Isolation of a Biologically Active Soluble Form of the Hemagglutinin-Neuraminidase Protein of Sendai Virus

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As a first step in establishing the three-dimensional structure of the Sendai virus hemagglutinin-neuraminidase (HN), we have isolated and characterized a potentially crystallizable form of the molecule. The sequence of HN, a surface glycoprotein, predicts a protein with an uncharged hydrophobic region near the amino terminus which is responsible for anchorage in the viral envelope. To avoid rosette formation (aggregation), which would preclude crystallization, this hydrophobic tail was removed from a membrane-free form of HN by proteolytic digestion. This digestion resulted in a single product with a molecular weight of about 10,000 less than native HN. N-terminal amino acid sequence analysis of cleaved HN (C-HN) indicated a single cleavage site at amino acid residue 131, resulting in a product consisting of the carboxyl-terminal 444 amino acids of HN. Functional analyses revealed that C-HN retained full neuraminidase activity and was able to bind erythrocytes, indicating that the N-terminal 131 residues were not necessary for these biological activities. Furthermore, this cleavage product retained the antigenic structure of intact HN, since monoclonal antibodies still bound to C-HN in enzyme-linked immunosorbent assay and Western (immuno-) blot analysis. Viewed by electron microscopy, the dimeric and tetrameric forms of intact HN form rosettes while C-HN maintains the oligomeric structure but no longer aggregates. Furthermore, the electron micrographs revealed a C-HN tetramer strikingly similar to the influenza virus neuraminidase in both size and gross structural features.

Two transmembrane glycoproteins form spike-like projections on the surface of Sendai virus, the hemagglutinin-neuraminidase (HN) and fusion (F) proteins. The nucleic acid sequences are known for both HN (3, 13) and F (4) and predict proteins of 575 and 565 amino acids, respectively. These proteins have a three-domain structure, typical of transmembrane viral proteins. This structure includes a large hydrophilic carbohydrate-containing domain external to the membrane, a small uncharged hydrophobic peptide spanning the membrane, and a small hydrophilic domain internal to the membrane. Both the HN and F proteins are anchored in the viral envelope and plasma membrane of infected cells by hydrophobic interactions between a region at the base of the spike and the membrane lipid bilayer. The F protein is anchored near the carboxyl terminus and has a cleaved signal sequence (4, 7, 30), whereas the HN protein is inserted in the opposite orientation and has no cleaved signal sequence (3).

The multiple functions of the HN protein include attachment of the virus to sialic acid-containing receptors on host cells, agglutination of erythrocytes, neuraminidase (NA) activity (26, 27, 34), and possibly a role in cell fusion (9, 14, 16-18, 22, 24). The F protein is synthesized as the inactive precursor F₀, which is cleaved intracellularly by a trypsin-like protease to produce the active form of two disulfide-linked subunits, F₁ and F₂. This active form of F is responsible for virus penetration of the host cell membrane, virus-induced hemolysis, and cell fusion (8, 15, 28, 29).

The features of the paramyxovirus glycoproteins are similar to those of the influenza virus NA and hemagglutinin proteins. The surface glycoproteins of both viruses possess the same functional capabilities and have one protein in an

inverted orientation in the membrane. In addition, limited amino acid homology has been found between the paramyxovirus F and the influenza virus hemagglutinin proteins (3), and secondary structural similarities exist for the paramyxovirus HN and the influenza virus NA proteins (10). These common features have led to the postulation of evolutionary relatedness between the paramyxovirus HN and the influenza virus NA and between the paramyxovirus F and the influenza virus hemagglutinin (3).

To understand the structural correlates to the binding, NA, and fusion functions, to expand our understanding of the antigenic structure, and to explore the possible evolutionary relationships between the orthomyxoviruses and paramyxoviruses, a description of these proteins at the atomic level is necessary. Furthermore, the three-dimensional description of the cell binding pocket may provide a basis for the design of new chemotherapeutic agents to prevent paramyxovirus infection (31).

Thus far the determination of the three-dimensional structures has not been possible because the hydrophobic regions of the isolated HN or F spikes aggregate in detergent-free aqueous media, resulting in rosette formation (26). In this report, we provide a description of an HN molecule in which the hydrophobic membrane-spanning region has been enzymatically removed. Analysis of the biological and structural properties of this nonaggregating form of HN indicate a biologically active homogeneous structure suitable for crystallization and three-dimensional structure determination.

MATERIALS AND METHODS

Virus growth and isolation of surface glycoproteins. The Enders strain of Sendai virus (20, 21) was grown in the allantoic cavity of 10-day-old embryonated eggs. Virus was concentrated and purified by differential centrifugation and

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sedimentation through sucrose gradients. Purified Sendai virions (20 mg/ml) in 0.01 M phosphate buffer, pH 7.2 (PB) were treated with an equal volume of disruption buffer (2% [vol/vol] Triton X-100 [Boehringer GmbH, Mannheim, Federal Republic of Germany] and 2 M KCl in PB) for 30 min at 22°C. The liberated nucleocapsids were removed by ultracentrifugation ($190,000 \times g$ for 1 h), resulting in a supernatant containing the HN, F, and M proteins. Dialysis of the supernatant against 0.01 M PB decreased the ionic strength, which caused precipitation of the M protein (27). The M protein was subsequently removed by ultracentrifugation ($50,000 \times g$ for 30 min), which resulted in a solution that contained only the F and HN proteins.

Isolation of a soluble form of HN. The solution containing HN and F (protein concentration, 5 mg/ml) was treated with acetylated trypsin (Sigma Chemical Co., St. Louis, Mo.; EC 3.4.21.4; 20 μ g/ml for 30 min at 37°C), and the resulting cleaved HN (C-HN) was purified by sucrose gradient centrifugation. Samples (2 ml) were layered onto 38-ml, 5-to-20% sucrose gradients containing a 1-ml Fluorinert FC-40 cushion (Instrumentation Specialties Co., Lincoln, Nebr.) and centrifuged in a VTi50 rotor at $220,000 \times g$ for 5 h at 4°C. A Haake-Buchler Auto Densi-Flow was used to collect 1-ml fractions. The distribution of HN in the gradients was determined by NA assay (1) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11, 19) of individual fractions. Fractions containing C-HN were pooled and dialyzed against 0.15 M NaCl–0.01 M PB to remove the sucrose. Whereas the majority of the F protein aggregated and sedimented on the Fluorinert cushion, a small amount remained in the C-HN fractions. The contaminating F was removed by passing the sample through a 5-ml DEAE-Sephadex column (Sigma) which had been equilibrated with 0.05 M PB. Under these conditions, the F protein remained adsorbed to the column while the C-HN passed through. Addition of the samples was followed by successive 2-ml washes (0.05 M PB) until protein could no longer be detected in the eluent by Coomassie blue staining of the sample spotted onto Whatman no. 1 filter paper. The samples were then concentrated by dialysis against 70% ammonium sulfate, and the resulting precipitate was suspended in 0.15 M NaCl.

Characterization of C-HN. To determine whether the hydrophobic anchor region of HN was removed, C-HN was subjected to amino acid sequence analysis by automated Edman degradation on a gas-phase sequencer (model 470A; Applied Biosystems, Foster City, Calif.).

Protein concentrations were determined by using the BCA Protein Assay reagent (Pierce Chemical Co., Rockford, Ill.).

The NA and hemagglutinating activities of C-HN were compared with those of the native HN protein. The NA activity was assayed by using fetuin as the substrate (1). The ability of C-HN to bind to chicken erythrocytes (cRBCs) was determined for both untreated erythrocytes and erythrocytes treated with a bacterial NA (39). Samples of a 5% (vol/vol) cRBC suspension in phosphate-buffered saline (pH 7.2) were treated for 2 h at 37°C with *Vibrio cholerae* receptor-destroying enzyme (Sigma) at a 1:20 (vol/vol) dilution in a calcium saline solution (7 mM CaCl_2 –20 mM H_3BO_3 –0.1 mM $\text{Na}_2\text{B}_4\text{O}_7$ –155 mM NaCl), washed in phosphate-buffered saline (pH 7.2), and adjusted to a concentration of 0.5% (vol/vol) in phosphate-buffered saline (pH 7.2). A 50- μ l sample of the cRBC suspension was added to 50 μ l of phosphate-buffered saline (pH 7.2) containing serial twofold dilutions of either C-HN or virus in 96-well polystyrene microdilution plates, and hemagglutination was scored after incubation for

30 min at 22°C. Hemagglutination inhibition (HI) tests were done by using standard procedures (23). Four agglutinating doses of virus or C-HN dimers were incubated for 30 min at 22°C with serial twofold dilutions of monoclonal antibody-containing ascitic fluid before the addition of erythrocytes.

The antigenic structure of C-HN was analyzed by Western (immuno-) blot analysis. The dimers and tetramers were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 7.5% gels (11) under nonreducing conditions, electrophoretically transferred to nitrocellulose sheets (33), and processed for immunodetection. Monoclonal antibodies specific for HN were used as first antibodies, and peroxidase-labeled, affinity-purified goat anti-mouse antibody was used as the second antibody (Bio-Rad Laboratories, Richmond, Calif.). Several of the monoclonal antibodies have been previously described (18, 22, 32).

Electron microscopy. The samples were adsorbed to glow-discharged parlodion grids, negatively stained with 1% uranyl acetate, and viewed in a Philips 301 electron microscope.

RESULTS

Isolation of a soluble form of the HN glycoprotein. Our initial strategy for isolating a soluble form of the HN glycoprotein paralleled the methods used for the isolation of the influenza virus NA (2) and hemagglutinin (5) proteins. These methods involved treatment of purified influenza virions with the proteases pronase and bromelain, which released the NA and hemagglutinin proteins, respectively. Treatment of Sendai virions in an analogous manner with pronase, bromelain, trypsin, protease Glu-C, protease Lys-C, protease Arg-C, or papain did not result in cleavage of either surface glycoprotein. On the basis of these preliminary findings, the knowledge that HN contains many potential sites for each protease (3), and the past work by Scheid et al. (30) showing that detergent-solubilized HN of simian virus 5 could be cleaved by chymotrypsin into a water-soluble HN fragment, HN of Sendai virus was removed by detergent disruption of the viral membrane prior to proteolytic cleavage. The products of such a disruption are shown in Fig. 1. Purified Sendai virions (Fig. 1, lane 1) were disrupted by treatment with Triton X-100 and KCl in 0.01 M PB. The liberated nucleocapsids were removed by ultracentrifugation, and the M protein was precipitated by decreasing the ionic strength, resulting in a supernatant that contained primarily the HN and F proteins (Fig. 1, lane 2). Treatment of this supernatant with the proteolytic enzyme trypsin resulted in an increased mobility of the HN protein, corresponding to a decrease in molecular weight of approximately 10,000, while the F protein had no apparent change in mobility (Fig. 1, lane 3). Treatment of HN with either pronase or papain also resulted in cleavage products of similar sizes, whereas treatment with chymotrypsin did not (results not shown). Thus, it appears that removal of HN from the viral membrane exposed a region sensitive to several proteases in an otherwise protease-resistant molecule.

HN has been shown to exist as disulfide-linked homodimers and homotetramers on the surfaces of intact virions (12). To determine the effect of cleavage on these oligomeric structures, the digestion was also visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions. The dimeric $[(\text{HN})_2]$ and tetrameric $[(\text{HN})_4]$ forms can be seen both in the intact virions (Fig. 1, lane 4) and in the purified surface protein preparation (Fig. 1,

FIG. 1. Polyacrylamide gel analysis of trypsin-treated surface glycoproteins visualized by Coomassie brilliant blue staining. The loading buffer used for lanes 1 to 3 contained 1% β -mercaptoethanol, whereas the loading buffer used for lanes 4 to 6 had none. Lanes: 1 and 4, 15 μ g of Sendai virus; 2 and 5, surface glycoproteins HN and F from 15 μ g of virus; 3 and 6, surface glycoproteins treated with trypsin. Indicated to the left of lanes 1 and 4 are the positions of the six structural proteins of Sendai virus, including the dimeric $[(HN)_2]$ and tetrameric $[(HN)_4]$ forms of HN.

lane 5). Analysis of cleaved HN (C-HN) revealed that these two forms were retained (Fig. 1, lane 6) and that HN molecules of both forms were susceptible to cleavage by trypsin. These results suggest that cysteine residues which participate in the intermolecular disulfide bonds of the dimeric and tetrameric structures are found in the cleaved form of HN.

To separate C-HN from the F protein, uncleaved HN, trypsin, and digestion products, the trypsin-treated samples were centrifuged through 5-to-20% sucrose gradients. The gradient fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions and assayed for NA activity. A representative gradient is shown in Fig. 2. Two peaks of NA activity were found (Fig. 2B, fractions 14 to 21 and 24 to 29), corresponding to the dimeric and tetrameric forms of C-HN (Fig. 2A). The F protein still contained a hydrophobic anchor region and therefore formed aggregates which were found near the bottom of the gradient (Fig. 2A, lanes 35 to 37). The observation that C-HN did not aggregate and sediment to the bottom of the gradient indicated that the hydrophobic membrane anchor region was no longer present.

To provide homogeneous populations of HN for crystallization, the fractions corresponding to each peak were pooled, thereby separating dimers from tetramers. The pooled gradient fractions were dialyzed to remove the sucrose, chromatographed on DEAE-Sephadex to remove any remaining F protein, and finally concentrated by ammonium sulfate precipitation. The purification resulted in a homogeneous preparation of dimeric C-HN (Fig. 3, lane 3), whereas the tetrameric preparation frequently contained a small amount of dimers (Fig. 3, lane 4). We estimate the final yield of purified cleaved dimers and tetramers to be 20% of the HN initially present in the virus. The final concentration of dimers and tetramers was typically adjusted to 7 to 8 mg/ml, which is sufficient for crystallization studies.

Amino-terminal sequence analysis of C-HN defines the trypsin cleavage site. Gradient analysis indicated that C-HN no longer aggregated and therefore did not contain a hydrophobic anchor region. To confirm this prediction and to

FIG. 2. Sucrose gradient separation of C-HN dimers and tetramers. (A) Polyacrylamide gel analysis, using nonreducing conditions, of gradient fractions 1 to 37, visualized by Coomassie brilliant blue staining; lane M (marker) contains trypsin-treated surface proteins prior to gradient separation. (B) NA activity of gradient fractions.

FIG. 3. Polyacrylamide gel analysis of purified preparations of C-HN dimers and tetramers. Lanes: 1, 15 μ g of Sendai virus; 2, surface glycoproteins HN and F from 15 μ g of virus; 3, C-HN dimers; 4, C-HN tetramers. Indicated to the left of lane 1 are the positions of Sendai virus structural proteins including the dimeric [(HN)₂] and tetrameric [(HN)₄] forms of HN.

determine the precise cleavage site(s), the N-terminal amino acid sequence of C-HN was determined. The sequence obtained is shown above the diagram in Fig. 4. This sequence correlates to amino acids 132 to 156 of the published sequence, indicating a single cleavage site at residue 131 and confirming that C-HN no longer contains the hydrophobic anchor region (residues 35 to 60).

Biological characterization of C-HN. To establish that C-HN has retained the functions of native HN, the biological activities and antigenic structure were examined. Using equivalent amounts of HN protein, we found NA activities of C-HN dimers, tetramers, and virus to be very similar (Table 1).

In our attempts to demonstrate the sialic acid-binding ability of C-HN, we assumed that C-HN would not cause hemagglutination because the proximity of the binding sites on a C-HN dimer or tetramer would make it sterically difficult to bind two erythrocytes. Quite unexpectedly, C-HN alone was able to cause hemagglutination (Table 1). With equivalent amounts of HN protein used as starting material, HN found in intact virions was approximately 250 times more efficient at agglutinating cRBCs than C-HN dimers or tetramers.

FIG. 4. Schematic representation of the amino acid sequence of HN. The results of sequence analysis of the new amino terminus are given above the diagram (residue numbers from the amino terminus are indicated). Potential trypsin cleavage sites (♦) are also indicated.

One possible explanation for this unexpected binding property is that the C-HN binding to erythrocytes was nonspecific. To discriminate receptor-specific binding from nonspecific binding, cRBCs were treated with a *V. cholerae*-derived NA and used for comparison with untreated erythrocytes in the hemagglutination assay. Neither virions nor C-HN oligomers bound to cRBCs which lacked receptors, indicating that binding was specific for sialic acid-containing receptors.

As an additional test of receptor-specific binding, we also measured the ability of anti-HN monoclonal antibodies to inhibit hemagglutination (Table 2). The results show that antibodies (M41 and M57) which inhibited binding of virions also inhibited binding of C-HN, whereas antibodies (M14 and M58) which had no HI activity with Sendai virions did not inhibit binding of C-HN. All four antibodies used in these assays bound equally well to both virions and C-HN as determined by enzyme-linked immunosorbent assay. These results suggest that the same region on the HN molecule participated in binding for both virions and C-HN. Therefore, taken together, the results shown in Tables 1 and 2 indicate that the agglutination observed for C-HN dimers and tetramers was due to receptor-specific binding and was dependent upon the ability of each dimer or tetramer to bridge the receptors on two erythrocytes.

To determine whether the proteolytic removal of the amino-terminal 131 amino acids of HN altered the antigenic structure, a panel of monoclonal antibodies specific for native HN were tested by Western blot analysis for binding to the dimeric and tetrameric forms of C-HN. A representative sample of this panel is shown in Fig. 5. In all cases, the monoclonal antibodies bound to both forms of C-HN. An anti-F monoclonal antibody (M64) failed to react with the C-HN blot, illustrating the purity of the preparation. These results suggest that the proteolytic cleavage and subsequent purification did not significantly alter the antigenic structure of HN and the cleaved and native forms of HN are antigenically indistinguishable. Therefore, the proteolytic cleavage of HN did not affect the biological properties or antigenic structure, providing evidence for the maintenance of the native structure of HN.

Electron microscopy of HN. The uncleaved HN and the C-HN dimers and tetramers were examined by electron microscopy to visualize gross structural features and determine whether removal of the amino-terminal 131 amino acids was sufficient to prevent aggregation. The sample of uncleaved HN contained many aggregates of HN (Fig. 6A). These aggregates were presumably formed by the attach-

TABLE 1. Biological activities of C-HN dimers and tetramers

HN preparation	Neuraminidase activity (A_{549}) ^a	Hemagglutination titer ^b	Hemagglutination titer ^c	% Reduction ^d
Virions	0.443	81,920	10,240	87.5%
C-HN dimers	0.606	320	<20	>93.8%
C-HN tetramers	0.476	320	<20	>93.8%

^a Replicate tubes containing equivalent amounts of HN protein (0.1 μ g/50 μ l) were incubated for 30 min at 37°C with 2.0 mg of fetuin and then assayed for free sialic acid. (HN was calculated to be 17% of total viral protein [25].)

^b Hemagglutination titers were recorded as the reciprocal of the highest dilution at which agglutination of the erythrocytes occurred after 30 min at 37°C. Equivalent amounts of each HN preparation (20 μ g/50 μ l) were used as the starting material.

^c Hemagglutination tests were done as described above except that the cRBCs were initially treated with a receptor-destroying enzyme derived from *V. cholerae* filtrate.

^d Percent reduction in hemagglutination titer comparing assays done using untreated erythrocytes and those using erythrocytes from which the sialic acid-containing receptors had been removed.

ment of the hydrophobic tails of HN molecules. By contrast, the C-HN samples showed many small unaggregated molecules containing two (Fig. 6B) or four (Fig. 6C) subunits, with each subunit measuring 5 to 6 nm in diameter. The tetramers are similar to the NA of influenza virus in that they possess a box-shaped head with four identical subunits arranged in fourfold symmetry. Additionally, the box-shaped head of the tetramer measures about 10 by 10 nm, which is similar to the NA of influenza virus (38). The dimers appear to equal one half of the tetrameric structures.

DISCUSSION

The primary goal of this study was to provide new information on the structure of the HN molecule and to gain insight on how the structure relates to its functions. A soluble form of HN with potential for crystallization and subsequent three-dimensional structural analysis was used. Large quantities of virus were used to isolate the surface proteins, and the hydrophobic membrane-spanning region was proteolytically removed from HN. Initial attempts to cleave HN directly from the surface of the virus, as was done for the influenza virus hemagglutinin and NA, were not successful. Tryptic cleavage of HN was possible only after HN had been removed from the viral membrane. The observation that the proteases pronase and papain were also able to cleave the membrane-free form of HN in the same region suggests that a previously protected region was made available to the proteolytic enzymes by disruption of the viral membrane. This increased susceptibility to proteolytic cleavage could be due to either a conformational change in HN or removal of HN from a membrane which physically prevented protease action.

It is interesting to note that there are 50 possible trypsin cleavage sites in HN (designated by the ♦'s in Fig. 4), 15 on the amino side and 34 on the carboxyl side of residue 131, yet the final result of tryptic cleavage was a single form with a

new amino terminus at residue 132. The homogeneity of the cleaved product suggests that the newly exposed stalk is trypsin-sensitive and that the portion of the molecule normally exposed to the extracellular environment is a trypsin-resistant globular head. The fact that the F protein requires cleavage by a host cell protease to be biologically active (28) suggests that the exposed portion of HN must have evolved to be protease resistant.

The native HN protein exists in dimeric and tetrameric forms at a ratio of 1.4:1.0 (12). Both forms of HN can be cleaved by trypsin and both polymeric forms are maintained after cleavage, indicating that the amino-terminal 131 residues are not required to maintain these structures. Separation of these two forms of HN provides a more homogeneous starting material which may facilitate crystallization.

It has been suggested that Sendai virus evolved from an influenza virus-like ancestor by concatenation of the genome segments to form the nonsegmented Sendai virus genome

TABLE 2. Anti-HN monoclonal antibodies inhibit the binding of C-HN dimers to erythrocytes

Antibody	HI ^a	
	Virions	C-HN Dimers
M41	12,800	200
M57	3,200	200
M14	<100	<100
M58	<100	<100

^a HI titers are expressed as the reciprocal of the highest dilution of antibody that completely blocked 4 hemagglutination units in a standard HI test.

FIG. 5. Western blot analysis of C-HN dimers and tetramers. Each monoclonal antibody (designated at the top) was used to probe blots of both virus (V) and C-HN. The viral proteins revealed by amido black staining are labeled at the left. Antibodies M2, M9, M41, M57, and M72 are specific for HN. Antibody M64, which is specific for F, was used as a negative control. Indicated at left are the positions of Sendai virus structural proteins, including the dimeric [(HN)₂] and tetrameric [(HN)₄] forms of HN.

FIG. 6. HN as seen in the electron microscope after negative staining with 1% uranyl acetate. (A) Aggregates of uncleaved HN. (B) C-HN dimers. (C) C-HN tetramers. Arrowheads indicate particularly clear examples. The molecules shown at the bottom of panels B and C were further magnified ($\times 3.3$) to show the structural details of dimers and tetramers, respectively.

(3). Although no significant sequence homology exists between the influenza virus NA and the Sendai virus HN (3), electron microscopy suggests that the morphological features of influenza virus NA and Sendai virus HN are similar, supporting an evolutionary relationship. The overall dimensions of the influenza NA head are 10 by 10 by 6 nm (38). The two-dimensional electron micrographs of C-HN tetramers reveal a similar size of approximately 10 by 10 nm. The

Sendai virus HN tetramers and the influenza virus NA both have box-shaped heads made from four identical subunits. However, the determination of the three-dimensional structure of HN is necessary to confirm an evolutionary relationship between the paramyxoviruses and orthomyxoviruses.

The cleaved and purified dimers and tetramers of HN retained biologic and antigenic capabilities, providing direct evidence that the amino-terminal 131 amino acids were not

necessary for these activities and locating all activities to a proposed globular head region. We propose that the residues 1 to 131 compose a long, slender stalk, the primary purpose of which is to connect the head to the virus membrane. We have also shown that the globular head region does not need to be anchored in a virion membrane to have cell-binding activity. Furthermore, all activities are present on both the dimers and tetramers, providing no clues for the existence of two different forms.

The hemagglutination titers for C-HN dimers and tetramers were 250-fold lower compared with virion-associated HN. This finding is analogous to the situation known for hemagglutination with immunoglobulin M and immunoglobulin G antibodies. Immunoglobulin M-class antibodies, due to their size as well as to the availability of 10 antibody-binding sites disposed at diametrical distances of 30 nm, hemagglutinate much more readily than immunoglobulin G-class antibodies, which have just two sites which are maximally spaced about 12 nm apart (36). Whereas Sendai virions are 150 to 200 nm in diameter and contain approximately 1,000 HN molecules on the surface, the distance between binding sites on C-HN oligomers can be estimated to be 8 to 10 nm. This estimation is based on the size of the HN heads (10 by 10 nm) deduced from electron micrographs and the minimum distance of approach for two erythrocytes (7.9 nm) based on electrostatic repulsion as well as their van der Waals attraction energy (37). The 250-fold decrease in hemagglutination can therefore be attributed to the fact that two erythrocytes must be within 10 nm for bridging to occur by C-HN oligomers, whereas the distance can be up to 200 nm for virion-mediated bridging.

Binding appears to be receptor specific, as indicated by the observation that C-HN no longer bound erythrocytes from which the receptors had been removed. Furthermore, binding of C-HN could be inhibited by antibodies shown to have HI activity with virions. The HI titers for C-HN were 10- to 100-fold less than for virions when the standard four hemagglutination units were used in the HI test. For virions, 6.1×10^{10} HN molecules are necessary for each hemagglutination unit, while for C-HN dimers, 4.8×10^{12} HN molecules are required. Therefore, the greater number of HN molecules present in a hemagglutination unit of dimers would require more antibody molecules to inhibit agglutination.

C-HN retained biological activity and antigenic reactivity, indicating that the cleavage and the purification of HN did not cause conformational changes that affected these properties. Thus, C-HN should be an appropriate substrate for structural analysis. Attempts to crystallize dimers and tetramers have not been successful. However, when C-HN dimers and Fab fragments derived from neutralizing monoclonal antibody to HN were mixed and subjected to vapor diffusion over 50% polyethylene glycol, needle-like crystals were formed (W. G. Laver, unpublished data). Thus, we may soon be able to obtain structural information for C-HN complexed with antibody as was done for the influenza virus NA (6, 35).

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