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JOURNAL OF VIROLOGY, Nov. 1994, p. 7654–7658
0022-538X/94/$04.00+0
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Vol. 68, No. 11

Mutations in the Fusion Peptide and Heptad Repeat Regions of the Newcastle Disease Virus Fusion Protein Block Fusion

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Received 19 May 1994/Accepted 18 July 1994

Nonconservative mutations were introduced by site-specific mutagenesis into the fusion peptide and the adjacent heptad repeat region of the fusion protein of Newcastle disease virus in order to determine the role of both regions in the fusion activity of the protein. Mutations in both regions that allowed for proper folding and intracellular transport of the protein blocked the fusion activity of the protein when assayed in the presence of the hemagglutinin-neuraminidase protein.

Membrane fusion, central to the entry of enveloped viruses, is mediated by one or more viral glycoproteins. Most of these fusion proteins have a conserved hydrophobic or apolar sequence, termed a fusion peptide or sequence, thought to be directly involved in the fusion event (28). As initially pointed out by Chambers et al. (3), many fusion proteins also have one or more heptad repeat regions which are often adjacent to the fusion sequence. The role of these regions in fusion is ill-defined.

Membrane fusion mediated by paramyxoviruses, such as Newcastle disease virus (NDV), usually requires two glycoproteins, the attachment protein (hemagglutinin-neuraminidase [HN] protein) and the fusion protein (F) (8, 9, 14). While the HN protein mediates attachment and has a yet-undefined role in subsequent membrane fusion (9, 23), the fusion protein is thought to be directly involved in the fusion event (9, 28). This protein is synthesized as a precursor (F_0) which is activated upon proteolytic cleavage to produce a disulfide-linked F_1 and F_2 (19, 20). The fusion peptide is at the new amino terminus of F_1 generated by the cleavage, and the heptad repeat is adjacent to the fusion peptide.

Mutational analysis of the paramyxovirus simian virus 5 fusion peptide showed that conservative mutations that allowed intracellular transport of the protein either had no effect on fusion or enhanced the fusion activity of the protein (7). There has been no reported mutational analysis of the adjacent heptad repeat region of a paramyxovirus fusion protein.

Expression of fusion proteins with nonconservative mutations in the fusion peptide or heptad repeat. Figure 1A shows the amino acid sequence of the fusion peptide and adjacent heptad repeat region of the NDV F_1 protein aligned with the comparable sequences from the fusion proteins of other representative paramyxoviruses. Point mutations resulting in single amino acid changes (Fig. 1B) were introduced into both regions of the protein by site-specific mutagenesis as previously described (10). The heptad repeat is predicted to form an α helix (3), as is the adjacent fusion peptide (18, 24). Figure 1C shows the entire region as an α helix with the position of the mutations indicated. Most of the mutations introduced were located on the hydrophobic or apolar side of the helix in the a or d position.

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The mutant proteins were expressed in Cos 7 cells by using simian virus 40-based vector as previously described (10, 23). Cells transfected with wild-type and mutant fusion protein genes were radioactively labeled with [^{35}S]methionine for 2 h at 48 h posttransfection, and labeled protein was precipitated with anti-Fu1a (Fig. 2A and B), a monoclonal antibody which reacts only with mature folded fusion protein (16). Precipitated proteins were electrophoresed in the absence (Fig. 2A, lanes 1 through 12, and 2B, lanes 1 through 8) or presence (Fig. 2A, lanes 13 through 24, and 2B, lanes 9 through 16) of reducing agent. In the absence of reducing agent, the uncleaved F_0 and cleaved but disulfide-linked F_1 and F_2 comigrate as a single band with an approximate molecular weight of 70,000 (Fnr), while in the presence of reducing agent, F_0 and F_1 are resolved. F_2 is not detected under these conditions (12, 17).

Mutant proteins A130K (resulting from an A-to-K mutation at position 130), A140K, N147K (Fig. 2A), G119K, A126K, A133K, and L154K (Fig. 2B) were precipitated by anti-Fu1a,

TABLE 1. Quantitation of expression and activities of fusion protein mutants

DNA	Surface expression[a]	Fusion activity[b]
Wild type	100	100
G119K	87	1
G123K	216	11
A126K	551	3
G128L	0	9
A130K	277	14
A133K	129	6
A140K	80	8
N147K	79	14
I151K	0	9
L154K	96	6
K155L	0	8
I158K	0	3

[a] Surface expression was quantitated as previously described (9, 10, 21). The values obtained for the wild type were set at 100%, and the values obtained for mutant proteins are expressed as percentages of the wild-type value. Values are averages for three separate experiments.

[b] Fusion activity was determined as previously described (21). The average size of syncytia obtained at 48 h with the wild-type fusion protein gene was set at 100%, and the values obtained for the mutant proteins are expressed as percentages of the wild-type value. Values are averages for three separate experiments.

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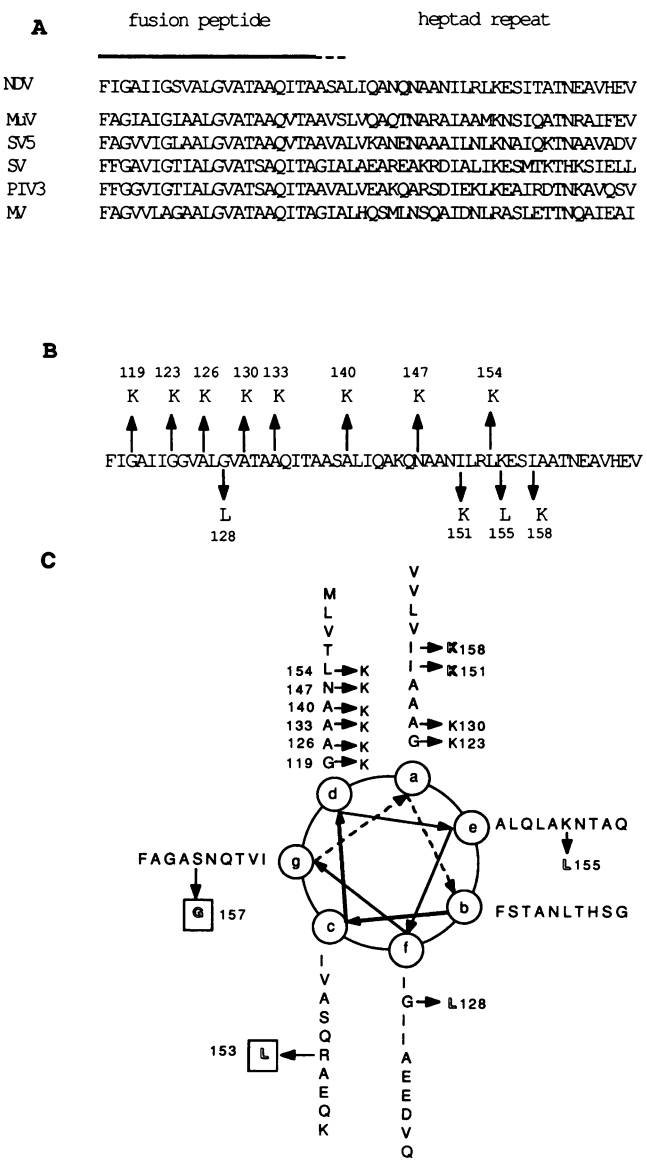

FIG. 1. Location of mutations in the fusion sequence and heptad repeat region. (A) The sequence at the amino terminus of the NDV (strain AV) F_1 protein is shown aligned with comparable sequences from representative paramyxovirus F_1 proteins (5, 13). The amino acid at the F_1 amino terminus is residue 117 in the entire sequence of the fusion protein of NDV. The solid bar above the sequences is the fusion sequence defined as the region of sequence homology across the family. The heptad repeat is the region just carboxy terminal to the fusion peptide. MuV, mumps virus; SV5, simian virus 5; SV, Sendai virus; PIV3, parainfluenza virus 3; MV, measles virus. (B) The positions of mutations are shown on the NDV sequence. Arrows pointing upward indicate mutations that result in proteins that are transported to the cell surface, while arrows pointing downward indicate changes that block transport (see text). (C) The fusion sequence and the heptad repeat region of the NDV F_1 protein are represented as an α helix beginning with the amino-terminal phenylalanine (F) in position b and the hydrophobic side of the helix in positions a and d. The positions of each of the 12 mutations reported here on the helix are represented by an arrow. Solid letters indicate changes that result in proteins that are transported to the cell surface, while open letters indicate changes that result in proteins that are not transported. Boxed letters indicate changes reported by Wang et al. (27).

while G128L, I151K, K155L, I158K (Fig. 2A), and G123K (Fig. 2B) were not precipitated. With the exception of L154K, all precipitated proteins appeared to be cleaved as efficiently as the wild type, since F_1 was detected in at least wild-type amounts.

In an attempt to detect other mutant proteins, extracts were also precipitated with anti-NDV antiserum. The anti-F antibodies in this serum also only recognize mature protein, not nascent protein (23). Like anti-Fu1a, anti-NDV antiserum did not precipitate G128L, I151K, K155L, and I158K mutant proteins (data not shown), a result that suggests that these mutant proteins are not properly folded. However, this serum did precipitate G123K, and the precipitated protein contained as much F_1 as the wild type did (Fig. 2C, lane 6). In addition, this serum precipitated F_1 in the L154K precipitate (Fig. 2C, lane 9).

In order to detect mutant proteins not reactive to conformation-sensitive antibodies, the expression of the mutant proteins was further characterized by Western blot (immunoblot) analysis with a polyclonal serum raised against a peptide with the sequence of the cytoplasmic tail of the fusion protein (27). Preliminary results with this antibody showed that, while reactivity of this antibody to wild-type protein on Western blots was low if the protein was boiled prior to electrophoresis (data not shown), proteins incubated at room temperature or heated to 50°C were readily detected. Furthermore, under these conditions, the antibody detected the cleaved F_1 and the uncleaved F_0, as well as larger oligomeric forms that were resistant to sodium dodecyl sulfate (SDS) as previously reported by Taylor et al. (25). In virion preparations, in the absence of reducing agent, three sizes of molecules were detected, a monomer-sized species (Fnr) and two larger species (140,000 and 210,000 kDa) which likely represent SDS-resistant oligomeric forms, while the monomer (F_1 and F_0) and a 140,000-kDa species are seen in the presence of reducing agent. Transfected cells also contained monomers as well as heterogeneous, higher-molecular-weight material which electrophoresed with molecular weights of approximately 140,000 and higher.

Western analysis detected the expression of G128L, I151K, K155L, and I158K mutant proteins which had not been detected in immunoprecipitations. As expected, only the F_0 forms of these mutant proteins were detected (Fig. 3A, lanes 14 and 18 through 20). The absence of cleavage of these mutants is a result consistent with failure to acquire mature epitopes recognized by the conformation-sensitive antibodies and strongly suggests that these mutant proteins are not transported to the cell surface. Western analysis of other mutant proteins confirmed the cleavage of mutant proteins A140K, N147K, G119K, G123K, A133K, and L154K, although the amount of F_1 detected in the L154K and G123K protein populations was less than the wild-type amount. Mutant proteins G128L, A130K, and A126K electrophoresed primarily as SDS-resistant oligomers (Fig. 3A, lanes 14 and 15, and 3B, lane 14), in contrast to the wild-type protein, suggesting that these mutations increased the SDS-resistant interactions of the proteins in oligomeric structures.

Surface expression of mutant proteins. To determine which mutant proteins were transported to the cell surface, immunofluorescence was performed on intact cells transfected with wild-type and mutant proteins as previously described (11). Figure 4 shows that all mutant proteins that could be detected by immunoprecipitations with conformation-specific antibodies were detected at the cell surface (G119K, G123K, A126K, A130K, A133K, A140K, N147K, and L154K). Mutant proteins not reactive to conformationally sensitive antibodies (G128L,

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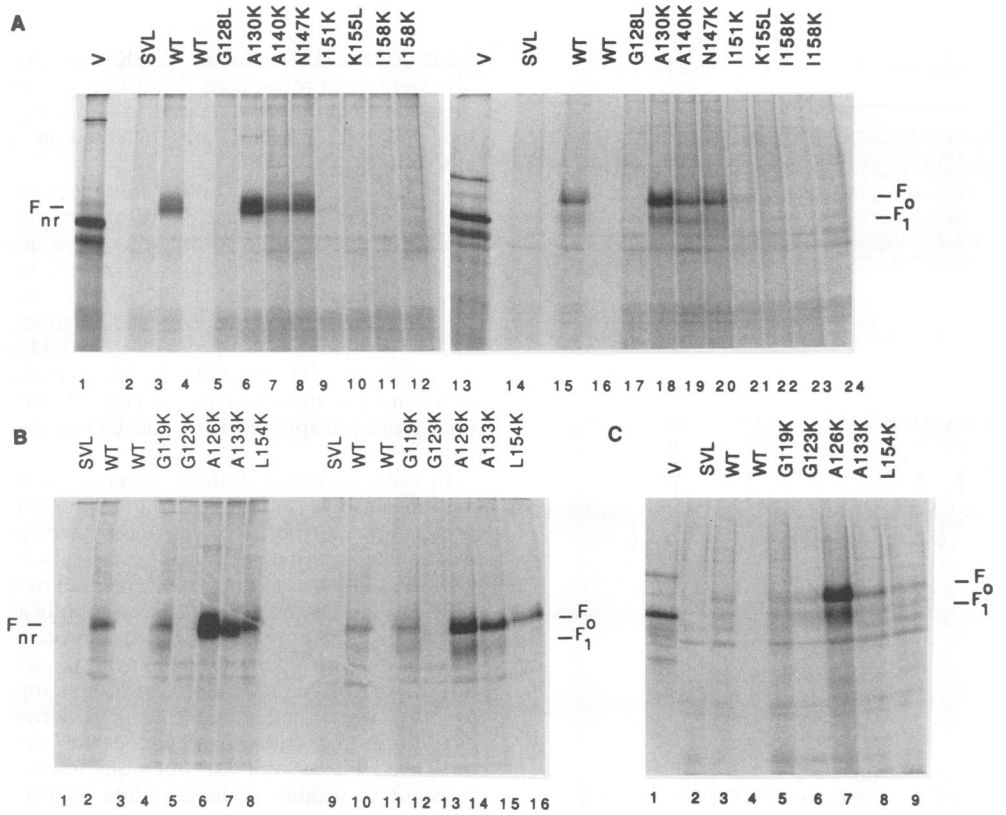
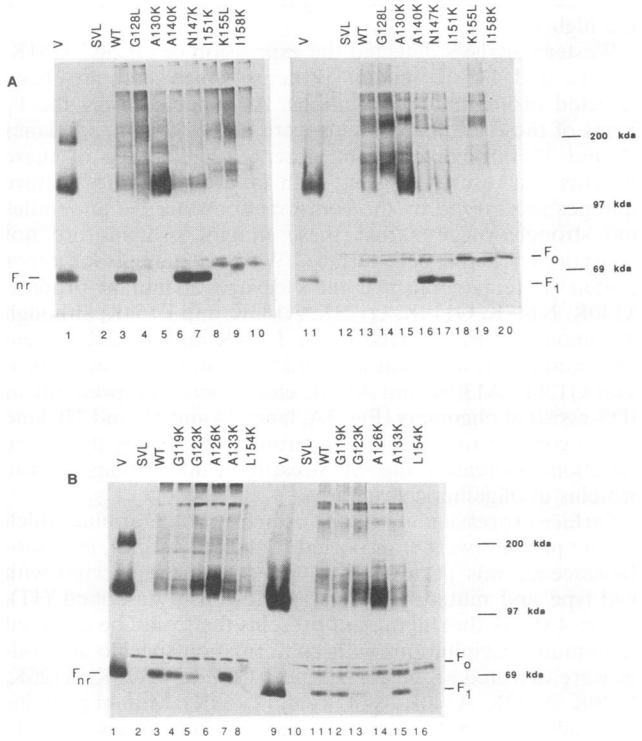


FIG. 2. Immunoprecipitation of mutant proteins with conformation-sensitive antibodies. At 48 h posttransfection by using DEAE-dextran, cells were radioactively labeled with [^{35}S]methionine for 3 h and lysed as previously described (23). Proteins present in extracts from 4×10^5 cells were precipitated with anti-Fu1a (A and B) or anti-NDV (C), and the precipitated protein was electrophoresed in the presence or absence of reducing agent. (A) Lanes: 1 to 12, nonreduced; 13 to 24, reduced. (B) Lanes: 1 to 8, nonreduced; 9 to 16, reduced. (C) All lanes contain reduced protein. V, virus-infected cells not immunoprecipitated; SVL, vector; wt, wild type. Mutants are indicated at the top of each lane. Lanes 4 and 16 in panel A, 3 and 11 in panel B, and 4 in panel C show precipitated proteins in wild-type-transfected extracts in the absence of antibody. F_0, uncleaved fusion protein; F_1, cleavage product; F_{nr}, cleaved and uncleaved fusion proteins which comigrate under nonreducing conditions.



I151K, K155L, and I158K) were not detected. All proteins detected at the surface were in either the a or d position in the α helix (Fig. 1C, solid letters), while the four mutants not transported to the surface were in various other positions on the helix (Fig. 1C, open letters). Interestingly, two mutations previously reported in this region are in the g and c positions on the helix (Fig. 1C, boxed letters) and result in proteins that are temperature sensitive in folding (27).

The surface expression of mutants was quantitated as previously described (15) (Table 1). All mutant proteins detected at the cell surface were present at approximately wild-type or higher levels. Some mutant proteins, particularly A126K and

FIG. 3. Western analysis of mutant proteins. At 48 h posttransfection, cells were lysed and proteins present were subjected to electrophoresis on 6% polyacrylamide gels. Prior to electrophoresis, extracts were incubated in gel sample buffer (23) at room temperature for 10 min. Proteins in lanes 1 to 10 in panel A and lanes 1 to 8 in panel B were electrophoresed in the absence of reducing agent; proteins in lanes 11 to 20 in panel A and lanes 9 to 16 in panel B were electrophoresed in the presence of reducing agent. Proteins were detected by Western analysis with anti-F_{tail} antibody (27). Positions of marker proteins are indicated at the side of the autoradiograph. V, virus proteins; SVL, vector; wt, wild type. Mutants are indicated at the top of the lanes. F_0, uncleaved fusion protein; F_1, cleavage product; F_{nr}, cleaved and uncleaved fusion proteins which comigrate under nonreducing conditions.

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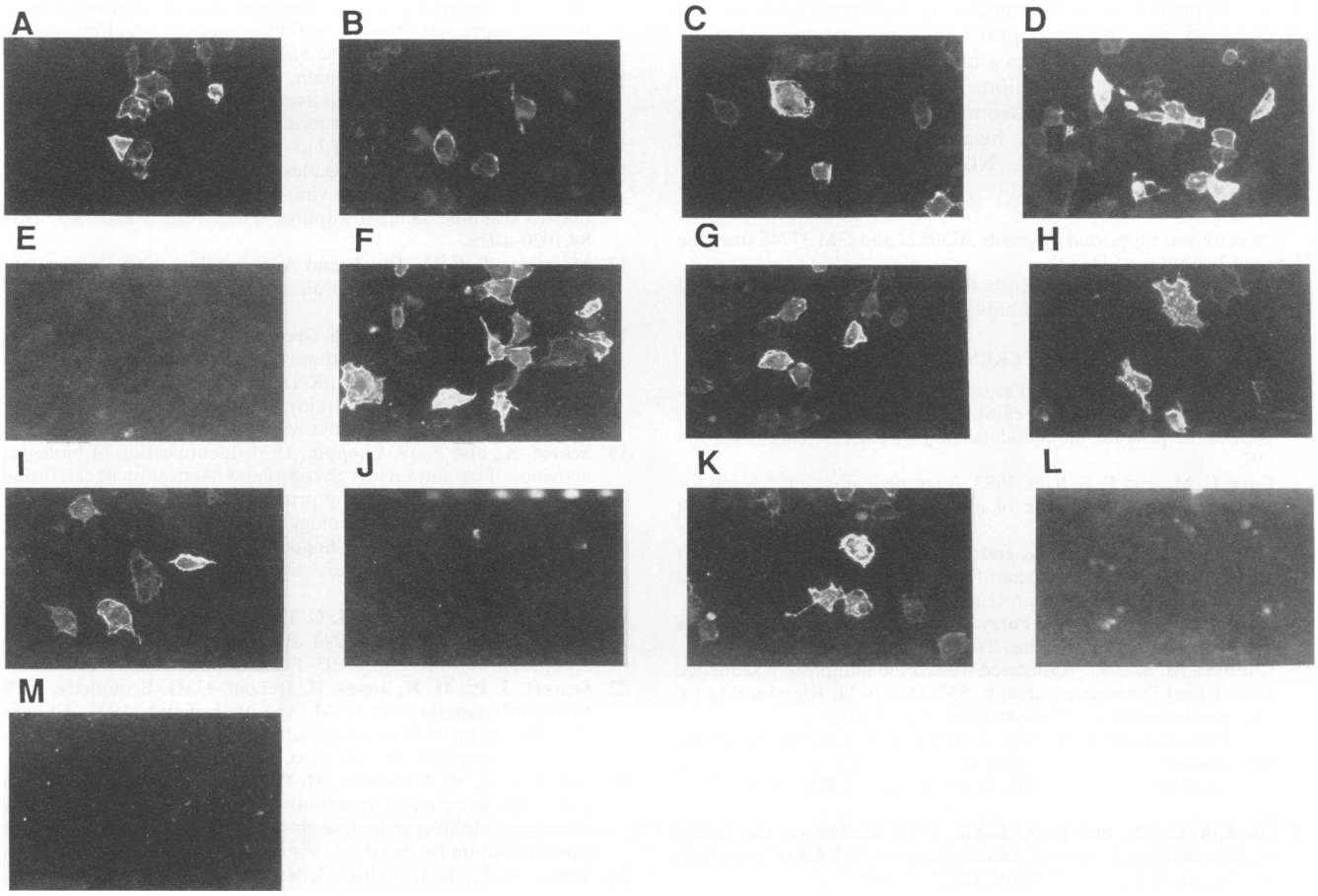

FIG. 4. Immunofluorescent detection of cell surface mutant proteins. At 48 h posttransfection, cells transfected with wild-type DNA (A) and mutant DNAs (B to M) were prepared for immunofluorescent detection of surface protein (10). Film was exposed for the same length of time for each panel. Panels: B, G119K; C, G123K; D, A126K; E, G128L; F, A130K; G, A133K; H, A140K; I, N147K; J, I151K; K, L154K; L, K155L; M, I158K.

A130K, were expressed at much higher levels than that of the wild type. The mechanism involved in this increased expression is now under active investigation.

Fusion activity of mutant proteins. As is typical of many paramyxovirus fusion proteins (8, 9, 14, 23), the NDV fusion protein does not direct membrane fusion unless coexpressed with the HN protein. Thus, to measure the fusion activity of the mutants, Cos cells were cotransfected with the mutant fusion protein genes as well as the wild-type HN protein gene. The size of syncytia formed with time after transfection was quantitated as previously described (23), and the results are shown in Table 1.

Clearly, nonconservative changes in the fusion peptide which did not block folding and transport eliminated fusion activity. The failure of these mutants to direct membrane fusion is likely due to direct effects on the insertion of the fusion peptide into membranes. It has been proposed that in several systems, the fusion peptide mediates membrane fusion by inserting obliquely into an adjacent membrane (6, 26). Thus, mutational changes which can block insertion or change the angle of insertion affect the fusion activity of the protein (26). Insertion of a charge would likely prohibit the insertion of this region of the protein into adjacent membranes just as insertion of a charge into the fusion peptide of the retrovirus transmembrane protein blocks fusion (1).

While introduction of a charged residue in several locations in the a position of the heptad repeat blocks transport, all mutants in the d position were transported to the cell surface. These mutations also block fusion. Several possible mechanisms may be envisioned. First, heptad repeats are characteristic of regions of molecules which form coiled-coil oligomeric structures (4, 21). We have found that these mutations do not block the formation of oligomers (unpublished data). However, a charge may disrupt a localized coiled-coil structure that might be required for fusion. Alternatively, alteration of this region of the fusion protein sequence may inhibit the activation of the fusion protein. The NDV fusion protein requires the presence of the HN protein to mediate membrane fusion. It has been hypothesized that the HN protein activates the F protein (2, 9, 23) in much the same way that acid pH activates the HA protein of influenza virus (2, 28–30) by changing the conformation of the fusion sequence and adjacent heptad repeat region. The introduction of nonconservative, charged amino acids into this region of the molecule may block this activation by interfering with a conformational change induced by the HN protein. Another possible mechanism is that this region of the fusion protein may also insert into membranes. Amphipathic α helixes can lie sideways along membranes with the side chains of the amino acids on the hydrophobic face penetrating the bilayer (22). Such an interaction of regions of

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the fusion protein with the adjacent membrane, in addition to insertion of the fusion peptide into membranes, may be required in the fusion process, and introduction of a charge would block such an interaction.

In summary, nonconservative mutations along one side of a predicted α helix in both the fusion peptide and the adjacent heptad repeat region of the NDV fusion protein block its fusion activity.

This work was supported by grants AI30572 and GM 37745 from the National Institutes of Health.

We thank Mark Peeples, Rush Presbyterian-St. Luke's Medical Center, for the generous gift of antibodies.

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