

Structural and Functional Relationship between the Receptor Recognition and Neuraminidase Activities of the Newcastle Disease Virus Hemagglutinin-Neuraminidase Protein: Receptor Recognition Is Dependent on Neuraminidase Activity

RONALD M. IORIO,^{1*} GISELA M. FIELD,^{1,2} JENNIFER M. SAUVRON,^{1,2} ANNE M. MIRZA,¹
RUITANG DENG,^{1†} PAUL J. MAHON,³ AND JOHANNES P. LANGEDIJK⁴

Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655-0122¹; Department of Biology and Biotechnology, Worcester Polytechnic Institute, Worcester, Massachusetts 01609²; Department of Biology, Assumption College, Worcester, Massachusetts 01615³; and Department of Mammalian Virology, The Institute for Animal Science and Health (ID-DLO), Lelystad, The Netherlands⁴

Received 5 September 2000/Accepted 20 November 2000

The terminal globular domain of the paramyxovirus hemagglutinin-neuraminidase (HN) glycoprotein spike has a number of conserved residues that are predicted to form its neuraminidase (NA) active site, by analogy to the influenza virus neuraminidase protein. We have performed a site-directed mutational analysis of the role of these residues in the functional activity of the Newcastle disease virus (NDV) HN protein. Substitutions for several of these residues result in a protein lacking both detectable NA and receptor recognition activity. Contribution of NA activity, either exogenously or by coexpression with another HN protein, partially rescues the receptor recognition activity of these proteins, indicating that the receptor recognition deficiencies of the mutated HN proteins result from their lack of detectable NA activity. In addition to providing support for the homology-based predictions for the structure of HN, these findings argue that (i) the HN residues that mediate its NA activity are not critical to its attachment function and (ii) NA activity is required for the protein to mediate binding to receptors.

The paramyxoviruses are enveloped, negative-stranded RNA viruses, including human parainfluenza viruses types 1 to 4, mumps virus, and the animal pathogens Newcastle disease virus (NDV), Sendai virus, and simian virus 5. The hemagglutinin-neuraminidase (HN) glycoprotein spike not only mediates receptor recognition but also possesses neuraminidase (NA) activity, the ability to cleave a component of those receptors, sialic acid (35). The presence of both receptor recognition and NA activities on the same protein is in contrast to influenza virus, in which the two activities reside on independent spike structures.

The HN spike is a type II homotetramer. The ectodomain consists of a long stalk, supporting a terminal globular head, in which reside the receptor recognition, NA, and antigenic sites (26, 42). All HN tetramers are pairs of dimers. In the case of the Australia-Victoria isolate of NDV (NDV-AV), the monomers in each dimer are disulfide linked via a cysteine at position 123 in the stalk region (26). NDV HN utilizes four of its six potential glycosylation sites. Elimination of two of them, G1 and/or G2, results in an increase in hemadsorption (HAD) activity (24).

Based on the conservation in HN of amino acids in the active

site of the influenza NA protein, the globular head of the HN spike has been predicted to have a six β -sheet propeller structures, similar to the influenza virus protein; putative NA active-site residues are contributed by five of the six β -sheets (5, 21). This prediction is consistent with the antibody escape mutant mapping of discontinuous epitopes on the NDV (17, 19), human parainfluenza virus type 3 (hPIV3) (4), and simian virus 5 (1) HN proteins.

In the influenza virus NA protein, residue D151 is thought to be important to catalysis by virtue of its position within hydrogen-bonding distance of the glycosidic oxygen of the substrate (2, 44). Several different substitutions for the corresponding aspartic acid at position 198 in NDV HN abolish NA activity (7), consistent with its having an analogous function in HN. This prediction is consistent with the properties of monoclonal antibodies (MAbs) to an antigenic site on NDV HN, called site 23. The binding of MAbs to this site can be blocked by a competitive inhibitor of NA activity (18). The characterization of antigenic variants has identified residues that flank D198 in the linear amino acid sequence (F193, S194, S200, H201, and H203) as contributing to antigenic site 23 (15, 18, 19, 23). In addition, several substitutions in the site modulate NA and attachment (15, 18). Finally, the NA activity of NDV-AV has been shown to exhibit cooperativity, a phenomenon that is eliminated in a site 23 variant carrying substitutions of F193L and S200L (23). All of these data suggest that antigenic site 23 and the NA active site are closely linked and may be topologically overlapping.

The results of another mutational analysis are also consis-

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, 55 Lake Ave., No., Worcester, MA 01655-0122. Phone: (508) 856-5257. Fax: (508) 856-5920. E-mail: Ronald.Iorio@umassmed.edu.

† Present address: Molecular & Cellular Virology, Animal Health Biological Discovery, Central Research Division, Pfizer Inc., Groton, CT 06340.

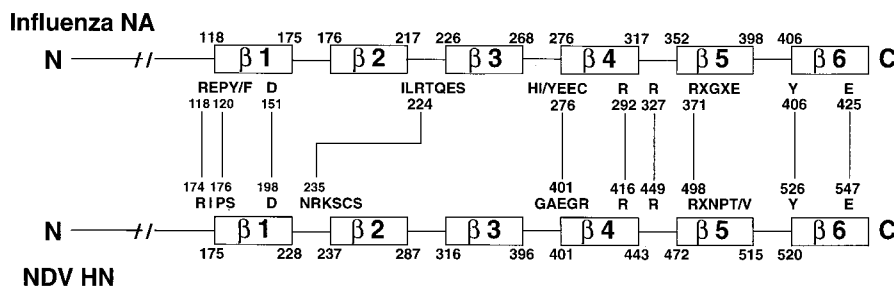


FIG. 1. Alignment of residues in the globular domain of NDV HN with the six β -sheets and NA active-site residues on the influenza virus NA protein. The numbers at the ends of each box are the first and last residues in each sheet. The residues in each sequence joined by lines are the NA active-site residues in influenza virus NA and the corresponding putative NA active-site residues in HN.

tent with the β -sheet propeller model. The longest linear stretch of amino acids completely conserved among all HN proteins is the NRKSCS sequence (residues 234 to 239 in NDV HN). Jorgensen et al. (20) first predicted the close proximity of this region to the NA site. Consistent with this, we have previously shown that substitutions for any of the first three residues in the sequence sharply diminish, although they do not eliminate, NA activity (25).

We have now completed an extensive site-directed mutational analysis of the role in NDV HN function of putative NA active-site residues as predicted by homology with influenza virus NA. Our findings are consistent with the homology-based predictions for the structure of the NA active site in the paramyxovirus HN protein. In addition, a total of seven residues have now been identified in the NDV HN protein for which substitution results in a loss of detectable NA and receptor recognition activity. The receptor-binding activity of these proteins can be partially restored by supplying NA activity either exogenously or by coexpression with another protein. Furthermore, a mutant with less than 5% of wild-type NA activity retains the ability to bind to receptors. These results suggest that the receptor-binding activity of HN is dependent on its NA activity and that less than 5% of the wild-type (wt) amount of NA is sufficient for the protein to mediate receptor recognition. The implications of this finding for the functional and topological relationship between the NA and receptor binding activities of HN are discussed.

MATERIALS AND METHODS

Recombinant plasmid vectors and site-directed mutagenesis. Construction of the NDV-AV HN pBluescript SK(+) (Stratagene Cloning Systems, La Jolla, Calif.) expression vectors and chimera CH1(+11) has been described (8, 25).

Site-directed mutagenesis was performed as described previously (6), using oligonucleotide primers obtained from Bio-Synthesis, Inc. (Lewisville, Tex.). The presence of the desired mutation was verified by sequencing of double-stranded DNA, using the Sequenase plasmid sequencing kit (United States Biochemical, Cleveland, Ohio), according to protocols provided by the company. Multiple clones were characterized for each substitution.

Transient-expression system and quantitation of cell surface HN. Wt and mutant HN proteins were expressed in BHK-21 cells using the vaccinia virus-T7 RNA polymerase expression system (9). Maintenance of cells, infection with the vaccinia virus recombinant, and transfection were performed as described (25), except that 1 μ g of each plasmid was used for transfection. Cell surface expression was quantitated by fluorescence-activated cell sorting (FACS) analysis, using a mixture of MAbs to at least five different antigenic sites on the globular head of HN (12, 13, 17, 19).

Functional assays. The HAd activity of HN proteins was determined by the ability of the expressed protein to adsorb guinea pig erythrocytes (Crane Labo-

ratories, Syracuse, N.Y.). HN-expressing monolayers were incubated for 20 min with a 2% suspension of erythrocytes in phosphate-buffered saline supplemented with 1% CaCl_2 and MgCl_2 . After extensive washing, adsorbed erythrocytes were lysed in 50 mM NH_4Cl , and the lysate was clarified by centrifugation. HAd activity was quantitated by measuring the absorbance at 540 nm and subtracting the background absorbance obtained with cells expressing the vector alone.

NA activity of cell surface HN was determined as described previously (25) on duplicate monolayers incubated for 30 min at room temperature with 625 μ g of neuraminidase (Sigma Chemical Co., St. Louis, Mo.) in 0.5 ml of 0.1 M sodium acetate (pH 6). This buffer system was also used to measure NA activity at pH 5.5, while NA activities at pH 6.5 and 7 were determined using 0.1 M sodium phosphate buffer.

Rescue of receptor-binding activity by NA activity. (i) **NA supplied from an exogenous source.** Mutated HN proteins were expressed at the cell surface as described above. The monolayer was washed with Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, Md.) and treated for 1 h at 37°C with DMEM containing 50 mU of *Vibrio cholerae* NA (VCNA) (Sigma). After extensive washing with DMEM, HAd activity was determined and quantitated as above.

(ii) **NA supplied by a coexpressed protein.** NA-deficient mutants lacking HAd activity were coexpressed with an hPIV3-NDV HN chimera, CH1(+11) (8). The HAd activity of monolayers coexpressing the chimera and each mutant was determined following treatment of the monolayer for 30 min at room temperature with 0.5 ml of DMEM containing an MAb that selectively inhibits the HAd activity of the chimera. This treatment was previously shown to completely block the HAd activity of monolayers coexpressing the chimera and wt HN. In this way, the percentage of the total HAd that was mediated by the previously HAd-deficient mutant could be determined.

Immunoprecipitation and SDS-PAGE. At 22 h posttransfection, BHK cells were starved for 1 h at 37°C in DMEM without cystine and methionine, supplemented with 7% dialyzed fetal calf serum, nonessential amino acids, vitamins, L-glutamine (2 mM), sodium bicarbonate (0.2%), penicillin, streptomycin, and gentamicin. All medium components were obtained from Life Technologies. Following starvation, the cells were labeled for 3 h at 37°C with 1 ml of medium containing 100 μ Ci of Expre- ^{35}S labeling mix (Dupont-New England Nuclear, Boston, Mass.) per ml and chased with medium for 90 min. The cells were lysed, and HN was immunoprecipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (26), except that the antigen-antibody complexes were collected with Ultralink-Immobilized Protein A Plus (Pierce, Rockford, Ill.). Digestion with peptidyl-N-glycosidase F (PNGase F) was performed as described previously (6).

RESULTS

Mutation of predicted NA active-site residues. The alignment of NDV HN with the influenza virus NA active site residues and six β -sheets is shown in Fig. 1, which has been adapted from the Colman et al. (5) model. By analogy, the NA site in HN is predicted to be formed by residues in five of the six β -sheets, all except β 3. In NDV HN, these include R174, P176, and D198 (sheet 1), R235 (near the beginning of sheet 2), E401 and R416 (sheet 4), R449 (in the loop between the fourth and fifth sheets), R498 (sheet 5), and Y526 and E547

TABLE 1. Functional profile of putative NA active-site mutants

Amino acid substitution	β -Sheet	Avg cell surface expression ^a (% of wt)	Avg NA ^b (% of wt)	Avg HAAd ^b (% of wt)
R174L	1	21.0 \pm 8.9	—	—
I175E	1	77.8 \pm 17.1	—	—
D198R ^c	1	89.8 \pm 5.0	—	—
K236R	2	66.8 \pm 3.6	—	—
R416L	4	27.4 \pm 6.4	—	—
R498L	5	116.9 \pm 22.6	4.2 \pm 1.7	36.1 \pm 3.1
N500D	5	112.6 \pm 5.9	107.0 \pm 27.3	94.7 \pm 16.2
V502A	5	106.9 \pm 17.9	107.5 \pm 11.8	132.5 \pm 10.5
Y526L	6	51.5 \pm 13.9	—	—
E547Q	6	80.0 \pm 16.1	—	—

^a Determined by FACS analysis and expressed as a percentage of wt HN. The data are averages of three independent experiments.

^b Expressed as a percentage of wt NDV HN. The data are averages of at least three independent experiments. —, no activity detected.

^c Taken from Deng et al. (7).

(sheet 6). In a number of instances, the putative NA active-site residues localize to a domain that is highly conserved among paramyxovirus HN proteins. These include the domains 174-RIPS-177, 234-NRKSCS-239, 399-GAEGR-403, and 498-RX-NPT/V-502.

With the goal of establishing the role of each of the above residues in HN function, proteins carrying the following substitutions for a conserved residue in one of the above regions of the protein were constructed: R174L, I175E, P176L, S177A, K236R, R416L, R449L, R498L, N500D, V502A, Y526L, and E547Q. The conserved domain 399-GAEGR-403 was not included in this analysis, since a previous study (36) had shown that substitutions for these residues either result in protein misfolding (G399A, E401D, and R403K) or do not affect HN function (A400G, G403A, and G403L).

We have previously shown that any of several substitutions for residue D198 completely abolish NA activity (7), consistent with its predicted role as the catalytic aspartic acid in the NA active site (5). Also, previous mutational analyses revealed that I175M and R235L substitutions result in a 12-fold and 15-fold reduction in NA activity, respectively (25, 37).

Cell surface expression of mutated proteins. The cell surface expression of each mutated protein was quantitated by FACS analysis, using a panel of anti-HN MAbs specific for at least five antigenic sites in the globular domain of the protein (Table 1). Of the mutated proteins that were detected at the cell surface, those carrying R498L, N500D, or V502A substitutions were expressed in amounts similar to that of the wt protein; I175E, K236R, Y526L, and E547Q proteins were expressed 50 to 80% as efficiently as wt HN, and R174L and R416L mutated HN were expressed at approximately 25% of the wt level. We have previously shown that HN carrying any of several different substitutions for D198 are expressed to at least 80% of the wt HN level (7).

Proteins carrying P176L, S177A, or R449L substitutions were not detectable at the cell surface. Since recognition of the mutated proteins by the panel of anti-HN MAbs is highly dependent on conformation, this strongly suggests that these proteins are retained intracellularly in a misfolded form. In any event, these proteins were not characterized further.

NA activity of mutated proteins. The NA activity of each of the mutated proteins expressed at the cell surface was determined (Table 1). As shown previously for D198 HN (7), substitutions of R174L, I175E, K236R, R416L, Y526L, and E547Q all result in a loss of detectable NA activity. NA activity cannot be detected even after a threefold-longer incubation period. On the other hand, three mutated proteins exhibit detectable NA activity. Notably, all carry substitutions in the putative NA-related conserved domain in β 5. Proteins carrying either the N500D or V502A substitution exhibit wt levels of NA activity. R498L HN exhibits markedly reduced NA activity, less than 5% of that of the wt protein. Though the three mutated proteins that exhibit NA activity are also those that are expressed at the surface at the highest levels, it seems unlikely that the failure to detect NA activity with the other expressed proteins is due solely to their relatively reduced cell surface expression. We have demonstrated NA activity for proteins expressed at as little as 25% of wt levels (25).

To determine whether any of the substitutions introduced alter the pH optimum of the NA activity of the protein, the activities of each mutated protein at pH 5.5, 6.5, and 7 were compared to its activity at pH 6, the pH optimum of the wt protein. These results are shown in Fig. 2 for proteins carrying N500D and V502A substitutions. Other than the slightly higher relative activity of V502A HN at pH 5.5, there is no significant difference in the pH profiles of the two mutated proteins and wt HN. All of the proteins that had no detectable activity at pH 6 also failed to exhibit activity at the other pHs. Similarly, the R498L HN also exhibited no change in its pH optimum, with its low activity at pH 6 often reduced to marginally detectable levels at the other pHs (data not shown). This suggests that the pH dependency of the protein is not significantly altered by any of the substitutions.

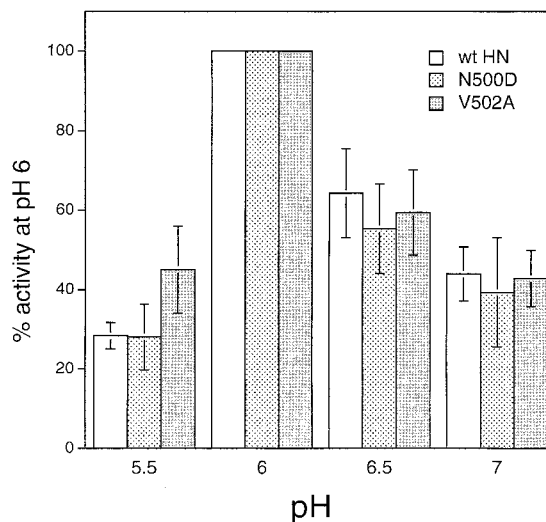


FIG. 2. NA activity of wt, N500D, and V502A HN as a function of pH. At 22 h posttransfection, the NA activity of the proteins expressed at the cell surface was assayed as described previously (25). After subtraction of the background absorbance obtained with the vector alone, the data were expressed as a percentage of the activity at pH 6 for each protein, the optimum pH for wt NA activity. The data represent the averages of at least three independent experiments.



FIG. 3. SDS-PAGE analysis of immunoprecipitated NA active-site mutants. At 22 h posttransfection, cells were starved, labeled, and chased prior to lysis and immunoprecipitation with a mixture of HN-specific MAbs. Cells express either vector (vec) alone (lane 1), wt NDV HN (lane 2), or NDV HN carrying mutation R174L (lane 3), I175E (lane 4), D198R (lane 5), K236R (lane 6), R416L (lane 7), R498L (lane 8), N500D (lane 9), V502A (lane 10), Y526L (lane 11), or E547Q (lane 12).

HAd activity of mutated proteins. The receptor recognition properties of the expressed, mutated proteins were evaluated by assaying their ability to absorb guinea pig erythrocytes. Without exception, all proteins that fail to exhibit detectable NA activity also fail to exhibit detectable HAd activity (Table 1). Thus, loss of receptor recognition activity correlates completely with loss of NA activity.

The three mutants for which NA activity can be demonstrated all exhibit HAd activity, with that of N500D HN being comparable to wt activity. The V502A protein hemadsorbs slightly more efficiently than wt HN. R498L HN hemadsorbs about one third as well as the wt protein, perhaps related to its markedly reduced NA activity.

NA and HAd deficiency correlate with an altered gel migration pattern. Figure 3 shows an SDS-PAGE analysis of the electrophoretic migration pattern of each of the expressed, NA active-site mutants compared to wt HN. Mutants R174L (lane 3), D198R (lane 5), K236R (lane 6), R416L (lane 7), Y526L (lane 11), and E547Q (lane 12) all exhibit an altered gel migration pattern relative to the wt protein (lane 2). These mutated proteins migrate as a distinct double band, one species comigrating with wt HN and a second migrating as a slower-moving, diffuse band. While this altered migration pattern correlates with the lack of detectable NA and HAd activity for these mutants, the migration rate and degree of diffuseness of the slower-migrating band vary depending on the mutant. For example, the defect, especially with respect to the retarded migration rate of the slower-moving form, is most pronounced for the D198R, K236R, and R416L proteins. It is less obvious for the R174L, Y526L, and E547Q proteins.

A migration defect is barely detectable for the I175E protein. It does not exhibit a distinct slower-migrating form, though it does migrate as a slightly more diffuse band than the wt protein. Though it exhibits the same phenotype as the other mutants, I175E HN is clearly a special case.

Each of the mutants for which NA and HAd are demonstrable exhibits a gel migration pattern indistinguishable from that of wt HN. These include the N500D and V502A proteins, which have essentially wt NA and HAd activity, as well as R498L HN, which has less than 5% of wt NA and approximately 36% of wt HAd activity.

Treatment with exogenous NA corrects the gel migration defects of NA-HAd-deficient mutants. To determine whether the gel migration anomalies exhibited by the NA active-site mutants are based on differences in the structure of their

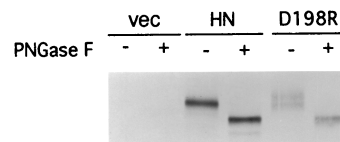


FIG. 4. PNGase treatment of wt and D198R HN. BHK cells were transfected with either vector (vec, lanes 1 and 2), wt HN (lanes 3 and 4), or D198R HN (lanes 5 and 6). The cells were starved, labeled, and lysed. The lysate from each well was split into two aliquots prior to immunoprecipitation. The immunobeads with bound precipitates were suspended in PNGase buffer (0.1 M sodium phosphate [pH 7.2], 25 mM EDTA) containing 0.8% SDS and boiled for 5 min. After cooling, the solution was adjusted to contain 0.1% SDS and 0.5% NP-40. One aliquot of each sample (lanes 2, 4, and 6) was digested with 200 mU of PNGase F for 16 h at 37°C prior to SDS-PAGE.

glycosyl groups, one mutant protein, D198R HN, was treated with PNGase F. This enzyme cleaves the *N*-glycan linkage of glycoproteins between asparagine and the carbohydrate chain (41). As shown in Fig. 4, after treatment with this enzyme, both wt HN and the D198R protein comigrate at a faster rate. This confirms that the altered migration rate of this mutant is due solely to glycosylation-based differences.

To begin to determine the relationship between the gel migration defects in the NA active-site mutants and their lack of NA activity, each radiolabeled protein was treated at the cell surface with exogenously added VCNA. Immunoprecipitation and SDS-PAGE confirm that the migrational defect is corrected by this treatment; the NA-treated proteins all comigrate with wt HN (Fig. 5A). This is consistent with the migration defects, being due to differences in sialic acid content between the mutants and wt HN.

Figure 5B compares the gel migration patterns of four of the mutated proteins to that of wt HN both with and without VCNA treatment. This allows us to compare the amount of

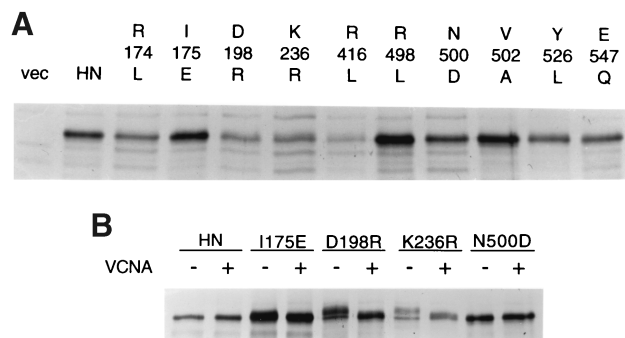


FIG. 5. SDS-PAGE analysis of NA active-site mutants treated with VCNA. (A) After starving, labeling, and chase as described for Fig. 3, monolayers were treated for 1 h at 37°C with 50 mU of VCNA. After extensive washing with DMEM, the cells were lysed and HN was immunoprecipitated for display on SDS-PAGE. Samples are as follows: vector (vec, lane 1), wt NDV HN (lane 2), and NDV HN carrying mutation R174L (lane 3), I175E (lane 4), D198R (lane 5), K236R (lane 6), R416L (lane 7), R498L (lane 8), N500D (lane 9), V502A (lane 10), Y526L (lane 11), or E547Q (lane 12). (B) Cells were transfected as follows: wt HN (lanes 1 and 2), I175E HN (lanes 3 and 4), D198R HN (lanes 5 and 6), K236R HN (lanes 7 and 8), or N500D HN (lanes 9 and 10). The precipitating antibody is a mixture of HN-specific MAbs. Samples were treated as in panel A (lanes +) or left untreated (lanes -).

sialic acid removed from each protein by the VCNA treatment. As one might expect, the migration pattern of wt HN is unaffected by the VCNA treatment (Fig. 5B, compare lanes 1 and 2); it has sufficient NA activity of its own. However, the slower-migrating forms of HN proteins carrying D198R and K236R substitutions are eliminated by treatment with VCNA (compare lanes 5 and 6 and lanes 7 and 8, respectively). The gel also again illustrates the marginal nature of the defect in I175E HN. Its migration is made slightly less diffuse by the enzyme treatment (compare lanes 3 and 4 in Fig. 5B), but the difference is certainly minor compared to mutants D198R and K236R. As a control, the migration pattern of the N500D protein is unaffected by the VCNA treatment, consistent with its wt level of NA activity (compare lanes 9 and 10 in Fig. 5B).

Rescue of receptor recognition activity by treatment with exogenous VCNA. We wondered whether the VCNA treatment might also be capable of restoring receptor recognition activity to the NA-HAd-deficient proteins. For these experiments, we focused only on those NA-HAd-deficient mutants that are expressed at the cell surface at least 50% as efficiently as the wt protein, i.e., mutants carrying I175E, D198R, K236R, Y526L, or E547Q substitutions (Table 1). The R174L and R416L mutants were not tested, as they are expressed only about 25% as well as wt HN.

Monolayers expressing the mutants were treated with exogenous VCNA, washed extensively to remove the enzyme, and assayed for their ability to adsorb erythrocytes. Figure 6A shows the amount of HAd activity exhibited by VCNA-treated monolayers expressing each mutant relative to that of the wt protein treated in the same way. VCNA treatment partially rescued the HAd activity of each mutant. The percentage of wt HAd activity varies from as little as 13.7% for D198R HN to as much as 40.2% for E547Q HN. The conversion of these two mutants from HAd deficient to HAd competent is shown in Fig. 6B. These data indicate that an otherwise HAd-deficient protein can be converted to a form competent to bind to receptors by exogenous NA treatment.

Rescue of receptor recognition activity by coexpression with another HN protein. Since NA treatment at the cell surface could partially restore the HAd activity of the mutants, a series of experiments were performed to determine whether the receptor recognition activity could be more efficiently rescued by NA activity contributed by another coexpressed protein. The HN protein chosen for these rescue assays was CH1(+11), an HN chimera in which the N-terminal 152 amino acid residues are derived from hPIV3 HN and the C-terminal 435 amino acids, including the entire putative globular domain, are derived from NDV HN (8) (Fig. 7A). This protein has approximately 10% of the NA activity exhibited by wt NDV HN.

To distinguish the HAd activity of the mutant from that of the chimera, an E347G mutation was introduced into each of the NA-HAd-deficient mutant proteins (site 14⁻). This mutation was previously shown to have no detectable effect on the receptor recognition, NA, or fusion properties of the virus but renders it unrecognizable to MAbs specific for site 14 (16, 19) (data not shown). MAbs to this site inhibit receptor recognition but not NA activity (12, 17, 18).

The HAd activity of monolayers coexpressing each 14⁻ mutant and the chimera was compared with that of monolayers coexpressing wt HN lacking site 14 and the chimera, following

treatment with site 14 MAb (Fig. 7B). When any NA-HAd-deficient mutant carrying the site 14 mutation is coexpressed with the chimera, extensive HAd activity is observed in the presence of the inhibiting antibody. Given the ability of the MAb to inhibit the HAd activity of the rescuing chimera, any HAd activity must be mediated by the previously receptor recognition-deficient molecules. In each instance, the HAd activities achieved in this system are greater than in the VCNA rescue. The most significant increases in this assay over the VCNA assay are for D198R HN (fourfold) and K236R HN (twofold). In the coexpression assay, all mutants hemadsorb between 40 and 60% of the wt-chimera control.

As additional controls, the HAd activity of wt HN and CH1(+11) coexpressing monolayers is completely blocked by pretreatment with the antibody; the presence of the site 14 mutation does not affect the HAd-deficient phenotype of the mutants; the site 14 MAb completely inhibits HAd activity of monolayers coexpressing each mutant with the chimera, when both retain the antigenic site; and antibody to site 1 that also inhibits HAd but not NA recognizes HN carrying the site 14 substitution and completely inhibits the HAd activity of monolayers coexpressing the site 14 mutants and the chimera. Also, we know that the chimera and NA-HAd-deficient proteins do not form oligomers, since they have N termini derived from heterologous HN proteins. This was verified by a sequential immunoprecipitation and Western blot protocol (data not shown). This means that the rescuing NA activity is not contributed by monomers in the same spike.

Relationship between the NA active site and antigenic site 23. Based on our previous demonstration of the topological proximity of antigenic site 23 and the NA active site (15, 18, 19, 23), we examined the ability of each of the NA active-site mutants to be recognized by an antibody to site 23 by immunoprecipitation and SDS-PAGE (Fig. 8). HN carrying either an R174L, D198R, or E547Q substitution is not immunoprecipitated by site 23 MAb, indicating that these residues contribute to antigenic site 23. It is important to note that these mutants are otherwise properly folded, as evidenced by their recognition by the conformation-dependent panel of MAbs used in the FACS analysis (Table 1) and the immunoprecipitations shown in Fig. 3, 5A, and 5B.

Loss of receptor recognition activity is not due to alteration in the structure of glycosyl groups. Rescue of the HAd activity and correction of the migration defect of the NA-HAd-deficient mutants by VCNA are reminiscent of a previous observation with the hemagglutinin of fowl plague virus, in which the protein is rendered receptor binding deficient in the absence of its NA protein (29). This suggests the possibility that the loss of receptor recognition function by the NA-deficient HN mutants might be due to failure to trim sialic acid from one or more glycosyl moieties near the receptor-binding site on HN.

This possibility was tested for the NA-HAd-deficient proteins carrying substitutions for D198 and I175. Each of the four glycosylation sites that are used in HN was individually deleted from the protein by the following amino acid substitutions: G1, S121N; G2, T343N; G3, T435A; and G4, T483N. Since G1 and G2 have been shown to be most closely associated with HN function (24), mutants carrying deletions of both of these sites were also constructed.

All of the glycosylation deletion mutants were expressed at

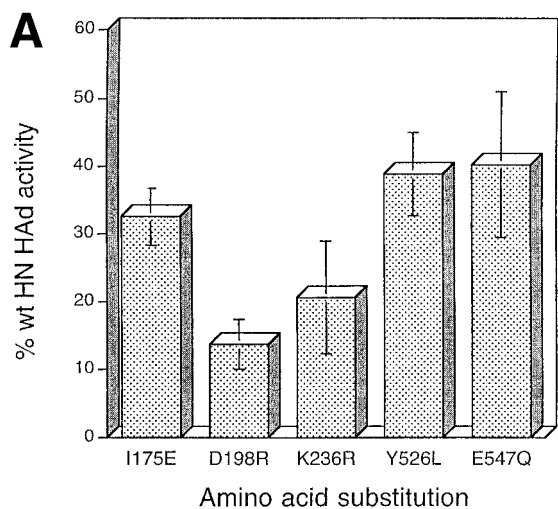
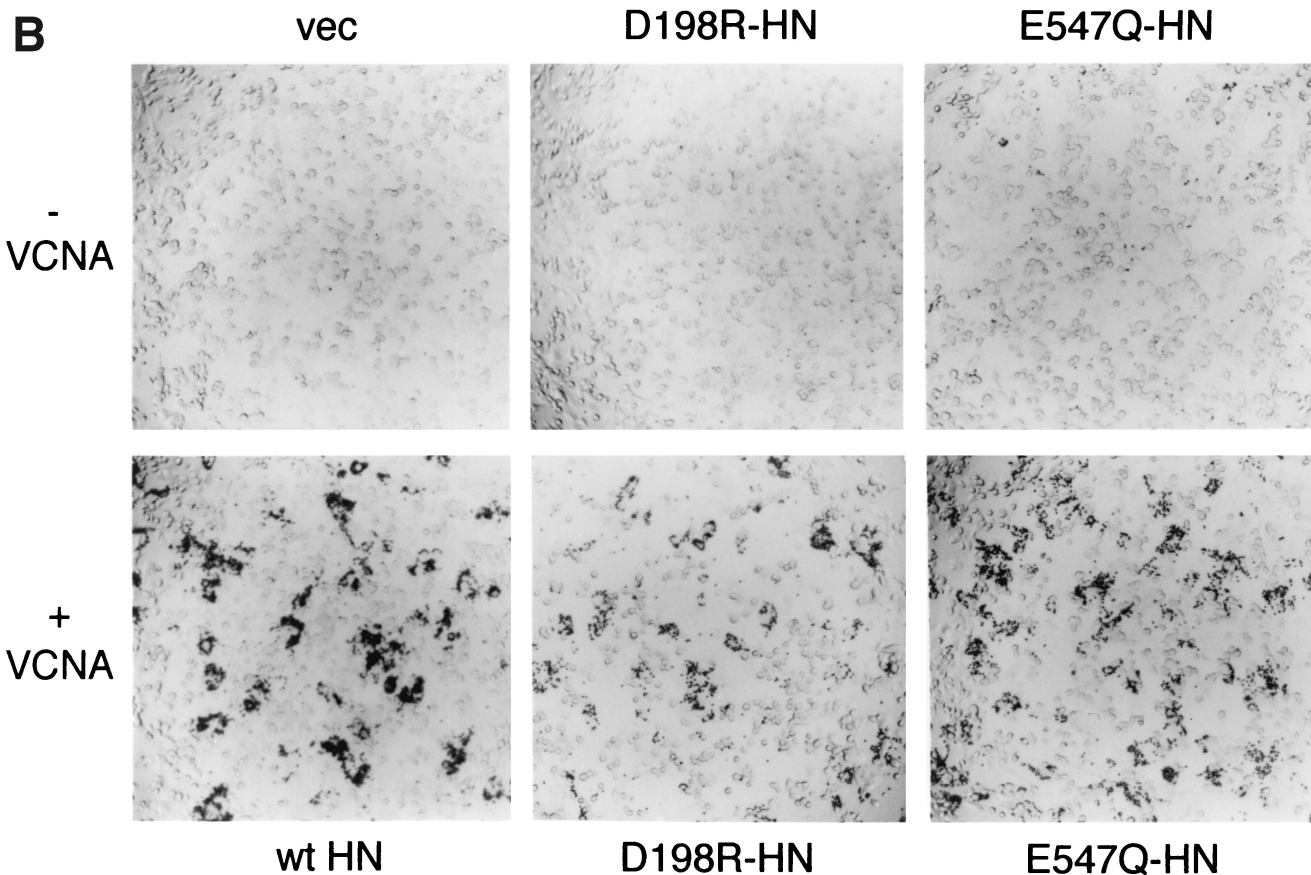


FIG. 6. Rescue of HAd activity of RR-deficient mutants by treatment with VCNA. Monolayers were washed four times with DMEM prior to incubation for 1 h at 37°C with 50 mU of VCNA. Following four washes with DMEM, cells were assayed for HAd activity. (A) HAd activity exhibited by each mutant following treatment at the cell surface with VCNA. Data are expressed as a percentage of the HAd activity exhibited by control wt HN monolayers treated in the same way. Each value represents the average of at least nine determinations. (B) HAd activity of untreated and VCNA-treated monolayers expressing D198R or E547Q HN. Untreated vector (vec)-expressing and VCNA-treated HN-expressing monolayers are shown for reference, though both are unaffected by the VCNA treatment.



the cell surface and had phenotypes indistinguishable from those of the corresponding parent mutant, i.e., undetectable NA and HAd activity (data not shown). These results indicate that the lack of receptor recognition activity is not due to interference by sialic acid residues on oligosaccharides near the receptor-binding site. Thus, though the receptor recognition-deficient mutants exhibit alterations of carbohydrate structure, their lack of attachment function is apparently unrelated.

DISCUSSION

Conserved residues in the paramyxovirus HN protein are homologous to and can be aligned with functional and structural active-site residues conserved in all the influenza virus NA proteins. On the basis of this homology, these HN residues are predicted to constitute the functional and framework residues of its NA active site, and the globular domain of the protein is predicted to have a structural motif similar to that of

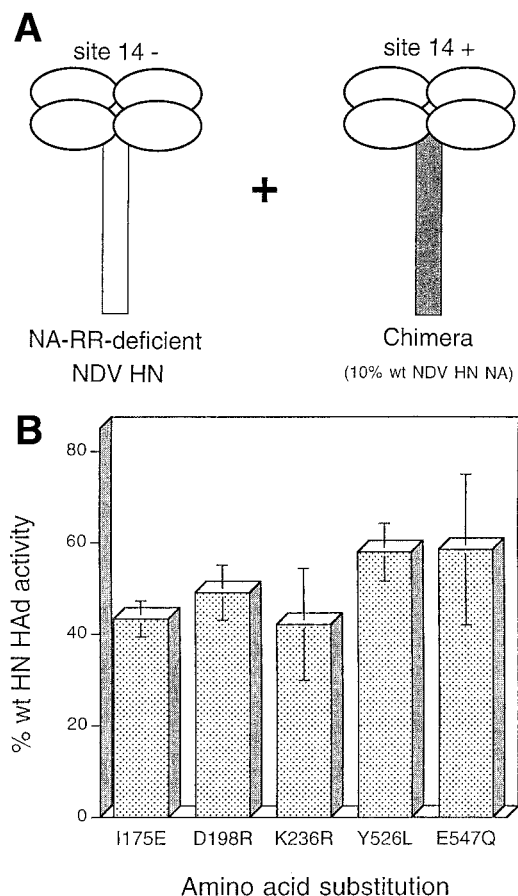


FIG. 7. Rescue of HA activity of RR-deficient mutants by coexpression with HN chimera CH1(+11). (A) Structures of the RR-deficient HN mutants and the rescuing chimera, CH1(+11). Clear and stippled areas are derived from NDV HN and hPIV3 HN, respectively. Each RR-deficient mutant carries a substitution (E347G) that renders it unrecognizable by antibody to site 14. In the CH1(+11) chimera, the N-terminal 152 amino acids are derived from hPIV3 HN and the C-terminal 435 amino acids are derived from NDV HN. The chimera exhibits approximately 10% of wt HN NA activity and is recognized by the site 14 MAb. (B) HA activity exhibited by each mutant carrying the site 14 mutation coexpressed with chimera CH1(+11) and treated for 30 min at room temperature with 200 μ g of site 14 MAb in 0.5 ml of DMEM. Data are expressed as a percentage of the HA activity of wt HN carrying the site 14 mutation and treated in the same way. Each bar represents the average of at least six determinations.

the influenza virus protein, i.e., a β -sheet propeller (5, 21). With the goal of testing these predictions and isolating HN proteins with altered NA activities, we have performed a site-directed mutational analysis of the role in NDV HN function of several of these invariant amino acids that are predicted to constitute its NA active site.

Of the predicted NA active-site residues, substitutions for five (R174, D198, R416, Y526, and E547) result in proteins lacking detectable NA activity, and substitutions for two more (R235 and R498) result in sharp reductions in NA (7, 25) (Table 1). Thus, substitutions for all seven putative NA active-site residues that allow cell surface expression of the protein result in complete or nearly complete loss of NA activity. In addition, substitutions for two other conserved residues, I175

and K236, whose relationship to the NA active site is not as clear in the homology alignment (5, 21), also result in complete loss of detectable NA activity (Table 1). Mutations at the residues corresponding to I175 and D198 in several other HN proteins also alter NA activity (11, 38, 45).

Thus, we have now shown that substitutions for a total of seven residues predicted to be either part of the NA active site or in close proximity to it completely abolish NA activity. This strongly suggests that the homology-based model for the structure of the globular domain of HN is correct. As originally proposed by Colman et al. (5), convergent evolution may have taken place, resulting in a similar NA active-site structure in influenza virus NA and the paramyxovirus HN protein, though they have divergent polypeptide sequences.

All mutated HN proteins for which NA activity cannot be demonstrated invariably also fail to mediate attachment to receptors. The absence of NA activity correlates completely with loss of detectable HA activity. This speaks to the long-standing question of the topological and functional relationship between the NA and attachment sites on HN. The simplest interpretation of this finding is that the NA active-site substitutions also directly abolish attachment function and that the NA site in HN also serves as the receptor-binding site.

However, this interpretation is inconsistent with our ability to demonstrate that NA-HA-deficient mutants can be rendered HA competent. When mutated proteins exhibiting this phenotype are supplied with NA activity, their ability to bind receptors is partially rescued. Thus, it seems unlikely that the residues that mediate NA activity also directly mediate attachment. Proteins carrying substitutions that abolish NA and HA activity can, in fact, be made competent for receptor recognition if supplied with NA activity. The lack of HA activity is secondary to and likely the result of the lack of NA. The more effective rescue of HA activity by the coexpressed protein, compared with exogenous NA, is probably an indication that the enzyme can act during intracellular transport.

A topological separation of the two activities is overwhelmingly supported by an extensive body of evidence from MAb functional inhibition studies (3, 10, 14, 17, 25, 27, 30, 32, 33) and analyses of escape and temperature-sensitive mutants (31, 39, 40, 43). However, if the two sites are, indeed, topologically distinct, this raises additional questions. First, if the NA active-site residues do not directly mediate attachment, where in the globular domain of the protein is the receptor-binding site? Second, if different sites mediate the two activities, why are



FIG. 8. SDS-PAGE analysis of immunoprecipitated NA active-site mutants with MAb to antigenic site 23. After starvation, labeling, and chase, HN was immunoprecipitated from lysates of labeled BHK cells expressing vector (vec) alone (lane 1), wt NDV HN (lane 2), or NDV HN carrying R174L (lane 3), I175E (lane 4), D198R (lane 5), K236R (lane 6), R416L (lane 7), R498L (lane 8), N500D (lane 9), V502A (lane 10), Y526L (lane 11), or E547Q (lane 12). The precipitating antibody is HN23c, specific for antigenic site 23 on HN.



FIG. 9. NA active site and antigenic site 23 overlap. The RASMOL molecular visualization program (34) was used to generate a model of the globular domain of the NDV HN monomer. Residues (P176, R235, E401, R416, R449, R498, and Y526) predicted to form the NA active site are shown in green; residues (F193, S194, S200, H201, and H203) at which substitutions have been identified in escape mutants selected by MAbs to site 23 are shown in magenta; and residues (R174, D198, and E547) that are part of both the NA site and site 23 are shown in yellow as well as labeled. Residues (P345, E347, Y350, and R353) at which substitutions have been identified in escape mutants selected by MAbs to sites 1 and 14, which inhibit only HAd, are shown in red. The interfaces with other monomers and the center of the tetramer are indicated by lines and an asterisk, respectively.

they not functionally independent, i.e., why does loss of NA also result in a loss of attachment function?

The first question can be approached by mapping epitopes recognized by MAbs that differentially affect the two activities. We have previously characterized MAbs to NDV HN that block attachment but not NA with neuraminlactose as the substrate (17). These antibodies have the potential to identify an attachment-relevant domain on HN. They map to two overlapping sites (1 and 14) on the protein and select escape mutants with substitutions for residues 345, 347, 350, and 353 at the end of the large loop β 3L23 in β -sheet 3 (19). These residues are predicted to be quite distant from the NA active site in the HN monomer (Fig. 9).

A role for β -sheet 3 in attachment was originally proposed by Colman et al. (5) based on the lack of a structural counterpart to it in the influenza virus NA protein and the fact that it does not include any putative NA active-site residues (Fig. 1). The structural model (21) suggests that this region is a large loop domain which could fold relatively independently of the

rest of the protein. Our findings are consistent with the idea that this region may be involved in attachment.

On the other hand, the conclusion that NA active-site residues are not directly involved in attachment can be questioned in light of other evidence. We have previously shown that mutants carrying I175M or I175V are HAd deficient, though they have 8 and 21% of wt NA activity, respectively (37). This is in contrast to I175E HN, shown here to be deficient for both activities. These data, along with the difference in its migration pattern compared to the other NA-deficient mutants, identifies I175 as being unique among the putative NA active-site residues targeted in this study. This could be related to its predicted position as one of the first residues in the first strand in β -sheet 1 in the HN globular domain (Fig. 1). Considering that it is situated right under the important active-site residue R174, I175 is probably very important to the structural integrity of the active site. Interestingly, the properties of I175E HN also suggest that the functional defects of the NA-HAd-deficient mutants are not causally related to their gel migration defects.

We have previously characterized MAbs to three overlapping sites on HN, sites 2, 12, and 23, that block both NA activity on neuraminlactose and attachment (17). This could be interpreted to mean that the NA and HAd sites are the same. Alternatively, the effect of these antibodies on attachment may be indirect, i.e., due to their effect on NA. Relevant to this point, all MAbs to NDV HN that inhibit NA also inhibit attachment (14, 18, 28, 47). The same can be said for MAbs to hPIV3 HN (3), though MAbs that inhibit NA but not attachment have been described for Sendai virus (32). Also, changes in NA activity have been known to affect attachment (15, 23).

While antibodies to overlapping sites 2, 12, and 23 on HN all block NA activity, additional studies strongly suggest that site 23 is unique in that it may directly overlap the NA active site. A low-molecular-weight competitive inhibitor of NA activity can block the binding of MAbs to site 23 but not those to any of the other sites (18). Previously identified site 23 residues at positions 193, 194, 200, 201, and 203 (15, 18, 19, 23) are in close proximity to residue D198, the putative catalytic aspartic acid in the NA active site. The overlapping nature of antigenic site 23 and the NA active site is supported by the demonstration that residue D198 contributes to the epitope recognized by a site 23 MAb (Fig. 8). Furthermore, two other putative NA active-site residues, R174 and E547, have now been identified as part of site 23 (Fig. 8). Residue E547 was certainly not obvious in this regard, as it is quite removed in the linear amino acid sequence from the other known site 23 residues.

The overlapping nature of the NA active site and antigenic site 23 is illustrated in Fig. 9, a RASMOL-generated depiction of an HN monomer. Residues contributing to the NA active site (green) and antigenic site 23 (magenta) are indicated. Residues R174, D198, and E547, which map to both of these sites, are labeled and colored yellow. As a reference, residues between 350 and 353 in β -sheet 3, selected by MAbs that inhibit only attachment but not NA on neuraminlactose, are indicated in red. This figure illustrates the topology of the overlap of the NA active site and site 23 and the separation of the receptor recognition and NA activities of HN.

Whatever the structural relationship between the receptor recognition and NA sites, it is clear that the two activities are

functionally intimately related; our data indicate that the former is completely dependent on the latter. While one cannot strictly rule out the possibility that any of the RR-deficient mutants we have characterized has a small amount of NA activity that is below the limit of detection in our assay, apparently none of them has a sufficient level to render HN attachment competent. What makes this study unique is that the substitutions introduced are in conserved domains that all very likely contribute to the structure of the NA active site. Previously, the relationship between NA and attachment had been addressed through the analysis of antibody escape and temperature-sensitive mutants that had altered activities. Only when NA is reduced to an undetectable level does the dependence of attachment on NA come into play. The threshold level of NA sufficient for attachment function could conceivably be significantly less than 1% of the wt level (25).

It still remains to be determined why loss of NA also results in loss of receptor-binding activity. The answer to this question may be related to the long-standing notion that NA is first and foremost a receptor-destroying activity. NA-minus mutants of influenza virus have been described (22, 46). In characterizing these mutants, Yang and Air (46) raised the issue of why influenza viruses, as well as some paramyxoviruses and coronaviruses, need such an activity, unlike the vast majority of other viruses. They postulated that a virus needs a receptor-destroying activity only if its receptor can be incorporated into the viral membrane along with the attachment protein. According to this hypothesis, the ability of NA-deficient NDV HN to mediate attachment to receptors on the target cell surface may be prevented by its association with receptors on the surface of the transfected cell, an association it cannot break due to its lack of receptor-destroying (NA) activity. Evidently, NA contributed exogenously or by another coexpressed protein can provide this activity and release HN to mediate attachment. It will be informative to rescue the mutations described here into an infectious clone of NDV to determine the effect of NA deficiency on the life cycle of the virus.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Greg Pettis. We thank Trudy Morrison for the NDV HN clone and Bernard Moss for the recombinant vaccinia virus.

This work was supported by a grant from the National Institutes of Health (AI-24770). R.D. was supported in part by the National Institutes of Health Training Program in Viral Immunology (AI-07272).

REFERENCES

- Baty, D. U., and R. E. Randall. 1993. Multiple amino acid substitutions in the HN protein of the paramyxovirus, SV5, are selected for in monoclonal antibody resistant mutants. *Arch. Virol.* **131**:217-224.
- Chong, A. K. J., M. S. Pegg, N. R. Taylor, and M. Von Itzstein. 1992. Evidence for a sialosyl cation transition-state complex in the reaction of sialidase from influenza virus. *Eur. J. Biochem.* **207**:335-343.
- Coelingh, K. L., C. C. Winter, E. D. Jorgensen, and B. R. Murphy. 1987. Antigenic and structural properties of the hemagglutinin-neuraminidase glycoprotein of human parainfluenza virus type 3: sequence analysis of variants selected with monoclonal antibodies which inhibit infectivity, hemagglutination, and neuraminidase activities. *J. Virol.* **61**:1473-1477.
- Coelingh, K. L., C. C. Winter, B. R. Murphy, J. M. Rice, P. C. Kimball, R. A. Olmsted, and P. L. Collins. 1986. Conserved epitopes on the hemagglutinin-neuraminidase proteins of human and bovine parainfluenza type 3 viruses: nucleotide sequence analysis of variants selected with monoclonal antibodies. *Virology* **60**:90-96.
- Colman, P. M., P. A. Hoyne, and M. C. Lawrence. 1993. Sequence and structure alignment of paramyxovirus hemagglutinin-neuraminidase with influenza virus neuraminidase. *J. Virol.* **67**:2972-2980.
- Deng, R., Z. Wang, R. L. Glickman, and R. M. Iorio. 1994. Glycosylation within an antigenic site on the HN glycoprotein of Newcastle disease virus interferes with its role in the promotion of membrane fusion. *Virology* **204**:17-26.
- Deng, R., Z. Wang, P. J. Mahon, M. Marinello, A. M. Mirza, and R. M. Iorio. 1999. Mutations in the NDV HN protein that interfere with its ability to interact with the homologous F protein in the promotion of fusion. *Virology* **253**:43-54.
- Deng, R., Z. Wang, A. M. Mirza, and R. M. Iorio. 1995. Localization of a domain on the paramyxovirus attachment protein required for the promotion of cellular fusion by its homologous fusion protein spike. *Virology* **209**:457-469.
- Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss. 1986. Eucaryotic transient expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* **83**:8122-8126.
- Gorman, W. L., D. S. Gill, R. A. Scroggs, and A. Portner. 1990. The hemagglutinin-neuraminidase glycoproteins of human parainfluenza virus type 1 and Sendai virus have high structure-function similarity with limited antigenic cross-reactivity. *Virology* **175**:211-221.
- Huberman, K., R. W. Peluso, and A. Moscona. 1995. Hemagglutinin-neuraminidase of human parainfluenza 3: role of the neuraminidase in the viral life cycle. *Virology* **214**:294-300.
- Iorio, R. M., J. B. Borgman, R. L. Glickman, and M. A. Bratt. 1986. Genetic variation within a neutralizing domain on the haemagglutinin-neuraminidase glycoprotein of Newcastle disease virus. *J. Gen. Virol.* **67**:1393-1403.
- Iorio, R. M., and M. A. Bratt. 1983. Monoclonal antibodies to Newcastle disease virus: delineation of four epitopes on the HN glycoprotein. *J. Virol.* **48**:440-450.
- Iorio, R. M., and M. A. Bratt. 1984. Monoclonal antibodies as functional probes of the HN glycoprotein of Newcastle disease virus: antigenic separation of the hemagglutinating and neuraminidase sites. *J. Immunol.* **133**:2215-2219.
- Iorio, R. M., and R. L. Glickman. 1992. Fusion mutants of Newcastle disease virus selected with monoclonal antibodies to the hemagglutinin-neuraminidase. *J. Virol.* **66**:6626-6633.
- Iorio, R. M., R. L. Glickman, and J. P. Sheehan. 1992. Inhibition of fusion by neutralizing monoclonal antibodies to the haemagglutinin-neuraminidase glycoprotein of Newcastle disease virus. *J. Gen. Virol.* **73**:1167-1176.
- Iorio, R. M., R. L. Glickman, A. M. Riel, J. P. Sheehan, and M. A. Bratt. 1989. Functional and neutralization profile of seven overlapping antigenic sites on the HN glycoprotein of Newcastle disease virus: monoclonal antibodies to some sites prevent viral attachment. *Virus Res.* **13**:245-262.
- Iorio, R. M., R. J. Syddall, R. L. Glickman, A. M. Riel, J. P. Sheehan, and M. A. Bratt. 1989. Identification of amino acid residues important to the neuraminidase activity of the HN glycoprotein of Newcastle disease virus. *Virology* **173**:196-204.
- Iorio, R. M., R. J. Syddall, J. P. Sheehan, M. A. Bratt, R. L. Glickman, and A. M. Riel. 1991. Neutralization map of the HN glycoprotein of Newcastle disease virus: domains recognized by monoclonal antibodies that prevent receptor recognition. *J. Virol.* **65**:4999-5006.
- Jorgensen, E. D., P. L. Collins, and P. T. Lomedico. 1987. Cloning and nucleotide sequence of Newcastle disease virus hemagglutinin-neuraminidase mRNA: identification of a putative sialic acid binding site. *Virology* **156**:12-24.
- Langedijk, J. P. M., F. J. Daus, and J. T. van Oirschot. 1997. Sequence and structure alignment of *Paramyxoviridae* proteins and discovery of enzymatic activity for a *Morbillivirus* hemagglutinin. *J. Virol.* **71**:6155-6167.
- Liu, C., and G. M. Air. 1993. Selection and characterization of a neuraminidase-minus mutant of influenza virus and its rescue by cloned neuraminidase genes. *Virology* **194**:403-407.
- Mahon, P. J., R. Deng, A. M. Mirza, and R. M. Iorio. 1995. Cooperative neuraminidase activity in a paramyxovirus. *Virology* **213**:441-444.
- McGinnes, L. W., and T. G. Morrison. 1995. The role of individual oligosaccharide chains in the activities of the HN glycoprotein of Newcastle disease virus. *Virology* **212**:398-410.
- Mirza, A. M., R. Deng, and R. M. Iorio. 1994. Site-directed mutagenesis of a conserved hexapeptide in the paramyxovirus hemagglutinin-neuraminidase glycoprotein: effects on antigenic structure and function. *J. Virol.* **68**:5093-5099.
- Mirza, A. M., J. P. Sheehan, L. W. Hardy, R. L. Glickman, and R. M. Iorio. 1993. Structure and function of a membrane anchor-less form of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus. *J. Biol. Chem.* **268**:21425-21431.
- Nishikawa, K., S. Isomura, S. Suzuki, E. Watanabe, M. Hamaguchi, T. Yoshida, and Y. Nagai. 1983. Monoclonal antibodies to the HN glycoprotein of Newcastle disease virus: biological characterization and use for strain comparisons. *Virology* **130**:318-330.
- Nishikawa, K., T. Morishima, T. Toyoda, T. Miyadai, T. Yokochi, T. Yoshida, and Y. Nagai. 1986. Topological and operational delineation of antigenic sites on the HN glycoprotein of Newcastle disease virus and their structural requirements. *J. Virol.* **60**:987-993.

29. **Ohuchi, M., A. Feldmann, R. Ohuchi, and H.-D. Klenk.** 1995. Neuraminidase is essential for fowl plague virus hemagglutination to show hemagglutinating activity. *Virology* **212**:77–83.
30. **Orvell, C., and M. Grandien.** 1982. The effects of monoclonal antibodies on biologic activities of structural proteins of Sendai virus. *J. Immunol.* **129**:2779–2787.
31. **Portner, A.** 1981. The HN glycoprotein of Sendai virus: analysis of site(s) involved in hemagglutinating and neuraminidase activities. *Virology* **115**:375–384.
32. **Portner, A., R. A. Scroggs, and D. Metzger.** 1987. Distinct functions of antigenic sites of the HN glycoprotein of Sendai virus. *Virology* **158**:61–68.
33. **Ray, R., and R. W. Compans.** 1986. Monoclonal antibodies reveal extensive antigenic differences between the hemagglutinin-neuraminidase glycoproteins of human and bovine parainfluenza 3 viruses. *Virology* **148**:232–236.
34. **Sayle, R. A., and E. J. Milner-White.** 1995. RASMOL: biomolecular graphics for all. *Trends Biochem. Sci.* **9**:374–376.
35. **Scheid, A., and P. W. Choppin.** 1973. Isolation and purification of the envelope proteins of Newcastle disease virus. *J. Virol.* **11**:263–271.
36. **Sergel, T., L. McGinnes, and T. Morrison.** 1993. Role of a conserved sequence in the maturation and function of the NDV HN glycoprotein. *Virus Res.* **30**:281–294.
37. **Sheehan, J. S., and R. M. Iorio.** 1992. A single amino acid substitution in the hemagglutinin-neuraminidase of Newcastle disease virus results in a protein deficient in both functions. *Virology* **189**:778–781.
38. **Shioda, T., S. Wakao, S. Suzu, and H. Shibuta.** 1988. Differences in bovine parainfluenza 3 virus variants studies by sequencing of the genes of viral envelope proteins. *Virology* **162**:388–396.
39. **Smith, G. W., and L. E. Hightower.** 1980. Uncoupling of the hemagglutinating and neuraminidase activities of Newcastle disease virus (NDV), p. 623–632. *In* B. Fields, R. Jaenish, and C. F. Fox (ed.), *Animal virus genetics*. Academic Press, New York, N.Y.
40. **Smith, G. W., and L. E. Hightower.** 1982. Revertant analysis of a temperature-sensitive mutant of Newcastle disease virus with defective glycoprotein: implications of the fusion glycoprotein in cell killing and isolation of a neuraminidase-deficient hemagglutinating virus. *J. Virol.* **42**:659–668.
41. **Tarentino, A. L., C. A. Gomez, and T. H. Plummer, Jr.** 1983. Deglycosylation of asparagine-linked glycans by peptide: N-glycosidase F. *Biochemistry* **24**:4665–4671.
42. **Thompson, S. D., W. G. Laver, K. G. Murti, and A. Portner.** 1988. Isolation of a biologically active soluble form of the hemagglutinin-neuraminidase protein of Sendai virus. *J. Virol.* **62**:4653–4660.
43. **Thompson, S. D., and A. Portner.** 1987. Localization of functional sites on the hemagglutinin-neuraminidase glycoprotein of Sendai virus by sequence analysis of antigenic and temperature-sensitive mutants. *Virology* **160**:1–8.
44. **Varghese, J. N., J. McKimm-Breschkin, J. B. Caldwell, A. A. Kortt, and P. M. Colman.** 1992. The structure of the complex between influenza virus neuraminidase and sialic acid, the viral receptor. *Proteins Struct. Funct. Genet.* **14**:327–332.
45. **Waxham, M. N., and J. Aronowski.** 1988. Identification of amino acids involved in the sialidase activity of the mumps virus hemagglutinin-neuraminidase protein. *Virology* **167**:226–232.
46. **Yang, P., A. Bansal, C. Liu, and G. M. Air.** 1997. Hemagglutinin specificity and neuraminidase coding capacity of neuraminidase-deficient influenza viruses. *Virology* **229**:155–165.
47. **Yusoff, K., M. Nesbit, H. McCartney, P. T. Emmerson, and A. C. R. Samson.** 1988. Mapping of three antigenic sites on the haemagglutinin-neuraminidase protein of Newcastle disease virus. *Virus Res.* **11**:319–333.