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Identification of Residues That Affect Oligomerization and/or Enzymatic Activity of Influenza Virus H5N1 Neuraminidase Proteins

Meiling Dai,[a] Hongbo Guo,[a] Jos C. F. M. Dortmans,[a] Jojanneke Dekkers,[a] Johan Nordholm,[b] Robert Daniels,[b] Frank J. M. van Kuppeveld,[a] Erik de Vries,[a] Cornelis A. M. de Haan[a]

Virology Division, Department of Infectious Diseases & Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands[a]; Department of Biochemistry and Biophysics, Center for Biomembrane Research, Stockholm University, Stockholm, Sweden[b]

ABSTRACT

Influenza A virus (IAV) attachment to and release from sialoside receptors is determined by the balance between hemagglutinin (HA) 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 K_m 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 (K_m 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.

IMPORTANCE

The IAV HA and NA glycoproteins are important determinants of host tropism and pathogenicity. However, NA is relatively understudied compared to HA. Analysis of soluble versions of these glycoproteins is an attractive way to study their activities, as they are easily purified from cell culture media and applied in downstream assays. In the present study, we analyzed the enzymatic activity of different NA ectodomains with three commonly used tetramerization domains and compared them with full-length NA proteins. By performing a mutagenic analysis, we identified several residues that affected NA assembly, activity, and/or substrate binding. In addition, our 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. NA ectodomains extended with a Tetrabrachion domain better mimicked the full-length proteins than when the other tetramerization domains were used.

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 the 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 nonfunctional (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 to H18) and 11 NA (N1 to 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

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Address correspondence to Cornelis A. M. de Haan, c.a.m.dehaan@uu.nl.

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 homotetrameric 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 various lengths. 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 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 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 (K_m value; positions 252 and 347).

MATERIALS AND METHODS

NA gene preparation. Human codon-optimized NA ectodomain (head plus stalk domain, amino acids 62 to 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 full-length NA (FL-NA) protein-coding plasmids were generated by replacement of the non-NA coding sequences with 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 (grams of DNA to grams of 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 Dulbecco's modified Eagle's medium (DMEM) containing 2% FCS for the full-length NA constructs. Five days posttransfection, cell culture media containing soluble NA were harvested and cleared by centrifugation, and the protein expression levels of the soluble NA proteins were analyzed by SDS-PAGE followed by Western blotting with horseradish peroxidase (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 bovine serum albumin (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) as described previously (34). Cells expressing full-length NA proteins were harvested at 36 h to 48 h posttransfection. Cells were lysed with phosphate-buffered saline (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 min, and protease inhibitor cocktail (Roche) was added to prevent protein degradation. Quantification of the protein expression levels was performed by Western blotting, 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) were used as secondary antibodies. All results were confirmed 2 to 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 using a procedure similar to one 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 (50 mM Tris-HCl, 4 mM $CaCl_2$, pH 6.0) in a flat-bottom 96-well black plate (Greiner Bio-One). Subsequently, a similar volume of reaction buffer containing 200 μM MUNANA was added to each well, mixed well, and incubated at 37°C for 60 min. The reaction was terminated with the stop solution (0.1 M 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 per nanogram) of the different NA proteins was determined from the linear parts of the resulting curves and graphed relative to an NA reference sample. Kinetic analysis was performed to determine the K_m values of the different NA proteins. NA samples, resulting in approximately 20% of the maximum RFU level after 1 h of incubation with 100 μM MUNANA, were incubated with different concentrations of MUNANA, ranging from 3.9 to 500 μM (using 2-fold serial dilutions) in a total volume of 100 μl. The fluorescence of 4-MU was measured at 37°C every 5 min for 40 min. The data were fitted to the Michaelis-Menten equation by nonlinear regression using Prism 6.05 software (GraphPad), and the K_m value was determined.

GALLEX TMD interaction system. The NA TMD interactions from the HN and VN strains were determined using the *Escherichia coli* GALLEX assay (36). The coding sequences for residues 2 to 35 and 2 to 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 to 34), the conserved N-terminal amino acids (residues 2 to 6), and either 1 or 8 juxtamembrane amino acids on the C-terminal side. SU101 cells, which contain 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 to an A_{600} of 0.1 and induced with 0.05 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2.5 h at 37°C until an A_{600} of ≈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 analysis of variance (ANOVA) in combination with Bonferroni's multiple-comparison test using Prism 6.05 software. The data shown are the means from three independent experiments. Error bars indicate standard deviations. The level of significance was determined as $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****).

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 to 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 an 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 HEK293T 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 masses (Fig. 1B).

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

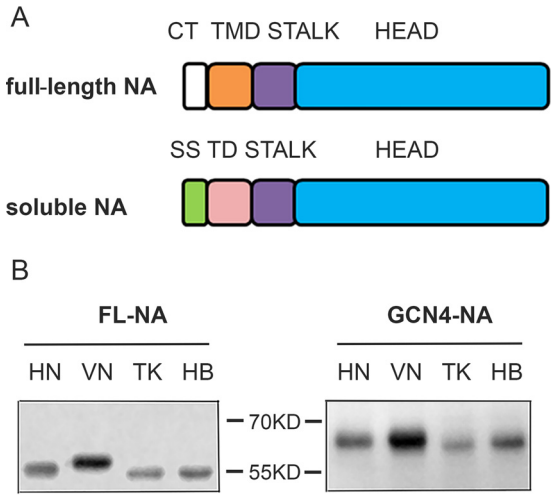

FIG 1 Expression of recombinant NAs. (A) Schematic representation of the recombinant full-length NA (FL-NA) and soluble NA proteins. The FL-NA protein contains the NA head, stalk, transmembrane domain (TMD), and cytoplasmic tail (CT). The soluble NA protein contains the head and stalk regions fused with 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 Materials and Methods. The position on the gel of the relevant molecular mass markers is shown.

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 Materials and Methods, the specific activities of the different NA proteins were calculated. As shown in Fig. 2C, the full-length proteins (HN, TK, and HB) were approximately 4 to ~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 those of the other NA proteins. However, the difference is much bigger between the soluble proteins (approximately 12-fold) than between the full-length proteins (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 K_m values of the different NA proteins. The K_m 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 K_m values than FL-NA VN, indicating that the VN protein has a lower binding affinity for sialic acid (Fig. 3A). Similar differences in the K_m 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 K_m values, which are significantly higher for the VN proteins than for the other proteins.

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

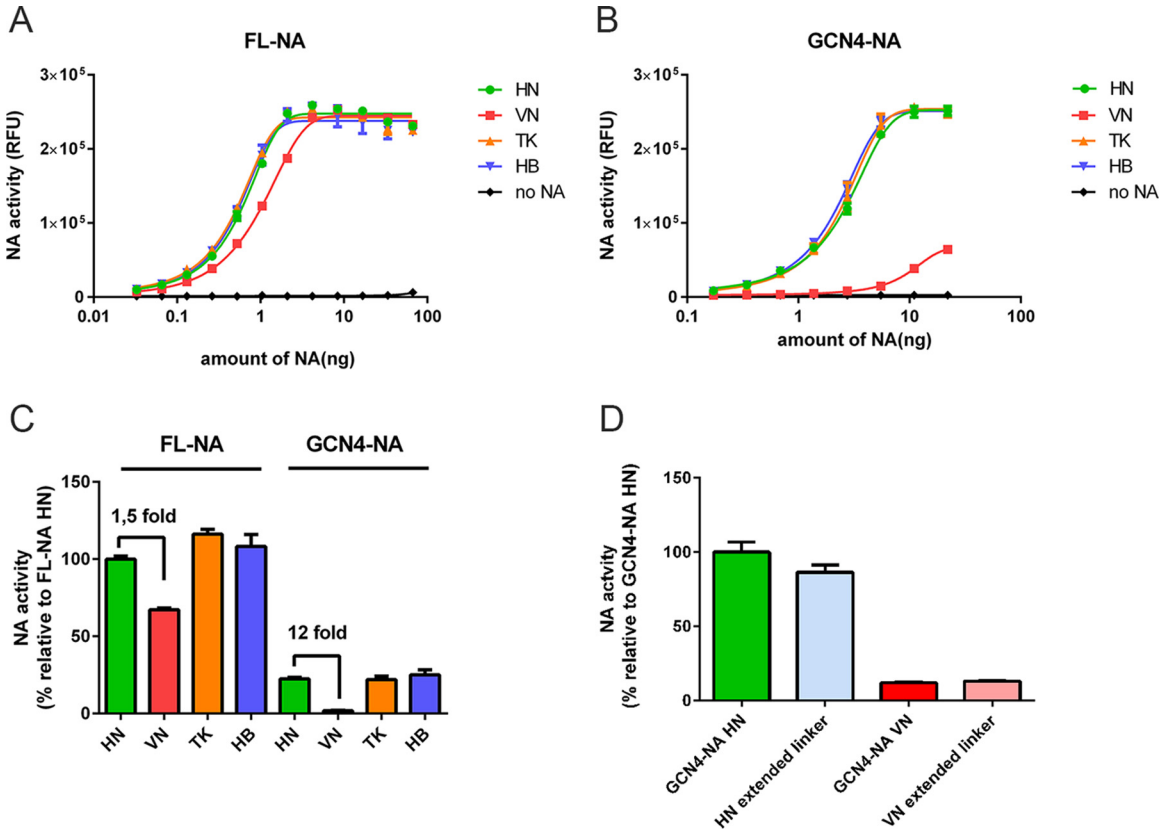

FIG 2 Activity of recombinant NA proteins derived from different H5N1 viruses. The NA enzymatic activity of preparations containing different amounts of the FL-NA (A) or GCN4-NA (B) proteins HN, VN, TK, and HB was determined using the MUNANA fluorometric assay (RFU, relative fluorescent units). (C) Specific activity (RFU/ng) of the different NA proteins was determined from the linear parts of the curves shown in panels 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 means from three independent experiments. Error bars indicate standard deviations.

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 were heat denatured only for a very short time (5 s), 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 could a minor protein species be detected at a lower position in the gel that likely corresponds to NA monomers. These results indicate

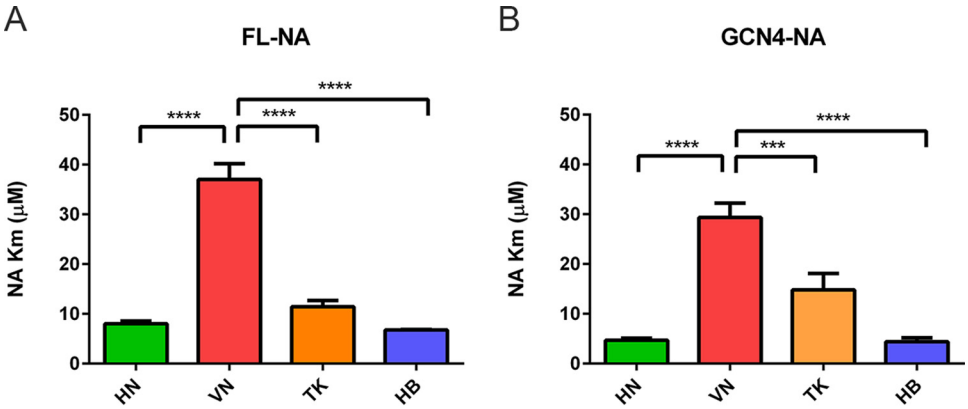

FIG 3 K_m values of FL- and GCN4-NA proteins. (A) K_m values of FL-NA proteins. (B) K_m values of soluble GCN4-NA proteins. All graphs represent the means from three independent experiments. Error bars indicate standard deviations. Significant differences by one-way ANOVA in combination with Bonferroni's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

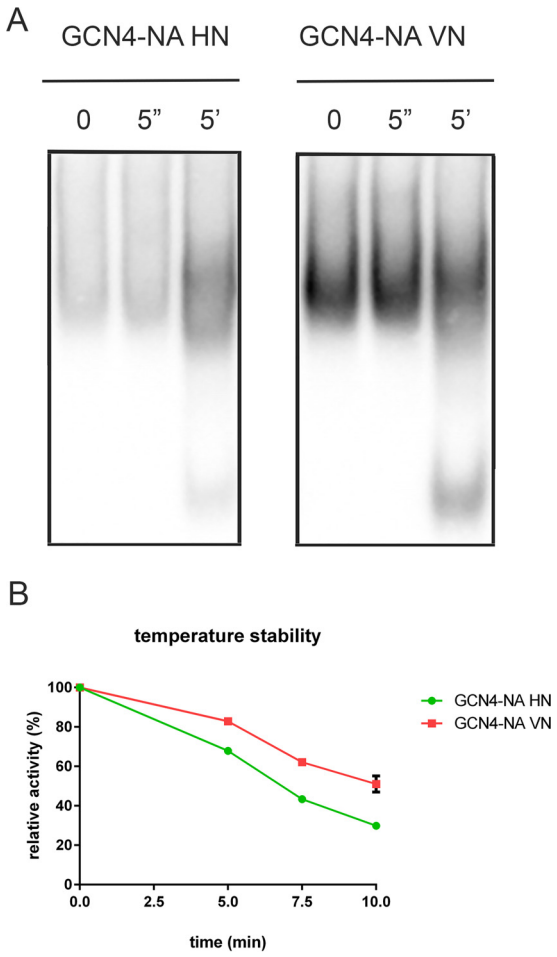

FIG 4 Stability of GCN4-NA HN and VN proteins. (A) BN-PAGE analysis of the GCN4-NA HN and VN proteins. NA samples were subjected to gel electrophoresis without boiling ("0") or after boiling for 5 s or 5 min. The positions of the presumed monomeric and tetrameric forms of NA in the gel are indicated.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

that the enzymatic difference between the two recombinant soluble NA proteins is not associated with differences in their oligomerization state *per se*. The thermostability of the two proteins next was compared by heating them to 50°C for different time periods and monitoring their enzymatic activity (Fig. 4B). The activity of both enzymes decreased after 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 is not explained by the VN protein being less thermostable.

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 (K84T, N95R, Y100H, H252Y, S343P, G347Y, E385G, and G454S; N2 numbering) domain (Fig. 5). Although the GCN4-extended HN and VN proteins differed dramatically in their K_m 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 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.

Identification of residues in NA that affect K_m. To identify the residues that are responsible for the different K_m values of the HN and VN proteins, we performed a mutagenic analysis. The residues that differ between the two proteins in the stalk and head domains were mutagenized one by one in the background of the GCN4-NA VN protein. All mutant VN proteins were expressed and their K_m values were determined (Fig. 7A). Of the 10 mutant NA proteins tested, only two (H252Y and G347Y) displayed K_m 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). In agreement with the TK and HB proteins also having a low K_m 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 positions 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 and B). The two substitutions that altered the K_m value also slightly increased the specific activity, but to a lesser extent that was not as significant as that of 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 an 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 and 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 alters the oligomerization of the head domain, as its substitution had a large effect on the NA-specific activity but not on the K_m 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

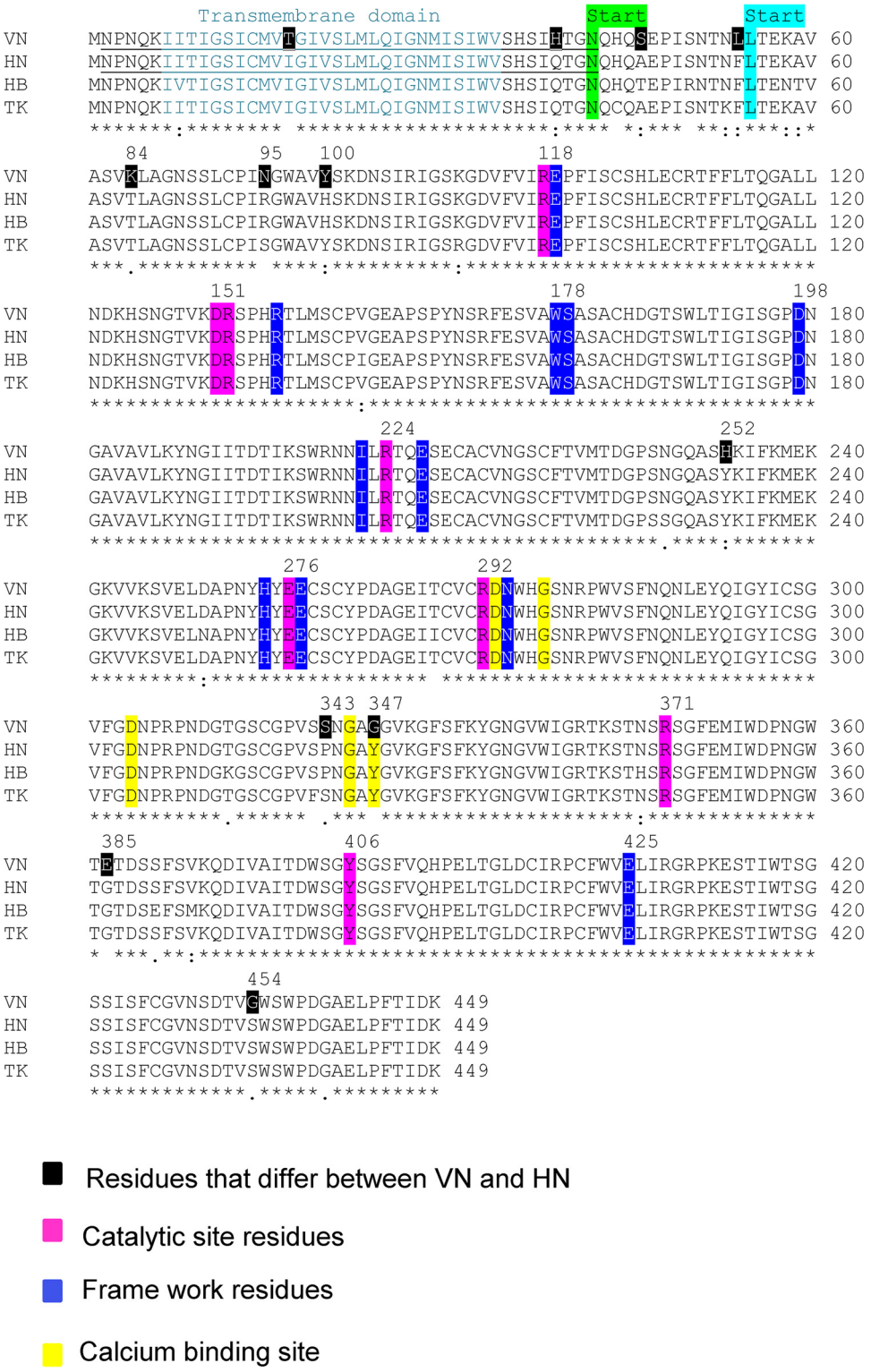

FIG 5 Alignment of the N1 proteins used in this study. Clustal Omega multiple-sequence alignment of the N1 proteins used in this study is shown. Residues that differ between the VN and HN proteins are indicated by the black boxes, and their numbering is indicated (N2 numbering is used from residue 84 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.

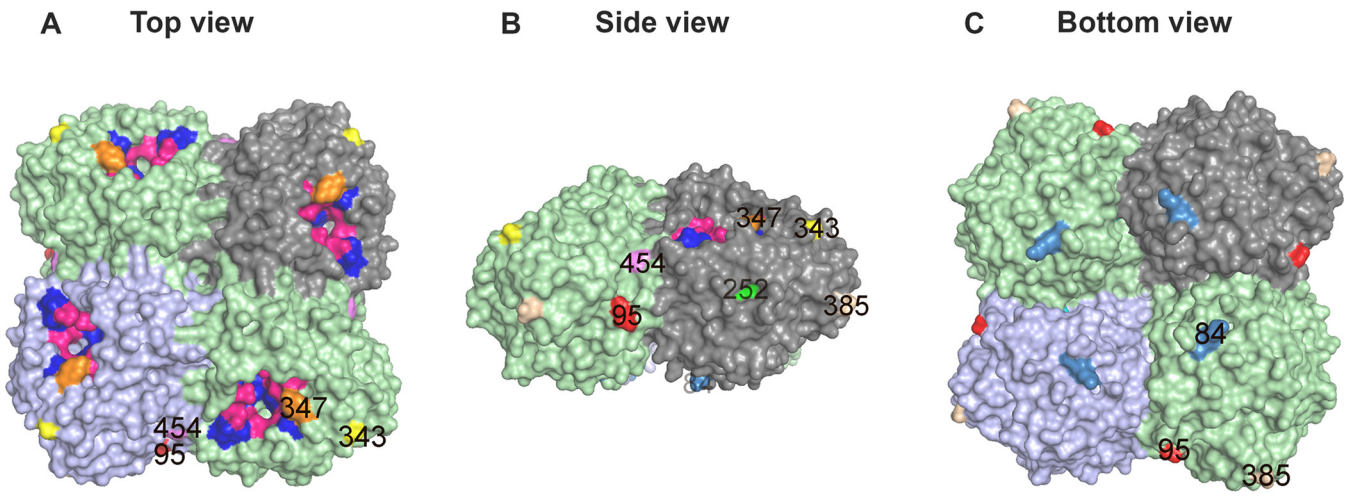

FIG 6 Structure of the H5N1 NA protein. Top (A), side (B), and bottom (C) views of the structure of NA protein of A/Vietnam/1203/04 generated with PyMol software are shown (PDB entry 2HTY) (52). This protein differs from the VN protein used in this study at only two positions (283N and 347Y). The residue at position 283 (not indicated) does not differ between the HN and VN N1 proteins. The NA catalytic site and the framework residues (as indicated in Fig. 5) are colored pink and blue, respectively. These residues do not overlap 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, and 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 HN proteins are not indicated as the structure thereof is not solved.

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 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

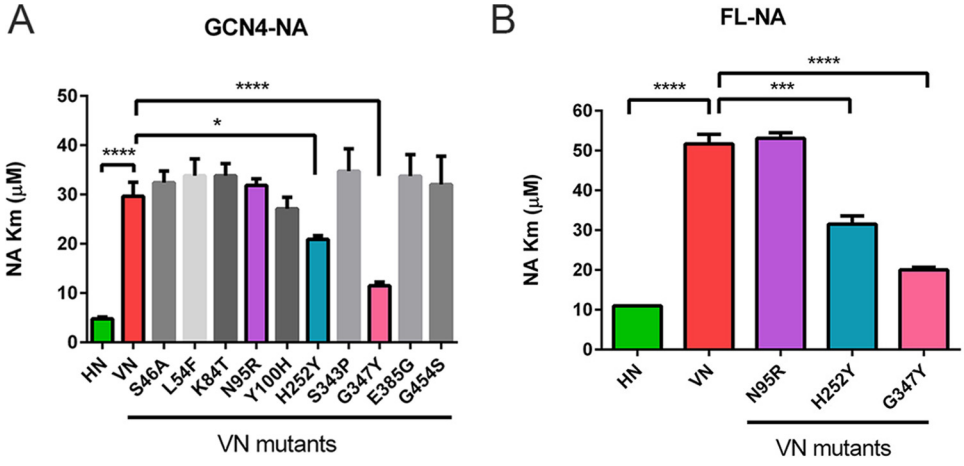

FIG 7 K_m values of mutant NA VN proteins. The indicated single-amino-acid changes were introduced into the background of the NA VN protein, and the K_m values of the resulting GCN4-NA (A) and FL-NA (B) proteins were determined. Wild-type VN and HN GCN4- and FL-NA were used as a reference. All graphs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

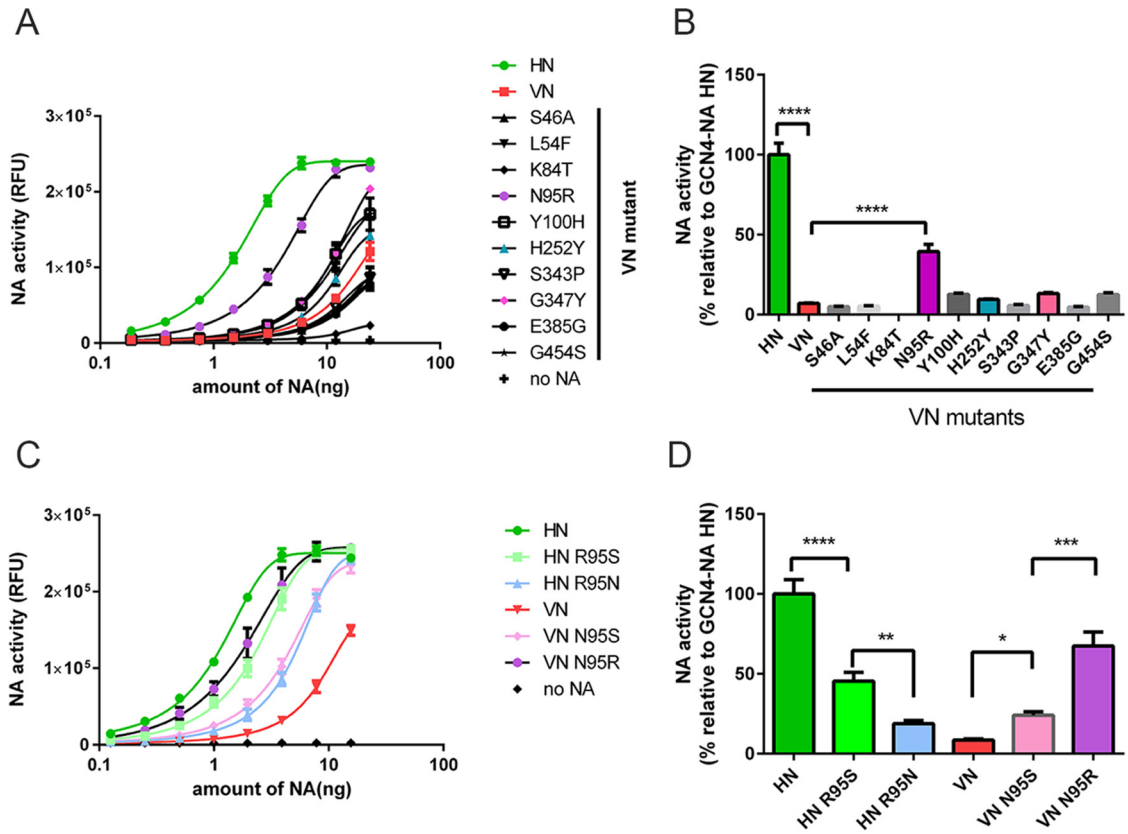

FIG 8 Enzymatic activity of mutant GCN4-NA VN and HN proteins. (A) Single-amino-acid changes were introduced into the background of the GCN4-NA VN protein, and preparations containing different amounts of these proteins were analyzed for their enzymatic activity, as described in the legend to Fig. 2. Wild-type VN and HN GCN4-NA were used as a reference. (B) The specific activity of the different NA proteins shown in panel 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 replaced 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. Wild-type VN and HN GCN4-NA were used as a reference. (D) The specific activity of the different NA proteins shown in panel C is graphed relative to the specific activity of the GCN4-NA HN protein. All graphs represent the means from three independent experiments. Error bars indicate standard deviations. Significant differences by one-way ANOVA in combination with Bonferroni's multiple-comparison test relative to GCN4-NA VN (B) or to VN N95S or HN R95S (D) are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

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.

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). Thus, as is the case 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 mea-

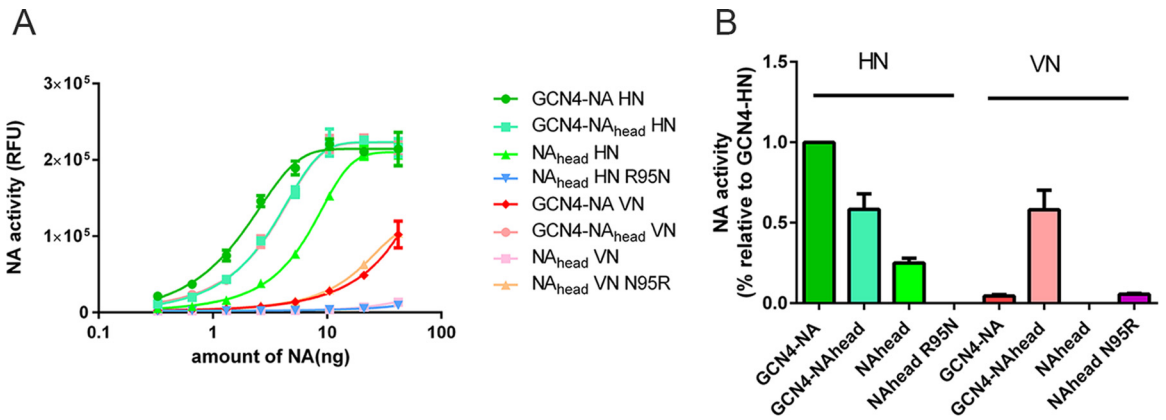

FIG 9 Enzymatic activity of NA proteins in the absence of the stalk and tetramerization domain. (A) Enzymatic activity of preparations containing different amounts of HN and VN GCN4-NA, GCN4-NA proteins lacking the stalk (GCN4-NA_{head}), NA head domains lacking the GCN4 domain (NA_{head}), and NA head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

sured. The interaction between the TMDs (residues 7 to 34) were determined in the presence of the 5 highly conserved N-terminal residues and either 1 (construct containing NA residues 2 to 35) or 8 (construct containing NA residues 2 to 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 that of the HN TMD, but when a 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 positions 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 of 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 the 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 specific activities similar to those of the GCN4-pLI-extended proteins, with the

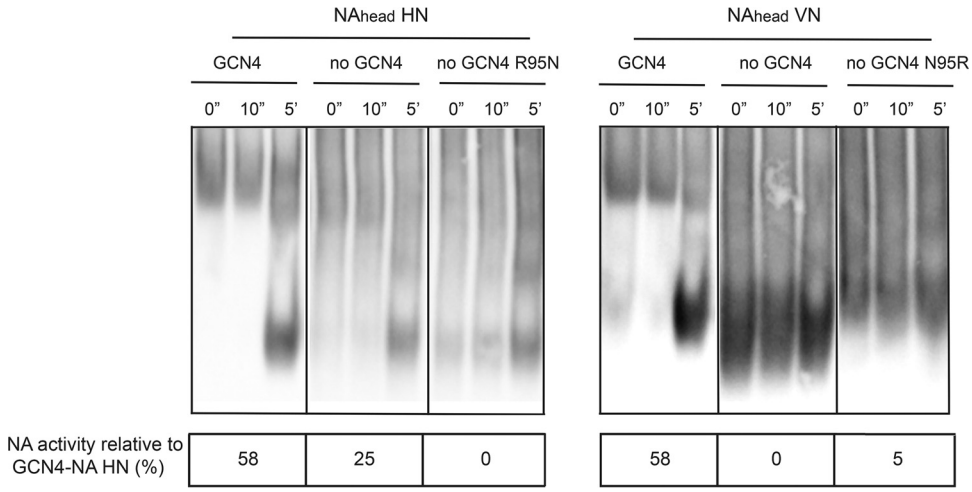

FIG 10 BN-PAGE analysis of HN and VN NA head domains. BN-PAGE analysis of the HN and VN NA head domains in the presence or absence of the GCN4 domain, with and without the substitution at position 95. NA samples were subjected to gel electrophoresis without boiling (“0”) or after boiling for 10 s or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

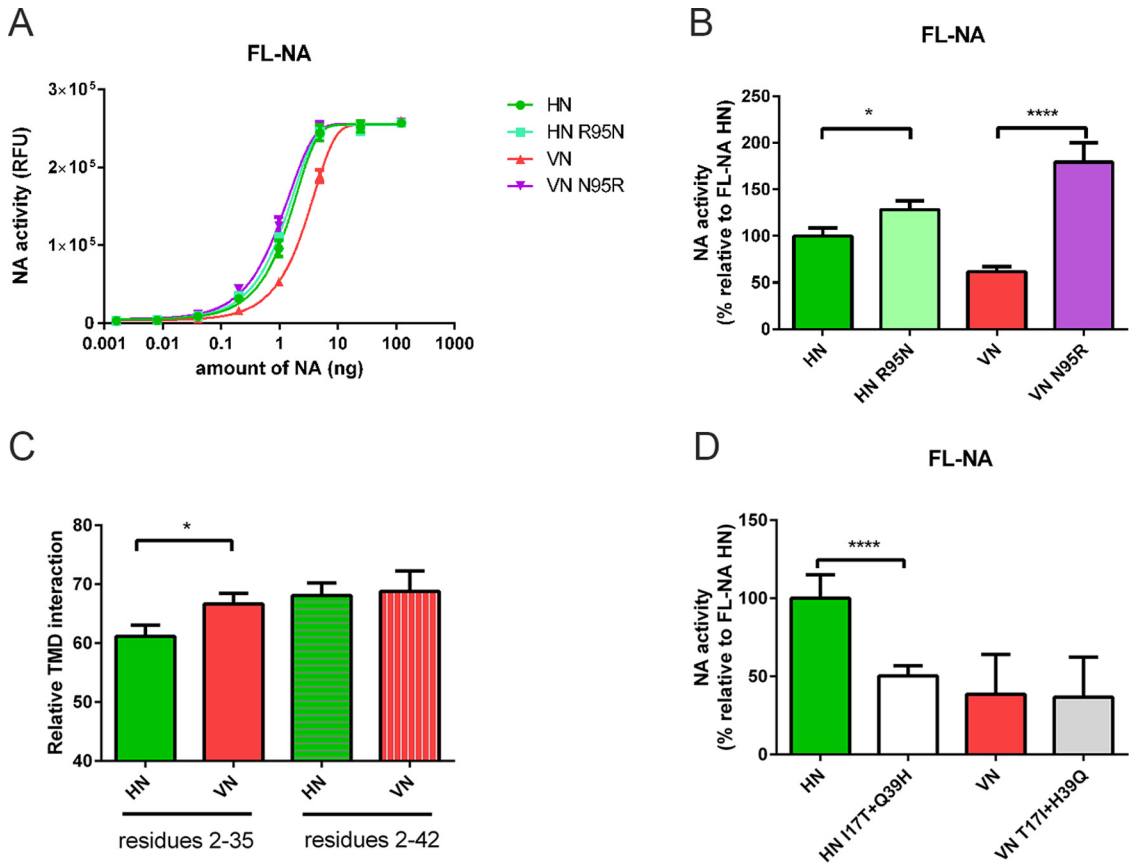

FIG 11 Substitution of residues located in the TMD and at position 95 in the FL-NA proteins. (A) Enzymatic activity of preparations containing different amounts of FL-NA HN and VN proteins with or without the indicated alteration of the residue at position 95. (B) The specific activity of the FL-NA proteins based on the results shown in panel 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 to 42) or 8 (residues 2 to 42) juxtamembrane C-terminal amino acids (see Fig. 5 for their sequences). (D) The specific activity of 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 means from three independent experiments. Error bars indicate standard deviations. Significant differences between the specific activity of the wild-type 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 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

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 K_m 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 of FL-NA proteins than the other coiled-coil tetramerization domains.

Finally, we studied the effect of changing the residues at positions 95, 252, and 347 in the background of the TE-NA HN and VN proteins. As shown in Fig. 13, replacement of the residues at positions 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. Alteration of the indicated residues in the VN protein affected the K_m to a similar extent as that observed for the GCN4-NA and FL-NA proteins (compare Fig. 13 and 7). Introduction of the reciprocal mutations in the HN protein did not affect the K_m value for the residues at positions 95 and 252, while the K_m 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 residue at position 347 for the K_m value. For the residue at position 252 we observe a background-dependent effect on the K_m value. However, the importance of this residue for the K_m value in the VN protein is observed for the FL-, GCN4-, and TE-NA proteins.

DISCUSSION

Here, we compared the enzymatic activities of four NA proteins derived from different H5N1 viruses that were expressed either as soluble proteins fused to different commonly used tetramerization domains or as full-length versions. The NA proteins differed in their K_m values, which depended on specific mutations in their

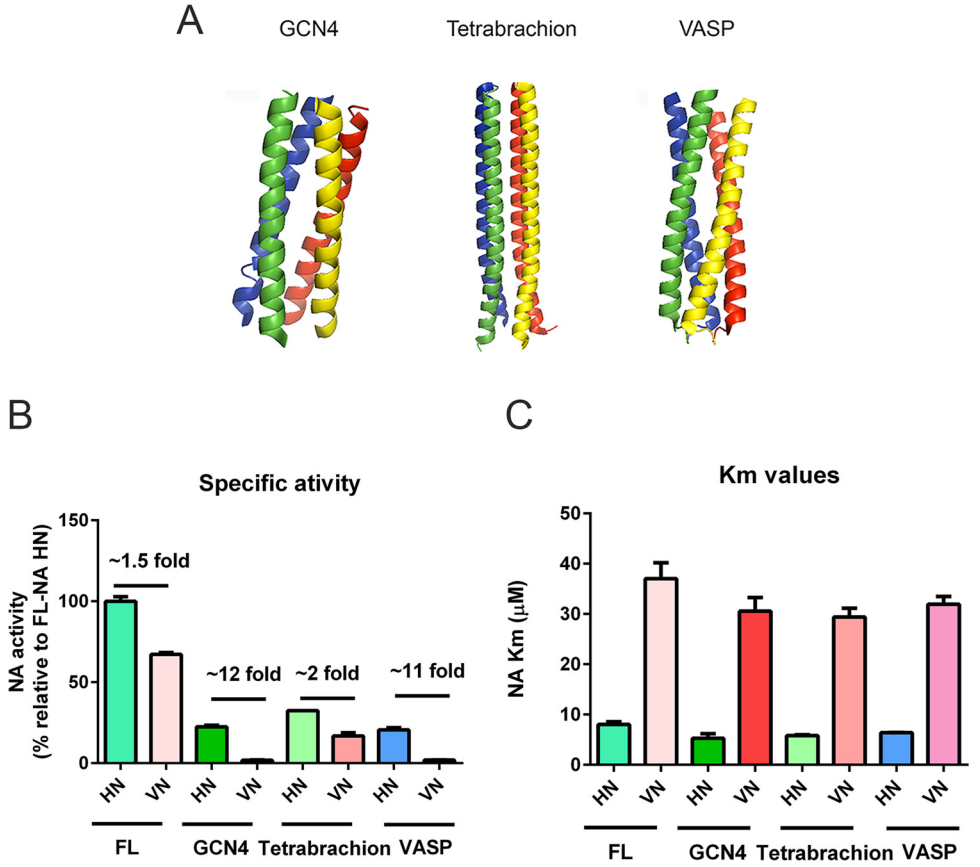

FIG 12 Enzymatic activity of HN and VN proteins extended with different tetramerization domains. (A) Structure of GCN4, Tetrabrachion, and VASP tetramerization domains generated with PyMol software are shown (PDB entries 1GCL, 1FE6, and 1USE) (31–33). (B) Specific activity of FL-NA proteins and of soluble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

head domains at positions 252 and 347, but their K_m 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 activ-

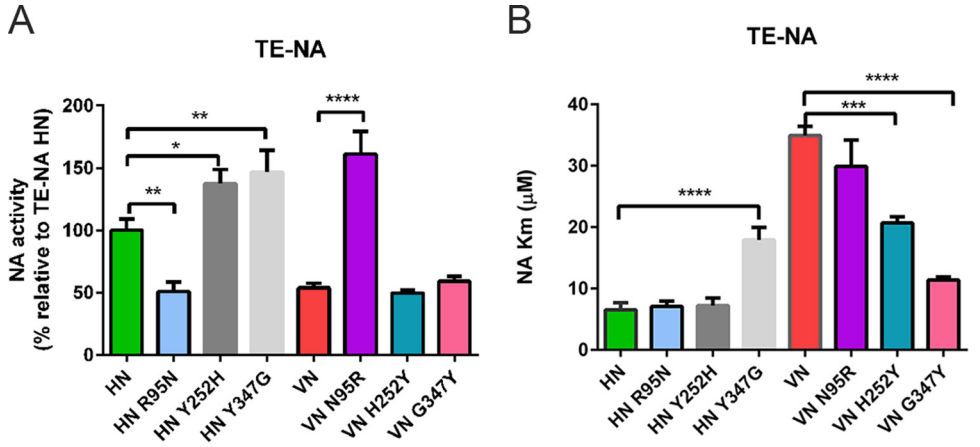

FIG 13 Specific activity and K_m values of mutant TE-NA proteins. (A) Specific activity of TE-NA HN and VN as well as the indicated mutant proteins is shown relative to the specific activity of TE-NA HN. (B) K_m values of the indicated wild-type and mutant TE-NA HN and VN proteins are shown. All graphs represent the means from three independent experiments. Error bars indicate standard deviations. Significant differences determined by one-way ANOVA in combination with Bonferroni's multiple-comparison test relative to the wild-type TE-NA HN and VN are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

ities of the NA proteins were explained by different intrinsic capacities of the NA ectodomains to oligomerize, which could be attributed largely 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 than 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 K_m 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 K_m 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 K_m values in different NA proteins (43). The effect on the K_m value may be explained by the residue at position 347 forming a calcium ion binding site together with other amino acids (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 toward 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 a 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 the K_m 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 K_m 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 indirectly affects 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 with this, 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 those 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 K_m value, of the resulting NA proteins. The full-length proteins displayed higher specific activities than the recombinant soluble NA proteins which carried nonnative 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). Therefore, they might 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 also contributes 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 the Tetrabrachion-extended proteins also 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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