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Site-Directed Mutagenesis of a Conserved Hexapeptide in the Paramyxovirus Hemagglutinin-Neuraminidase Glycoprotein: Effects on Antigenic Structure and Function

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The sequence NRKSCS constitutes the longest linear stretch in the amino acid sequence of the hemagglutinin-neuraminidase (HN) glycoprotein of the paramyxoviruses that is completely conserved among all viruses in the group. We have used site-directed mutagenesis and expression of the mutated HN protein of one member of the group, Newcastle disease virus, to explore the role of this highly conserved sequence in the structure and function of the protein. Any substitution introduced for each of four residues in the sequence, N-234, R-235, K-236, or S-237, results in a drastic decrease in neuraminidase activity relative to that of the wild-type protein. Only substitutions for the terminal serine residue in the sequence had comparatively little effect on this activity. These findings are consistent with prior computer-based predictions of protein secondary structure which had suggested that this domain corresponds to one in the β-sheet propeller structure of the neuraminidase protein of influenza virus closest to the center of the sialic acid binding site and forms part of the enzyme active site. Four of the substitutions, N-234→Y and K-236→E, →Q, and →S, apparently cause a local alteration in the antigenic structure of the protein. This is evidenced by (i) the diminished recognition of the protein only by monoclonal antibodies thought to bind at the neuraminidase active site, among an extensive panel of conformation-specific antibodies, and (ii) the slower rate of migration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis for all except the K-236→Q mutation. One of the mutations, K-236→S, completely abolishes the ability of the protein to promote cellular fusion when coexpressed with the fusion protein. The latter cannot be explained by a decrease in the relative hemadsorption activity of the protein and suggests that the globular head of the protein may contribute to this process beyond providing receptor recognition.

The paramyxoviruses are a group of enveloped, negative-stranded, RNA-containing viruses that includes mumps virus, Newcastle disease virus (NDV) and the parainfluenza viruses (including Sendai virus, parainfluenza virus 3, and simian virus 5) (18). Virions are characterized by the presence of two types of spikes protruding from the virion envelope. These are the hemagglutinin-neuraminidase (HN) and fusion (F) glycoprotein spikes. HN is responsible for the apparently opposite functions of attachment to sialic acid-containing cellular receptors and release from the same moiety, catalyzed by the neuraminidase (NA) activity associated with the protein (4). Following exposure of a hydrophobic sequence by proteolytic cleavage, the F protein mediates both virus-cell and cell-cell fusion (9, 25).

For many paramyxoviruses, including NDV, membrane fusion also requires the participation of the HN spike (8, 19, 22). The most obvious contribution of HN to the fusion process is its ability to mediate attachment to cellular receptors. Consistent with this requirement is the demonstration that mutations in HN that influence the strength of its interaction with cellular receptors also influence the extent of syncytium formation (12). In addition to its receptor recognition properties, the requirement for HN in fusion apparently also involves a virus-specific interaction with the F protein spike (10).

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The structure of HN has not been elucidated. However, the three-dimensional structures of several influenza virus NAs have been determined (1, 3, 6, 32–34). All exhibit the same folding pattern, six β-sheets, each composed of four antiparallel strands, a motif commonly referred to as the β-sheet propeller.

Jorgensen et al. (17) made the initial observation that a region of the HN glycoprotein of paramyxoviruses might have some homology with the influenza virus protein. HN residues 212 to 303 were predicted to correspond, at the level of secondary structure, to two of the β-sheets of the influenza virus NA. One domain in particular, the sequence NRKSCS at residues 234 to 239 in NDV's HN, was predicted to be closest to the sialic acid binding site in the three-dimensional structure of the protein. This hexapeptide is also the longest linear stretch of amino acids that is totally conserved among the HN proteins of all the paramyxoviruses (23). Colman et al. (5) have recently expanded the comparison between the influenza NA and paramyxovirus HN proteins to suggest that the NRKSCS domain constitutes just one of a number of active site elements, specifically the arginine residue in the sequence, that are conserved in the two proteins.

Since NRKSCS constitutes the only hydrophilic region of NDV's HN that has not yet been shown to coincide with an antigenic site (16), studies using monoclonal antibodies (MAbs) and antibody-selected variants that might have provided information concerning its role in HN structure and function have not been possible. Therefore, we have performed mutational analysis to determine the effect of individ-

ual amino acid substitutions in this domain on the antigenic structure and various functions attributed to the HN protein.

MATERIALS AND METHODS

Site-directed mutagenesis and expression of the mutated proteins in COS-7 cells. The construction of the expression vector SVL-HN and the protocols for the introduction of site-directed mutations into the HN gene by using mismatched oligonucleotides (Oligos, Etc., Inc., Wilsonville, Oreg.), the preparation of plasmid DNA, and the maintenance of COS-7 cells (American Type Culture Collection, Rockville, Md.) have all been described previously (28). The wild-type (wt) and mutated HN proteins were expressed from SVL-HN vectors transfected into the cells by the DEAE-dextran method (21). Assays were performed 44 to 46 h posttransfection.

Labeled transfected cell lysates and immunoprecipitation. Transfected COS cells were labeled with EXPRE^{35}S^{35}S Labeling Mix (DuPont, New England Nuclear, Boston, Mass.). HN was immunoprecipitated with a mixture of MAbs, and the complexes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (21).

PNGase F digestion. To deglycosylate immunoprecipitated proteins, immunobeads with bound immunoprecipitate were digested with peptidyl-*N*-glycosidase F (PNGase F) (30). Briefly, they 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. The solution was allowed to cool and adjusted to contain 0.1% SDS and 0.5% Nonidet P-40. One aliquot of each sample was digested with 200 mU of PNGase F for 16 h at 37°C, prior to electrophoresis under reducing conditions.

Quantitation of cell surface expression of HN by fluorescence-activated cell sorter (FACS) analysis. Transfected cells were washed with phosphate-buffered saline (PBS [10 mM Na_2HPO_4, 3 mM KCl, 1.8 mM KH_2PO_4, 8.5% NaCl]) containing 5% fetal calf serum prior to incubation at room temperature for 30 min with a mixture of hybridoma supernatants containing MAbs to several sites on HN, one of which recognizes a linear epitope defined by HN residues 341 to 355 (16). After being washed twice with PBS plus serum, the cells were incubated as described above with a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Kirkegaard and Perry Labs, Gaithersburg, Md.). Following two more washes, the cells were detached by treatment with 62.5 μM EDTA in PBS, pelleted by centrifugation, washed again, and resuspended in 0.5 ml of PBS containing 1% fetal calf serum. They were fixed by incubation for 7 min at 4°C with 200 μl of 4% paraformaldehyde. After being washed with PBS plus serum, cells were resuspended in 0.4 ml of PBS for analysis in a FACScan flow cytometer model 440 (Becton-Dickinson, San Jose, Calif.), with cells transfected with the vector alone used to set the background level. Transfection efficiency was determined directly from the percentage of fluorescing cells. The amount of cell surface HN expression was calculated by multiplication of the mean fluorescence intensity per cell by the transfection efficiency.

Hemadsorption assay. The receptor recognition properties of the mutated proteins expressed at the surface of transfected cells were evaluated by determination of their ability to adsorb chicken erythrocytes (Crane Laboratories, Syracuse, N.Y.). Transfected monolayers in 35-mm plates were incubated for 20 min at room temperature with 4×10^7 erythrocytes in PBS containing 1% $CaCl_2$ and 1% $MgCl_2$. After removal of unadsorbed erythrocytes by extensive washing, the adsorbed erythrocytes were eluted by treatment for 2 h at 37°C with 1 mU of *Vibrio cholerae* NA (Calbiochem, La Jolla, Calif.), pelleted and lysed in 50 mM NH_4Cl. After removal of membranes by centrifugation, hemoglobin was quantitated by determination of the A_{560} in an automated enzyme-linked immunosorbent assay reader. Background obtained with vector alone was subtracted, and the data were corrected for differences in transfection efficiency.

NA assay. The NA activities of the mutated proteins expressed at the surface of transfected cells were determined as described previously (28). After subtraction of the background absorbance obtained with vector alone, the data were corrected for differences in the amount of HN expressed at the surface of the monolayer. Thus, the data shown represent the specific NA activity.

Fusion assay. The wt or mutated HN genes were excised from the appropriate SVL-HN vector with *Sac*I and *Xba*I and ligated into pBluescript SK(+) (Stratagene Cloning Systems, La Jolla, Calif.), which had been cut with the same enzymes, to generate pBSK-HN. The F gene of the NDV-AV strain was removed from the plasmid pSV103-NDV-F (generously provided by Robert Lamb) by digestion with *Xho*I and ligated into the same site in the pBSK vector to generate pBSK-F. Plasmid DNA was prepared from transformed JM109 cells and purified using Qiagen maxi-preps (Chatsworth, Calif.).

The HN and F proteins were coexpressed in BHK-21 cells (American Type Culture Collection, Rockville, Md.) driven by T7 RNA polymerase, which was provided by infection with the recombinant vaccinia virus vTF7-3 (7), a gift of Bernard Moss. Monolayers, seeded 24 h earlier at 4×10^5 cells per 35-mm plate, were infected with the recombinant vaccinia virus at a multiplicity of infection of 10. After 1 h at 37°C, the monolayers were washed with Optimem and transfected with 0.5 μg of DNA and LipofectACE reagent, according to protocols suggested by the supplier (Bethesda Research Laboratories, Gaithersburg, Md.). After 4 to 5 h at 37°C, an equal volume of medium containing twice the concentration of serum was added and the monolayers were incubated for an additional 17 h at 37°C. After being washed with PBS, the cells were fixed with methanol and stained with Giemsa Accustain (Sigma Chemical Co., St. Louis, Mo.), prior to microscopic examination for syncytium formation.

RESULTS

Effect of substitutions in NRKSCS on cell surface expression of HN. A total of 15 individual amino acid substitutions were introduced in the conserved NRKSCS domain of HN by using mutagenesis primers having single- or double-nucleotide mismatches as shown in Table 1. Substitutions were not introduced for the cysteine residue in the domain because it was felt that substitution for this residue would be likely to leave an unpaired cysteine elsewhere in the protein and result in aberrant folding.

As determined by FACS analysis with a mixture of anti-HN MAbs (16), most of the mutated proteins were transported to the cell surface, but with various degrees of efficiency relative to that of the wt protein. Cell surface expression ranged from a low of approximately 25% of that of the wt (S-237→T) to levels indistinguishable from that of the wt protein (>90% of that of the wt for N-234→Y- and K-236→E-mutated HN). No HN was detected at the surface of COS cells expressing proteins having R-235→E, R-235→Q, S-239→I, or S-239→N mutations. Since recognition of HN by MAbs in the mixture is highly conformation dependent, this strongly suggests that HN having these mutations is not transported to the cell surface in an antigenic form.

TABLE 1. Mutations in the NRKSCS domain and their effect on HN transport to the cell surface

Mutation	Nucleotide mismatch(es)[a]	Cell surface HN[b]
N-234→D	A-791→G	38.3 ± 10.0
N-234→Q	A-791→C + T-793→A	50.4 ± 2.6
N-234→Y	A-791→T	94.0 ± 5.6
R-235→E	C-794→G + G-795→A	ND[c]
R-235→L	G-795→T	37.1 ± 12.2
R-235→Q	G-795→A	ND
K-236→E	A-797→G	95.5 ± 0.6
K-236→Q	A-797→C	79.5 ± 10.7
K-236→S	A-790→T + A-791→C	79.2 ± 2.2
S-237→A	T-800→G	69.9 ± 3.0
S-237→T	T-800→A	25.6 ± 2.3
S-239→A	A-806→G + G-807→C	35.8 ± 5.1
S-239→I	G-807→T	ND
S-239→N	G-807→A	ND
S-239→T	G-807→C + T-808→C	46.4 ± 4.9

[a] Mutagenesis primers have the nucleotide mismatch or mismatches encoding the site-directed amino acid substitution shown in the first column. Each primer was a 17-mer with the mismatched nucleotides at the center. The numbering is modified (16) from the published sequence of the HN gene of the Australia-Victoria isolate of NDV (20).

[b] Values are expressed as a percentage of that of the wt and represent a minimum of seven determinations.

[c] HN was not detected at the cell surface.

Immunoprecipitation and SDS-PAGE analysis of NRKSCS-mutated proteins. Lysates of radioactively labeled transfected COS cells were immunoprecipitated with a mixture of MAbs specific for HN and analyzed by SDS-PAGE under reducing conditions (Fig. 1A). Many of the mutations that resulted in reduced amounts of HN at the cell surface, e.g., N-234→D, S-239→A, and, especially, S-237→T, resulted in mutant proteins that are also immunoprecipitated from cell lysates in smaller amounts than the wt protein is.

Most of the mutated HN proteins that were immunoprecipitated by the mixture of MAbs migrate at the same rate as the wt protein. This includes the R-235→L-mutated HN (data not shown). However, three of the mutations, N-234→Y, K-236→E, and K-236→S, result in proteins that migrate at a slower rate in SDS-PAGE (Fig. 1A). To determine whether the slower migration rate of any of these proteins is due to differential utilization of a glycosylation site or sites in the protein, immunoprecipitates from cells expressing each mutated protein were digested with PNGase F. This glycosidase cleaves the *N*-glycan linkage between asparagine and the carbohydrate chain (30). Figure 1B shows that, even after digestion with PNGase F, the K-236→E- and K-236→S-mutated proteins still migrate in the gel at a slower rate than the wt protein. Similar results were obtained with N-234→Y-mutated HN (data not shown). This indicates that the slower migration rate of the mutated proteins is not attributable to a change in the glycosylation pattern of the protein. This is especially important with respect to the K-236→S-mutated protein because this substitution adds a potential *N*-linked glycosylation site at residues 234-NRS-236, which is apparently not utilized.

All four of the mutated proteins (R-235→E, R-235→Q, S-239→I, and S-239→N [Table 1]) that were not detected at the cell surface by the panel of anti-HN MAbs were also not immunoprecipitated by the same MAbs from transfected COS cell lysates (Fig. 1A). The simplest explanation for this finding is that the presence of these substitutions may interfere with the proper folding of the protein, although this has not formally been proven. These mutated proteins were not characterized further.

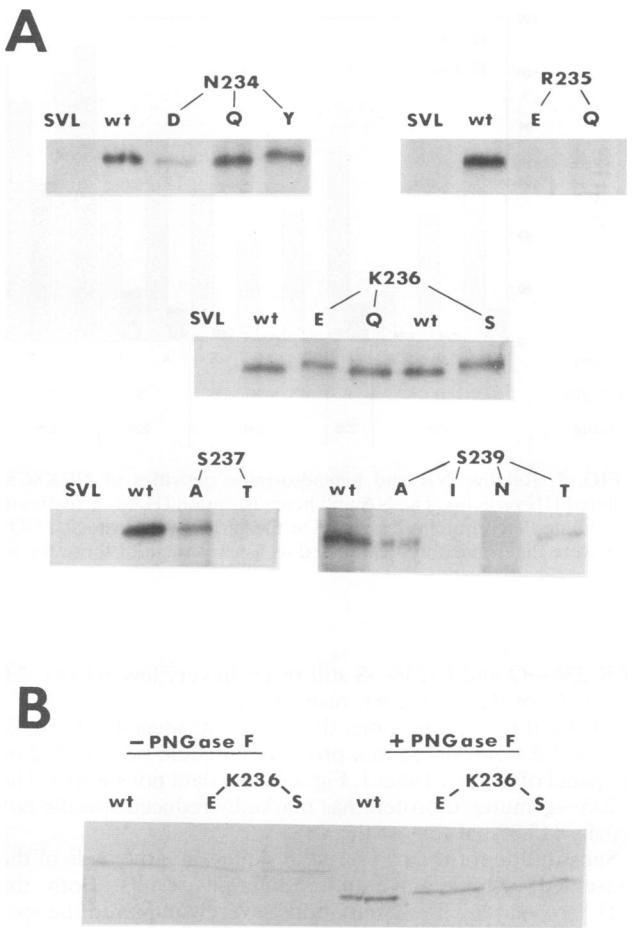

FIG. 1. (A) Immunoprecipitation of NRKSCS-mutated HN proteins by a mixture of MAbs. At 42 to 44 h after transfection with the vector alone (SVL lanes), SVL-HN (wt lanes), or mutated HN genes, COS cells in 60-mm dishes were labeled for 2 h and lysed and the HN protein was immunoprecipitated with a mixture of MAbs. The immunoprecipitates were separated by SDS-PAGE under reducing conditions. (B) Immunoprecipitation of HN from lysates of labeled COS cells expressing wt HN or K-236→E- or K-236→S-mutated HN. The immunoprecipitates were either untreated or digested with PNGase F before resolution by SDS-PAGE under reducing conditions.

Specific NA activity of the mutated proteins. The specific NA activity of the mutated proteins expressed at the surface of COS cells was determined (Fig. 2). Substitutions at the N and K residues in the domain result in the lowest specific NA activities. Both N-234→Q and N-234→Y substitutions result in proteins having only about 3% of the wt specific NA activity. The most severe reduction in specific activity was observed with cells expressing the K-236→E-mutated protein. This protein is transported to the cell surface at 95% of the wt level (Table 1) yet has less than 1% of its NA activity (Fig. 2). One possibility is that this is due to the introduction of a change in charge at the position, which might be expected to have important implications for the interaction of the active site with the substrate. However, more conservative substitutions

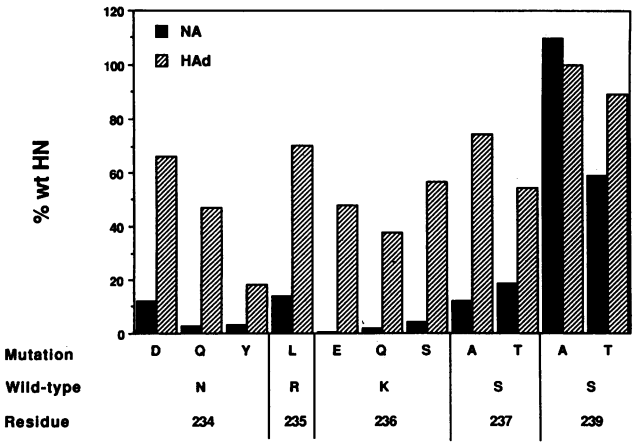

FIG. 2. Relative NA and hemadsorption activities of NRKSCS-mutated HN proteins. The NA and hemadsorption (HAd) activities of the mutated HN proteins expressed at the surface of transfected COS cells were determined and expressed as a percentage of those for wt HN.

TABLE 2. Recognition of HN proteins mutated in the NRKSCS domain by site-specific monoclonal antibodies[a]

wt or mutation	MAb specificity at site:					
	1	12	2	23	3	4
wt	102.0	95.2	85.5	87.7	99.2	83.3
N-234→Q	118.5	116.6	159.5	80.7	93.5	62.8
N-234→Y	115.1	88.1	88.7	23.5	115.3	91.3
R-235→L	83.2	115.9	109.7	78.1	129.7	64.4
K-236→E	101.3	87.6	103.9	21.8	117.7	90.3
K-236→Q	123.1	74.6	119.0	26.3	103.5	70.5
K-236→S	111.2	79.6	90.8	24.8	95.0	86.8
S-237→T	96.2	99.7	91.8	70.8	75.8	65.1
S-239→A	74.8	70.7	88.8	66.8	86.6	61.3

[a] Values reflect mean fluorescence (determined by FACS analysis), which is expressed as the percentage of the mean fluorescence obtained with MAb to site 14 that recognizes a linear epitope.

of K-236→Q and K-236→S still result in very low activity, 2.0 and 4.3% of that of the wt, respectively.

Of the three substitutions that were introduced for R-235, only R-235→L resulted in a protein that could be detected by the panel of MAbs (Table 1, Fig. 1A, and data not shown). The R-235→L-mutated protein has markedly reduced specific NA activity (13.8% of that of the wt).

Substitution for the two serine residues on either side of the conserved cysteine gave quite different results. Both the S-237→A and S-237→T mutations severely impaired the specific NA activity of the protein, both exhibiting <20% of the wt specific activity (Fig. 2). However, mutation of the second serine in the domain at position 239 had comparatively little effect on NA activity. An S-239→A substitution at this position has no detectable effect on specific NA activity, indicating that the presence of a hydroxyl group at this position is not a requirement for NA activity. Indeed, introduction of the slightly larger, hydroxyl-containing threonine side group in this position results in a 40% reduction in activity. S-239→I and S-239→N substitutions apparently result in the formation of misfolded forms of the protein. These results suggest that this residue may be more important for the proper folding of HN than for its catalytic activity.

Hemadsorption properties of the mutated proteins. The receptor recognition properties of the mutated proteins expressed at the surface of COS cells were evaluated by assaying their ability to adsorb chicken erythrocytes. While most of the mutated proteins show various degrees of reduction in hemadsorption activity, only two are markedly lower than 50% of the wt level, N-234→Y (18.0%) and K-236→Q (37.6%) (Fig. 2). Thus, there does not appear to be a direct correlation between the effects of mutations in the NRKSCS domain on the sialidase and receptor recognition activities of HN.

Recognition of the mutated proteins by a panel of conformation-specific MAbs. To probe the effect of mutations in the NRKSCS conserved domain on the antigenic structure of HN, the ability of MAbs to each of six conformational antigenic sites on HN (sites 1, 2, 3, 4, 12, and 23) to recognize several of the mutated proteins was determined by FACS analysis. For each mutated protein, the data shown in Table 2 are percentages of the mean fluorescence obtained with an antibody to antigenic site 14 that recognizes a linear epitope defined by HN residues 341 to 355 (16).

The only conformation-specific antibody that shows more than a 50% reduction in binding to any of the mutated HN proteins is the one to antigenic site 23. Four of the mutated proteins are recognized only about 25% as efficiently by this antibody as they are by the one that recognizes a linear epitope. There is a correlation between this property and the altered migration of the protein in SDS-PAGE. Three of the four proteins weakly recognized by the site 23 MAb, the N-234→Y-, K-236→E-, and K-236→S-mutated HN proteins, are those that exhibited slower migration rates by SDS-PAGE. Only one, K-236→Q, comigrated with the wt protein by SDS-PAGE (Fig. 1A).

Ability of the mutated proteins to promote cellular fusion when coexpressed with the F protein. Next, the effect of the substitutions in the NRKSCS domain on the ability of HN to complement the F protein in the promotion of cellular fusion was assayed. However, for several reasons, chief among them a high background level of syncytia in COS cell monolayers, a different expression system was utilized. The wt or mutated forms of HN were coexpressed with the wt F protein in BHK cells driven by T7 RNA polymerase. This cell line is especially suitable for assay of fusogenic activity because the induction of fusion in it by various isolates of NDV has been extensively characterized (2).

First, we expressed wt HN and wt F both individually and together to determine whether both HN and F are required for the promotion of fusion by NDV in this expression system. Figure 3 shows monolayers expressing either HN (panel B) or F (panel C). Neither appears different from the control monolayer expressing the vector alone (panel A). Only when HN and F are coexpressed is significant syncytium formation observed (panel D). This confirms that the findings obtained with other expression systems (8, 22) also apply to vaccinia virus-T7 RNA polymerase-driven expression in BHK cells.

The number of syncytia in a transfected monolayer is directly dependent on the transfection efficiency. To eliminate this variable, we chose to evaluate the extent of fusion in transfected monolayers by comparing the average number of nuclei per syncytium in monolayers expressing the mutated

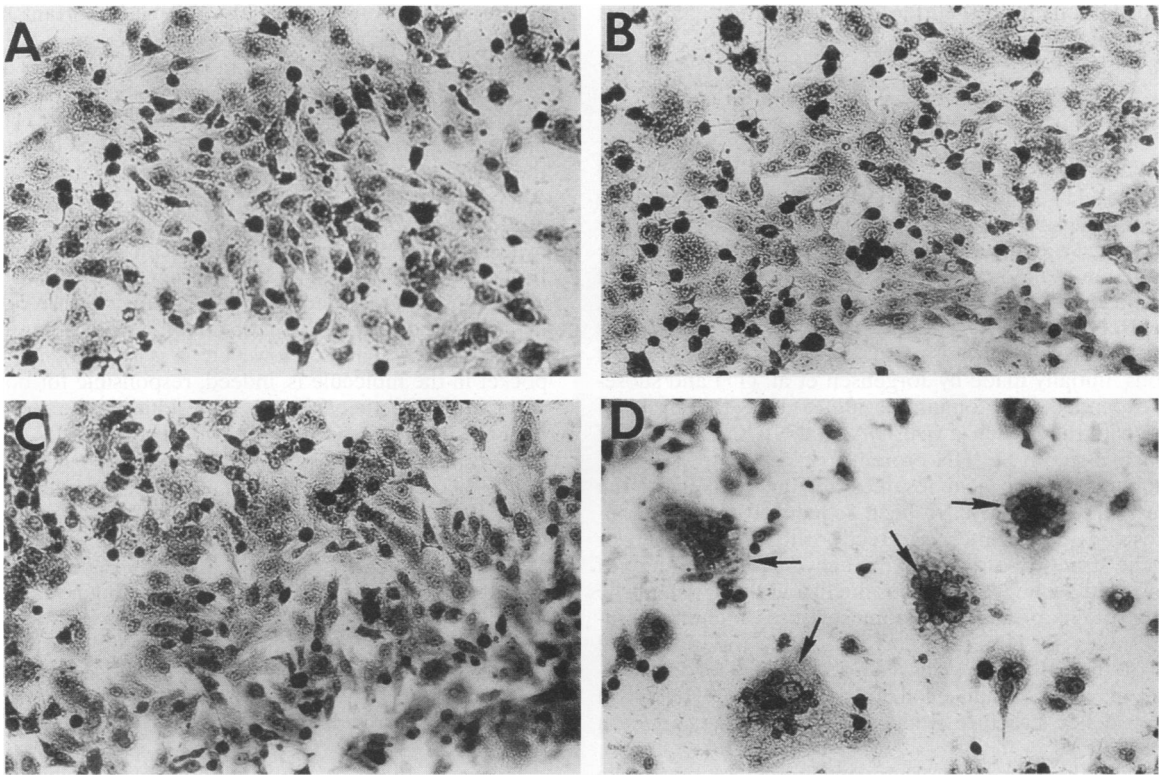

FIG. 3. Syncytium formation in BHK cells requires the participation of both the HN and F proteins. BHK cell monolayers were infected with the recombinant vaccinia virus and transfected with pBSK (A), pBSK-HN (B), pBSK-F (C), or pBSK-HN and pBSK-F (D). After 4 to 5 h at 37°C, an equal volume of medium containing twice the concentration of serum was added and the monolayers were incubated for an additional 17 h at 37°C. The monolayers were then fixed, and the nuclei were stained. Syncytia are indicated by arrows.

proteins and wt F with the average number of nuclei per syncytium in those expressing the wt forms of both proteins (Fig. 4). Most of the mutated HN proteins were indistinguishable from wt HN in their ability to complement the F protein in the promotion of fusion. They induced the formation of syncytia that had, on average, at least 85% of the number of nuclei in syncytia induced by wt HN and F. This includes the N-234→D-, N-234→Q-, R-235→L-, K-236→Q-, S-237→A-, S-239→A-, and S-239→T-mutated proteins (Fig. 4).

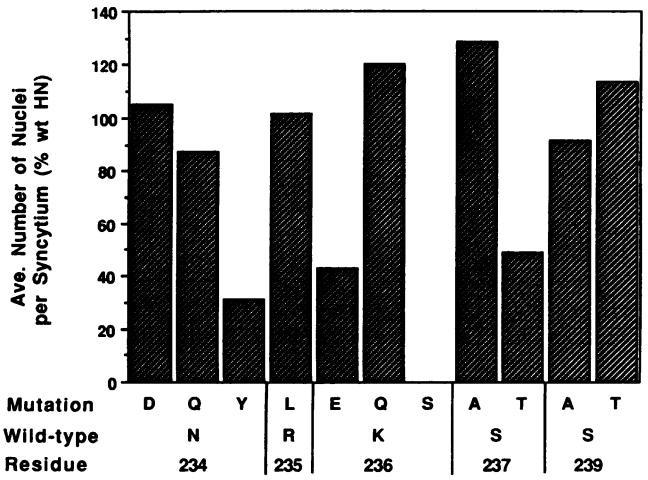

FIG. 4. Effect of mutations in NRKSCS on the ability of HN to take part in the promotion of fusion. The HN proteins mutated in the NRKSCS domain as indicated were coexpressed with wt F protein in BHK cell monolayers. The average number of nuclei per syncytium, as a percentage of that obtained with wt HN, is shown. A minimum of 15 syncytia were scored for each mutated HN protein.

Four of the mutated HN proteins show a diminished capacity to complement the F protein in the promotion of fusion. Three promote the formation of syncytia which, on average, have less than 50% of the number of nuclei in syncytia formed in monolayers expressing wt HN. These include N-234→Y (31.1%), K-236→E (43.3%), and S-237→T (48.8%).

One substitution, K-236→S, results in a protein that fails to promote syncytia containing more than three nuclei when coexpressed with F protein (Fig. 4). This is clearly not entirely due to a defect in transport of the protein to the cell surface (79.2% of that of the wt [Table 1]). Several proteins which are transported at even lower relative efficiencies (Table 1) promote syncytium formation quite effectively (Fig. 4).

Similarly, the failure of the K-236→S-mutated protein to promote fusion is also not due solely to its reduced ability to recognize cellular receptors (56.9% of wt hemadsorption activity [Fig. 2]). Five other mutated proteins have lower hemadsorption activities yet still promote fusion.

For three of the mutated proteins, N-234→Y, K-236→E, and K-236→S, reduced fusogenic activity may be related to a slower rate of migration by SDS-PAGE relative to that of wt HN (Fig. 1A) and weaker recognition by MAbs to site 23 (Table 2). On the other hand, the decreased fusogenic activity of the S-237→T-mutated HN may be related to the fact that cells expressing this mutated form of HN have the lowest

amount of HN at the cell surface, approximately 25% of wt levels (Table 1).

DISCUSSION

The sequence NRKSCS is the single longest domain in the paramyxovirus HN protein that is completely conserved among all viruses in the group. Such conservation of a hexapeptide sequence suggests that the region is important to the structure and/or function of the protein. We have examined the effect of several individual mutations for each residue in this domain, with the exception of the cysteine, on the various functions attributed to the HN protein. Our findings strongly support predictions, initially made by Jorgensen et al. (17) and subsequently expanded by Colman et al. (5), on the basis of homology with the influenza virus NA, that NRKSCS is part of the NA active site in the HN protein.

A number of amino acid residues are invariant in all the influenza virus NAs that have been sequenced (5). The conserved residues either interact directly with the substrate or stabilize the structure of the enzyme active site. Alignment of the amino acid sequences of the influenza virus NAs and the HN glycoproteins of several paramyxoviruses identifies conserved residues in HN that correspond to the functional residues of the influenza virus NA active site, leading Jorgensen et al. and Colman et al. to propose that HN has a β-sheet propeller structure similar to those of the influenza virus NA proteins.

Residues in the active site of influenza virus NA that make direct contact with the substrate are called functional residues. R-235 is the only residue in the NRKSCS domain of HN that is predicted to correspond to one of the functional residues in the active site of the influenza virus NA (5). In the β-sheet propeller model, R-235 in HN is proposed to be the counterpart of either R-224 or R-152 in influenza virus NA. The former contacts the glycerol side chain of sialic acid, and the latter hydrogen bonds to the carbonyl group of the *N*-acetyl moiety. A substitution of R-235→L abolished more than 85% of the NA activity of the wt protein, consistent with its predicted role as an NA active site functional residue.

However, substitutions for N-234 and K-236 resulted in the most severe reductions in NA activity. Apparently, these residues are more important to the activity of the protein than the arginine residue between them. It may be that N-234 and K-236 are important to the framework structure of the NA active site in HN.

Support for this comes from screening the ability of MAbs to conformational epitopes on HN to bind to the protein. There is an increasing body of evidence to support the idea that site 23 is contiguous with part of the NA active site (15). Indeed, a predicted interaction between an HN NA active site residue and one in site 23 (15) was found to be plausible in the Colman model (5). The demonstration that substitutions at N-234 and K-236 markedly diminish the ability of site 23 MAbs to recognize the mutated protein and that several alter the migration of the protein on SDS-PAGE is consistent with an alteration of the local topology of the NA active site, induced by these mutations.

Substitutions for S-237 also result in diminished NA activity but to a lesser extent. Thus, the presence of a serine at this position is apparently less stringently required for NA activity. Unlike S-237, the NA activity of the protein tolerates an A or T substitution for the S at position 239 (Fig. 2). However, two proteins mutated to I or N at this position were not immunoprecipitated by a mixture of MAbs predominantly specific for conformational epitopes. S-239 may be important for proper folding of the protein in a more global sense.

These findings are consistent with a separation of the NA and receptor recognition sites, in agreement with data from a number of other approaches (11, 24, 29, 31, 35). There is no direct correlation between the effect of NRKSCS mutations on the relative levels of specific NA activity and hemadsorption. All of the proteins carrying either N-234, R-235, or K-236 mutations have relative hemadsorption activities greater than fivefold higher than their relative specific NA activities (Fig. 2). In fact, the K-236→E-mutated protein has less than 1% of the wt amount of NA activity yet retains almost 50% of its hemadsorption activity, a more than 50-fold difference in the two relative activities. However, it is still possible that one pocket in the molecule is, indeed, responsible for both attachment and NA activity but that all residues in the pocket are not equivalent with respect to the two functions.

Previously, we have shown that an S-194→P mutation in a MAb-selected variant of NDV, although reducing NA activity to 17% of that of the wt (15), had no effect on the induction of fusion from within by the virus (12). However, another laboratory subsequently reported that NA-deficient, S-194→P-mutated HN has twice the fusion-promoting activity of wt HN in an HN and F, COS cell transient coexpression system (26). Our data for the NRKSCS-mutated proteins strongly support our original findings. For example, N-234→Q-mutated HN promotes syncytium formation slightly less efficiently than wt HN (Fig. 4), despite having only 2.7% of its specific NA activity (Fig. 2).

However, three of the mutated HN proteins (N-234→Y, K-236→E, and S-237→T) promote syncytium formation significantly less efficiently than the wt protein, and one (K-236→S) fails to promote the formation of syncytia at all when coexpressed with the F protein in BHK cell monolayers (Fig. 4). This decreased fusogenic activity is probably not due to a defect in HN's attachment function. K-236→S-mutated HN retains more than 50% of the wt level of hemadsorption activity.

This suggests that the globular head of the HN spike provides a function, in addition to attachment, that is required for the promotion of cellular fusion. This is consistent with previous results obtained with MAbs that bind to the globular head of the protein (21) and inhibit fusion (14) with no apparent effect on attachment (13). Previous mutations that had separated these two functions of HN, i.e., attachment and fusion promotion, were not localized to the globular head of the protein (26, 27).

For three of the four fusion-defective mutants, all except S-237→T, the diminished fusogenic activity correlates with an altered migration rate of the protein by SDS-PAGE (Fig. 1A) and diminished recognition by an MAb that binds at the NA active site (Table 2). Thus, it appears that the altered fusogenic properties of the mutated proteins stem from a localized change in the conformation of the protein, perhaps one that affects another domain important to the fusion process.

In summary, our findings support earlier predictions, on the basis of homology between HN and influenza virus NA, that the sequence NRKSCS of HN is part of the NA active site in the molecule and suggest that some mutations in the domain may alter the architecture of the site and the local topology of HN, resulting in changes in its antigenic structure and ability to take part in syncytium formation.

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