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Carbohydrate Modifications of the NDV Fusion Protein Heptad Repeat Domains Influence Maturation and Fusion Activity

Lori McGinnes, Theresa Sergel, Julie Reitter, and Trudy Morrison[1]

Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655

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The amino acid sequence of the fusion protein (F) of Newcastle disease virus (NDV) has six potential N-linked glycosylation addition sites, five in the ectodomain (at amino acids 85, 191, 366, 447, and 471) and one in the cytoplasmic domain at amino acid 542. Two of these sites, at positions 191 and 471, are within heptad repeat (HR) domains implicated in fusion activity of the protein. To determine glycosylation site usage as well as the function of added carbohydrate, each site was mutated by substituting alanine for the serine or threonine in the addition signal. The sizes of the resulting mutant proteins, expressed in Cos cells, showed that sites at amino acids 85, 191, 366, and 471 are used. This conclusion was verified by comparing sizes of mutant proteins missing all four used sites with that of unglycosylated F protein. The role of each added oligosaccharide in the structure and function of the F protein was determined by characterizing stability, proteolytic cleavage, surface expression, and fusion activity of the mutant proteins. Elimination of the site in F_2 at amino acid 85 had the most detrimental effect, decreasing cleavage, stability, and surface expression as well as fusion activity. The protein missing the site at 191, at the carboxyl terminus of the HR1 domain, also showed modestly reduced surface expression and negligible fusion activity. Proteins missing sites at 366 and 471 (within HR2) were expressed at nearly wild-type levels but had decreased fusion activity. These results suggest that all carbohydrate side chains, individually, influence the folding or activity of the NDV F protein. Importantly, carbohydrate modifications of the HR domains impact fusion activity of the protein.

INTRODUCTION

Newcastle disease virus (NDV), a prototype paramyxovirus, encodes two glycoproteins, the hemagglutinin–neuraminidase (HN) protein and the fusion protein (F) (reviewed in Lamb and Kolakofsky (1996); Morrison and Portner (1991)). The HN protein serves to attach virus to cells while the F protein is directly responsible for membrane fusion. As with all paramyxovirus fusion proteins, the NDV F protein is synthesized as a precursor, F_0, which, for activity, must be proteolytically cleaved to disulfide-linked F_1 and F_2, placing a fusion peptide sequence at the new amino terminus (Lamb and Kolakofsky, 1996). Typical of most viral glycoproteins, the fusion protein is modified by the covalent attachment of N-linked oligosaccharide chains. While the role of carbohydrates in the expression and function of some paramyxovirus F proteins has been analyzed (Alkhatib *et al.,* 1994; Bagai and Lamb, 1995; Hu *et al.,* 1995; Pastey and Samal, 1997; Segawa *et al.,* 2000), the number of oligosaccharide chains added to the NDV F protein is unknown, as is the importance of each to the structure and function of the protein. This question is particularly interesting for the NDV F protein, since two of the glycosylation addition sites are located within heptad repeat domains implicated in membrane fusion. In addition, because of the likely disulfide linkage between F_1 and F_2 (Iwata *et al.,* 1994), a carbohydrate addition site within F_2 is likely in close proximity to one of the heptad repeats and could also affect the structure and function of this domain.

Many fusion proteins have heptad repeat (HR) sequences important for fusion activity. The paramyxovirus F proteins have two characterized heptad repeat motifs, one located adjacent to the fusion peptide, HR1, and the other adjacent to the transmembrane domain, HR2 (Buckland and Wild, 1989; Chambers *et al.,* 1990). Mutational analyses of these regions have shown them to be important in fusion activity of the protein as well as the folding of the protein (Buckland *et al.,* 1992; Reitter *et al.,* 1995; Sergel-Germano *et al.,* 1994). Further implicating HR domains in fusion, studies of peptides with sequences from these regions have found that they inhibit fusion if added prior to fusion activation (Ghosh *et al.,* 1997; Ghosh and Shai, 1998; Lambert *et al.,* 1996; Rapaport *et al.,* 1995; Wild and Buckland, 1997; Yao and Compans, 1996; Young *et al.,* 1997, 1999). Furthermore, mixtures of HR1 and HR2 peptides form complexes, an association that has been suggested as reflecting an interaction within the intact protein upon activation of fusion (Baker *et al.,* 1999; Joshi *et al.,* 1998; Young *et al.,*

[1] To whom correspondence and reprint requests should be addressed. Fax: (508) 856-5920. E-mail: trudy.morrison@umassmed.edu.

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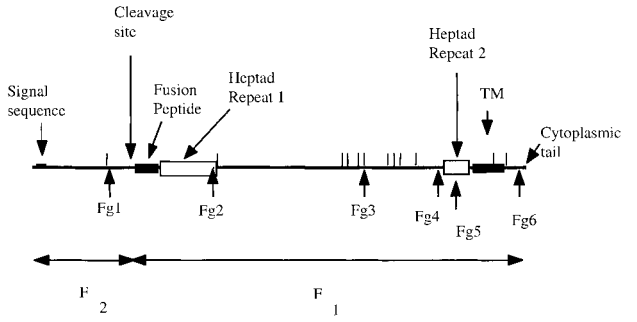

FIG. 1. Location of glycosylation signals in the primary sequence of the NDV F protein. The figure shows a linear diagram of the sequence of the NDV F protein with important domains indicated. Glycosylation addition sites are indicated below, Fg1–Fg6. Vertical lines show locations of cysteine residues. Heptad repeats, open boxes; transmembrane (TM) domain and fusion peptide, closed boxes.

1999). All studies with peptides, however, were accomplished with either synthetic peptides or peptide sequences synthesized in bacteria and thus were not glycosylated. Clarification of the function of these domains within the intact protein requires characterization of the role of the glycosylation addition sites on the formation of the prefusion form of the protein as well as the fusion activity of the protein.

N-linked oligosaccharide chains have been variously implicated in protein folding, protease resistance, protein solubility, and protein activity (Doms *et al.,* 1993). To determine the role of this modification of the HR1 and HR2 regions in the NDV F protein, we characterized the effect of elimination of these addition sites, as well as other sites within the protein sequence, on the maturation and activity of the protein. We found that the sites in the HR1 and HR2 domains are used, as are two other addition sites within the F protein ectodomain. Loss of carbohydrate in the HR1 domain influenced folding and severely inhibited fusion activity. Carbohydrate loss in the HR2 domain had little effect on folding but did depress fusion activity. Loss of the single F_2 site, which may influence the folding of the HR1 domain, also affected maturation of the protein.

RESULTS

Glycosylation site usage

The six glycosylation addition sites in the NDV F protein sequence are indicated in Fig. 1 (McGinnes and Morrison, 1986). Of the five sites in the ectodomain, one is located in the F_2 protein at amino acid 85 while the others are in F_1 at amino acids 191 (in the HR1 domain), 366, 447, and 471 (in the HR2 domain). There is also an addition site at amino acid 543 in the cytoplasmic tail. Each of the sites was altered individually by site-specific mutagenesis to generate six mutants, Fg1–Fg6, indicated in Fig. 1. Expression of mutant proteins was characterized by Western analysis in the absence or presence of reducing agent (Figs. 2A and 2B, respectively) and by immunoprecipitation of radioactively labeled proteins (Fig. 2C). In the absence of reducing agent, the antibody detected the monomeric form of the protein as well as large sodium dodecyl sulfate (SDS)-resistant, oligomeric forms migrating at the top of the resolving gel. These SDS-resistant forms of the fusion protein, previously reported (Collins and Mottet, 1991; Taylor *et al.,* 1993) for wild-type proteins, were significantly increased in amount by mutations Fg1 and Fg2. The sizes of monomeric forms of mutant proteins Fg1, Fg2, Fg3, and Fg5 were slightly smaller than those of the wild-type, suggesting that elimination of these addition sites resulted in loss of a carbohydrate side chain. The size of the protein encoded by Fg4 mutant was the same as that of the wild-type, indicating that this site is not used. As previously reported, the Fg6 mutant protein was also the same size as the wild type, as expected, since this addition site is in the cytoplasmic domain and inaccessible to oligosaccharyl transferase (Sergel and Morrison, 1995).

Electrophoresis of proteins in the presence of reducing agent resolved the fusion protein into both F_0 and F_1 (Figs. 2B and 2C). All mutant proteins were cleaved, since the F_1 protein was detected, but to varying extents. The F_1 protein encoded by the Fg1 mutant comigrated with wild-type F_1, a result consistent with the location of this mutation in the F_2 polypeptide. The F_1 polypeptides of the Fg2, Fg3, and Fg5 mutant proteins were smaller than the F_1 of wild-type F_1, a result consistent with the loss of a side chain in each of the mutant F_1 polypeptides.

These results suggest that sites 1, 2, 3, and 5 are used for carbohydrate addition. To verify this conclusion, a mutant missing these four sites was constructed (Fg1,2,3,5) and expressed in Cos cells along with the single mutants as well as a mutant missing two of the sites (Fg1,2) (Fig. 2C). In addition, mRNA derived from the wild-type gene was translated in a cell-free system in the absence of membranes in order to generate an unglycosylated F protein. The Fg1,2,3,5 mutant protein migrated slightly faster than the unglycosylated wild-type protein made in a cell-free reaction devoid of membranes, demonstrating that elimination of these four sites deglycosylated the protein. That the Fg1,2,3,5 protein is slightly smaller than the unglycosylated wild-type protein made in a cell-free translation reaction is consistent with signal sequence cleavage of the Fg1,2,3,5 protein.

The amount of F protein detected after elimination of two or four oligosaccharides was much less than that detected for the single mutants, suggesting that loss of two or more side chains resulted in a protein with a shorter half-life than wild type. In addition, the amounts of Fg1 protein detected, particularly in Western analysis, which detects steady state levels, were somewhat less

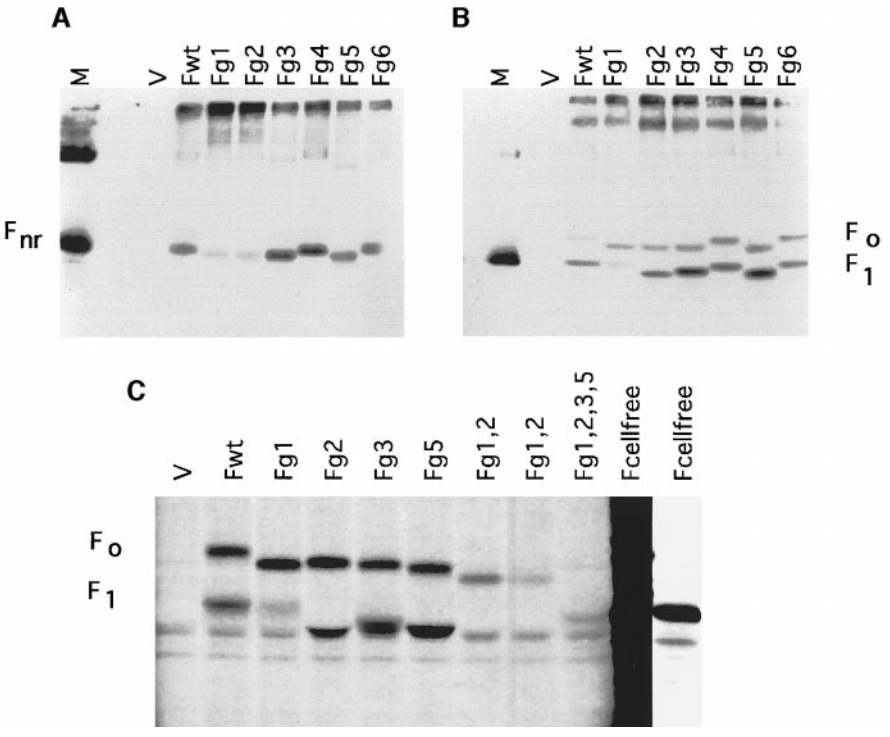

FIG. 2. Expression of mutant proteins. (A and B) Western analysis of mutant proteins using anti-F tail for detection. Proteins present in cells transfected with DNAs encoding wild-type and mutant proteins for 48 h were resolved on polyacrylamide gels in the absence (A) or presence (B) of reducing agent. (C) Wild-type and mutant proteins immunoprecipitated, using anti-F tail antibody, from extracts derived from cells radioactively labeled for 2 h and chased for 4 h. Precipitated proteins were electrophoresed on polyacrylamide gels in the presence of reducing agent. The last two lanes show products of a reticulocyte cell-free reaction directed by wild-type (wt) F protein mRNA in the absence of dog pancreas membranes in order to synthesize unglycosylated F protein. The last lane is a shorter exposure of the adjacent lane in order to resolve the cell-free products.

than the amounts of protein detected for other single mutations, suggesting that loss of this single oligosaccharide also destabilized the protein.

Oligomer formation

We have previously reported that NDV F protein sediments on sucrose gradients as a trimer (Reitter *et al.,* 1995). This conclusion was also indicated by crosslinking studies of the SV5 F protein (Russell *et al.,* 1994). To determine if the absence of any carbohydrate side chain influences this oligomer formation, the sedimentation of mutant proteins was characterized on 10–45% sucrose gradients. The proteins in each gradient fraction were detected by Western analysis (Fig. 3). Figure 3A shows the sedimentation of the wild-type protein, while Figs. 3B–3F show the sedimentation of mutant proteins, Fg1–Fg5, respectively. No significant differences from wild-type protein were detected in the sedimentation of any of the mutant proteins.

Surface expression of mutant proteins

To determine if mutant proteins were transported to the cell surface, immunofluorescence was performed on intact cells transfected with wild-type and single mutant proteins. All mutant proteins were detected at the cell surface (not shown). Quantitation of surface expression was accomplished in two ways, by flow cytometry and by surface immunoprecipitation of radioactively labeled transfected cells as previously described (Sergel and Morrison, 1995). Analysis by flow cytometry (Fig. 4) showed that the numbers of positive cells detected after transfection with each of the mutants was similar to those with the wild type, but the intensity of fluorescence was somewhat lower for all mutants. The most severe effects were detected for Fg1 protein, while the least severe for Fg5 protein. Surface immunoprecipitation results are shown in Table 1 and expressed as a percentage of wild-type protein. This assay also showed that the most severe effects on surface expression were seen upon elimination of the more amino-terminal glycosylation sites.

The double mutant protein, Fg1,2, and the unglycosylated F protein were not detected at the surface (not shown).

Fusion activity of mutant proteins

To determine the fusion activity of mutant proteins, Cos cells were transfected with the mutant fusion protein genes as well as the wild-type HN protein gene. Two different fusion assays were done. First, the size of syn-

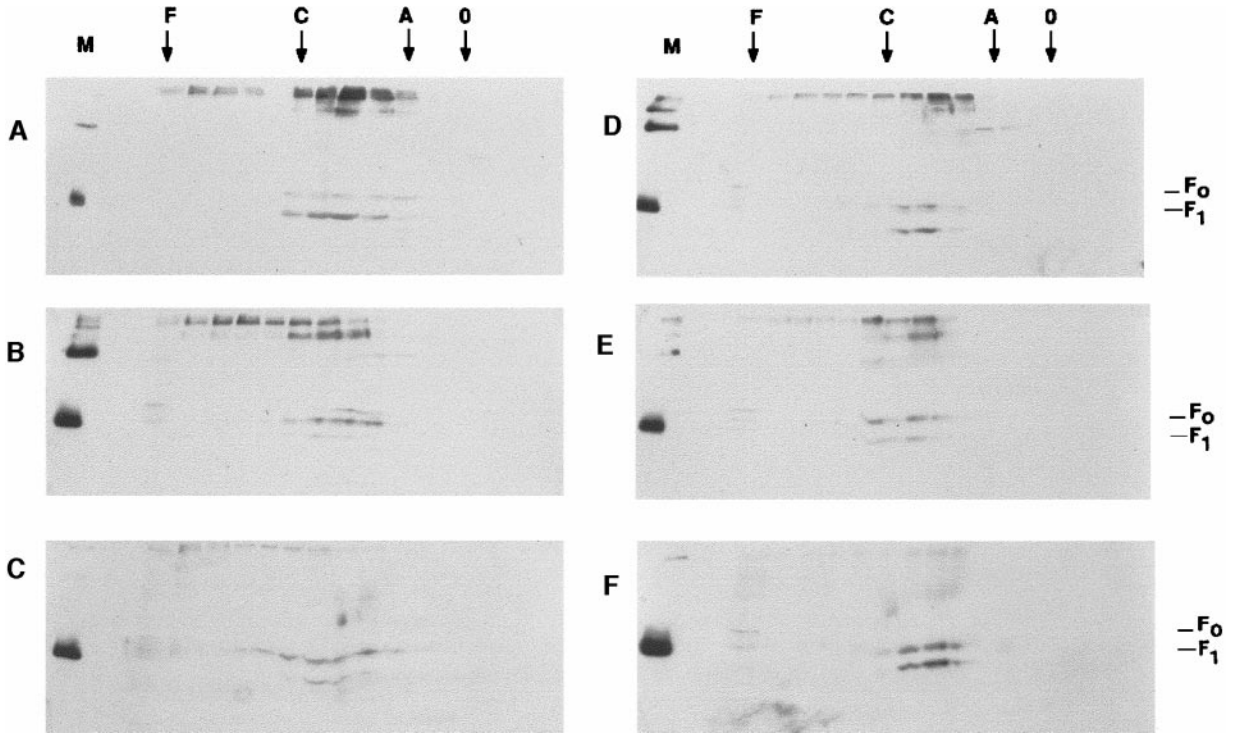

FIG. 3. Sucrose gradient analysis of mutant proteins. At 48 h posttransfection, cells were lysed and extracts layered onto a 10–45% sucrose gradient as described under Materials and Methods. Proteins present in each fraction were precipitated with trichloroacetic acid (TCA), incubated at 50°C for 10 min, and electrophoresed in the presence of reducing agent, and the F protein in each fraction was detected by Western analysis using anti-F tail antibody. The positions of marker proteins are indicated at the tops of A and D: F, ferratin; C, catalase; A, aldolase; O, bovine serum albumin. (A) wild type, (B) Fg1, (C) Fg2, (D) Fg3, (E) Fg4, (F) Fg5. Marker F protein (M) present in virions is shown at the side of each panel.

cytia formed by each of the mutants with time after transfection was quantitated as previously described (Sergel *et al.,* 1993) (Fig. 5). Clearly, mutation in site 4 had no effect on syncytia formation, since the activity was comparable to that of the wild type. Mutations eliminating carbohydrate chains 1 or 2 resulted in proteins with virtually no ability to form syncytia. Mutations eliminating chains 3 or 5 resulted in proteins with reduced syncytia size. The syncytia forming activities, normalized to wild-type levels, are shown in Table 1. Elimination of oligosaccharide chains located closer to the amino terminus of the F_0 protein had a more severe effect on fusion than elimination of the side chains closer to the transmembrane domain.

Fusion was also assayed using a content mixing assay, an assay that measures a step earlier than syncytia formation. This assay, similar to ones previously reported (Nussbaum *et al.,* 1994), measures the mixing of cytoplasms of fusing cells. In this assay, cells transfected with a plasmid encoding the β-galactosidase gene under the control of a tet sensitive transactivator were mixed with cells transfected with HN and F cDNAs as well as a plasmid encoding the tet responsive transactivator of transcription. β galactosidase synthesis is induced only when cells from the different populations fuse (Fig. 6). That this assay is specific for fusion directed by the HN and F proteins was shown by the findings that little enzyme activity was present when only F cDNA was present or when only HN cDNA was present. Furthermore, there was little activity after transfection with HN and an F cDNA that encodes an uncleaved F protein (F115G) (Li *et al.,* 1998). Activity was present only after protease activation of the F115G protein (not shown). This assay is also dependent upon the levels of expression of F proteins. Content mixing increased linearly with increasing concentrations of F DNA and a fixed amount of HN DNA (Fig. 6A).

Like wild-type F protein, expression of the fusion protein mutants in the absence of the HN protein did not result in any detectable fusion activity (Fig. 6B). However, after cotransfection with HN protein cDNA, there was variable activity with less activity the closer the mutated site was to the amino terminus (Fig. 6B).

As shown in Fig. 4 and Table 1, at a fixed concentration of F DNA all mutant F proteins were expressed at the surface at levels variously lower than those for wild-type F protein. To determine if lower surface expression could account for the decreased fusion activities of the mutant proteins, we measured the surface expression of the wild-type F protein using different concentrations of wild-type F DNA (Fig. 7) and correlated those results with levels of fusion detected at each concentration of DNA (Fig. 6A). Using this

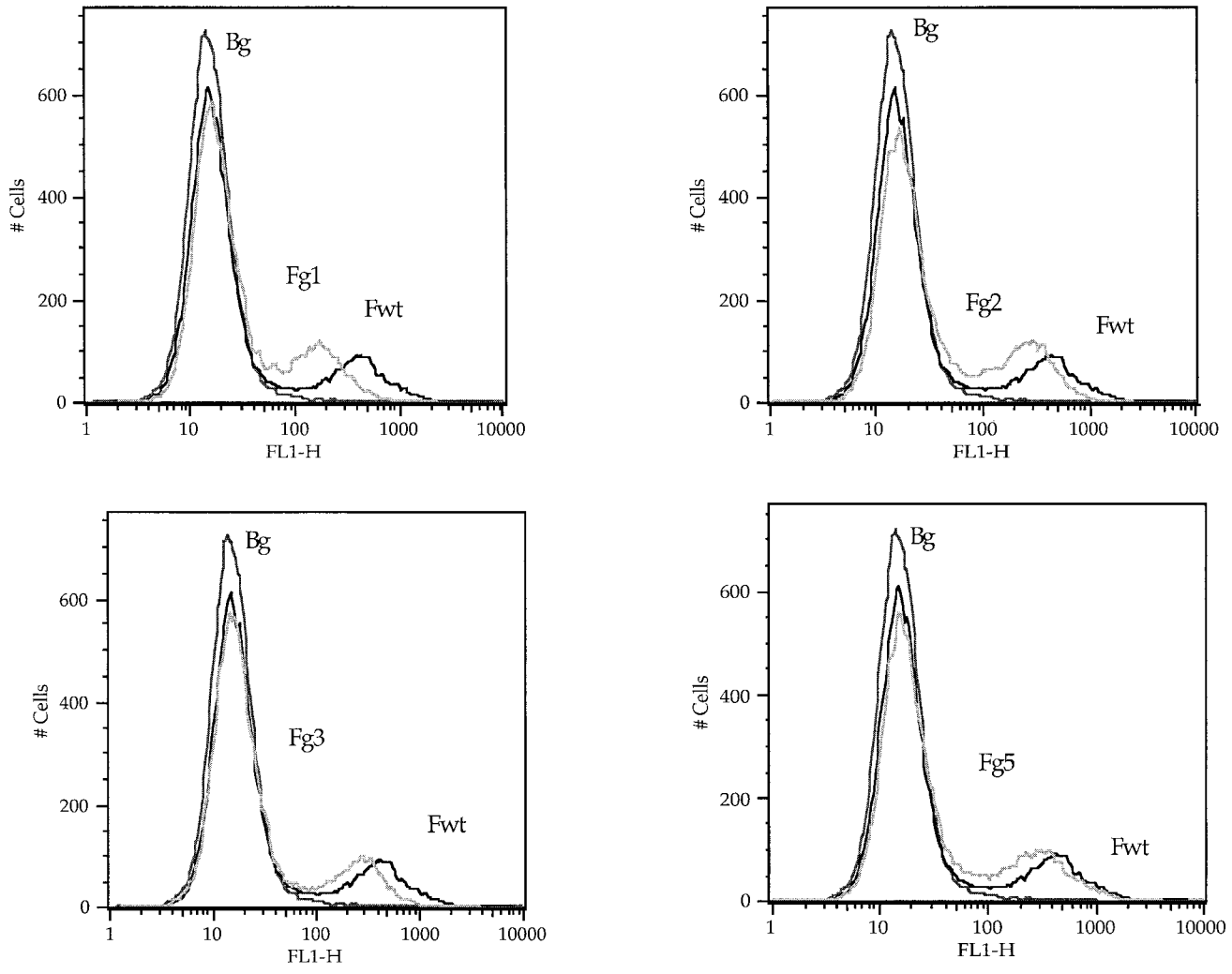

FIG. 4. Flow cytometry of cells expressing mutant proteins. Cells transfected with 0.5 μg DNA/35-mm plate were processed for analysis by flow cytometry as described under Materials and Methods. The primary antibody was anti-NDV antibody. Each panel shows background (cells transfected with vector alone and incubated with both primary and secondary antibody), and F wild type (Fwt) as well as a mutant. The numbers of positive cells detected after transfection of each of the mutants were comparable to the number detected after F wild-type transfection.

data, it is clear that if the decreased fusion activities of Fg2, Fg3, and Fg5 were due only to decreased levels of surface expression, then fusion activity of these mutants should be approximately 65, 70, and 75%, respectively, that of wild-type F protein. These levels are considerably higher than the measured values of 15, 26, and 51% (Table 1), particularly for the Fg2 and Fg3 mutant proteins. Similarly, the fusion activity of Fg1 should be approximately 40%, also considerably higher than the 5% measured amount of fusion. However, decreased fusion activity of Fg1 may also be due to deceased cleavage. Figure 2 shows that the amount of F_1 detected was less than that for wild type. However, when four times as much mutant DNA as wild type was used in a content-mixing assay, the Fg1 mutant protein still directed only 7% of the level of content mixing as wild-type F protein.

DISCUSSION

Role of oligosaccharides in maturation of NDV F protein

The mature conformation of the fusion proteins of paramyxoviruses is unknown. Furthermore, F proteins undergo one and or more conformational shifts prior to the onset of fusion. One change occurs with cleavage of the F protein and has been defined by an increase in hydrophobicity of the protein (Hsu *et al.,* 1981). Another conformational change likely occurs upon activation of fusion and has been proposed to include the interaction of the HR1 and HR2 domains of the protein (Baker *et al.,* 1999; Young *et al.,* 1999). Very likely contributing to the F protein conformation both before and after conformational shifts are the modifying oligosaccharide side chains. Definition of oligosaccharide site usage as well

TABLE 1

Quantitation of Surface Expression and Fusion Activity

DNA	Surface[a]	Surface[b]	Syncytia formation[c]	Content mixing[d]	Syncytia/cell surface[e]	Content mixing/ cell surface[f]
Wild-type	100	100	100	100	1.00	1.00
Fg1	36	40	4	5	0.10	0.12
Fg2	57	55	8	15	0.14	0.27
Fg3	129	71	24	26	0.34	0.37
Fg5	96	78	40	51	0.51	0.65

[a] Surface expression was determined by surface immunoprecipitation as described under Materials and Methods. The values obtained for the wild type were set at 100% and the values obtained for mutant proteins are expressed as a percentage of the wild type. Values are the average of four separate experiments.

[b] Surface expression determined by FACS analysis. The values are intensity of signal as a percentage of the wild type.

[c] Fusion activity, measured in the presence of HN protein expression, was determined as described under Materials and Methods. The average size of syncytia obtained at 72 h with the wild-type fusion protein gene was set at 100% and the values obtained for the mutant proteins are expressed as a percentage of the wild type. Values are the average of three separate experiments.

[d] Values represent β-galactosidase activity relative to amounts found in HN + F wild-type transfections.

[e] Values represent the size of syncytia (as percentage of the wild type) divided by surface expression (FACS analysis) (percentage of the wild-type protein).

[f] Values represent β-galactosidase activity relative to that of the wild type divided by surface expression (FACS analysis) (percentage of the wild-type protein).

as the role of each side chain in the folding and activity of the protein is, therefore, a key component to understanding F protein structure and function. This question is particularly relevant for the NDV F protein, since two of the glycosylation addition sites within the NDV F protein sequence are within the HR domains implicated in at least one conformational shift (Baker *et al.,* 1999).

One of the important functions of oligosaccharide chain addition, which occurs during synthesis of the molecule (Gilmore, 1993), is the folding of the nascent chain. Elimination of each side chain individually showed that the oligosaccharides have varying effects on F protein folding with the most severe effects occurring upon loss of the more amino-terminally located oligosaccharide chains. Loss of the side chain in the F_2 region of the molecule likely significantly impaired folding. The mole-

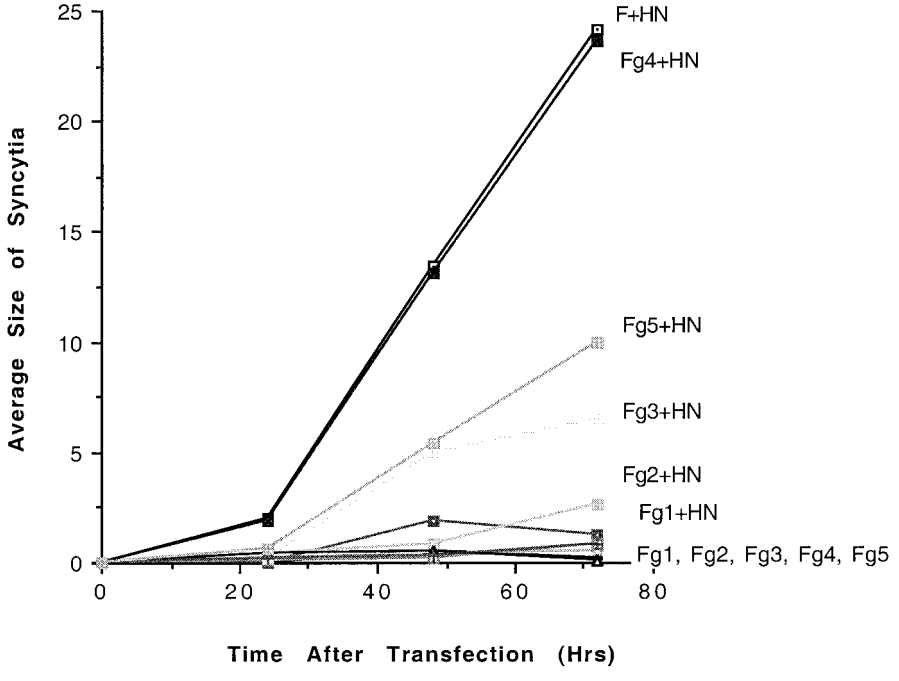

FIG. 5. Kinetics of syncytia formation by mutant proteins. The size of syncytia formed with time after transfection of plasmids encoding HN protein as well as the wild-type F protein or the glycosylation mutants was determined as described under Materials and Methods. Syncytia formed by the mutant proteins expressed alone are also shown.

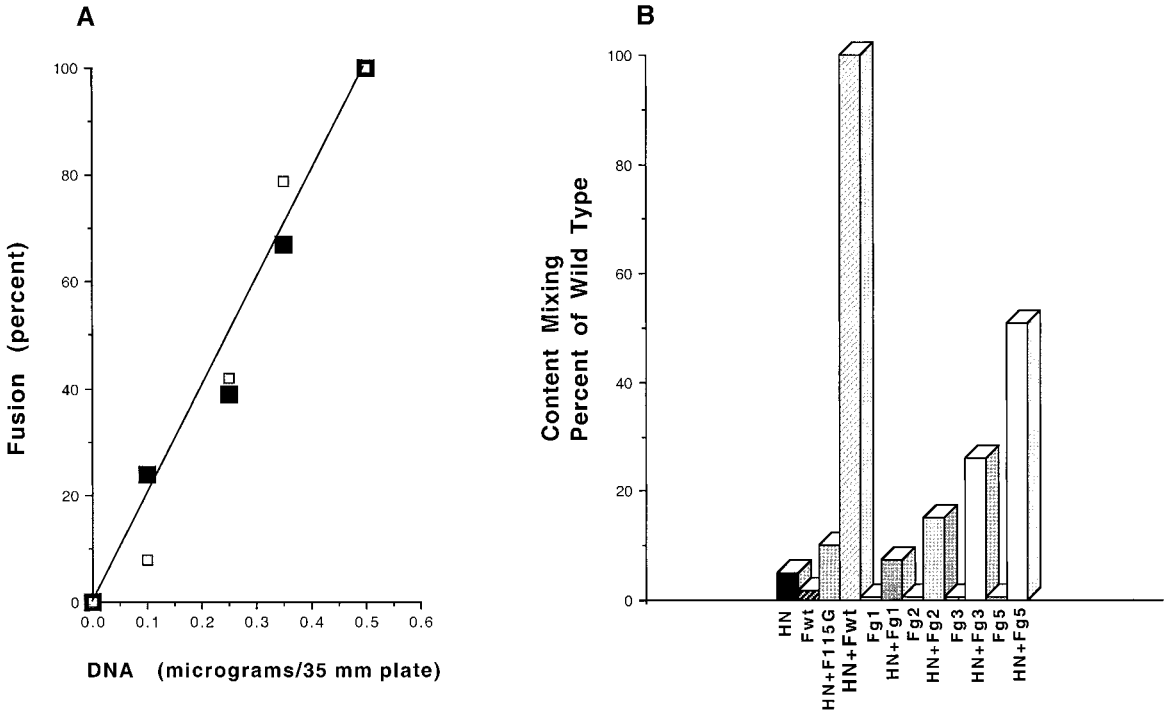

FIG. 6. Content mixing mediated by wild-type and mutant proteins. Fusion as measured by content mixing was accomplished as described under Materials and Methods. (A) Enzyme activity obtained with increasing amounts of wild-type F (Fwt) protein controlled by amounts of DNA added to the transfection with a constant amount of HN protein expression (0.5 μg/plate of HN DNA). Values are represented as a percentage of fusion activity measured at 0.5 μg/plate of F DNA). Different symbols represent results of two different experiments. (B) Enzyme activity with values expressed as a percentage of activity obtained with HN and Fwt protein expression. DNAs used for each transfection are indicated at the bottom of the figure. F115G is a mutant with an alteration in the cleavage site preventing cleavage by furin enzymes in the trans-Golgi membranes (Li *et al.,* 1998). Results are from a single experiment and were comparable to those of two other experiments.

cule was more susceptible to degradation and had an increased tendency to aggregate. Furthermore, there was less cleavage into F_1 and F_2 subunits and reduced detection at the cell surface, both properties indicating inefficient maturation. Failure to add oligosaccharide did not, however, affect the oligomerization of the molecule, suggesting that folding was not entirely blocked.

The oligosaccharide at the g1 site may influence the conformation of the F_1 region of F_0 as well as the F_2 region. The g1 site is located 9 amino acid residues on the carboxyl-terminal side of the single cysteine residue within the F_2 polypeptide, the residue that must covalently link F_2 with F_1. While the linkage of cysteine residues in the NDV F has not been determined, studies of the Sendai virus F protein have demonstrated that a cysteine residue within the F_2 domain is linked to the most amino-terminal cysteine residue in F_1 (Iwata *et al.,* 1994). This conserved cysteine residue is at amino acid 199 in the NDV F_1 sequence. If a similar cysteine linkage occurs in the NDV F protein, then the Fg1 oligosaccharide would be in close proximity to the HR1 domain of F_1 and may, therefore, play a role in the conformation of the HR1 domain.

Loss of the g2 site at amino acid 191 in the F_1 also affected conformational markers similarly, although to a lesser extent than loss of the g1 site. This mutant molecule was also expressed at the surface at levels slightly lower than those of wild type and had a tendency to aggregate. This site is located at the carboxyl terminus of the HR1 domain and, therefore, may play a role in the formation of the prefusion form of the HR1 domain. Interestingly, it is located 8 amino acids on the amino-terminal side of the cysteine residue at position 199 that likely links F_1 and F_2 and, therefore, could also play a role in positioning the molecule for disulfide bond formation between F_1 and F_2.

Glycosylation at sites g3 and g5 had little effect on stability and cleavage and only small effects on surface expression. Nor did the mutant molecules show increased tendency to aggregate. Thus neither of these oligosaccharides detectably influenced the conformation of the prefusion protein. These results were surprising given previous studies of SV5 F protein glycosylation (Bagai and Lamb, 1995). This F protein has a glycosylation addition site in the same position as the g5 site in the NDV F protein. Elimination of this site in the SV5 protein severely depressed surface expression, suggesting a significant role in the prefusion conformation of the SV5 protein. In addition, the bovine respiratory syncytial virus F protein has a glycosylation addition site in the HR2 domain and elimination of this site also interfered with surface expression (Pastey and Samal, 1997). These

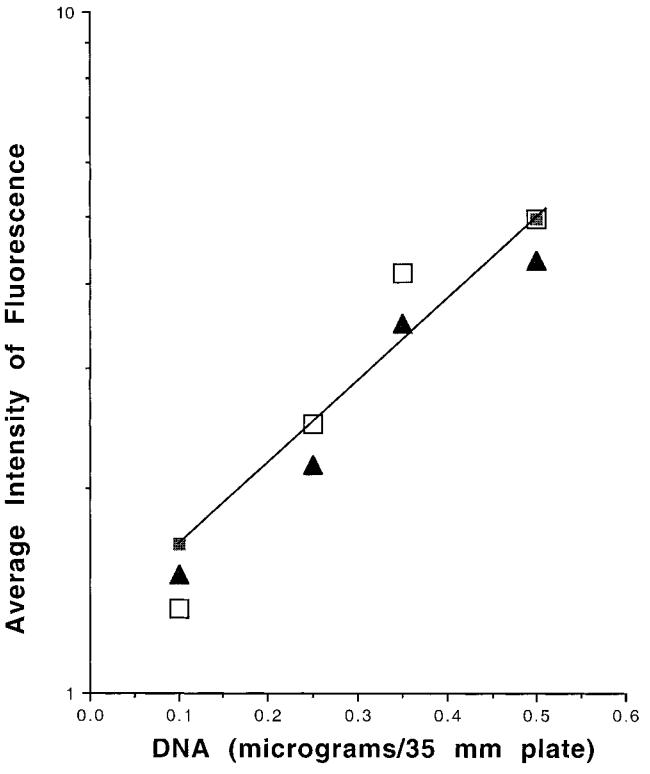

FIG. 7. Surface expression of F protein with increasing amounts of DNA. Cos cells transfected with increasing amounts of DNA were subjected to flow cytometry as described in the legend to Fig. 4. Results show approximate average intensity of fluorescence of positive cells observed at each DNA concentration. Different symbols represent duplicate experiments.

differences may indicate some structural variability between the different fusion proteins.

Oligosaccharides and fusion activity

The HR domains within the paramyxovirus F proteins play an important role in the fusion activity of the proteins. The interactions between the two domains are thought to pull the donor and target membranes into close proximity (Baker *et al.,* 1999; Weissenhorn *et al.,* 1999). That the two domains also function in subsequent steps in fusion has also been considered (Joshi *et al.,* 1998; Peisajovich *et al.,* 2000). Thus, carbohydrate modifications of either HR domain may well play a role in the fusion activity of the protein, and, indeed, all mutant proteins had decreased fusion activity. The activity of Fg1 may be due to the inefficient cleavage and surface expression, and, therefore, direct effects of the oligosaccharide on fusion cannot be determined. The Fg2 mutant protein also directed very little fusion but the degree of inhibition could not be solely due to poor surface expression (Table 1). This oligosaccharide is located at the carboxyl terminus of the HR1 domain and on the amino-terminal side of a region that has been proposed to have a fusion peptide-like activity in the Sendai virus F protein (Peisajovich *et al.,* 2000). Reduced fusion activity of molecules missing this site indicates that the carbohydrate in this domain of the NDV F protein contributes to conformation of this region or conformational shifts in ways that are critical to fusion activity of the protein. Interestingly, if the structure of the NDV F protein in this region is similar to the structure of the peptide complex of SV5 HR1 and HR2, then the oligosaccharide would be located within a groove of the HR1 trimer predicted to interact with the HR2 (Baker *et al.,* 1999). The presence of the oligosaccharide in this position should decrease the affinity of HR2 for HR1. Other F protein sequences have a proline residue in this position, a residue that would also destabilize the structure in this region. Perhaps decreasing the affinity of the two regions promotes disassembly of the complex required for subsequent fusion steps (Peisajovich *et al.,* 2000).

Elimination of the g5 oligosaccharide reduced fusion activity by approximately 40 to 50%, indicating that this carbohydrate has some role in the fusion activity of the protein. The oligosaccharide side chain is added to an "e" position of the helix formed by this sequence (Young *et al.,* 1997). As predicted by the structure of the SV5 HR1–HR2 complex, an oligosaccharide side chain in this position would not directly participate in HR1–HR2 contacts (Baker *et al.,* 1999). It could, however, influence the interactions of the HR2 domain with the HR1 domain, directly or indirectly, accounting for the decrease in fusion activity of this mutant protein.

Mutant Fg3 also expressed low fusion activity; thus, this oligosaccharide side chain is also important for fusion activity. It is located within a cysteine-rich region, which is characteristic of all fusion proteins (Morrison and Portner, 1991). The role of this uncharacterized region in fusion activity of F proteins is unknown, but these results argue that this region has, at least, an indirect role in conformation or conformational shifts involved in fusion.

Glycosylation site usage

Preassembled core oligosaccharides are covalently attached by oligosaccharyl transferase to nascent glycoproteins as the addition sequence is translocated into the lumen of the roush endoplasmic reticulum (RER) (Kornfeld and Kornfeld, 1985). Oligosaccharides are added at asparagines in the sequence NXT or NXS, where X is any amino acid except proline. However, not all such sequences are used for carbohydrate addition. Failure to use a site has been attributed to localized folding events on the nascent chain, which block access of the oligosaccharide transferase to the addition site (McGinnes and Morrison (1997) and references therein). The addition site at amino acid 447 in the NDV F protein sequence is not used. We have previously shown that a site in the NDV HN protein is unused due to the forma-

tion of an intramolecular disulfide bond between two cysteine residues in the sequence that bracket the addition site, blocking access of the site (McGinnes and Morrison, 1997). Glycosylation addition site 4 in the F protein sequence is not, however, bracketed by cysteine residues; thus, a similar mechanism is not likely operative in this case. However, other folding events in the vicinity of this site may well bock carbohydrate addition. Failure to use the site is puzzling since it is conserved in F proteins of all strains of NDV sequenced, suggesting that the sequence has some functional importance (Toyoda *et al.,* 1989). Furthermore, sites in a very similar location in the SV5 F protein (Bagai and Lamb, 1995) and in the Sendai virus F (Segawa *et al.,* 2000) are utilized, suggesting that the conformation of these two F proteins in this region may be different from that of the NDV F protein.

MATERIALS AND METHODS

Cells, vectors, and viruses

Cos-7 cells, obtained from the American Type Culture Collection, were maintained in Dulbecco's modified Eagle medium supplemented with nonessential amino acids, vitamins, penicillin/streptomycin, and 10% fetal calf serum. NDV HN and F genes, characterized previously (Sergel *et al.,* 1993), were expressed in Cos cells using pSVL obtained from Pharmacia.

Site-specific mutagenesis

The F gene was ligated into a *Xba*I and *Sac*I cut M13-mp10 for mutagenesis. A single-stranded template was prepared and mutagenesis reactions were done using the mutagenesis kit from Amersham Corp. The appropriate oligomers, of 21 to 27 nucleotides in length, were used to change the serine or threonine in the addition site to alanine. The entire gene of the resulting mutant DNA was sequenced to verify that the rest of the gene remained unchanged by the mutagenesis reaction. The mutations made in the F protein are shown in Fig. 1. The pSVL-Fg1 mutant had the threonine at amino acid 87 changed to an alanine. The threonine at amino acid 193 was changed to alanine in the pSVL-Fg2 mutant, the serine at amino acid 368 was changed to alanine in mutant pSVL-Fg3, the serine at amino acid 449 was changed to alanine in mutant pSVL-Fg4, the serine at amino acid 473 was changed to alanine in pSVL-Fg5, and the threonine at amino acid 543 was changed to an alanine in mutant pSVL-Fg6.

Transfections

Transfections using Lipofectin or Lipofectamine (GIBCO BRL) were done essentially as recommended by the manufacturer. Cos cells were plated at 3×10^5 per 35-mm plate. Twenty to twenty-four hours later, a mix of DNA in 0.1 ml OptiMem (GIBCO BRL) and 10 μl of transfection reagent in 0.2 ml of OptiMem was incubated at room temperature for 45 min, diluted with 0.7 ml OptiMem, and added to a plate previously washed with OptiMem. Cells were incubated for 4 to 5 h and then 2 ml of Cos cell media was added.

Antibodies

Anti-F tail, used for immunoprecipitation of the fusion protein in cytoplasmic extracts and for Western blots, was raised against a synthetic peptide with the sequence of the cytoplasmic tail of the fusion protein previously described (Wang *et al.,* 1992) and prepared by the Peptide Core Facility of the University of Massachusetts Medical School. Antibody used for cell surface immunoprecipitations was anti-NDV, a polyclonal antiserum raised in rabbits against UV-inactivated virions by standard protocols.

Western analysis of mutant proteins

Cell extracts were diluted in sample buffer and loaded onto 10% polyacrylamide gels. After electrophoresis, the gels were subsequently equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 5% methanol, pH 8.2) and transferred to Immobilon-P (Millipore Corp.) membranes. The membrane was blocked in PBS containing 0.5% Tween 20 and 10% nonfat dry milk for 2 h at room temperature or overnight at 4°C. Membranes were washed in PBS/Tween 20 and incubated with primary antibody diluted in PBS/Tween 20 and 0.5% nonfat milk for 2 h at room temperature. Membranes were washed and then incubated in secondary antibody, anti-rabbit IgG coupled to horseradish peroxidase (Boehringer Mannheim) diluted in PBS/Tween and 0.5% nonfat milk, for 1 h at room temperature. Membranes were washed extensively and bound antibody was detected using the ECL Western blotting detection reagent system (Amersham).

Sucrose gradients

At 48 h posttransfection, cells were lysed in RSB buffer, 1% Triton X-100, and 10 mM iodoacetamide. The resulting extracts were layered on top of 10–45% continuous sucrose gradients made in RSB buffer containing 0.1% Triton X-100. Gradients were spun in an SW41 rotor for 19 h at 38,000 rpm at 17°C and collected into 16 equal fractions. Proteins present were precipitated with TCA and electrophoresed on polyacrylamide gels for Western analysis. Marker proteins used were ferratin, catalase, aldolase, and bovine serum albumin (MW 450,000, 240,000, 158,000, and 68,000, respectively). The location of marker proteins in the gradients was detected by Coomassie blue staining of the gradient fractions.

Detection of cell surface molecules with antibody

Immunofluorescence. Cos cells were plated on 35-mm plates containing glass coverslips (Corning) and transfected as described above. The cells were washed twice with PBS and incubated at 4°C in PBS containing 3% bovine serum albumin (BSA), 0.02% sodium azide, and anti-NDV antibody (diluted 1:100) for 1 h. Cells were washed three times with PBS containing BSA and azide and incubated with PBS containing BSA, azide, and fluorescein-conjugated anti-rabbit IgG (Southern Biotechnology) for 1 h. Cells were washed and a drop of DABCO (25 mg/ml in PBS and glycerol) was added.

Precipitation of antibody–surface protein complexes. Cells transfected for 48 h were radioactively labeled with [^{35}S]methionine for 2 h and then chased with nonradioactive media for 16 h. Anti-NDV antibody was incubated with intact cells and unbound antibody was washed away prior to cell lysis as previously described (Morrison *et al.,* 1990). Cells were lysed in RSB buffer (0.01 M Tris–HCl, pH 7.4, 0.01 M NaCl) containing 1% Triton X-100 and 0.5% sodium deoxycholate, 8 μg/ml DNase, and 2 mg/ml iodoacetamide. Immune complexes were isolated using Protein A coupled to beads. Cell surface immune complexes were electrophoresed on 10% polyacrylamide gels. The resulting autoradiographs were scanned with a microdensitometer to quantitate the amount of F protein precipitated.

Flow cytometry. Cells were transfected with concentrations of DNA that were within the linear range of expression of viral protein in order to detect differences between mutant protein expression. Transfected cells were removed from plates with cell detachment buffer (Sigma) after a 1-min pulse in trypsin (50 μg/ml), washed in PBS containing 1% BSA and 0.02% azide (FACS buffer), and incubated with anti-NDV antibody for 1 h at 4°C. After cells were washed three times with FACS buffer, they were incubated for 1 h at 4°C with goat anti-rabbit IgG coupled to Alexa dye 488 (Molecular Probes). After three washes in FACS buffer, cells were resuspended in PBS containing 2% paraformaldehye and subjected to flow cytometry (University of Massachusetts Medical School Flow Cytometry Facility).

Fusion assays

Fusion was measured by measuring syncytia formation and content mixing. To quantitate syncytia formation, Cos cells were cotransfected with wild-type or mutant fusion protein genes and the wild-type HN protein gene using Lipofectin. At 48 h posttransfection, the number of nuclei in 40 fusion areas were counted to determine the average size of syncytia at each time point as previously described (Sergel *et al.,* 1993). Values obtained after transfection of the vector alone were subtracted.

To measure content mixing, a plasmid encoding a tet responsive transcriptional activator, tTA (Clontech) was transfected alone with HN and F cDNAs. A separate population of cells was transfected with a plasmid encoding the β-galactosidase protein under the control of the tet responsive transcriptional activator (Clontech). After 30 h, Cos cells transfected with the β-galactosidase gene were trypsinized and then plated on Cos cells expressing HN and F protein as well as the tet responsive transactivation. At 45 h posttransfection, when fusion was evident, the monolayers were lysed and extracts assayed for β-galactosidase activity. Activity due to background fusion typical of Cos cells was measured after transfecting cells with comparable amounts of vector alone and values obtained were subtracted from values obtained with cells expressing HN and wild-type F or mutant F proteins.

Cell-free translation

Transcription and translation reactions were done as recommended by the Promega Protocols and Application Guide. Transcription reactions (50 μl) contained 0.5 μg of DNA and transcription buffer (Promega), DTT (10 mM), GTP (0.05 mM), UTP, ATP, and CTP (each at 0.5 mM), RNasin (1 u/μl), CAP analogue (0.5 mM), and SP6 polymerase (0.8 u/μl) and were incubated for 40 min at 37°. Cell-free translation reactions (25 μl) contained rabbit reticulocyte extract (17.4 μl) (Promega), amino acids (20 μM each) minus methionine, [^{35}S]methionine (10 μCi, New England Nuclear), RNasin (1 u/μl), potassium acetate (96 mM), and 1 μl of transcription reaction and were incubated for 1 h at 30°.

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