ORIGINAL ARTICLE

Functional significance of the hemadsorption activity of influenza virus neuraminidase and its alteration in pandemic viruses

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Abstract Human influenza viruses derive their genes from avian viruses. The neuraminidase (NA) of the avian viruses has, in addition to the catalytic site, a separate sialic acid binding site (hemadsorption site) that is not present in human viruses. The biological significance of the NA hemadsorption activity in avian influenza viruses remained elusive. A sequence database analysis revealed that the NAs of the majority of human H2N2 viruses isolated during the influenza pandemic of 1957 differ from their putative avian precursor by amino acid substitutions in the hemadsorption site. We found that the NA of a representative pandemic virus A/Singapore/1/57 (H2N2) lacks hemadsorption activity and that a single reversion to the avian-virus-like sequence (N367S) restores hemadsorption. Using this hemadsorption-positive NA, we generated three NA variants with substitutions S370L, N400S and W403R that have been found in the hemadsorption site of human H2N2 viruses. Each substitution abolished hemadsorption activity. Although, there was no correlation between hemadsorption activity of the NA variants and their enzymatic activity with respect to monovalent substrates, all four hemadsorption-negative NAs desialylated macromolecular substrates significantly slower than did the hemadsorption-positive counterpart. The NA of the 1918 pandemic virus A/Brevig Mission/1/18 (H1N1) also differed from avian N1 NAs by reduced hemadsorption activity and less efficient hydrolysis of macromolecular substrates. Our data indicate that the hemadsorption site serves to enhance the catalytic efficiency of NA and they suggest that, in addition to changes in the receptor-binding specificity of the hemagglutinin, alterations of the NA are needed for the emergence of pandemic influenza viruses.

Introduction

Influenza A viruses carry two surface glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA) which recognise the same host cell molecule, sialic acid. HA mediates virus binding to sialic acid-containing cell-surface receptors to initiate infection (reviewed in ref. [28, 42]). NA is an enzyme that cleaves sialic acid from glycoconjugates on extra-cellular inhibitors, cells and progeny virions and thus facilitates virus access to receptors on cell membrane, promotes release of viral progeny and prevents its receptor-mediated self-aggregation [2, 10, 30, 35]. X-ray analysis of NAs from several influenza A and B viruses revealed that the catalytic site is a deep pocket on the NA surface formed by amino acid residues that are conserved among NA types and subtypes [4, 11, 41, 49].

Viruses that carried either HA (H3N2, 1968) or both HA and NA (H1N1, 1918; H2N2, 1957) derived from avian influenza viruses caused three influenza pandemics in the last century (reviewed in ref. [54]). The 1918 virus is believed to be an avian-like virus derived in toto from an unknown animal host [45]. The 1957 pandemic was caused by a reassortant virus that contained genes of HA, NA and PB1 from an H2N2 avian virus and the remainder from a currently circulated human H1N1 virus. The 1968 pandemic virus acquired H3 HA and PB1 from an avian virus

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and the rest of the genes including NA from the contemporary human H2N2 virus. Thus, the NAs of both H2N2 and H3N2 human viruses represent descendants of the avian NA that was introduced into humans in 1957. A shift of the receptor-binding specificity of the avian virus HA from Neu5Acα2-3Gal recognition to Neu5Acα2-6Gal recognition is thought to be a prerequisite for the generation of pandemic viruses [25, 26, 47], however, no functional changes in the avian NAs of 1918 and 1957 viruses have been identified so far.

It has been known for some time that the NA of the avian influenza viruses has in addition to the catalytic site a separate sialic acid binding site and displays hemadsorption activity which cannot be blocked by the inhibitors of the catalytic site [16, 20, 23]. The amino acid residues responsible for hemadsorption were identified by sequencing monoclonal escape mutants of N9 NA that lost this activity [53] and by site-directed mutagenesis of N2 and N1 NAs [16, 20, 34]. The crystal structure of the complex of the N9 NA with two sialic acid residues bound to both the catalytic site and the hemadsorption site was resolved [48]. The hemadsorption site is a shallow pocket located in the vicinity of the deep catalytic site and formed by three surface peptide loops. Six residues on these loops directly interact with the sialic acid residue in the hemadsorption site (see Fig. 1a, b). With a few exceptions, five of these amino acids (367S, 370S, 372S, 400N and 403W) are conserved among the avian virus NAs of all nine antigenic subtypes [20, 48]. Kobasa et al. [20] examined representative NAs of most antigenic subtypes from avian, human and swine viruses. They found that all avian virus NAs possessed a high level of hemadsorption activity whereas N1 and N2 NAs of human viruses displayed much weaker activity. This finding correlated with the conservation of the amino acids forming the hemadsorption site of NA in avian viruses and a lack of such conservation in human and swine viruses [20, 48]. Taken together, these notions suggested that the hemadsorption site of the NA has some role in virus replication in birds but is either not essential or disadvantageous for the virus replication in humans. However, abrogation of the hemadsorption activity of an avian virus NA by site-directed mutagenesis did not significantly affect viral replication in the duck intestine under experimental conditions [20]. Unlike most avian viruses, H9N2 viruses from Asian poultry were found to possess HAs that displayed a human-virus-like receptor specificity and NAs that harboured amino acid substitutions in the hemadsorption site [1, 24, 29]. Sequence analysis revealed that mutation of the NA hemadsorption site of H9N2 viruses resulted from a positive selective pressure to change suggesting that a loss of the site increased virus fitness in land-based poultry [29].

Neither the function of the NA hemadsorption site in avian viruses, nor the exact time and mechanisms of its disappearance after interspecies transmission are known. It was noticed that substitution S370L reduced hemadsorption activity of avian N2 NA with concomitant 50% reduction of the NA enzymatic activity [20]. However, several other single or multiple amino acid substitutions in N1 and N2 NAs abolished hemadsorption activity without apparent effects on enzymatic activity [16, 53]. These results argued against a consistent role of the hemadsorption site in the catalytic activity of the NA. A possible role of this site in the virus attachment to receptors in birds was suggested [38, 48]. No evidence in support of this hypothesis is available; moreover, failure of antibodies to NA to inhibit virus attachment [23] disagrees with it.

In this study, we wished to specify the functional role of the NA hemadsorption site. To address this question, we generated several variants of the NA of the pandemic virus strain A/Singapore/1/57 (H2N2) that differed from each other by amino acid substitutions in the key positions of the hemadsorption site and by hemadsorption activity. We analyzed the enzymatic activity of the NAs expressed in Cos-7 cells and of corresponding recombinant viruses and concluded that the hemadsorption site serves to enhance the catalytic activity of NA.

Materials and methods

Viruses, cells and reagents

Influenza virus A/Singapore/1/57 (H2N2) was from the repository of the Institute of Virology, Marburg. Madin–Darby canine kidney (MDCK) and African Green Monkey Kidney Fibroblast (Cos-7) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 1% glutamine. 2′-(4-methylumbelliferyl)-N-acetylneuraminic acid (MU-NANA), 3′-sialyl-N-acetyllactosamine (3SLN), 6′-sialyl-N-acetyllactosamine (6SLN) and bovine fetuin were obtained from Sigma (St. Louis, MO, USA). Peroxidase-conjugated anti-rabbit antibodies were from DAKO (Hamburg, Germany). Human airway mucins were collected from the apical surface of differentiated human tracheo-bronchial epithelial cultures prepared as described previously [14, 30]. Synthetic sialylglycopolymers 3SL-PAA and 6SLN-PAA, which contained 20 mol% of 3′-sialyllactose (3SL) and 6SLN, respectively, attached to a soluble polyacrylamide carrier [9] were kindly provided by Nikolai Bovin and Alexander Tuzikov, Institute of Bio-Organic Chemistry, Moscow, Russia. pHW2000 plasmid [17] was kindly provided by Eric Hoffmann and Robert Webster, St. Jude Children's Research Hospital,
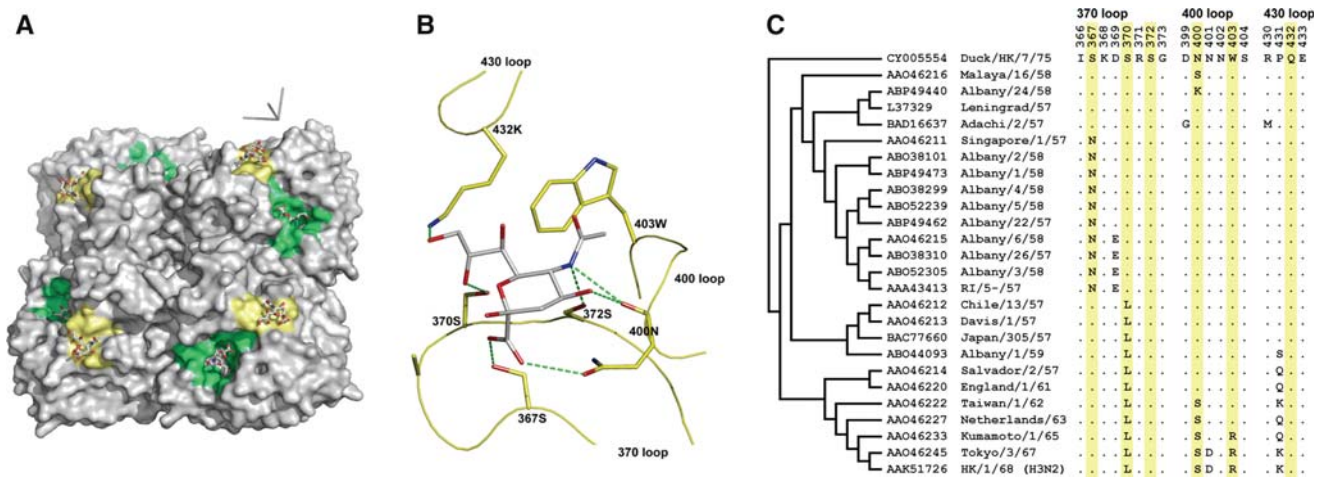
Fig. 1 Hemadsorption site of the NA and its alteration in the H2N2 pandemic viruses. **a** Molecular surface of N9 NA tetramer with sialic acids bound to the catalytic (*green*) and hemadsorption (*yellow*) sites (1MWE, [48]). **b** Positions of the three peptide loops forming the hemadsorption site are indicated. The side chains of amino acids contacting sialic acid are shown and labelled using the N2 numbering system. The carbon atoms of the protein and sialic acid are coloured *yellow* and *grey*, respectively. Nitrogens are *blue* and oxygens are *red*. *Dashed lines* depict polar contacts. The models in Figs. 1 and 5 were generated using DeLano Scientific PyMOL release 0.99 (DeLano, W.L. The PyMOL Molecular Graphics System, http://pymol. sourceforge.net). **c** Amino acid substitutions in the NA hemadsorption site of human viruses with respect to the closely related avian virus A/Duck/Hong Kong/7/75 (H3N2). All non-redundant NA sequences of H2N2 viruses from 1957 to 1958 and few representative sequences of the viruses isolated after 1958 are shown. Amino acids contacting sialic acid in the hemadsorption site are highlighted. The minimum evolution phylogenetic tree was build for nucleotide sequences using MEGA4 software [44]

Memphis, TN, USA. Anti-H9N2 antibodies were kindly provided by Wolfgang Garten, Institute of Virology, Marburg.

Cloning and site-directed mutagenesis

The full-length gene of the NA of A/Singapore/1/57 (H2N2) virus (SG/57) was amplified by RT-PCR from isolated RNA and ligated into the pHW2000 plasmid using *Bsm*BI restriction sites as described previously [17, 18]. Amino acid substitutions in the hemadsorption site of the NA-pHW2000 plasmid were introduced using Quikchange site directed mutagenesis kit [Stratagene, (La Jolla, CA, USA)]. The coding sequences of the original and mutated NAs were subcloned into pCAGGS/MCS plasmid [20, 33] using *Xho*I/*Sma*I restriction sites. The full length NA gene of A/Brevig Mission/1/18 (H1N1) virus (Genbank AF250356) was synthesized commercially (Genscript Corporation, Piscataway, NJ, USA). Plasmids pHH21-FPV-NA and *pol*I*Sap*I-Os/Italy-NA encoding NA genes of A/Chicken/FPV/Rostock/34 (H7N1) [50] and A/Ostrich/Italy/984/00 (H7N1) were provided by Ralf Wagner and Bjoern Keiner, Institute of Virology, Marburg. The N1 NA genes were PCR-amplified and cloned into pCAGGS/MCS vector using *Eco*RI/*Not*I restriction sites. The coding sequence of the M gene of A/Hong Kong/1/68 (H3N2) was subcloned from pHW2000-M plasmid [25] into *Xho*I/*Sma*I sites of the pCAGGS/MCS plasmid. The identities of all plasmids were confirmed by sequencing.

Assays of expression level and hemadsorption activity of the NAs

Monolayers of Cos-7 cells grown in 96-well plates were transfected with pCAGGS-NA plasmids using Lipofect-amine 2000 (Invitrogen, Karlsruhe, Germany). Empty pCAGGS plasmid was used for a mock-transfected control. Forty-eight hours after transfection, the cell monolayers were fixed with 4% paraformaldehyde for 20 min at 4°C and incubated with serial twofold dilutions of rabbit anti-H9N2 virus antibodies, followed by peroxidase-conjugated anti-rabbit IgG antibodies. Levels of bound antibody were determined with the peroxidase substrate *o*-phenylenedi-amine and quantified by measuring absorbance at 490 nm. To calculate the relative expression level of the NAs with respect to that of the NA of A/Singapore/1/57 (as presented in the Table 1), we used ratios of corresponding absor-bencies at anti-H9N2 antibody dilution 1/1,600 corrected for the absorbance of mock-transfected cells.

The hemadsorption activities of the NAs were deter-mined in parallel with the assay of NA expression levels using replicate transfected cultures of Cos-7 cells 48 h post transfection. The cell monolayers were incubated with 1% suspensions of either chicken or human

Table 1 Hemadsorption and enzymatic activities of N2 neuraminidases expressed in Cos-7 cells

NA[a]	Expression relative to SG/57[b]	Hemadsorption activity (CRBCs per ten Cos-7 cells)	Enzymatic activity (pmole sialic acid released per minute per culture)						
			MU-NANA 0.1 mM	3SLN 0.1 mM	6SLN 0.4 mM	Fetuin 0.3 mM	Human mucin 0.1 mM	3SL-PAA 0.1 mM	6SLN-PAA 0.4 mM
SG/57	1	0.68 (0.60)	230 (44)	280 (26)	56 (5.4)	110 (27)	4.9 (1.2)	160 (20)	30 (6.3)
N367S (av-like)	0.99 (0.11)	72 (15)*	230 (37)	270 (29)	59 (4.3)	180 (32)*	25 (1.0)*	340 (49)*	130 (22)*
N367S-S370L	1.0 (0.09)	0.39 (0.33)	230 (36)	210 (14)*	49 (7.3)	96 (35)	4.3 (1.4)	110 (15)*	27 (5.3)
N367S-N400S	0.99 (0.14)	0.48 (0.38)	230 (36)	260 (41)	58 (5.8)	130 (19)	5.0 (1.3)	180 (27)	35 (8.9)
N367S-W403R	0.98 (0.09)	0.58 (0.42)	220 (31)	290 (35)	54 (14)	120 (14)	5.5 (0.9)	170 (23)	31 (3.3)

All data represent mean values and SD (in parenthesis) of three to four independent experiments with assay performed in duplicate ($n = 6$–8). Hemadsorption activity is expressed as number of chicken red blood cells (CRBCs) adsorbed per ten Cos-7 cells (see Fig. 2). Concentrations of bound sialic acid used in the assay are indicated under the name of corresponding substrate

* $P < 0.05$ (unpaired two-sided t test versus SG/57)

[a] NA designations reflect sequence differences with SG/57

[b] Determined by cell ELISA

erythrocytes for 30 min at 4°C. Non-bound erythrocytes were removed by washing, and bound erythrocytes were stained using diaminobenzidine peroxidase substrate solution. The cultures were fixed with methanol, and nuclei of Cos-7 cells were stained with 1% Alcian Blue in 3% acetic acid. To quantify hemadsorption activity, we determined the average number of red blood cells adsorbed per ten Cos-7 cells by analysis of at least 250 Cos-7 cells per replicate culture under an Olympus IMT-2 microscope with a 20× objective.

Enzymatic activity of expressed NA

Monolayers of NA-expressing Cos7-cells in 96 well plates were incubated with sialic acid-containing substrates in Ca-TBS buffer (4 mM CaCl$_2$, 0.02 M Tris, 0.85% NaCl; pH 7) at 37°C. Hydrolysis of MU-NANA was quantified by measuring fluorescence of released 4-methylumbelliferone as described previously [27]. Sialic acids liberated from non-fluorescent substrates were determined at different time points (from 5 min to 6 h) by thiobarbituric acid assay [43, 52]. Specific NA activities (pmole sialic acid released per minute per culture) were calculated in the linear range of released sialic acid-versus-time plots.

Generation of recombinant viruses

Recombinant viruses rgHAD− and rgHAD+ which contained, respectively, the gene of hemadsorption-negative (HAD−) wild-type SG/57 NA and its hemadsorption-positive (HAD+) N367S mutant were generated by using the eight plasmid reverse genetic system as described previously [17]. The seven other genes of the viruses were derived from the 1968 pandemic virus A/Hong Kong/1/68

(H3N2) because corresponding plasmids were already available from our previous study [25]. The viruses were plaque purified, grown in MDCK cells and stored in aliquots at −80°C. The identities of all viral genes were confirmed by sequencing.

Enzymatic activity of recombinant viruses

The stocks of recombinant viruses rgHAD+ and rgHAD− were analyzed for their content of the NA and M1 proteins by SDS-PAGE under non-reducing conditions and immuno-blotting using anti-H9N2 influenza virus antibodies, peroxidase-labelled secondary antibodies and ECL substrate (Pierce, Rockford, IL, USA). To study NA enzymatic activities, the stocks were adjusted to equal NA content and incubated with sialic acid-containing substrates. The liberation of sialic acid was quantified at different time points as described above for cell-expressed NAs. To determine the ability of the viral NA to destroy sialic acid receptors on cells, we used a hemagglutination-elution assay. Serial twofold dilutions of viruses in 50 μl PBS in U-bottomed microtiter plates were incubated for 1 h at 4°C with 50 μl of 0.5% suspensions of human erythrocytes. Disaggregation of virus-agglutinated cells was monitored at 37°C.

Adsorption of desialylated/resialylated red blood cells

Human erythrocytes that carry either Neu5Acα2-3Gal or Neu5Acα2-6Gal moieties were prepared as described previously [13, 36]. In brief, 100 μl of 10% human red blood cells were desialylated using 100 mU *Vibrio cholerae* sialidase (Behring AG, Marburg, Germany) and resialylated for 60 min at 37°C using either 0.5 mU rat α2-3-(N)-

sialyltransferase or 2.5 mU rat α2-6-(*N*)-sialyltransferase (Calbiochem, Bad Soden, Germany) in the presence of 1.5 mM CMP-sialic acid (Sigma, St. Louis, MO, USA). Resialylated erythrocytes were used to perform hemadsorption assay as described above.

Results

The NAs of pandemic H2N2 virus strains from 1957 carry amino acid substitutions in the hemadsorption site

The NA gene of the H2N2/1957 pandemic virus originated from an avian precursor; this gene was later inherited by the H3N2/1968 pandemic virus [40, 54]. Previous studies indicated that the NAs of H2N2 and H3N2 human viruses isolated after 1966 had multiple amino acid changes in the hemadsorption site and lacked hemadsorption activity [20, 29, 34, 48]. However, it remained obscure whether changes in the structure and activity of the hemadsorption site had already occurred at the onset of the H2N2 pandemic.

To address this question, we analyzed the N2 NA sequences currently available from the National Center for Biotechnology Information Influenza Virus Resource (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). With the exclusion of two virus strains, all sequenced human pandemic viruses isolated in 1957 and 1958 harboured substitutions in the conserved positions of the hemadsorption site, either S367N, or S370L (Fig. 1c). In the structural model of the N9 NA complex with two sialic acid molecules [48], the side chain hydroxyls of 367S and 370S of the protein form hydrogen bonds with the carboxyl oxygen and the 8-OH group of the sialic acid, respectively (Fig. 1b). Substitutions S367N and S370L affect these hydrogen bonds and, in addition, might create sterical obstacles for the accommodation of the sialic acid in the hemadsorption site. Three viruses from 1957 contained in addition to the 367/370 substitution a second change (D369E or P431Q). Neither 369 nor 431 of the N9 NA contacts the bound sialic acid residue. However, the side chain of D369 faces towards the solvent [49] and could potentially interact with either the sialic acid or the penultimate sugar residue in the hemadsorption site of N2 NA. Substitution P431Q could change the orientation of the 430 loop and affect the positions of residues 432 and 403 that interact with the sialic acid.

A/Leningrad/57 and A/Adachi/2/57 lacked substitutions in any of the six positions that contact sialic acid in the hemadsorption site. The HA of the former virus has avian-virus-like receptor specificity and is therefore believed to be a mutant derived in the laboratory by passaging in embryonated hen's eggs [26]. The sequence of the HA and passage history of the second virus are not known.

Two virus isolates from 1958, A/Malaya/16/58 and A/Albany/24/58, had substitutions N400S and N400K, respectively. Amino acids in this position are not conserved among the NAs of different subtypes [20, 48], however, both main chain and side chain atoms of 400N interact with the sialic acid residue in the N9 NA (Fig. 1b), suggesting that substitutions in this position could affect the stability of the complex. All sequenced viruses isolated after 1958 harboured the S370L substitution and accumulated additional substitutions in the hemadsorption site. In particular, the substitution W403R that occurred after 1965 could affect interactions of the protein with the 5*N*-acetyl moiety of the sialic acid (Fig. 1b). As a result, five substitutions in the hemadsorption site (S370L, N400S, N401D, W403R, P431K) separate the NA of the late H2N2 virus isolate A/Tokyo/3/67 and the NA of H3N2 pandemic virus A/Hong Kong/1/68 from a putative avian precursor.

Effects of amino acid substitutions in the second sialic acid binding site on hemadsorption and enzymatic activities of N2 NA

To assess phenotypic effects of the substitutions in the hemadsorption site of human H2N2 virus, we focused on the NA of the H2N2 pandemic virus A/Singapore/1/57. The hemadsorption site of this NA differs from that of a putative avian ancestor by a single amino acid substitution S367N (Fig. 1c). As reported previously [19, 21], the NA of SG/57 displayed high enzymatic activity, preferentially cleaved α2-3-linked sialic acids and supported replication of an avian reassortant virus in the duck intestine. Thus, apart from the substitution in position 367, the NA of SG/57 represents a typical avian virus NA. We amplified the full-length NA gene of SG/57 by RT-PCR from isolated RNA, cloned the cDNA into the mammalian expression plasmid pCAGGS/MCS and introduced a single-point substitution, N367S, that restored the sequence of the avian hemadsorption site. Using this avian-virus-like NA, we prepared three NA variants with amino acid substitutions S370L, N400S and W403R, respectively (Table 1). This set of five NAs modelled the NA hemadsorption site of the putative avian precursor of the 1957 pandemic viruses (variant N367S) and of distinct H2N2 viruses that circulated in the first years of the pandemic and afterwards (Fig. 1c).

To study properties of the NA variants, we transfected cultures of Cos-7 cells with pCAGGS-NA plasmids. Forty-eight hours later, we analyzed the cells for NA expression level, hemadsorption and enzymatic activities. All NAs were expressed in the same amount on the cell surface (Table 1). Human and chicken erythrocytes efficiently bound to the cells expressing the avian-virus-like NA

(N367S). By contrast, neither the NA of SG/57 nor any of the other three NAs with human-virus-like amino acid substitutions in the hemadsorption site showed significant hemadsorption (Fig. 2; Table 1). These data suggested that the majority of the earliest available virus isolates from the 1957 pandemic, as well as subsequent isolates, lacked high hemadsorption activity of avian NAs owing to mutations in the hemadsorption site.

We next compared enzymatic activities of NAs expressed in Cos-7 cells using several substrates (Table 1). The low molecular mass substrates 2′-(4-methylumbelliferyl)-N-acetylneuraminic acid (MU-NANA), 3′-sialyl-N-acetyl-lactosamine (3SLN) and 6′-sialyl-N-acetyllactosamine (6SLN) contained one sialic acid residue per molecule. The macromolecular substrates fetuin, human airway mucin, synthetic sialylglycopolymers 3SL-PAA and 6SLN-PAA [9] harboured multiple copies of sialyloligosaccharide moieties. Fetuin and mucin contained both 3-linked and 6-linked sialic acid residues, whereas sialylglycopolymers were monospecific and contained either 3-linked (3SL-PAA) or 6-linked (6SLN-PAA) residues. All five NAs cleaved 3SLN much faster than 6SLN in accord with the

known linkage specificity of avian virus NAs [5, 16, 19]. Four NAs (SG/57, N367S, N367S-N400S and N367S-W403R) desialylated each of the monovalent sialosides with identical efficiency, indicating that amino acid substitutions in the hemadsorption site had no effect on the enzymatic activity of the adjacent catalytic site. The variant N367S-S370L hydrolyzed 3SLN with about 30% lower efficiency than did other NAs. A similar effect was reported by Kobasa and colleagues [20] who found that substitution S370L decreased enzymatic activity of avian N2 NA against MU-NANA. Despite a lack of correlation between NA hemadsorption activity and hydrolysis of monovalent sialosides, all four hemadsorption-negative NAs desialylated macromolecular substrates 2–5 times less efficiently than did the hemadsorption-positive avian-virus-like NA variant N367S. These findings indicated that amino acid substitutions in the hemadsorption site of 1957 pandemic viruses resulted in reduced enzymatic activity as compared to the putative avian precursor. Interestingly, in most instances, the reduction was not caused by a direct effect of the mutation on the function of the adjacent catalytic site.
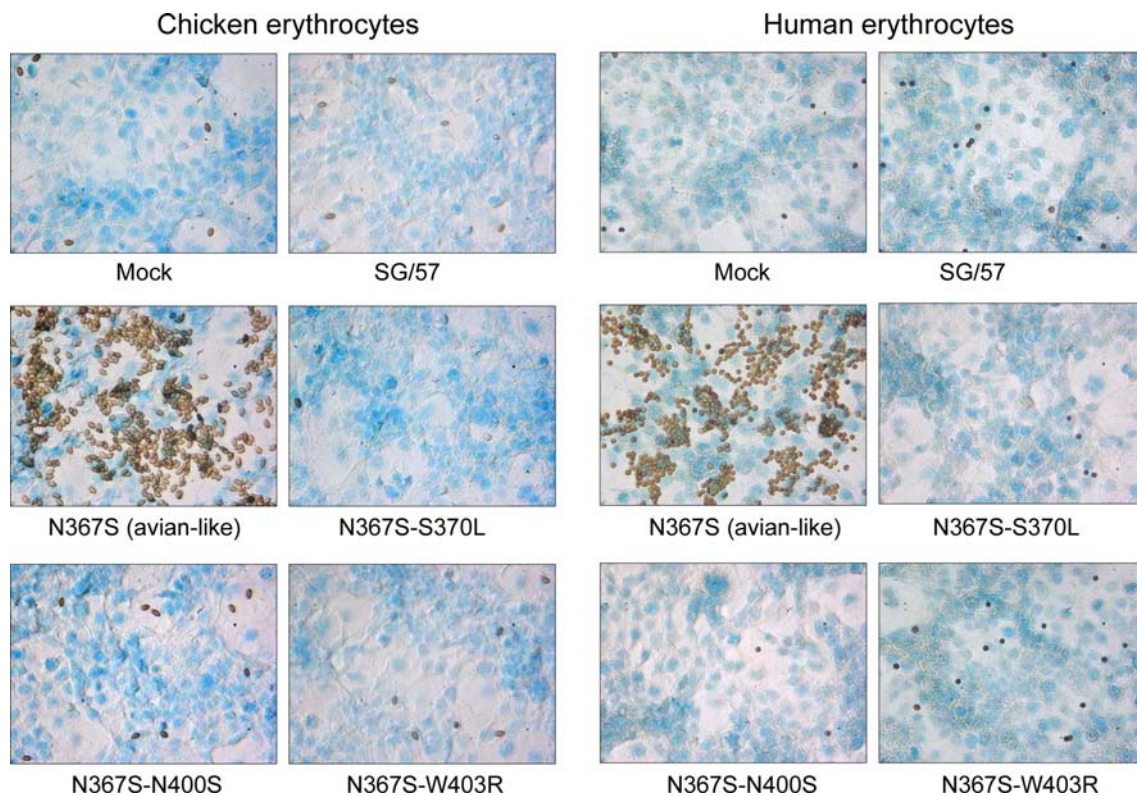
Fig. 2 Hemadsorption activity of N2 neuraminidases. Cos-7 cells were transfected with pCAGGS-NA plasmids coding for either SG/57 NA or its variants with indicated amino acid substitutions. Empty pCAGGS plasmid was used as a control (mock). Forty-eight hours post transfection, the cells were incubated with chicken (*left panel*) and human (*right panel*) erythrocytes, which are commonly used to characterize binding specificity of influenza viruses. The cells were stained and photographed using Olympus IMT-2 microscope equipped with Nikon DS-2Mv camera. Objective, 20×. Data are representative of five or more independent experiments

Enzymatic activity of recombinant viruses containing hemadsorption-positive and hemadsorption-negative NAs

To corroborate the results obtained in the experiments with expressed NAs, we tested the effect of the NA hemadsorption activity on the catalytic activity in the context of complete virus particles. Using the 8-plasmid reverse genetics system [17], we generated two recombinant viruses, rgHAD− and rgHAD+, which contained the hemadsorption-negative NA of SG/57 and its hemadsorption-positive mutant N367S, respectively. The seven other gene segments of each virus were derived from the 1968 pandemic virus A/Hong Kong/1/68 (H3N2) [25]. We determined the ratio of NA to matrix protein in the viruses by immunoblotting. No differences were found between rgHAD− and rgHAD+ (Fig. 3a), indicating that amino acid 367 had no effect on NA incorporation into virions. Based on immunoblotting analysis, we adjusted viral stock suspensions to identical NA concentrations (Fig. 3a) and compared their enzymatic activity (Fig. 3b). The pattern of viral enzymatic activities agreed with that observed in the case of NAs expressed in Cos-7 cells. Thus, viruses did not differ in their capacity to hydrolyze monovalent substrates MU-NANA, 3SLN and 6SLN, however, the virus with hemadsorption-negative NA desialylated macromolecular substrates significantly more slowly than did rgHAD+.

To compare the ability of the NAs to cleave sialic acid from receptors expressed on the cell surface, we studied virus elution from human erythrocytes. We incubated the viruses with erythrocytes for 1 h at 4°C and monitored disaggregation of virus-agglutinated cells at 37°C. The viruses standardized with respect to their content of NA and M proteins (Fig. 3a) displayed identical hemagglutination activity at 4°C (Fig. 3c, top panel). Complete disaggregation typically occurred between 6 and 8 h of incubation in the case of rgHAD+ (Fig. 3c, bottom panel) and between 24 and 48 h in the case of rgHAD− (data not shown).These experiments indicated that the virus with hemadsorption-negative NA destroyed receptors on red blood cells more slowly than did its hemadsorption-positive counterpart.

Based on these data, we conclude that the presence of the functional hemadsorption site enhances the capacity of the virus-associated NA to remove sialic acid from both soluble macromolecular substrates and cells.

Hemadsorption site binds to both α2-3-linked
and α2-6-linked sialic acids

To characterize binding specificity of the hemadsorption site with respect to two major natural sialic acid determinants (Neu5Acα2-3Gal and Neu5Acα2-6Gal), we employed human erythrocytes that were desialylated using *V. cholerae* sialidase and then resialylated using either α2-3- or α2-6-sialyltransferases. As expected, desialylated erythrocytes lost their ability to bind to Cos-7 cells expressing hemadsorption-positive NA variant N367S (Fig. 4). Resialylation of erythrocytes partially restored their binding although modified erythrocytes bound less efficiently than native red blood cells. It seems likely, that reduced hemadsorption activity of resialylated cells was at least partially determined by reduced levels of incorporation of sialic acid as compared to that of native erythrocytes [36]. However, as both α2-3- and α2-6-resialylated erythrocytes showed substantial hemadsorption activity, we conclude that the hemadsorption site of the NA can bind to sialic acids attached to the penultimate sugar chain by either linkage type.

To understand the molecular basis for the ability of the hemadsorption site to bind to both 3-linked and 6-linked sialic acids, we compared the available crystal structure of the hemadsorption site of N9 NA [48] with that of the receptor-binding site of H3 HA [15] (Fig. 5). In the case of HA, amino acid 226 on the 220-loop and amino acid 190 on the 190-helix are known to interact with asialic parts of 3-linked and 6-linked receptors and to be primarily responsible for the linkage recognition (see reviews [28, 42]). There is no structural equivalent of the 190-helix in the hemadsorption site of the NA. Furthermore, the 370-loop of NA is located in a relatively low position of the hemadsorption site as compared to the corresponding 220-loop of the receptor-binding site of HA. For example, the distance between the glycosidic oxygen of the bound sialic and the closest amino acid in the receptor-binding site of the HA (glutamine in position 226) is 3.8 A; the corresponding distance to the closest amino acid in the second sialic acid binding site of the NA (position 369) is about 2 A longer. Thus, the ability of the hemadsorption site to accommodate both α2-3- and α2-6-linked sialic acids can be explained by the lack of close amino acid counterparts for the interaction with the penultimate to sialic acid saccharide residue(s).

Hemadsorption and enzymatic activities of the NA
of the 1918 pandemic virus

The N1 NA of human influenza viruses originates from the NA of the virus that caused the pandemic in 1918 [39]. The earliest tested human H1N1 virus isolates, A/PR8/34 and A/Rome/49, showed weak, if any, hemadsorption activity [20]. To characterize properties of the 1918 NA, we synthesized the NA gene of A/Brevig Mission/1/18 (H1N1) using the sequence determined by Reid and colleagues [39] (Genbank AF250356). We compared hemadsorption and catalytic activities of this NA with those of two N1 NAs of
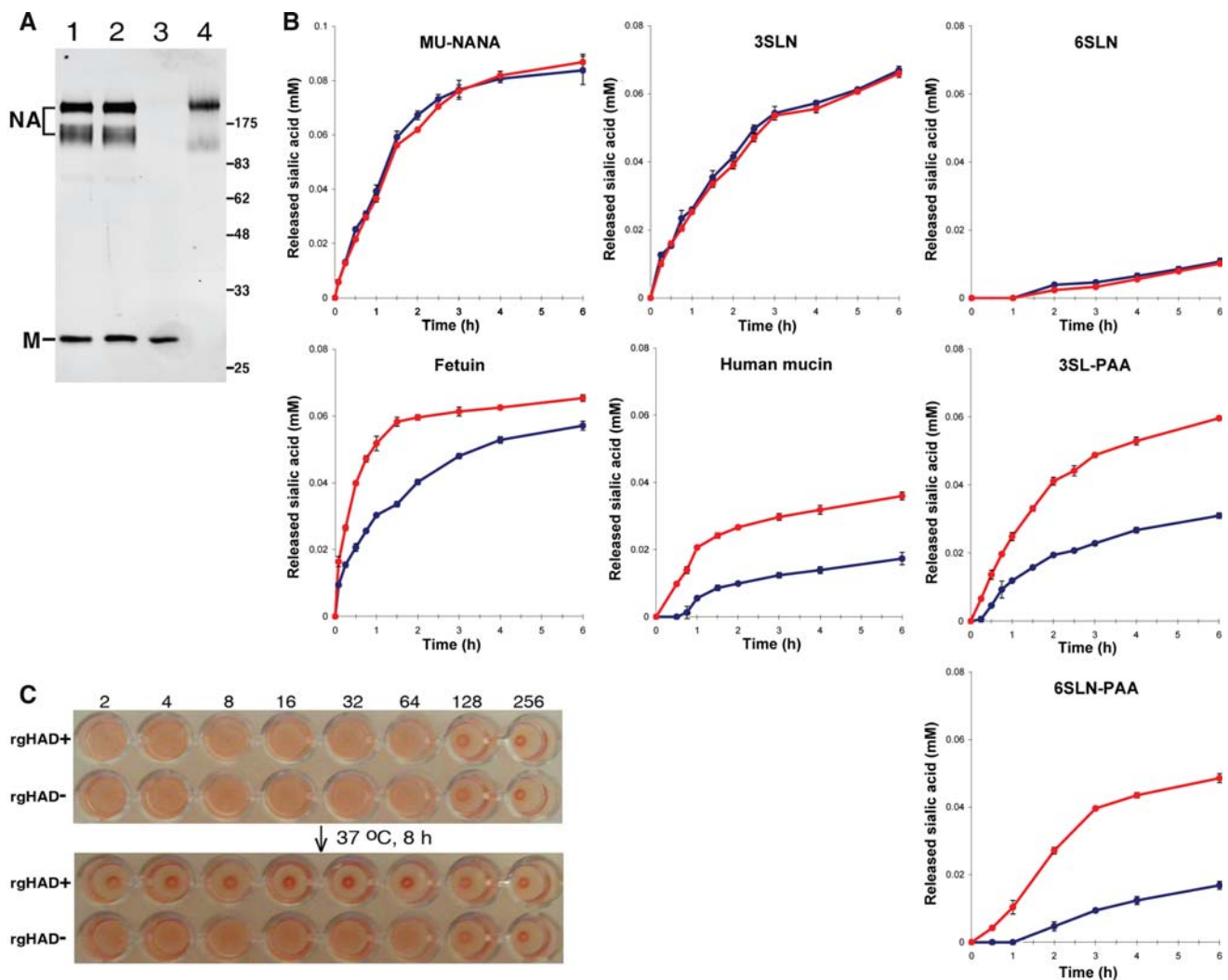
Fig. 3 Neuraminidase activity of the recombinant viruses rgHAD− and rgHAD+. Viral suspensions were adjusted to the same NA content (**a**) and compared for their enzymatic activity with respect to soluble substrates (**b**) and cells (**c**). Data are representative of five or more independent experiments. **a** Western blot analysis of rgHAD− (*lane 1*) and rgHAD+ (*lane 2*) using anti-H9N2 influenza virus antibodies. Lysates of 293T cells transfected with pCAGGS plasmids expressing M1 protein of A/Hong Kong/1/68 (*lane 3*) and NA of A/Singapore/1/57 (*lane 4*) were included as controls. **b** rgHAD− (*blue*) and rgHAD+ (*red*) were incubated with substrates containing 0.1 mM sialic acid at 37°C, and concentrations of released sialic acid were determined at different time points. To account for substrate-specific differences in viral neuraminidase activity, 20-fold lower virus concentration was used in the case of 3SLN, 3SL-PAA and MU-NANA than in the case of other four substrates. Mean values and SD of two replicate samples in one experiment are shown. **c** Serial twofold dilutions of the viruses were incubated with equal volumes of 0.5% human erythrocytes at 4°C for 1 h (*top panel*), followed by incubation at 37°C for 8 h (*bottom panel*)

avian viruses, A/Chicken/FPV/Rostock/34 (H7N1) (FPV) and A/Ostrich/Italy/984/00 (H7N1) (Os/Italy) using assays with transfected Cos-7 cells. The FPV NA was chosen because it is close to the 1918 NA both phylogenetically [39] and by the time of circulation; the Os/Italy NA represented a more distant NA. The expression levels of three N1 NAs on the cell surface were identical in these experiments as judged by the results of cell ELISA with antibodies against FPV (data not shown). The NA of 1918 virus displayed substantial hemadsorption with human erythrocytes, although the level of the hemadsorption

activity was reproducibly lower than that of avian N1 NAs (Fig. 6). The 1918 NA showed much weaker hemadsorption with chicken erythrocytes (five to ten times lower than avian NAs). Thus, similar to the NAs of 1957 pandemic viruses, the NA of 1918 virus had reduced hemadsorption activity. However, in the case of 1918 NA the reduction was less pronounced than in the case of 1957 NA.

The enzymatic activity of 1918 NA, when assayed with 3SLN as substrate, was identical to that of FPV NA and about 30% lower than that of the NA of Os/Italy (Table 2). Interestingly, the 1918 NA desialylated 6SLN two times
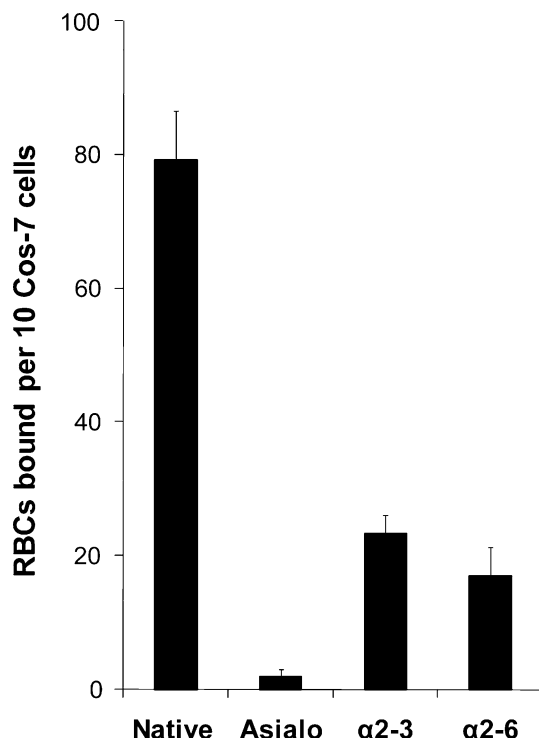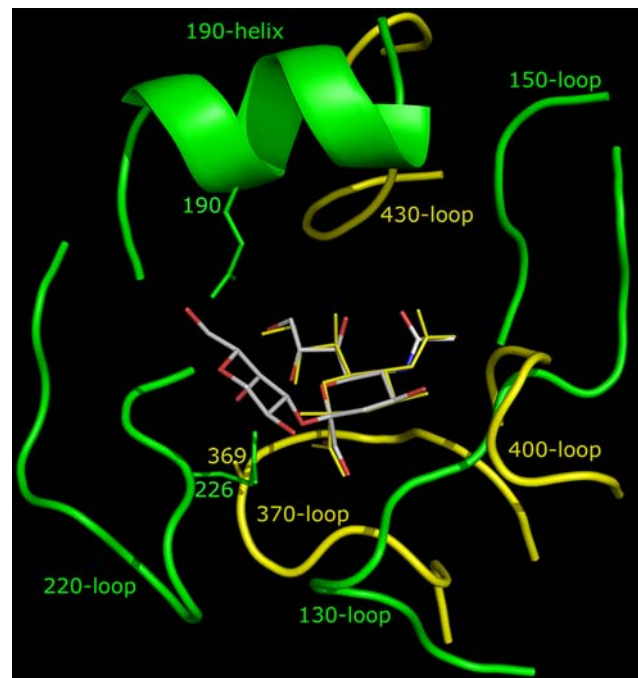
Fig. 4 NA hemadsorption to native and modified erythrocytes. Cos-7 cells expressing the hemadsorption-positive NA variant N367S were probed with native human erythrocytes, erythrocytes desialylated with *V. cholerae* sialidase, and erythrocytes resialylated using α2-3- and α2-6-sialyltransferases. Data show mean numbers of red blood cells attached per ten Cos-7 cells and are representative of three independent experiments

Fig. 5 Comparison of the structures of the NA hemadsorption site of the N9 NA with bound sialic acid [48] and of the HA receptor-binding site of A/duck/Ukraine/63 (H3N8) with bound Neu5Acα2-3Gal disaccharide [15]. The ligands are shown as stick models. Hemadsorption site (*yellow*) and receptor-binding site (*green*) are superimposed over the bound sialic acid residues. Positions of peptide loops forming the sites are indicated

faster than did avian N1 NAs. All three NAs cleaved 6SLN-PAA with identical efficiency; however, the 1918 NA displayed a reduced activity with respect to the three other polyvalent substrates tested. To compare the capacity of N1 NAs to discriminate between monovalent and polyvalent substrates, we calculated ratios of specific enzymatic activities 3SL-PAA/3SLN and 6SLN-PAA/6SLN (Table 2). Both ratios were at least twofold smaller in the case of the 1918 NA than in the case of the avian NAs, indicating that the polyvalency of the substrate has a lower impact on the catalytic efficiency of the 1918 NA. These results agree with the data on N2 NA mutants which suggest that hemadsorption activity of the NA enhances its catalytic activity with respect to polyvalent macromolecular substrates without changing hydrolysis rates of monovalent substrates.

Discussion

In this study, we addressed a long-standing question about the functional significance of the hemadsorption site present in the NA of avian influenza viruses [20, 23, 48]. To

this end, we cloned the hemadsorption-negative NA of the human pandemic virus A/Singapore/1/57 (H2N2) and generated its hemadsorption-positive avian-virus-like variant as well as three other hemadsorption-negative human-virus-like variants with single amino acid substitutions at different positions of the hemadsorption site. We next used this panel of five NA variants to determine how substitutions that abolish hemadsorption activity will affect the enzymatic activity of the NA. All hemadsorption-negative variants turned out to have a significantly lower enzymatic activity than the hemadsorption-positive counterpart with respect to all macromolecular substrates tested. Importantly, three out of four hemadsorption-negative variants (S367N, N400S and W403R) did not differ from the hemadsorption-positive NA in their capacity to hydrolyze low molecular weight monovalent sialosides. This finding indicated that the reduced catalytic activity of these variants resulted primarily from the loss of the hemadsorption activity and not from direct effects of the substitutions in the hemadsorption site on the structure of the adjacent catalytic site.

Similarly to influenza virus NA, some bacterial NAs, such as the NAs from *V. cholerae*, *Clostridium perfringens* and *Micromonospora viridifaciens* have lectin domains in addition to the catalytic domain. For example, the *N*-lectin-like
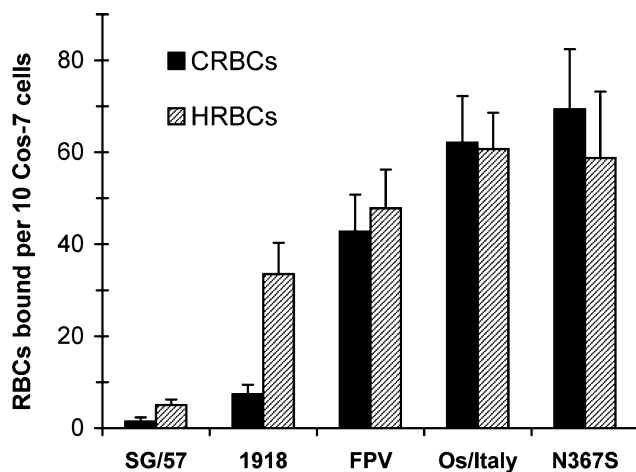
Fig. 6 Hemadsorption activity of N1 neuraminidases. Cos-7 cells were transfected with pCAGGS-NA plasmids coding for the neuraminidases of A/Brevig Mission/1/18 (H1N1), A/chicken/FPV/Rostock/34 (H7N1) and A/Ostrich/Italy/984/99 (H7N1). pCAGGS plasmids expressing N2 NAs of SG/57 and its hemadsorption-positive mutant (N367S) were used as negative and positive controls, respectively. Forty-eight hours post transfection, the cells were probed with chicken (*closed bars*) and human (*hatched bars*) erythrocytes. Data are mean values and SD of replicate samples in one experiment representative of five independent experiments. The values of hemadsorption activity of the 1918 NA differ from corresponding values of each other NAs tested ($P < 0.05$, unpaired two-sided t test)

domain of *V. cholerae* NA binds to sialic acid [31]. Functions of these lectin domains are not yet understood, but it is believed that they increase the catalytic efficiency of the enzymes (see ref. [31, 32] and references therein). We provide here direct experimental support to this hypothesis by demonstrating that an influenza virus NA with a functional hemadsorption site has a higher enzymatic activity than its hemadsorption-negative single-point mutants. Thobhani et al. [46] reported that bacterial NAs, which have additional lectin domains, hydrolyze polyvalent substrates with significantly greater catalytic efficiency than corresponding monovalent sialosides. The authors explained this effect by enhanced affinity of such NAs for polyvalent substrates. Our

results are consistent with this mechanism. We therefore postulate that the hemadsorption site of influenza virus NA serves to enhance the catalytic efficiency of the NA by binding natural multivalent substrates and thus recruiting and keeping them in the vicinity of the enzymatic site.

Hausmann et al. [16] assayed enzymatic activity of N1 NA using 3'-sialyllactose and 6'-sialyllactose and found that site-directed mutations of the hemadsorption site had no effect. Our data explains the failure of this and other previous studies [20, 53] to uncover the functional role of the NA hemadsorption site as neither study used polyvalent substrates. Bousse, Takimoto et al. [7, 8, 56] discovered recently that paramyxoviruses Newcastle disease virus (NDV) and Sendai virus have a second sialic acid binding site on their hemagglutinin–neuraminidase (HN) protein. They demonstrated that amino acid substitutions in these sites of either virus have no effect on the NA activity assayed using 3'-sialyllactose. It will now be interesting to test, whether the second binding site on the HN of paramyxoviruses could facilitate desialylation of multivalent substrates.

Two earlier reports revealed that erythrocytes bound to hemadsorption-positive N9 and N1 NAs could not be released by the viral NA activity, but were released by broadly active bacterial sialidases [3, 16]. These data suggested that the hemadsorption site can bind to an unidentified species or linkage of sialic acid that is not sensitive to cleavage by the catalytic site of the NA. The N1 NA was previously found to bind to both 3-linked and 6-linked receptors [16]. We have shown here that the N2 subtype NA also binds to both linkage types. Analysis of the structural features of the NA hemadsorption site (Fig. 5) explains the inability of the hemadsorption site to differentiate between the 6- and 3-linked sialic acids and predicts that this site can have a relatively broad binding specificity. The biological significance of these findings remains to be determined. In any case, binding to non-cleavable substrate moieties does not seem to be a

Table 2 Enzymatic activities of N1 neuraminidases expressed in Cos-7 cells

NA	Enzymatic activity (pmole sialic acid released per minute per culture)						Relative enzymatic activity[a]	
	3SLN 0.1 mM	6SLN 0.4 mM	Fetuin 0.3 mM	Human mucin 0.1 mM	3SL-PAA 0.1 mM	6SLN-PAA 0.4 mM	3SL-PAA/ 3SLN	6SLN-PAA/ 6SLN
1918	300 (16)	86 (6.2)	90 (6.1)	3.2 (0.3)	120 (22)	67 (7.7)	0.41 (0.05)	0.78 (0.01)
FPV	310 (43)	37 (7.9)*	120 (11)*	4.6 (0.8)*	230 (28)*	76 (2.4)	0.75 (0.15)*	1.72 (0.09)*
Os/Italy	410 (23)*	34 (11)*	140 (13)*	5.3 (0.6)*	300 (43)*	86 (16)	0.74 (0.09)*	2.87 (0.24)*

All data are mean values and SD (in parenthesis) of two to four independent experiments with assay performed in duplicate ($n = 4$–8). Concentrations of bound sialic acid used in the assay are indicated under the name of corresponding substrate

* $P < 0.05$ (unpaired two-sided t test versus 1918 NA)

[a] Ratio of specific enzymatic activities determined with indicated polyvalent and monovalent substrates

prerequisite for the hemadsorption-mediated enhancement of the catalytic efficiency, as the enhancement effect was observed in the case of mono-specific sialylglycopolymers 3SL-PAA and 6SLN-PAA (Fig. 3b) that do not carry non-cleavable sialic acid residues.

In our experiments with recombinant viruses, the virus with hemadsorption-positive NA did not display a higher hemagglutinating activity and slower elution from red blood cells than did the hemadsorption-negative counterpart. These results indicate either that the hemadsorption site of the NA does not significantly contribute to the HA-mediated binding to cellular receptors or that the hemadsorption-dependent enhancement of receptor cleavage outperforms the hemadsorption-mediated enhancement of attachment. Thus, our data do not support the hypothesis that the hemadsorption site of influenza virus NA plays a significant role in the virus attachment to target cells [38, 48].

Two influenza pandemics in the last century were caused by viruses that derived both HA and NA from avian influenza viruses (H1N1, 1918; H2N2, 1957) (reviewed in ref. [40, 54]). A shift of the receptor-binding specificity of the HA from Neu5Acα2-3Gal recognition to Neu5Acα2-6Gal recognition is believed to be essential for the emergence of these pandemic viruses [25, 26, 47], whereas functional changes in their NAs have not been identified. For example, it was known that the N2 NA of human viruses isolated after 1967 harboured substitutions in the hemadsorption site and lacked hemadsorption activity [20, 34], however, due to a lack of sequencing and phenotypic data it was not clear what happened with the hemadsorption activity of the N2 NAs in the first year of the 1957 pandemic. Furthermore, analyses of H2N2 and H3N2 viruses isolated from humans between 1957 and 1987 revealed a gradual change in the relative activity of the N2 NA with respect to 3- and 6-linked sialic acids and in the absolute enzymatic activity [5, 19, 21]. Nevertheless, the first phenotypic changes were observed in viruses from the early 1960s, whereas NAs of a few tested H2N2 virus isolates from 1957 appeared to fully retain enzymatic characteristics of their avian precursor. In this study, we show for the first time that the NA of viruses that caused the influenza pandemic in 1957 differed from their putative avian ancestor by amino acid substitutions in the hemadsorption site, lack of hemadsorption activity and reduced catalytic activity. Substitutions in the different positions of the hemadsorption site (367, 370, and 400) emerged in independent N2 lineages (see Fig. 1), suggesting that the hemadsorption site was under positive selective pressure to change and that it was destroyed at some time points after initial avian-to-human transmission when the pandemic virus started to spread in the human population. Two of the substitutions, S367N and W400N, only affected the hemadsorption activity, whereas substitution S370L also had marginal direct effect on the activity of the catalytic site as judged by changes in the hydrolysis rate of some monovalent substrates (Table 1 and ref. [20]). Interestingly, only the lineage with S370L change, that showed the lowest enzymatic activity against all tested substrates continued to circulate after the 1957–1958 pandemic, possibly owing to a better fitness of the NA in humans. Viruses with the S370L substitution isolated after 1958 accumulated additional mutations in the hemadsorption site (Fig. 1c). Given that the S370L change was sufficient to abolish hemadsorption activity, the functional significance of these mutations remains unclear. Because the hemadsorption site overlaps with one of the NA antigenic sites [53], we speculate that these additional mutations in the HAD site of hemadsorption-negative human NAs could represent the antigenic drift of the NA.

In contrast to the NA of the 1957 pandemic virus, the only available sequence of the N1 NA of the 1918 pandemic virus [39] shows no substitutions of the six key amino acids in the hemadsorption site that contact bound sialic acid. Nevertheless, this NA displayed significantly decreased hemadsorption activity and lowered enzymatic activity than two avian N1 NAs tested. While further studies are required to confirm these findings and specify their molecular mechanisms, our data suggest that the NA of A/Brevig Mission/1/18 might have differed already from its putative avian ancestor in birds.

The role of the viral NA in the virus life cycle is mitigation of unfavourable HA interactions with receptors by removing sialic acids from the mucous blanket, the cell surface and progeny virions [2, 10, 30, 35]. Because of their opposing activities, a functional balance between HA and NA is required to ensure that NA destroys decoy receptors and inhibitors that can bind to the HA but does not excessively compromise virus entry into susceptible cells by premature destruction of functional receptors. This balance depends on the host- and tissue-specific patterns of sialic acid-containing receptors and inhibitors present at the site of viral replication. The balance between HA and NA can be disturbed by various events, such as virus transmission to a new host species or gene reassortment with acquisition of a new HA/NA combination; the resulting decrease in the viral fitness could be overcome by compensatory mutation of the HA, NA or both proteins (for a review, see ref. [51]). One can speculate, therefore, that alteration of the enzymatic activity of the NA of the 1957 pandemic viruses (and likely of the 1918 pandemic viruses) served to compensate for the changes in the receptor-binding activity of the viral HA and to convert a virus originally adapted to the intestinal epithelium of birds [21, 54] to its new environment in the human respiratory tract. The NA of the third pandemic of the 20th century (H3N2, 1968) originated from the contemporary human H2N2

virus, and hence lacked hemadsorption activity and had significantly reduced enzymatic activity [5, 20, 21]. Thus, in this case the balance seems to have been restored by acquiring the NA gene directly from a human virus rather than by a gradual adaptation of an avian virus NA by point mutations.

Recent human infections by H5N1, H7N7 and H9N2 avian influenza viruses raise fears of a new pandemic and highlight the lack of full understanding of adaptive changes in the avian virus genes required for the emergence of a pandemic virus [12, 37]. The importance of HA adaptation for efficient binding to Neu5Ac2-6Gal-terminated receptors in human airway epithelium is well recognized providing a marker for assessing the pandemic potential of avian viruses [6, 22, 29, 55]. Our results show that genetic and functional changes in the NA represent another indicator of ongoing adaptation of avian influenza viruses to humans.

Note added in proof Since the submission of our manuscript, the new swine-like H1N1 influenza virus was detected in humans. Our analysis of available NA sequences (NCBI Influenza Virus Resource, GenBank sequences from 2009 H1N1 influenza outbreak) indicate that this virus contains a mutation S372N in the NA that likely reduces or abolishes its hemadsorption activity.

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