Characterization of an Alternate Form of Newcastle Disease Virus Fusion Protein

Homer Pantua,3 Lori W. McGinnes,1 John Leszyk,2 and Trudy G. Morrison1,3*

Department of Molecular Genetics and Microbiology,1 Department of Biochemistry and Molecular Biology,2 and Program in Immunology/Virology,3 University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655

Received 17 December 2004/Accepted 27 May 2005

The sequence and structure of the Newcastle disease virus (NDV) fusion (F) protein are consistent with its classification as a type 1 glycoprotein. We have previously reported, however, that F protein can be detected in at least two topological forms with respect to membranes in both a cell-free protein synthesizing system containing membranes and infected COS-7 cells (J. Virol. 77:1951–1963, 2003). One form is the classical type 1 glycoprotein, while the other is a polytopic form in which approximately 200 amino acids of the amino-terminal end as well as the cytoplasmic domain (CT) are translocated across membranes. Furthermore, we detected CT sequences on surfaces of F protein-expressing cells, and antibodies specific for these sequences inhibited red blood cell fusion to hemagglutinin-neuraminidase and F protein-expressing cells, suggesting a role for surface-expressed CT sequences in cell-cell fusion. Extending these findings, we have found that the alternate form of the F protein can also be detected in infected and transfected avian cells, the natural host cells of NDV. Furthermore, the alternate form of the F protein was also found in virions released from both infected COS-7 cells and avian cells by Western analysis. Mass spectrometry confirmed its presence in virions released from avian cells. Two different polyclonal antibodies raised against sequences of the CT domain of the F protein slowed plaque formation in both avian and COS-7 cells. Antibody specific for the CT domain also inhibited single-cycle infections, as detected by immunofluorescence of viral proteins in infected cells. The potential roles of this alternate form of the NDV F protein in infection are discussed.

Newcastle disease virus (NDV) is a major agricultural pathogen that causes a fatal respiratory and neurological disease in poultry (21). This virus, a member of the Paramyxoviridae family, initiates infection by fusion of the viral membrane with host cell plasma membranes (13). Virus spread is facilitated by cell-cell membrane fusion. NDV, as well as other paramyxoviruses, encodes two spike glycoproteins, the hemagglutinin-neuraminidase (HN) protein, which mediates attachment of the virion to sialic acid-containing receptors, and the fusion (F) protein, which directly mediates membrane fusion (reviewed in reference 13). Primary sequence and structural analyses indicate that the F protein is a classical type 1 glycoprotein with an amino-terminal signal sequence, a hydrophobic transmembrane domain near the carboxyl terminus, and a 25- to 30-amino-acid cytoplasmic domain (CT) (13, 19). The F protein is synthesized as a precursor, Fp, which undergoes proteolytic cleavage to form disulfide-linked amino-terminal F2 and carboxyl-terminal F1 polypeptides, and cleavage is required for fusion activity (reviewed in reference 13).

There are now several examples of both cellular (4, 10, 15, 31) and viral glycoproteins that are found in different topological forms with respect to membranes. Examples of viral glycoproteins with alternate membrane topologies include the hepatitis B virus L protein (reference 14 and references therein), the transmissible gastroenteritis virus M protein (8), and the hepatitis C virus envelope glycoproteins (18, 23). We previously reported that synthesis of the NDV F protein in a cell-free protein-synthesizing system containing membranes resulted in at least two topological forms of the protein with respect to membranes (17). The properties of one form were entirely consistent with a type 1 fully glycosylated F protein. The other was a partially translocated or polytopic form in which approximately 200 amino acids of the amino terminus as well as the CT domain of the protein were translocated across membranes (17). Importantly, we detected this second, polytopic form of F protein in COS-7 cells expressing the F protein (17) and provided evidence that it was involved in cell-cell fusion, either directly or indirectly.

Extending these results, we report that the second form of the F protein is also found in F protein-expressing avian cells, which are the natural host cells of NDV. We have detected this second form of the F protein in virions released from both infected COS-7 and avian cells. We report evidence that this second form of the F protein may have role in virus-cell fusion.

MATERIALS AND METHODS

Cells, virus, and plasmids. COS-7 cells, obtained from the American Type Culture Collection, were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with nonessential amino acids, vitamins, penicillin-streptomycin, and 10% fetal calf serum. East Lansing Line (ELL-0) chicken fibroblasts (UMNSAH/DF-1), obtained from American Type Culture Collection, were maintained in DMEM supplemented with penicillin-streptomycin and 10% fetal calf serum.

NDV strain AV (virulent) and strain B1 (avirulent) stocks (21) were prepared by growth in eggs by standard protocols. AV stocks formed plaques in COS-7 cells, while B1 did not, consistent with the expected phenotypes of the two strains of NDV. The F protein gene carried by purified NDV strain B1 virus was sequenced to verify the absence of a furin recognition sequence. The NDV F and
Transfected (UT), lane 1.

Infections and virus purification. COS-7 or ELL-0 cells were plated at 10^5 per 35-mm plate. After 20 h, a mixture of DNA (0.5 µg) in 0.1 ml OptiMEM (Gibco/Invitrogen) and 5 µl of lipofectamine in 0.2 ml of OptiMEM was incubated at room temperature for 45 min, diluted with 7.0 ml of OptiMEM, and added to a 35-mm plate previously washed with OptiMEM. The cells were incubated for 5 h, the lipofectamine-DNA complexes were removed, and then 2 ml of supplemented DMEM was added.

Antibodies. Anti-NDV is a polyclonal antiserum raised in rabbits against UV-inactivated virions by standard methods. Anti-HR1 is raised against a glutathione S-transferase (GST) fusion protein that contained sequences from amino acid 130 to 173 of the F protein cloned in frame with the carboxyl terminus of GST (16). Anti-Tfi1 523–553 was raised against a synthetic peptide with the sequence of the cytoplasmic domain of the fusion protein (amino acids 523 to 553) as previously described (29) and prepared by the Peptide Core Facility of the University of Massachusetts Medical School. Anti-Tfi1 was raised against a GST fusion protein containing the sequence between amino acids 96 and 117 cloned in frame with the carboxyl terminus of GST. Anti-Tfi1 was raised against a GST fusion protein containing the sequence between amino acids 540 and 553 cloned in frame with the carboxyl terminus of GST. The GST fusion polypeptides were purified by standard methods recommended in the Novagen Applications Guide. Antibody was raised in rabbits by standard methods.
Fig. 2. Detection of cytoplasmic tail sequences on the cell surfaces of transfected and infected avian cells by immunofluorescence. Panels B-E show results with NDV-infected avian cells. Panels F-L show results with transfected avian cells. Uninfected avian cells (panel A) and avian cells infected with NDV strain AV (panels B and D) or strain B1 (panels C and E) were incubated with anti-NDV (panels A to C) or anti-Ftail 540–553 (panels A, D, and E). Infected cells incubated with anti-NDV and anti-Ftail antibodies were digitally exposed for 3 s. Avian cells transfected with vector alone (panel F) or transfected with pCAGGS-Fwt (panels G and J), pCAGGS-Fd523-553 (panels H and K), or pCAGGS-Fwt and pCAGGS-HN (panels I and L), were incubated with anti-NDV (panels F-I) and anti-Ftail 540–553 (panels F and J to L). Transfected cells incubated with anti-NDV and anti-Ftail antibodies were digitally exposed for 3 s. Images were acquired using a ×60 objective and OPEN LAB software.

Ftail antibodies, or preimmune sera for 1 h at room temperature prior to adsorption. The virus and antibody mixes were then added to cells and incubated for 45 min at 37°C. After adsorption, the virus-antibody mixture was removed and cells were washed once in PBS. For plaque assays, cells were then overlayed with agar diluted to 1% in DMEM and supplemented with nonessential amino acids, vitamins, penicillin-streptomycin, sodium bicarbonate, and 10% fetal bovine serum. Plaques were counted after 48 h of incubation at 37°C. Cells were fixed in methanol and stained with Giemsa stain (Sigma) diluted 1:20 in...
FIG. 3. Detection of cytoplasmic tail sequences on the cell surfaces of infected and transfected COS-7 cells by immunofluorescence. Panels B to E show results with NDV-infected COS-7 cells. Panels F to P show results with transfected COS-7 cells. Uninfected COS-7 cells (panel A) and COS-7 cells infected with NDV strain AV (panels B and D) or strain B1 (panels C and E) were incubated with anti-NDV (panels A, B, and C) or anti-Ftail 540–553 (panels A, D, and E). Infected cells incubated with anti-NDV and anti-Ftail antibodies were digitally exposed for 3 s. COS-7 cells transfected with vector alone (panel F), transfected with pCAGGS-Fwt (panels G and J), pCAGGS-Fd523-553 (panels H and K), or pCAGGS-Fwt and pCAGGS-HN (panels I and L, M, N, O, and P) were incubated with anti-NDV (panels F to I) or anti-Ftail 540–553 (panels F and J to L). Transfected cells incubated with anti-NDV and anti-Ftail antibodies were digitally exposed for 3 s using OPEN LAB software. Intact cells expressing the HN and F proteins were incubated with anti-NDV and phalloidin-Alexa568 (panels M and N, respectively). Cells expressing HN and F proteins were incubated in 0.1% Triton X-100 and then anti-NDV and phalloidin-Alexa568 (panels O and P, respectively).
RESULTS

Two forms of F protein are expressed in infected avian cells. Because a second form of the paramyxovirus F protein has not been previously found in other systems, it seemed possible that the alternate form of the NDV F protein could be an unusual property of protein expression in COS-7 cells. Thus, we asked if this second form of the NDV F protein could be detected in avian cells, cells that are the natural host cell for NDV.

Unambiguous detection of the second, partially translocated form of the F protein by Western analysis required the expression of an F protein that was not cleaved, since the partially translocated F protein comigrated on polyacrylamide gels with the F1 cleavage product, F1 protein (17). Therefore, we utilized the B1 strain of NDV, a strain that encodes an F protein missing a furin recognition sequence at the cleavage site. The B1 F protein, synthesized in tissue culture cells, is not proteolytically cleaved (13, 19, 21). NDV strain AV encodes an F protein with a furin recognition site, and the F protein present in cells infected by NDV strain AV is proteolytically cleaved into F1 and F2 (13, 19, 21).

Avian cells (ELL-0 cells), as well as COS-7 cells, were infected with NDV strain B1 or NDV strain AV for 12 or 16 h, and the F proteins present in the resulting cell extracts were characterized by Western analysis using F-protein-specific antibodies. As previously reported (17), two forms of the F protein from B1-infected COS-7 cells were detected (Fig. 1A, lanes 3 and 6), the fully translocated F0 protein (68 kDa) and a 59-kDa protein. As expected, extracts from AV-infected COS-7 cells contained two polypeptides, a 68-kDa F0 and a 59-kDa F1 (Fig. 1A, lanes 2 and 5). Proteins from ELL-0 cells contained a 68-kDa host band, which obscured the F0 polypeptide from both B1- and AV-infected cells (Fig. 1B, lanes 1 to 6). However, ELL-0 cell infection with B1 virus resulted in the synthesis of significant amounts of a 59-kDa polypeptide (Fig. 1B, lanes 3 and 6), a polypeptide the size of the partially translocated F protein. These results show that the second form of the F protein can be detected in B1-infected avian cells.

To eliminate the possibility that anti-F protein antibody cross-reacted with NP, which is also 59 kDa, avian and COS-7
cells were transfected with NP cDNA, and proteins present in the resulting cell extracts were detected by Western analysis using anti-NDV and anti-HR1 antibodies. The results show that NP is recognized only by anti-NDV antibody and not by anti-HR1 antibody (Fig. 1C). Thus, the 59-kDa protein detected with anti-F antibodies in B1-infected cells is not due to a cross-reaction of the antibodies with NP.

Surface expression of cytoplasmic tail sequences. Our analysis of the cell-free translation products directed by F-protein mRNA also showed that the F protein cytoplasmic tail was translocated across membranes (17), leading to the proposal that the partially translocated F protein may be a polytopic protein with the carboxyl terminus as well as the amino terminus of the protein exposed on cell surfaces. In support of this idea, we found that CT sequences could be detected on surfaces of COS-7 cells expressing the NDV F protein using a polyclonal antibody raised against a peptide with the F protein CT sequence (amino acids 523 to 553) (17).

We asked if F-protein CT sequences could be detected on infected or transfected avian cell surfaces (Fig. 2). We also raised another polyclonal antibody specific for the carboxy-terminal half of the F-protein CT domain (amino acids 540 to 553) to eliminate the possibility that our previous results were due to a nonspecific effect of the anti-Ftail antibody (Fig. 2 and 3).

ELL-0 and COS-7 cells were infected with NDV AV or B1 strains. In addition, ELL-0 and COS-7 cells were transfected with vector alone, wild-type F (Fwt) protein cDNA, cDNA encoding an F protein with a cytoplasmic tail deletion (F523-553), or a mixture of Fwt-protein and HN-protein cDNAs. Intact cells were incubated with anti-Ftail 540–553 antibody, and binding of antibody was assessed by cell surface immunofluorescence. Both infected and transfected ELL-0 cells, as well as COS-7 cells, bound this anti-Ftail antibody (Fig. 2 and 3, panels E, J, and L). In contrast, binding of anti-Ftail antibody to cells expressing the F523-553 protein was negative (Fig. 2 and 3, panel K), although these cells were positive for anti-NDV antibody (Fig. 2 and 3, panel H), which further confirms specificity of the anti-Ftail antibody for the CT sequences. None of the antibodies bound to uninfected or vector-transfected cells (panels A and F). Binding of anti-NDV antibody was detected in parallel cultures (Fig. 2 and 3, panels B, C, G, H, and I).

Detection of CT sequences on surfaces of infected or transfected cells was not due to permeabilization of cells during antibody binding. Under the same conditions, fluorescence-labeled phalloidin, which binds to actin, did not stain intact cells (Fig. 3N), although phalloidin readily stained actin after permeabilizing cells in 0.1% Triton X-100 (Fig. 3P).

To confirm expression of CT sequences on avian cell surfaces, binding of the anti-Ftail 540-553 antibody was also analyzed by flow cytometry. Figure 4 shows that cells expressing the F protein bound anti-Ftail 540-553 antibody, while cells expressing the F523-553 or HN proteins were negative (Fig. 4B and D). These results show that CT sequences can be detected on the surfaces of both avian and COS-7 cells expressing the NDV F protein using two different anti-Ftail antibodies.

Two forms of F protein are in virions. We next asked if the polytopic form of the F protein was present in virions. NDV strain B1 virions were generated from infected ELL-0 or COS-7 cells. Virions from B1 strain AV were also prepared in parallel. Figure 5 shows Western analysis of B1 virion proteins, as well as AV virion proteins, using two different F-protein-specific polyclonal antibodies. F-protein-specific bands detected were similar to those found in the infected cell extracts. As expected, F0 and F1 polypeptides were detected in AV virions by using anti-HR1 and anti-Ftail antibodies (Fig. 5A). Similarly, F0 was detected in B1 virions grown from both COS-7 and ELL-0 cells using anti-HR1 and anti-Ftail antibodies. Interestingly, a 59-kDa polypeptide was also detected in B1 virions by both anti-HR1 and anti-Ftail antibodies (Fig. 5A). To determine if the 59-kDa polypeptide was due to unexpected cleavage of the F0 protein, we used anti-F1 antibody, an antibody specific to the F1 polypeptide. Detection of the F1 polypeptide was confirmed by mass spectrometry.
FIG. 6. Anti-Ftail antibodies alter plaque morphology. COS-7 (panels A to F) and avian cells (panels G to L) were infected with untreated NDV strain AV (panels B and H) or NDV that was preincubated with preimmune sera (panels C and I), anti-NDV (panels D and J), anti-Ftail 540–553 (panels E and K), or anti-Ftail 523–553 (panels F and L). Uninfected monolayers, panels A and G. Plaques were counted and stained with 1:20 Giemsa and distilled water after 48 h of incubation at 37°C.
TABLE 2. Effect of anti-NDV and anti-Ftail antibodies on plaque formation in COS-7 and ELL-0 cells

| Treatment | COS-7 | ELL-0 |
|-----------|-------|-------|
|           | Expt 1 | Expt 2 | Expt 1 | Expt 2 |
| UI | 0 | 0 | 0 | 0 |
| No Ab | $3.45 \times 10^{10}$ | $5.33 \times 10^{10}$ | $4.53 \times 10^{10}$ | $3.78 \times 10^{10}$ |
| Pre | $3.25 \times 10^{10}$ | $5.13 \times 10^{10}$ | $4.25 \times 10^{10}$ | $3.75 \times 10^{10}$ |
| Anti-NDV | $<1.0 \times 10^{9}$ | $<1.0 \times 10^{9}$ | $<1.0 \times 10^{9}$ | $<1.0 \times 10^{9}$ |
| Anti-Ftail 540–553 | 5.00 | $10^{10}$ | 3.00 | $10^{10}$ |
| 523–553 | 2.15 | $10^{10}$ | 2.18 | $10^{10}$ |
| 540–553 | 3.40 | $10^{10}$ | 3.08 | $10^{9}$ |
| 523–553 | 3.78 | $10^{10}$ | 3.75 | $10^{10}$ |

* Dilutions of $10^{-9}$ for NDV strain AV were preincubated with anti-NDV and anti-Ftail antibodies for 1-h prior to adsorption. NoAb, without antibody; Pre, preincubated with preimmune sera; UI, mock infected. Antibody was 150 μg protein per ml.

* Plaques are pinpoint.

polypeptide in B1 virions would suggest that the 59-kDa polypeptide was due to cleavage of the F₂ polypeptide. Figure 5B, shows that the F protein from the AV virions was cleaved, generating an F₂ polypeptide, F₂ is not present in B1 virions, indicating that the F protein was not cleaved in these virions.

To verify this conclusion, NDV strain B1 virions were purified from supernatants of avian infected cells, and the polypeptides that migrated with a molecular weight of 59 kDa were analyzed by mass spectrometry. As expected, peptides derived from the viral NP and P proteins were present in the band. In addition, F-protein-specific peptides were present (Table 1), supporting the conclusion that a form of the F protein comigrated with the NP and P proteins. Furthermore, peptides from both the F₂ and F₁ regions of the protein were detected. F₁-specific peptides detected were from the CT domain of the F protein and from the HR1 domain. Surprisingly, F₂ peptides detected were from the signal sequence of the F protein. These findings suggest that the p59 F polypeptide contains sequences from the uncleaved F₀ polypeptide and that the signal sequence was not cleaved from the alternate form of the F protein. These results are consistent with the presence of a second form of F protein, an uncleaved protein with a molecular weight of 59 kDa.

**Effect of anti-Ftail antibodies on plaque formation.** Since the second form of the F protein was detected in virions generated from both infected avian and COS-7 cells, we asked if it has a role in plaque formation. We asked if anti-Ftail antibodies could neutralize virus infectivity in a plaque assay. Egg-grown NDV strain AV was incubated with preimmune sera, anti-NDV, or anti-Ftail antibody (both anti-Ftail 523–553 and anti-Ftail 540–553) and then plated on monolayers of avian or COS-7 cells. After adsorption, unbound virus and antibody were removed and the cells were washed prior to addition of agar overlays. Thus, effects of the antibody should reflect effects on the initial virus cell interaction and not subsequent cell-cell fusion. After 40 h, plaques were visible on control plates (Fig. 6B, C, H, and I). As expected, anti-NDV neutralized virus infectivity, and no plaques were observed (Fig. 6D and J). Anti-Ftail 540–553 reduced the titer approximately 2- to 18-fold in COS-7 cells and 20-fold in ELL-0 cells compared to that for the preimmune sera and no-antibody controls (Table 2). Most importantly, however, plaques formed after incubation with both anti-Ftail antibodies were pinpoint (Fig. 6E, F, K, and L), much smaller than those produced by untreated virus or virus incubated with preimmune serum (Fig. 6B, C, H, and I). These results suggest that the anti-Ftail antibodies slow plaque formation. This result is not likely due to a nonspecific effect of antibody raised against a GST fusion protein. Incubation of virus with antibody raised against HR1 sequence coupled to a GST protein did not inhibit plaque size or number (unpublished observations).

**Effect of anti-Ftail antisera on a single cycle of infection.** The effect of anti-Ftail antibodies on plaque size suggested that the anti-Ftail antibodies, bound prior to virus attachment, inhibited virus entry. To test this possibility by an alternative approach, effects of anti-Ftail antibody on a single cycle of infection were monitored by immunofluorescence. Virus incubated with anti-Ftail antibody, anti-NDV antibody, anti-β-galactosidase, or preimmune sera, as described in the legend to Fig. 6, was then bound to cell surfaces. After adsorption, unbound virus and antibody were removed and cells were washed and incubated for 9 h at 37°C. Infected cells were detected by the presence of proteins on cell surfaces that bound anti-NDV antibody. Figure 7C and D show the presence of virus proteins on infected cell surfaces after infection with untreated virus, while uninfected cells were negative for anti-NDV staining (panels A and B). Incubation of virus in preimmune serum or nonspecific antiserum had no effect on the infection (panels E, F, G, and H), while incubation with anti-NDV antibody inhibited infection as expected (panels I and J). Incubation of virus with anti-Ftail 540–553 inhibited infection (Fig. 7K and L), consistent with results of the plaque assay.

This inhibition by anti-Ftail antibody was not likely due to an inhibition of virus binding. Cells cotransfected with HN- and F-protein cDNAs were incubated with avian red blood cells in the presence or absence of antibody. As expected, anti-NDV blocked the binding of red blood cells (RBCs) to these monolayers, while binding of RBCs in the presence of anti-Ftail antibodies was unaffected (Table 3).

**DISCUSSION**

We have previously reported that the NDV F protein, synthesized in a cell-free protein-synthesizing system containing membranes, inserted itself into membranes in at least two ways (17). One form of the protein (p68) was typical of a type 1 glycoprotein anchored in membranes by a transmembrane domain located near the carboxyl terminus. The second form (p59) had properties consistent with the partial translocation of the protein, translocation of the F₂ domain and the amino terminus of the F₁ domain. In addition, we reported evidence for the translocation of the CT domain of the protein. Based on these observations, we suggested that the second form of the F protein was a polypeptide protein that spanned membranes at least twice. We also reported the detection of this second form of the F protein in infected and transfected COS-7 cells. A polypeptide consistent with the size of this second form was detected by Western analysis of proteins in extracts of infected cells. In addition, CT sequences were detected on the surfaces of cells expressing the NDV F protein.
FIG. 7. Anti-Ftail antibodies block infection. COS-7 cells were infected at a multiplicity of infection of 10 with untreated egg-grown NDV strain B1 (panels C and D) or NDV strain B1 that was preincubated with preimmune sera (panels E and F), anti-β-galactosidase (panels G and H), anti-NDV (panels I and J), or anti-Ftail 540–553 (panels K and L) as described in Materials and Methods. Uninfected monolayers are shown in panels A and B. After 9 h of infection, cells were incubated with Hoechst stain, in order to visualize nuclei of all cells in the monolayer, and then anti-NDV antibody. Binding of anti-NDV antibody was visualized by Alexa 568 coupled to goat antirabbit antisera. Images were acquired with a ×20 objective using OPEN Lab software. Hoechst-stained images were digitally exposed for 0.5 s, and anti-NDV images were digitally exposed for 2.5 s. Identical results were obtained in two separate experiments.
Because of the unexpected nature of our findings and because this second form of the F protein has not been found in other paramyxovirus systems, we were concerned that formation of a polytopic NDV F protein in COS-7 cells could be attributed to a translocation defect in primate cells of a glycoprotein normally expressed in avian cells. However, as described above, we detected this second form of the F protein in avian cells, both in infected cells and in cells transfected with F-protein cDNA. First, we detected by Western analysis a polypeptide the size of the polytopic F protein (p59), as well as the fully translocated Fp protein (p68), in tissue culture-grown avian cells infected with an avirulent strain of NDV, strain B1, as well as in purified virions derived from these cells. Cells infected with this virus should express only uncleaved protein (13, 19–21), and indeed, virus released from cells infected with this virus does not form plaques in the absence of trypsin. However, it was possible that the F protein was cleaved, accounting for the p59 form of the protein. To verify that the p59 polypeptide was the F protein and that it included the full-length polypeptide rather than only a cleaved form of the protein, we analyzed, by mass spectroscopy, the p59 material from tissue culture-derived, purified virus particles. As expected, the material contained NP and P protein. Significantly, F-protein-specific peptides were detected in this band, indicating that detection of F-protein sequences by Western analysis was not due to a cross-reaction of anti-F-protein antibodies to another protein. Furthermore, peptides from the F2 region of the protein, the HR1 domain, and the CT domain were detected, indicating that sequences from the entire Fp protein were present in this polypeptide.

Interestingly, the F2 sequences detected in the p59 material by mass spectroscopy were from the signal sequence of the protein, suggesting that the signal sequence was not cleaved from this polypeptide. This surprising finding suggests that the second form of the F protein is anchored in membranes not only by sequences in the HR1 domain and the transmembrane domain but also the signal sequence. The presence of peptides from the signal sequence also indicates that the material is not from contaminating fully translocated Fp protein (p68), since it has been reported that the signal sequence is cleaved from the fully translocated F protein (9). It should be noted that the two peptides detected from the signal sequence are overlapping but have several amino acid differences. Heterogeneity in the signal sequence is not unexpected, since comparisons of signal sequences from F proteins of many different strains of NDV have shown that the sequence of this region of the F protein is highly variable (28).

Another indication for the existence of the alternate form of the F protein was the detection, on avian cell surfaces, of sequences from the CT domain of the F protein of both the AV and B1 strains of NDV. To eliminate the possibility that our previous detection of surface-expressed CT sequences in COS-7 cells (17) was due to an unusual cross-reactivity of that anti-Ftail antibody preparation, we prepared a second polyclonal antibody by different protocols using only the carboxy-terminal half of the F protein CT domain as an immunogen. This antibody also detected surface-expressed F-protein CT sequences. Furthermore, deletion of the CT domain of the F protein eliminated the binding of both antibodies to transfected ELL-0 and COS-7 cell surfaces (17; these results) and shows that the antibodies were not binding to an unrelated polypeptide. Thus, the existence of an unusual form of the F protein is indicated by Western analysis and mass spectroscopy of proteins in cell extracts and virions and by detection of CT sequences on infected and transfected cell surfaces.

The functional significance of a second form of the F protein is a key question. It is possible that the alternate F protein is an aberrant form that has no direct role in the virus infection. Alternatively, the expression of this form of the F protein may serve to down-regulate the expression of the fully translocated F protein while not having a direct role in infection. However, detection of the second form of the F protein in purified virions released from both infected avian cells and COS-7 cells led us to explore a role of this polypeptide in membrane fusion.

Current models for type 1 fusion proteins propose that these proteins are initially folded into a metastable conformation (2, 7, 11). Upon activation of fusion, it is proposed that these proteins undergo a series of conformational changes that involve, first, the insertion of a fusion peptide into the target membrane, followed by the association of heptad repeat domains into a very stable, coiled-coil structure. The structure of this coiled coil and the anchoring of the protein to both the target and viral membrane by the fusion peptide and the transmembrane domain, respectively, result in pulling the target and viral membrane into close proximity (2, 7, 11). The membrane merger proceeds first by hemifusion and then by pore formation and expansion (6).

We previously reported that antibody specific for CT sequences inhibited red blood cell fusion to cells expressing the NDV HN and F proteins (17). The antibody inhibited hemifusion, suggesting that antibody binding interfered with the initial stages in the onset of fusion. Here we explored the effects of anti-Ftail antibodies on virus entry. To limit effects of the antibody to virus-cell fusion and not cell-cell fusion, we removed unbound antibody after virus binding. We showed that two different antibodies specific for CT sequences considerably slowed plaque formation, suggesting a role of surface-exposed CT sequences in virus-cell fusion. Furthermore, in a single cycle of infection, preincubation of anti-Ftail antibody with virions eliminated infection. These combined results suggest that antibody bound to the surface-exposed CT sequences inhibited both virus-cell fusion (Fig. 6 and 7) and cell-cell fusion (17).

Since the antibody inhibited hemifusion, initial steps of fusion are affected. It is possible that binding of the antibody to
CT sequences sterically inhibits conformational changes in the fully translocated F protein required for initiation of fusion and that the CT domain has no direct role in the fusion process. Alternatively, the CT domain could be involved in the formation of the metastable form of the fully translocated F protein or the activation of that form required for fusion. The presence of the anti-Ftail antibody could inhibit this activation.

Previous reports indicate that CT domains of paramyxovirus F proteins do have a role in fusion (1, 5, 25–27, 30). Deletion of the CT domain of the NDV F protein interferes with complete fusion (25). It has been proposed that this domain affects the conformation of the ectodomain (25, 30). Surface-exposed CT domains may also have additional roles. Clarification of the role of surface-expressed CT domains in fusion as well as potential roles of the amino-terminal regions of the alternate F protein requires further investigation.

ACKNOWLEDGMENT

This work was supported by grant AI30572 from the National Institutes of Health.

REFERENCES

1. Bagai, S., and R. A. Lamb. 1996. Truncation of the C00H-terminal region of paramyxovirus SV5 fusion protein leads to hemifusion but not complete fusion. J. Cell Biol. 135:73–84.
2. Baker, K. A., R. E. Dutch, R. A. Lamb, and T. S. Jardetsky. 1999. Structural basis for paramyxovirus-mediated membrane fusion. Mol. Cell 3:309–319.
3. Dolgainiuc, V., L. McGinnes, E. J. Luna, and T. G. Morrison. 2003. Role of the cytoplasmic domain of the Newcastle disease virus fusion protein in association with lipid rafts. J. Virol. 77:12968–12979.
4. Dunlop, J., P. C. Jones, and M. E. Finbow. 1995. Membrane insertion and assembly of ductin: a polytopic channel with dual orientations. EMBO J. 14:3699–3616.
5. Dutch, R. E., and R. A. Lamb. 2001. Deletion of the cytoplasmic tail of the fusion protein of paramyxovirus simian virus 5 affects the fusion pore enlargement. J. Virol. 75:5363–5369.
6. Earp, L. J., S. E. Delos, H. E. Park, and J. M. White. 2004. The many mechanisms of viral membrane fusion. Curr. Top. Microbiol. Immunol. 285:25–66.
7. Eckert, D. M., and P. S. Kim. 2001. Mechanisms of viral membrane fusion and its inhibition. Annu. Rev. Biochem. 70:777–810.
8. Escors, D., E. Camafetta, J. Ortego, H. Laude, and L. Enjuanes. 2001. Organization of two transmissible gastroenteritis coronavirus membrane protein topologies within the virion and core. J. Virol. 75:12228–12240.
9. Gorman, J. J., A. Nestorowicz, S. J. Mitchell, G. L. Corino, and P. W. Selleck. 1989. Characterization of the sites of proteolytic activation of Newcastle disease virus membrane glycoprotein precursors. J. Biol. Chem. 266:12522–12531.
10. Hegde, R. S., J. A. Mastronardo, M. R. Scott, K. A. DeFea, P. Tremblay, M. Torchia, S. J. DeArmond, S. B. Prusiner, and V. R. Lingappa. 1998. A transmembrane form of the prion protein in neurodegenerative disease. Science 279:827–834.
11. Jardetsky, T. S., and R. A. Lamb. 2004. A class act. Nature (London) 427:307–308.
12. Lahm, H. W., and H. Langen. 2000. Mass spectrometry: tool for the identification of proteins separated by gel electrophoresis. Electrophoresis 21: 2105–2114.
13. Lamb, R. A., and D. Kolakofsky. 2001. Paramyxoviridae: the viruses and their replication, p. 1305–1340. In D. M. Knipe and P. M. Howley (ed.), Fields virology, 3rd edition ed., vol. I. Lippincott Williams & Wilkins, Philadelphia, Pa.
14. Lambert, C., and R. Prange. 2001. Dual topology of the hepatitis B virus large envelope protein. J. Biol. Chem. 276:22265–22272.
15. Lu, Y., J. I. R. Turnbull, A. Bragin, K. Carvelth, A. S. Verkman, and W. R. Skach. 2000. Reorientation of Aquaporin-1 topology during maturation in the endoplasmic reticulum. Mol. Biol. Cell 11:2973–2985.
16. McGinnes, L. W., K. Gravel, and T. G. Morrison. 2002. The NDV HN protein alters the conformation of F protein at cell surfaces. J. Virol. 76: 12622–12633.
17. McGinnes, L. W., J. N. Reitter, K. Gravel, and T. G. Morrison. 2003. Evidence for mixed membrane topology of the Newcastle disease virus fusion protein. J. Virol. 77:1951–1963.
18. Migliaccio, C. T., K. E. Follis, Y. Matsuura, and J. H. Nunnberg. 2004. Evidence for a polytopic form of the E1 envelope glycoprotein of hepatitis C virus. Virus Res. 105:47–57.
19. Morrison, T. G. 2003. Structure and function of a paramyxovirus fusion protein. Biochim. Biophys. Acta 1614:73–84.
20. Nagai, Y. 1993. Protease-dependent virus tropism and pathogenicity. Trends Microbiology 1:81–87.
21. Nagai, Y., M. Hamaguchi, and T. Toyoda. 1989. Molecular biology of Newcastle disease virus. Prog. Vet. Microbiol. and Immunol. 8:16–84.
22. Niwa, H., K. Yamamura, and M. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 106:193–199.
23. Pavio, N., D. R. Taylor, and M. M. C. Lai. 2002. Detection of a novel unglycosylated form of hepatitis C virus E2 envelope protein that is located in the cytosol and interacts with PKR. J. Virol. 76:1265–1272.
24. Sergel, T., L. W. McGinnes, M. E. Peeples, and T. G. Morrison. 1993. The attachment function of the Newcastle disease virus hemagglutinin-neuraminidase protein can be separated from fusion promotion by mutation. Virol. 193:717–726.
25. Sergel, T., and T. Morrison. 1995. Mutations in the cytoplasmic domain of the fusion glycoprotein of Newcastle disease virus depress syncytia formation. Virology 210:264–272.
26. Seth, S., A. L. Goodman, and R. W. Compans. 2004. Mutations in multiple domains activate paramyxovirus F protein-induced fusion. J. Virol. 78:8513–8523.
27. Tong, S., M. Li, A. Vincent, R. W. Compans, E. Fritsch, R. Beier, C. Klenk, M. Ohashi, and H. D. Klenk. 2002. Regulation of fusion activity of the cytoplasmic domain of a paramyxovirus F protein. Virology 301:322–333.
28. Toyoda, T., T. Sakaguchi, H. Hirota, B. Gotoh, K. Kuma, T. Miyata, and Y. Nagai. 1989. Newcastle disease virus evolution. II. Lack of gene recombination in generating virulent and avirulent strains. Virology 169:273–282.
29. Wang, C., G. Ragu, T. Morrison, and M. E. Peeples. 1992. Intracellular processing of the paramyxovirus F protein: critical role of the predicted amphipathic alpha helix adjacent to the fusion domain. J. Virol. 66:4161–4169.
30. Wanning, D. L., C. J. Russell, T. S. Jardetsky, and R. A. Lamb. 2004. Activation of a paramyxovirus fusion protein is modulated by inside-out signaling from the cytoplasmic tail. Proc. Natl. Acad. Sci. USA 101:9217–9222.
31. Zhang, J.-T., M. Duthie, and V. Ling. 1993. Membrane topology of the N-terminal half of the hamster P-glycoprotein. J. Biol. Chem. 268:15101–15110.