like glycoproteins.

***Recombinant soluble NA proteins***

So far most work about NA catalytic parameters has been done with whole virus preparations or (incompletely) purified NA head domains. However, viral NAs can become intrinsically unstable once they are cleaved from the membrane-anchored region (29) and rapidly lose activity. This will obviously impede subsequent analysis of NA enzymatic activity. Therefore, recombinant soluble NA proteins have been generated not only for structure determination but also for the investigation of enzyme activity by us and other groups (30-34). The use of recombinant soluble glycoproteins provides several advantages when compared with NA purification from live virus. Importantly, there is no need to cultivate potentially dangerous viruses. The recombinant proteins can be immediately produced when the NA sequence is known and available. Moreover, the soluble glycoproteins can be easily purified when compared to their membrane-anchored counterparts and then used for downstream assays. Care should be taken however that the recombinant soluble NA proteins mimic their full length counterparts as much as possible. Therefore, in **Chapter 2** and **Chapter 3**, we analyzed the optimal recombinant protein expression approach with respect to the tetramerization domain used and the length of the NA ectodomain-coding sequence. Our results indicate that the particular oligomerization domain used did not affect the Km value of the resulting NA proteins, but clearly affected the specific enzymatic activity (**Chapter 2** and **3**). NA ectodomains extended with a Tetrabrachion domain, which forms a nearly parallel four-helix bundle, better mimicked the enzymatic properties of full-length proteins than when coiled-coil tetramerization domains were used, which probably distort the stalk and head domain. This was particularly observed when an NA ectodomain was used, which displayed a low intrinsic ability to oligomerize and which included part of the stalk domain (**Chapter 2**). Nevertheless, inclusion of the stalk domain proved to be important for high level expression of several NA proteins, including N9 (**Chapter 3**) and N1 of H1N1pdm09 virus (unpublished results). The stalk domain probably plays an important role in folding and oligomerization of the NA ectodomain and thereby in transport through and secretion from cells of soluble NA proteins (35). In this respect, it is important to

mention that the stalk domain may contain cysteine residues involved in inter-protomer disulfide bridging as well as several N-linked glycans that probably affect folding and oligomerization.

***NA cleavage assays***

At present, the substrate most widely used for detecting NA enzymatic activity and inhibition thereof is MUNANA, initially described by Potier *et al.* (36). We also used this substrate in **Chapters 2-5.** This monovalent substrate is very suitable for determining NA activity in drug inhibition assays or to determine NA kinetic parameters such as specific activity and Km values. Because of its small size, it is less suitable to determine antibody inhibition titers, while in addition MUNANA is not useful to differentiate between preference for α2,3- or α2,6-linked SIAs. However, the N-glycolyl derivative of MUNANA used in **Chapter 4** allowed us to identify a residue in the NA protein of H1N1pdm09 (position 432), mutation of which resulted in reduced cleavage of this substrate. N-glycolyl derivatives of SIA (Neu5Gc) are found in most mammals, including swine, mice and monkeys, but not in humans and ferrets (37). In most birds species analyzed to date, Neu5Gc is either not detectable or only found in trace amounts (38). Cleavage of Neu5Gc-containing substrates is presumably of importance for swine viruses, including the precursor of the H1N1pdm09 virus.

NA enzyme activity has also been extensively investigated with several monovalent substrates, including (fluorogenic forms of) 3'SiaLac (Neu5Acα2,3Galβ1-4Glc); 6'SiaLac (Neu5Acα2,6Galβ1-4Glc); 3'SiaLacNAc (Neu5Acα2,3Galβ1-4GlcNAc) and 6'SiaLacNAc (Neu5Acα2,6Galβ1-4GlcNAc) (34, 39, 40). Generally, those studies revealed that the NA proteins showed preference for the α2,3-linkage over the α2,6-linkage of SIA either with purified NA heads (41), virus like particles (VLPs) containing NA (40) or whole virus (39). Similar results were obtained in **Chapters 3-5**, when analyzing the preference of cleavage of N9 and N1 proteins derived from avian, human or laboratory-adapted IAVs. NA proteins derived from the latter two types of viruses appeared, however, relatively better in cleavage of α2,6-linked sialosides. However, when analyzing the substrate specificity of the NA proteins in **Chapters 3-5**, we did not use monovalent substrates, but we rather used solid phase cleavage assays in which the SIA-receptor containing structures are presented in a multivalent form.

Generally, a cell surface is densely covered with various carbohydrates containing terminal SIAs that are usually attached to other biomolecules like proteins or lipids. Thus, SIAs are always presented in a multivalent configuration, since multiple glycans may be attached to a biomolecule or a multimeric complex thereof and because a large number of SIA-receptor containing molecules are presented on the cell surface. Also soluble decoy receptor proteins such as mucins contain a large number of sialylated glycans and thus often present receptors in a multivalent form. Influenza viruses adhere to host cells and decoy receptors through multivalent interactions of HA proteins with SIAs. Although the individual interactions between HA proteins and carbohydrates are weak (low affinity), the multiple simultaneous interactions lead to high avidity. IAV particles are released from non-functional, multivalent sialosides by the action of their NA proteins. Analysis of NA enzymatic activity using multivalent presentation of substrates therefore probably provides better insights into

its characteristics then when only monovalent substrates are used. Indeed, for several proteins analyzed in **Chapters 3-5** differences in specific activity could only be observed when using solid phase cleavage assays in which fetuin carrying multiple glycans was used as substrate. Both for N9 (**Chapter 3**) and N1 proteins derived from human (**Chapter 4**) or avian (**Chapter 5**) IAVs, these differences could be attributed to substitutions in the 2nd SIA-binding site, which for the N9 protein resulted in detectable differences in sialoside binding via this site.

Cleavage of SIAs from fetuin and transferrin was analyzed using lectins with different specificities (**Chapters 3-5**). These enzyme linked lectin assays (ELLAs) (42-44) can thus provide insight into the substrate specificity of the NA proteins analyzed. In **Chapter 3**, we also analyzed the substrate specificity of N9 proteins using a glycan array-based neuraminidase assay (45, 46). Also on glycan arrays, the synthetic glycans are presented in a multivalent form since they are coated to the array surface. The (dramatically) preferred cleavage of α2,3- over α2,6-linked SIAs by the N9 proteins as determined by the fetuin- and transferrin-based ELLAs was confirmed by the glycan array analysis of NA cleavage. Moreover, the glycan array assay provided insight into NA cleavage fine specificity, with both N9 proteins preferentially cleaving multi-antennary glycans carrying multiple LacNAc repeats. The importance of such glycans for IAV replication *in vivo* remains to be established. The preference for cleavage of these glycans may be explained by the increased multivalency resulting from the presentation of SIAs on multi-antennary glycans and by the increased accessibility of these SIAs on the elongated glycan chains. Of note, the preferred cleavage of these glycans correlated well with increased binding of these glycans by N9 via the 2nd SIA binding site. While the glycan array analysis provided insight into the receptor fine specificity, it may be less suitable than the glycoprotein-based ELLA to determine the NA specific activity as many different amounts of NA and several replicates are needed to reliably determine these values.

## 2) Mechanisms of tuning of IAV NA activity

***NA expression, folding and oligomerization***

The NA protein plays an important role in the IAV life cycle and transmission and is a major target of the immune system. Its activity probably needs to be balanced with the receptor-binding properties of the HA protein. It therefore makes sense that IAVs NA protein functionality, especially enzyme activity, may be modulated via various mechanisms during IAV evolution. Our studies provided insight into several mechanisms by which NA enzymatic activity can be modulated. For example, in **Chapter 4**, we identified several mutations in the NA protein of H1N1pdm09 viruses that affected NA protein expression levels, particularly the V241I and N270K substitutions that significantly increased or decreased expression and secretion of recombinant soluble NA proteins, respectively (Supporting information contains an alignment of the NA proteins analyzed in this thesis including the N2 and H1N1pdm09 NA numbering of the residues, which is used in Chapters 2,3,5 and 4, respectively). In agreement herewith, the V241I substitution was also shown to enhance cell surface expression of full length NA proteins (47). We did not study in detail the mechanism by which these mutations affect NA protein expression levels. Possibly, these mutations affect

the translation, folding and oligomerization rates of the NA proteins. Folding and oligomerization is a prerequisite for transport of these proteins through the secretory pathway. Of note, IAVs might also modify the NA protein expression levels by tuning the replication and/or transcription levels of the NA protein encoding gene segments (48).

NA is present at the cell and virion surface as a tetramer and it loses enzymatic activity when it is presented as monomer or dimer (49). It is speculated that intracellular monomeric NA probably lacks a functionally active conformation, which can only be achieved in the tetrameric oligomer (50). Therefore, residues that affect NA folding or oligomerization can also change sialidase activity by affecting the formation of the active conformation. In **Chapter 2**, we identified a residue (position 95) that is of importance for oligomerization of the NA head domain. Substitutions at position 95 were found to have a significant effect on the oligomerization capacity of the NA head domain of avian H5N1 virus and on the specific activity of the resulting protein, but not on the substrate affinity. These effects were most prominent, when recombinant soluble NA proteins were used lacking an N-terminal oligomerization domain or were fused to the GCN4 coiled coil oligomerization domain linked by the stalk domain. Mutations at position 95 resulted in smaller differences in enzyme activity in the context of the full-length protein. This is possibly achieved by the transmembrane domain promoting the proper assembly of the stalk region and oligomerization of the head domain (51). The importance of the stalk domain for tetramerization was also shown by Zanin and coworkers (52), who identified a mutation in the NA stalk (position 66) of H1N1pdm09-lineage swine IAV, which negatively affected NA protein tetramerization and enzymatic activity.

***Virion incorporation and substrate accessibility***

Changes in NA folding and oligomerization rates and/or in NA cell surface expression levels likely also affect the incorporation of NA into virus particles. Alternatively, incorporation of NA into virus particles might also be modified via other mechanisms. Hensley and coworkers showed that antibody-driven antigenic variation in HA led to the acquisition of compensatory mutations in NA that not only decreased NA activity per virion by directly altering the enzymatic activity of NA, but also by reducing NA incorporation into virions (53). It was not studied however, whether these mutations resulted in decreased virion incorporation directly or by altering NA protein expression levels.

NA activity in cells or virions can be modified *in cis* by mutations in NA itself (53, 54), but also *in trans* by (alterations in) other gene segments and their protein products, such as NP, M1 and the viral polymerase proteins PB2 and PB1. For instance, Christopher and coworkers found an adaptive mutation in the NP segment that selectively decreases both the expression and packaging of the NA gene segment. This decreased segment packaging was associated with increased *in vivo* fitness and contact transmission (55, 56). Moreover, it is proposed that the pleomorphic nature of influenza virus particles affects neuraminidase functioning *in vivo*. For example, recombinant PR8 mutant viruses containing mutations within M1 that resulted in filamentous particles displayed higher neuraminidase activities than the spherical wild-type PR8 virus (57). The important role of the M segment on NA activity and viral fitness has also been discovered by others, who found that introduction of

the pandemic M segment alone into PR8 virus by reverse genetics resulted in an increase in the neuraminidase activity of the PR8-based viruses, which was accompanied with a potent effect on transmissibility (58).

NA activity in the virion context may also be altered by changing the accessibility of substrates to NA. Upon adaptation of avian IAVs from waterfowl to poultry, NA stalk deletions of up to 27 amino acids have been observed. These NA deletions are regarded as a chicken adaptive feature and have been shown to be involved in IAV host range, cell tropism, and increased virulence (59-62). The shortened NA stalk was associated with reduced enzyme activity as measured by the rate of virus elution from red blood cells (RBCs). The NA enzymatic activity was not affected when using the small soluble MUNANA substrate (63). The reduction in RBC elution was suggested because of reduced accessibility of the NA active site to SIAs present at the cell surface (63, 64). Although it has been reported that NA assembly is not dramatically affected by deletions in the stalk, a minimal stalk length of 10 amino acids is needed for the head domain to fold (51). The small (5 amino acid) stalk deletion in the novel N9 protein studied in **Chapter 3** had no impact on virus infectivity or replication in vitro or in vivo compared to that of a virus with a full-length stalk, suggesting that the accessibility to substrates was not compromised for this N9 protein (65). Only when a larger deletion (19 to 20 amino acids) was introduced in the H7N9 virus, enhanced virulence in mice was observed (65). How the N9 and N1 (61, 63) stalk lengths are affecting virulence is not known.

***NA enzymatic cleavage***

Enzymatic activity may also be changed by directly modifying the capacity of the NA proteins to cleave substrates. This may be achieved by different kinds of mutations. Enzyme activity can be affected by changes in the NA active site involving either catalytic or framework residues. Generally, NA active site residues are highly conserved among all the NAs, except for inhibitor-resistant NA proteins, which may contain substitutions in highly conserved regions in the NA active site. For example, oseltamivir resistant clinical virus isolates normally carry one or more substitutions of framework residues 119, 198, 274, 294, or even of the catalytic residue at position 292 in the NA active site (66-69). These mutations generally compromise NA enzymatic activity and cause growth disadvantages for the mutant viruses (66-69). These detrimental effects may be compensated by mutations at other positions in NA that for example increase cell surface expression or enzymatic activity to restore viral fitness (47, 69, 70). NA enzymatic activity may also be affected by residues that are not located in the NA active site. In **Chapter 2 and 4**, we identified several residues outside the NA active site (residues 252 and 347 in H5N1 NA, and residue 432 in H1N1pdm09 NA) that affected the MUNANA Km value. The residue at position 432 in H1N1pdm09 may affect the conformation of the active site and thereby the Km value (and the ability of NA to cleave at low pH) as it is located immediately adjacent to the NA active site. The residue at position 252 may indirectly modify the active site via hydrogen bonding with the residue at position 274 (71), which may also explain the importance of the 252 residue in sensitivity of the NA protein towards oseltamivir (71, 72). The effect of the substitution at position 347 on the Km value has also been observed by others (73) and may

be explained by this residue being part of a calcium binding site next to the active site (74). Binding of calcium ions has previously been shown to be important for NA activity and temperature stability (75, 76). Besides the calcium binding site next to the active site, NA proteins also contain a calcium binding site at the four-fold symmetry axis. The residue at position 106 in the H1N1pdm09 protein was shown to be important for calcium-dependent stability of NA, but did not affect the Km value of the NA protein (**Chapter 4**). Mutations that affected the Km value of NA for MUNANA, did not affect the specific activity of NA for this substrate, however.

***2nd SIA-binding site***

In **Chapter 3, 4,** and **5**, we show that the enzymatic cleavage by NA can also be tuned by modification of its 2nd SIA-binding site. The 2nd SIA-binding site is a shallow pocket composed by six amino acids S367, S370, S372, N400, W403, and K432 (77, 78), located in three loops, which as the name implies is capable of binding SIAs. We consistently found that substitutions located adjacent to or in the 2nd SIA binding site can significantly affect enzymatic on the multivalent glycoprotein fetuin in solid phase cleavage assays, while cleavage of MUNANA was often only minimally affected. In **Chapter 3**, we observed that the novel N9 protein displayed reduced binding to and enzymatic activity on multivalent substrates when compared to avian N9 protein from 2008. Furthermore, we identified a single substitution in N9 (T401A) located in the 400-loop of the 2nd SIA-binding site, adjacent to a SIA contact residue, to be responsible for the reduced binding to and enzymatic activity on multivalent substrates. Also in **Chapter 5,** non-SIA-contact residues were identified in the 370 or 430 loops of the 2nd SIA-binding site of N1, which are responsible for increased cleavage of sialosides linked to fetuin. Apparently, avian IAVs can modify the enzymatic activity of their NA proteins via mutation of non-SIA-contact residues, a feature that had not been recognized so far. In **Chapter 4** and **5** we showed that also mutations of SIA-contact residues (369/372, 403 and 432) can affect the catalytic activity for multivalent, but not monovalent substrates. This was even observed for human IAVs (H1N1pdm09) that already contained a mutated 2nd SIA binding site. Apparently, the 2nd SIA-binding of this virus may contain residual activity even when a putative SIA contact residue is already mutated. Both in **Chapter 4** and **5** we showed an important role for the K432 residue in the 430 loop for enzymatic activity of NA. Interestingly, while the residue at position 432 appears to be very important for enzymatic activity, it is less conserved than the other SIA-contact residues in the 2nd SIA-binding site of the NA proteins of avian IAVs (79). These results indicate that IAVs have a many options to tune their NA activity via mutation of the 2nd SIA-binding site.

In **Chapter 3** we showed that increased cleavage of substrates by N9, resulting from a single mutation in the 2nd SIA-binding site, correlated with increased binding to these substrates via the 2nd SIA-binding site. This result confirms a previously made suggestion that binding via this site may serve to enhance the catalytic efficiency of NA as changes in hemadsorption activity of N2 correlated with changes in the ability of N2 to cleave SIA from multivalent, but not monovalent substrates (80). The enhanced catalytic efficiency of NA towards multivalent substrates via the 2nd SIA-binding site is probably mediated by recruiting and keeping complex substrates close to the active site, thereby contributing to increased substrate affinity. Unfortunately, we have not yet been able to determine Km values for

fetuin in the solid phase cleavage assay due to technical difficulties. While both N9 proteins displayed detectable SIA-binding activity when complexed to nanoparticles, this was not the case for any of the N1 proteins, indicating these interactions to be of very low avidity (**Chapter 4** and **5**). Nevertheless we consistently observed also for the N1 proteins that changes in the 2nd SIA-binding site affected cleavage of multivalent substrates, indicating that also very weak binding via the 2nd SIA-binding site may contribute to NA catalytic activity. Enhancement of catalytic efficiency resulting from catalytic and carbohydrate-binding domains interacting simultaneously with a polyvalent substrate has been observed for many glycoside hydrolases. Most of these enzymes contain lectin domains, including eukaryotic and bacterial NA proteins (81) as well as other viral receptor-destroying enzymes (82, 83). Importantly, the presence of a functional 2nd SIA-binding site was also shown to enhance the capacity of virus-associated NA to remove sialic acid from both soluble macromolecular substrates and cells (80). The importance of the 2nd SIA-binding site for replication and transmission in vivo, however, remains enigmatic. Disruption of the 2nd SIA-binding site in N2 decreased replication of the recombinant virus in cell culture, but surprisingly did not affect replication in ducks (77).

## 3) HA/NA balance and virus evolution

***HA/NA balance***

HA binds to host cell receptors and initiates internalization of virus into the endosome, where it mediates membrane fusion at acidic pH (84). After virus replication, nascent viruses remain attached to the cell via HA binding unless NA removes SIAs from host cell receptors to facilitate the release of progeny virions (85). NA activity also serves to prevent irreversible attachment of virions to decoy receptors such as mucins. Thus, an optimal balance between HA receptor binding and NA receptor destruction is of great importance for viral replication, transmission and fitness along with HA receptor specificity and HA stability (12, 45, 64, 86-89). The importance of a functional balance between HA and NA was illustrated by Zanin and coworkers (89), who isolated H1N1pdm09 viruses from swine with high HA binding affinity for α2,6-SIA but almost undetectable NA activity. These viruses exhibited abolished aerosol transmission and significantly impaired transmission via direct contact in ferrets compared to highly homologous H1N1pdm09 viruses with better-balanced HA and NA activities. Moreover, viruses with low NA activity were significantly inhibited by human mucus either in normal human bronchial epithelial cells or MDCK cells (89).

Despite that many studies have shown the importance of a functional balance with respect to SIA interactions between HA and NA for replication and transmission, it is not well established what this balance actually comprises. Analysis of receptor binding by recombinant HA protein and cleavage thereof by recombinant NA proteins by the Paulson and Wilson laboratories (90) revealed an apparent functional balance between the HA and NA proteins of human viruses, but not swine viruses, as determined by glycan array analysis. Human viruses containing HA proteins displaying increased receptor binding also contained NA proteins with increased enzymatic activity. Likewise, in **Chapter 3** we observed an apparent functional balance for the H7N9 viruses from 2008 and 2013. The 2013 virus with

carrying an H7 protein with decreased receptor-binding avidity also contained an N9 protein decreased sialidase activity.

A functional balance was not reported for all human viruses analyzed by the Paulson and Wilson laboratories by using recombinant soluble HA and NA proteins (45). This may make sense in view of the many other mechanisms by which IAVs may modulate the overall NA activity in virus particles or infected cells. Nevertheless, it will be interesting to analyze in more detail the receptor-binding properties of the HA proteins corresponding to the NA proteins analyzed in this thesis. The results shown in **Chapter 4** furthermore suggest that a functional balance between the HA and NA proteins may not only exist with respect to their interactions with SIA receptors, but possibly also for other HA and NA properties such as low pH or thermostability. Mutations in N1 of H1N1pdm09 virus were identified that affected calcium-dependent thermostability (positions 106 and 369) or low pH activity (position 432). These mutations may compensate for/be accompanied with mutations in HA that resulted in increased stability and a reduced activation pH (91, 92). Increased stability of HA has been associated with improved aerosol transmission (93), while higher NA activity at low pH resulting in increased sialidase activity in endosomes enhanced virus replication (94, 95).

The use of recombinant HA and NA proteins is very informative with respect to HA receptor-binding avidity and specificity and NA receptor-cleavage activity and specificity. Nevertheless complementary approaches are needed for an accurate analysis of HA/NA balance. Bio-layer interferometry was recently shown to be an attractive tool to study HA/NA balance in virus particles (96). Similar, improved assays have also been developed at the Virology Division of the Utrecht University (unpublished results Guo, de Vries, and de Haan). In these assays, virus binding to sialoside receptors and subsequent release thereof resulting from the cleavage of receptors by NA activity can be quantified in real time. NA-dependent release after virus binding was shown to depend on the HA and NA proteins as well as on the identity of the receptor used and the density thereof. Biolayer interferometry will allow us to determine and subsequently to link the HA/NA balance of different viruses to their replicative abilities in different (animal) models. Interestingly, studies using intact virus particles in combination with complex multivalent substrates in solid phase cleavage assays indicate that the HA protein may also be of importance for the observed NA activity of virus particles. For example, more efficiently cleavage of fetuin was found with a recombinant virus than with recombinant soluble NA protein although these preparations have the same MUNANA activity (unpublished data). The increased NA activity in this assay is most likely explained by the HA-mediated binding to fetuin. This result is in agreement with a recent study Kosik and coworkers (97) who found that antibodies targeting the HA protein may interfere with cleavage of fetuin by NA in virus particles.

***Virus evolution***

Virus transmission to a new host species, containing a different sialome, or gene reassortment resulting in the acquisition of a new HA/NA combination can result in the balance between HA and NA being disturbed and needing a reset. Such events may thus result in the selection of mutations in HA and/or NA that tune the HA/NA balance to achieve optimal replication and transmission. Tuning of the balance between HA and NA is probably

required for the emergence of a pandemic IAV. For instance, a gradual change was observed in the cleavage of the N2 protein of H2N2 and H3N2 human pandemic viruses from α2,3- to α2,6-linked SIAs. This change is corresponding with the preferred binding of the H2 and H3 proteins to α2,6-linked SIAs (87). Substitution I275V in N2 was identified by Kobasa *et al.* to be responsible for the enhanced cleavage of α2,6-linked SIAs. This substitution was maintained in all later human as well as swine viruses (98). Moreover, the NAs of the majority of human H2N2 viruses circulating during the influenza pandemic of 1957 differ from their avian precursor by amino acid substitutions in the 2nd SIA binding site, and those NA alterations are suggested to be required for the emergence of pandemic influenza viruses in addition to changes in the HA receptor-binding specificity (80). In agreement herewith, the conservation of the 2nd SIA-binding site in avian IAVs is also lost in human N1 proteins of seasonal H1N1 from before 2009 and H1N1pdm09 (77, 99) (**Chapter 4**). Consequently, N1 and N2 proteins of swine and human IAVs display severely reduced binding compared to their avian counterparts using hemadsorption and STD-NMR assays (80, 100) . In contrast to the widespread assumption that such substitutions in NA are only obtained after acquisition of functional changes in HA, in **Chapter 3,** our phylogenetic analyses showed that mutation of the 2nd SIA-binding site in N9 occurred prior to substitutions in HA causing the altered receptor-binding properties of the novel H7N9 viruses, suggesting that mutations in the 2nd SIA-binding site may have enabled and even driven the acquisition of altered HA receptor-binding properties, and contributed to the spread of the novel H7N9 viruses.

The wealth of viral sequence information that has come available after the emergence of the H1N1pdm09 virus allowed us to study the evolution of its NA protein after introduction in the human population in detail in **Chapter 4**. It is clear that the earliest H1N1pdm09 isolates already contain a mutated 2nd SIA-binding site, in which one of the SIA contact residues is substituted. With time, additional mutations were acquired in this site, resulting in initially increased (N369K) and subsequently decreased (K432E) cleavage of fetuin-linked sialosides. The consequences of the altered enzymatic properties of the H1N1pdm09 N1 proteins for replication in vitro and vivo remain to be established as well as the link thereof with the receptor-binding properties of the corresponding HA proteins. Recombinant H1N1pdm09 viruses carrying mutated NA proteins are currently being generated in collaboration with Carles Martinez and Adolfo Garcia-Sastre (Mount Sinai, USA).

It is not always clear what drives the selection of mutations in NA as these mutations often affect multiple NA characteristics (**Chapter 4**). For example, some mutations (e.g. K432E) may be selected as they reduce cleavage of multivalent substrates or as they increase the ability of NA to cleave substrates at low pH. Antigenic drift of NA or HA may also be a selective force. The N270K mutation in N1 of H1N1pdm09 virus, which reduces NA protein expression, is probably selected as it resulted in the only appreciable antigenic change that could be detected using polyclonal antisera. Also other mutations observed in H1N1pdm09 (e.g. the N369K mutation, which affects NA protein stability and receptor cleavage) may have been selected to escape human NA-specific antibodies, as some of them have been reported to abolish the binding of monoclonal antibodies to NA (101). Antigenic changes in HA may also drive evolution of NA. For example, it has been shown that amino acid

substitutions in HA responsible for escape from neutralizing monoclonal antibodies can also modulate HA avidity for host cells receptors (102). Furthermore, substitutions in HA resulting in antigenic drift have been shown to be primarily located around the receptor binding site (103-105) and may therefore also affect receptor binding by HA. Such changes in HA can therefore also select for compensatory changes in NA, which may in addition result in NA antigenic variation and acquisition of drug resistance (53). For the HA protein of H1N1pdm09 virus, the antigenic drift appears very limited so far.

The functional HA/NA balance can also be disturbed by NA inhibitor treatment. NA-inhibitor resistant mutants usually acquire mutations in the NA active site that decrease the enzymatic activity of these proteins in the absence of inhibitors (106-108). These mutations may be compensated by decreasing receptor binding by HA (109-111) or by the acquisition of so called “permissive” mutations in NA (54, 69) that increase cell-surface expression and NA activity. Substitutions at position 369 and 241 that increased receptor cleavage and protein expression were identified previously as permissive mutations that confer robust fitness on H1N1pdm09 viruses containing the H275Y oseltamivir-resistance mutation (47, 70, 112). These permissive mutations first emerged in 2010 and are now present in almost all circulating A(H1N1)pdm09 viruses. These mutations may increase the risk of the H1N1pdm09 virus acquiring NA inhibitor resistance mutations. Similarly, also for the seasonal H1N1 influenza viruses that evolved prior to 2009, several permissive substitutions were identified that played an important role in increasing overall NA activity and in reducing the H275Y generated defects thereof. Likely, these mutations facilitated the emergence of oseltamivir-resistant H1N1 influenza viruses independent of the use of oseltamivir (54, 69, 113). However, this scenario is less likely for recent H1N1pdm09 viruses, as several substitutions acquired by NA had large negative effects on cleavage of multivalent substrates (K432E) or on protein expression levels (N270K).

## Outlook and future prospects

This thesis provides comprehensive insights into the (tuning of) enzymatic activity of NA proteins of different subtypes, using a well-established recombinant protein approach. Among others, it firmly establishes an important role for the 2$^{nd}$ SIA-binding site in NA enzymatic activity. It has to be kept in mind, however, that NA protein enzymatic activity (and HA receptor binding) should also be investigated in the context of virus particles and in cells to fully understand and characterize the HA/NA balance. This can for example be done using biolayer interferometry, which can measure virus binding to and release from immobilized receptor analogs on a biosensor surface in real time. These HA/NA interactions and their balance should ideally be investigated using different types of receptors, including ones that mimic as much as possible the substrates that are encountered in vivo, such as cell surface mimics and mucus.

The studies presented in this thesis also highlight some complexities of HA/NA balance during virus evolution. Different NA phenotypic properties of H1N1pdm09 virus were found to be intertwined, with several NA substitutions affecting more than one phenotypic

characteristic. The phenotypic changes of NA are probably also linked to the properties of the HA protein and corresponding HA/NA balance, which makes evolution of HA and NA more difficult to understand. As a next step, different NA phenotypes need to be linked to the in vivo properties of the corresponding viruses, such as virus replication, transmission, and pathogenesis. Altogether, these studies may not only increase our fundamental knowledge of the IAV infection process, but possibly may ultimately also help us to combat these viruses.

## Supporting information

```
                                                  start
VN          MNPNQKIITIGSICMVTGIVSLMLQIGNMISIWVSHSIHTGNQHQSEP------------
HN          MNPNQKIITIGSICMVIGIVSLMLQIGNMISIWVSHSIQTGNQHQAEP------------
HB          MNPNQKIVTIGSICMVIGIVSLMLQIGNMISIWVSHSIQTGNQHQTEP------------
WSN         MNPNQKIITIGSICMVVGIISLILQIGNIISIWISHSIQTGNQNHTGICNQGS-------
CA/09       MNPNQKIITIGSVCMTIGMANLILQIGNIISIWISHSIQLGNQNQIETCNQSV-------
IN/15       MNPNQKIITIGSICMTIGMANLILQIGNIISIWVSHSIQIGNQSQIETCNQSV-------
Spain       MNPNQKILCTSATAIVIGTIAVLIGIANLGLNIGLHLKPSCNCSHSQPEATNTS------
Anhui       MNPNQKILCTSATAIIIGAIAVLIGIANLGLNIGLHLKPGCNCSHSQPETTNTS------

N2 numbering                    start head          95         106
VN          ---------------ISNTNLLTEKAVASVKLAGNSSLCPINGWAVYSKDNSIRIGSKGD
HN          ---------------ISNTNFLTEKAVASVTLAGNSSLCPIRGWAVHSKDNSIRIGSKGD
HB          ---------------IRNTNFLTENTVASVTLAGNSSLCPIRGWAVHSKDNSIRIGSKGD
WSN         ITYKV----------------VAGQDSTSVILTGNSSLCPIRGWAIHSKDNGIRIGSKGD
CA/09       ITYENNTWVNQTYVNISNTNFAAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSVRIGSKGD
IN/15       ITYENNTWVNQTYVNISNTNFAAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSVRIGSKGD
Spain       QTIINNYYNETNITQISNTNIQMEERASRSFNNLTKGLCTINSWHIYGKDNAVRIGENSD
Anhui       QTIINNYYNETNIT-----NIQMEERTSRNFNNLTKGLCTINSWHIYGKDNAVRIGESSD
CA/09 numbering                                 95         106

N2 numbering    119                             151 155
VN          VFVIREPFISCSHLECRTFFLTQGALLNDKHSNGTVKDRSPHRTLMSCPVGEAPSPYNSR
HN          VFVIREPFISCSHLECRTFFLTQGALLNDKHSNGTVKDRSPHRTLMSCPVGEAPSPYNSR
HB          VFVIREPFISCSHLECRTFFLTQGALLNDKHSNGTVKDRSPHRTLMSCPIGEAPSPYNSR
WSN         VFVIREPFISCSHLECRTFFLTQGALLNDKHSRGTFKDRSPYRALMSCPVGEAPSPYNSR
CA/09       VFVIREPFISCSPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSR
IN/15       VFVIREPFISCSPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSR
Spain       VLVTREPYVSCDPDECRFYALSQGTTIRGKHSNGTIHDRSQYRALISWPLSSPPTVYNSR
Anhui       VLVTREPYVSCDPDECRFYALSQGTTIRGKHSNGTIHDRSQYRALISWPLSSPPTVYNSR
CA/09 numbering 120                             151

N2 numbering    178
VN          FESVAWSASACHDGTSWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECAC
HN          FESVAWSASACHDGTSWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECAC
HB          FESVAWSASACHDGTSWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECAC
WSN         FESVAWSASACHDGMGWLTIGISGPDDGAVAVLKYNGIITETIKSWRKNILRTQESECTC
CA/09       FESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECAC
IN/15       FESVAWSASACHDGINWLTIGISGPDSGAVAVLKYNGIITDTIKSWRNNILRTQESECAC
Spain       VECIGWSSTSCHDGKSRMSICISGPNNNASAVVWYNRRPVAEINTWARNILRTQESECVC
Anhui       VECIGWSSTSCHDGKSRMSICISGPNNNASAVVWYNRRPVAEINTWARNILRTQESECVC
CA/09 numbering 179                 201

N2 numbering                    252                 274             292
VN          VNGSCFTVMTDGPSNGQASHKIFKMEKGKVVKSVELDAPNYHYEECSCYPDAGEITCVCR
HN          VNGSCFTVMTDGPSNGQASYKIFKMEKGKVVKSVELDAPNYHYEECSCYPDAGEITCVCR
HB          VNGSCFTVMTDGPSNGQASYKIFKMEKGKVVKSVELNAPNYHYEECSCYPDAGEIICVCR
WSN         VNGSCFTIMTDGPSDGLASYKIFKIEKGKVTKSIELNAPNSHYEECSCYPDTGKVMCVCR
CA/09       VNGSCFTVMTDGPSNGQASYKIFRIEKGKIVKSVEMNAPNYHYEECSCYPDSSEITCVCR
IN/15       VNGSCFTIMTDGPSDGQASYKIFRIEKGKITKSVEMKAPNYHYEECSCYPDSSEITCVCR
Spain       HNGVCPVVFTDGSATGPADTRIYYFKEGKILKWESLAGTAKHIEECSCYGERTGITCTCR
Anhui       HNGVCPVVFTDGSATGPADTRIYYFKEGKILKWESLTGTAKHIEECSCYGERTGITCTCR
CA/09 numbering   241    248             264   270  275
```

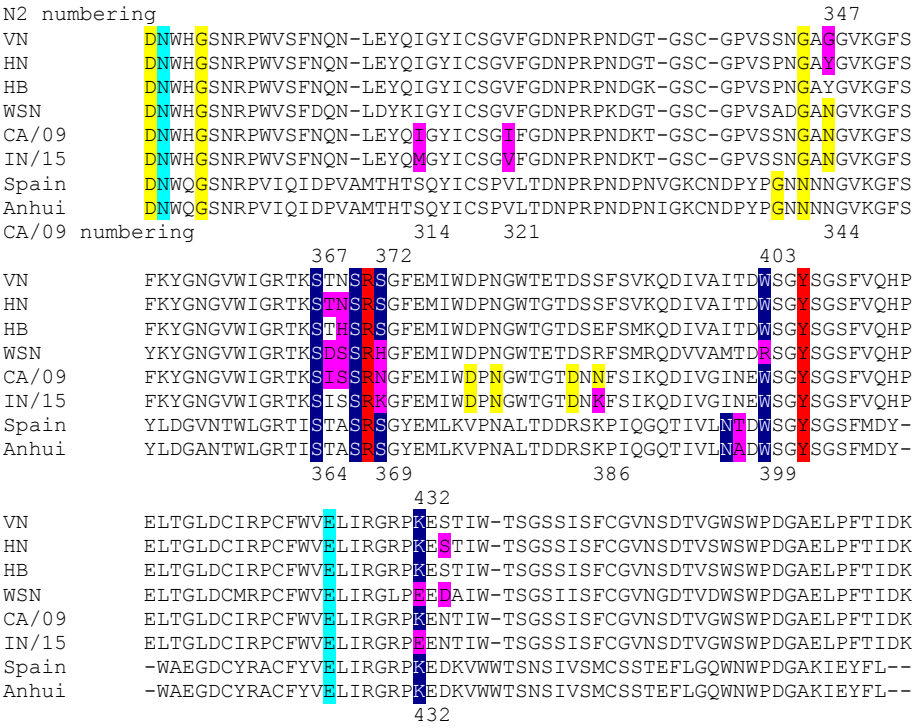



**Figure 1. Alignment of several NA proteins analyzed in this thesis**. The transmembrane domain (identified with http://www.cbs.dtu.dk/services/TMHMM/) is indicated with blue letters. Start of the NA protein ectomain and of the NA protein head domain in the recombinant proteins is indicated. Catalytic and framework residues in the active site are shown in red and light blue, respectively, while residues forming different calcium binding sites are colored yellow (79). SIA-contact residues in the 2nd SIA-binding site, based on the N9 crystal structure (78), are colored dark blue. Several residues in NA, which were mutated in this thesis, are colored purple. VN, HN, HB and TK refer to NA proteins of H5N1 viruses studied in Chapter 2 and 5. CA/09 and IN/15 refer to NA proteins of H1N1pdm09 viruses studied in Chapter 4 and 5, while WSN refers to the NA protein of the laboratory-adapted WSN virus used in Chapter 5. Spain and Anhui refer to the N9 proteins used in Chapter 3. Above the aligned sequences the N2 numbering of the head domain is shown, which was used in Chapter 2, 3 and 5. The CA/09 (H1N1pdm09 NA) numbering used in Chapter 4 is shown below the aligned sequences.

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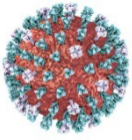
# SAMENVATTING

# Nederlands



## Samenvatting

Griepvirussen veroorzaken ziekte bij mens en dier. Deze membraan-bevattende virussen met een gesegmenteerd, negatief-strengig RNA genoom behoren tot de familie Orthomyxoviridae. Deze virusfamilie bevat 4 genera van griepvirussen, waaronder de influenza A virussen, die het onderwerp van studie zijn in dit proefschrift. Influenza A virussen zijn zoönotische pathogenen die in verschillende species circuleren, waaronder veel soorten vogels, varkens en de mens.

Van oorsprong komen influenza A virussen bij watervogels voor. Infecties van deze vogels zijn over het algemeen asymptomatisch. Sommige van deze virussen hebben echter de kans gezien om over te springen naar pluimvee of varkens, waarbij ze endemisch zijn geworden in verschillende delen van de wereld. Infectie van deze dieren kan leiden tot ernstige ziekte en grote economische schade veroorzaken. Bij mensen veroorzaken influenza A virussen seizoensgebonden epidemieën van respiratoire infecties en af en toe pandemieën. De jaarlijkse epidemieën kunnen resulteren in miljoenen infecties en daardoor ernstige schade toebrengen aan de volksgezondheid en de economie. De pandemieën worden veroorzaakt door diervirussen die de gastheer-barrière overwonnen hebben en overdraagbaar zijn geworden tussen mensen. De afgelopen 100 jaar hebben zich vier influenza A virus pandemieën voorgedaan, waarbij er miljoenen doden zijn gevallen, namelijk de Spaanse griep in 1918, de Aziatische griep in 1957, de Hongkong griep in 1968 en de “Mexicaanse” griep in 2009.

Het membraan van het influenza A virus partikel bevat twee belangrijke glycoproteïnen, het hemagglutinine (HA) en het neuraminidase (NA), waarvan er respectievelijk 16 (H1-16) en 9 (N1-9) subtypes bekend zijn. Zowel HA als NA herkennen siaalzuur-receptoren. Siaalzuren zijn eindstandige modificaties van suikerketens die voorkomen op alle cellen in ons lichaam en in de mucuslaag direct boven de epitheelcellen van ons luchtwegstelsel en maagdarmkanaal. Er is een enorme variatie in deze siaalzuren en de suikerketens waaraan ze verbonden zijn. Om een cel te infecteren moeten de HA eiwitten van het virus binden aan siaalzuren op het oppervlak van de cel, waarna het viruspartikel kan worden opgenomen. Vogelgriepvirussen prefereren binding aan zogenaamde α2,3-gebonden siaalzuren terwijl humane virussen bij voorkeur binden aan α2,6-gebonden siaalzuren. Geïnfecteerde cellen maken vervolgens nieuwe virusdeeltjes, die vrijkomen als het siaalzuur-klievende NA eiwit er tenminste voor zorgt dat er geen siaalzuren meer op het oppervlak van een cel zitten waaraan deze partikels blijven binden. Daarnaast voorkomt de enzymatische activiteit van het NA dat virusdeeltjes aggregeren door aan elkaar te binden. Het HA kan ook binden aan siaalzuren die vast zitten op niet-functionele receptoren die bijvoorbeeld veel voorkomen op mucus-eiwitten. Irreversibele binding van virussen aan mucus zal verhinderen dat de virusdeeltjes de onderliggende epitheelcellen kunnen bereiken en infecteren. De enzymatische activiteit van NA is daarom ook van belang voor de penetratie van de mucuslaag door niet-functionele receptoren te verwijderen.

Doordat HA bindt aan siaalzuur-receptoren en NA deze klieft, denkt men dat een functionele balans tussen HA en NA van belang is voor virusreplicatie en transmissie. Bijvoorbeeld, de

combinatie van een slecht-bindend HA eiwit met een hoog actief NA eiwit zal mogelijk de binding van viruspartikels aan cellen verstoren, terwijl een lage NA-activiteit in combinatie met een sterk-bindend HA kan leiden tot virusaggregatie. Functionele veranderingen in HA moeten dus gecompenseerd worden door veranderingen in NA en omgekeerd. Verder is er een enorme variatie aan siaalzuur-receptoren, die in verschillende mate worden herkend en gekliefd door HA en NA. Allerlei diersoorten verschillen in de opmaak van hun siaalzuur-receptoren en virussen die aangepast zijn aan verschillende diersoorten kunnen daarom uiteenlopen in de activiteit van hun HA en NA eiwitten. Dus, ook als een virus overstapt naar een andere gastheer met een ander opmaak van siaalzuur-receptoren zijn er functionele veranderingen in HA en NA nodig. Terwijl HA-receptor interacties tot in detail bestudeerd zijn, is er over het algemeen veel minder bekend over de moleculaire determinanten van (veranderingen in) de specificiteit en enzymatische activiteit van de influenza A virus NA eiwitten. Het doel van het onderzoek beschreven in dit proefschrift was om deze determinanten op te helderen.

In hoofdstuk 2 hebben we onderzocht wat de optimale methode is om recombinante NA eiwitten te maken, die door cellen worden uitgescheiden, met als doel om de enzymatische activiteit van deze NA eiwitten te kunnen bestuderen. Het tetramere NA eiwit bevat een korte cytoplasmatische staart, een transmembraandomein, een steel en een globulair domein met het katalytische centrum. Om uitscheidbare eiwitten te maken moeten het cytoplasmatische staartje en het transmembraandomein verwijderd worden. Toevoeging van een oligomerizatiedomein is nodig om tetramere en enzymatisch actieve NA eiwitten te maken. Onze resultaten laten zien dat zowel het specifieke oligomerizatiedomein als de lengte van het NA ectodomein van invloed is op de enzymatische activiteit van het recombinante NA eiwit. Door de NA eiwitten van verschillende H5N1 virussen te vergelijken identificeerden we verschillende residuen in NA die van invloed zijn op de enzymatische activiteit door hun belang voor de oligomerizatie van het globulaire domein of voor de affiniteit van NA voor het substraat.

In hoofdstuk 3, hebben we de geoptimaliseerde NA expressie methode gebruikt om de enzymatische activiteit van N9 eiwitten van verschillende H7N9 virussen te bestuderen. In 2013 verscheen een nieuw H7N9 virus dat regelmatig mensen infecteert. Het HA eiwit van dit virus vertoont verminderde binding aan α2,3- en een bescheiden verbeterde binding aan α2,6-gebonden siaalzuren ten opzichte van het HA van een H7N9 virus uit 2008. Vergelijking van de enzymatische activiteit van de overeenkomende N9 eiwitten liet zien dat deze eiwitten niet verschillen in hun vermogen om een monovalent substraat te klieven. Klieving van multivalente substraten, zoals bijvoorbeeld siaalzuren gelinkt aan suikerbomen van een glycoproteïne, was echter verminderd door verminderde binding van het N9 uit 2013 aan dergelijke substraten. Deze verminderde binding was het gevolg van een mutatie in een siaalzuur-bindingsplaats gelegen naast het katalytische centrum. Fylogenetische analyse toont aan dat deze mutatie in de nieuwe H7N9 virussen voorafging aan (en mogelijk aanleiding gaf tot) de mutaties in H7 die leidden tot de veranderde receptor binding van dit virus.
In hoofdstuk 4 hebben we op geleide van een fylogenetische analyse de evolutie van de N1 eiwitten van het “Mexicaanse” griepvirus onderzocht na introductie in de humane populatie

in 2009. Op basis hiervan hebben we verschillende N1 eiwitten tot expressie gebracht en vervolgens in detail gekarakteriseerd. Onze resultaten laten zien dat de N1 eiwitten binnen een bepaalde bandbreedte fluctueren wat betreft expressie niveaus, enzymatische activiteit en pH gevoeligheid als het gevolg van mutaties. Deze mutaties worden mogelijk geselecteerd omdat ze bijdragen aan antigene drift of omdat ze de balans tussen HA en NA herstellen. Opvallend genoeg waren verschillende mutaties van invloed op meer dan één NA eigenschap. Dit suggereert dat gedurende virusevolutie er een continue wisselwerking is tussen verschillende NA eigenschappen. Het N1 eiwit van een virus uit 2015 vertoonde uiteindelijk een fenotype dat vergelijkbaar was met dat van het N1 eiwit van een van de eerste virussen uit 2009, alleen was het eiwit antigeen gezien veranderd.

In hoofdstuk 5 hebben we de enzymatische activiteit van de N1 eiwitten van verschillende aviaire en humane griepvirussen vergeleken en bestudeerd in hoeverre de verschillen in enzymatische activiteit het resultaat waren van mutaties in de siaalzuur-bindingsplaats gelegen naast het katalytisch centrum. De resultaten laten zien dat N1 eiwitten van humane virussen relatief beter zijn dan aviaire virussen in het klieven van α2,6-verbonden siaalzuren. Verschillen in enzymatische activiteit konden gedeeltelijk verklaard worden door mutaties in de siaalzuur-bindingsplaats. Niet alleen mutaties van residuen die contact maken met siaalzuur bleken van invloed te zijn op de enzymatische activiteit, maar ook van nabij gelegen residuen die niet direct contact maken met siaalzuur. Mutaties buiten de siaalzuur-bindingsplaats hadden over het algemeen weinig effect op de enzymatische activiteit. De NA activiteit van griepvirussen kan dus gemodificeerd worden zonder het katalytisch centrum te veranderen, maar door de selectie van mutaties in de siaalzuur-bindingsplaats.

In hoofdstuk 6 worden de resultaten en inzichten verkregen in de voorgaande hoofdstukken geanalyseerd en bediscussieerd in een breder perspectief aan de hand van de volgende drie onderwerpen: I) bestuderen van NA activiteit en substraat specificiteit, II) mechanismes waardoor de influenza A virus NA activiteit gemoduleerd kan worden en III) de balans tussen HA en NA en virus evolutie. Uiteindelijk hebben we aanbevelingen gedaan voor vervolgonderzoek. Om de balans tussen HA en NA goed in kaart te brengen en te kunnen begrijpen zal niet alleen nodig zijn om de HA en NA eiwitten afzonderlijk en in detail te bestuderen, maar ook om deze balans ook in de context van virus partikels te analyseren.

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# CURRICULUM VITAE

## Education

**2013-2017**: PhD in Virology
Virology Division, Department of Infectious Diseases & Immunology,
Faculty of Veterinary Medicine, Utrecht University, The Netherlands

**2010-2013**: MSc in Veterinary Medicine
Faculty of Veterinary Medicine, Northwest A&F University, Shaanxi, China

**2006- 2010**: BSc in Veterinary Medicine
Faculty of Animal Science & Technology,
Anhui Science & Technology University, Anhui, China

## Presentations

Oral presentation — Emergence of novel H7N9 influenza A virus is accompanied with reduced neuraminidase activity resulting from mutation of the 2nd sialic acid-binding site. 6th European Congress of Virology, Hamburg, Germany. 2016

Poster presentation — Emergence of novel H7N9 influenza A virus is accompanied with reduced neuraminidase activity resulting from mutation of the 2nd sialic acid-binding site. 5th International Influenza Meeting, Muenster, Germany. 2016

Poster presentation — Emergence of novel H7N9 influenza A virus is accompanied with reduced neuraminidase activity resulting from mutation of the 2nd sialic acid-binding site. Veterinary Science Day 2016, Utrecht, The Netherlands.

## Honors & Awards

2017 Scholarship for Biobusiness Summer School.

2016 Young Scientists Travel Grant Award, 6$^{th}$ European Congress of Virology.

2013 China Scholarship Council (CSC) Scholarship

## List of Publications

1. **Dai M**, McBride R, Dortmans J, Peng W, Bakkers M.J.G, de Groot RJ, van Kuppeveld FJ, Paulson J.C, de Vries E, de Haan CA. 2017. Mutation of the 2nd Sialic Acid-Binding Site Resulting in Reduced Neuraminidase Activity Preceded Emergence of H7N9 Influenza A Virus. Journal of Virology. 91(9). pii: e00049-17.
2. **Dai M**, Guo H, Dortmans J, Dekkers J, Nordholm J, Daniels R, van Kuppeveld FJ, de Vries E, de Haan CA. 2016. Identification of residues that affect oligomerization and/or enzymatic activity of influenza virus H5N1 neuraminidase proteins. Journal of Virology. 90(20):9457-70.
3. de Vries E, Guo H*, **Dai M***, Rottier PJ, van Kuppeveld FJ, de Haan CA. Rapid Emergence of Highly Pathogenic Avian Influenza Subtypes from a Subtype H5N1 Hemagglutinin Variant. (2015). Emerging Infectious Diseases. 21(5):842-6. *second co-authors.
4. **Dai M**, de Vries E, Wennekes Tom, Jan Bosch Berend, Rimmelzwaan Guus, van Kuppeveld FJ, de Haan CA. Evolution of the phenotypic properties of the neuraminidase protein of the new pandemic influenza A(H1N1) virus after its introduction in the human population. Manuscript in preparation.
5. **Dai M**, de Vries E, van Kuppeveld FJ, de Haan CA. Important role of the 2nd sialic acid-binding site for the enzymatic activity of the influenza A virus neuraminidase protein. Manuscript in preparation.
6. Ding L, Huang Y, **Dai M**, Zhao X, Du Q, Dong F, Wang L, Huo R, Zhang W, Xu X, Tong D. Transmissible gastroenteritis virus infection induces cell cycle arrest at S and G2/M phases via p53-dependent pathway. (2013). Virus Research. 178(2):241-51.
7. Huang Y, Ding L, Li Z, **Dai M**, Zhao X, Li W, Du Q, Xu X, Tong D. Transmissible gastroenteritis virus infection induces cell apoptosis via activation of p53 signalling. (2013). Journal of General Virology. 94(Pt 8):1807-17.