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Intracellular Processing of the Newcastle Disease Virus Fusion Glycoprotein

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The fusion glycoprotein (Fo) of Newcastle disease virus is cleaved at an intracellular site (Nagai et al., Virology 69:523–538, 1976) into F_1 and F_2. This result was confirmed by comparing the transit time of the fusion protein to the cell surface with the time course of cleavage of Fo. The time required for cleavage of half of the pulse-labeled Fo protein is ca. 40 min faster than the half time of the transit of the fusion protein to the cell surface. To determine the cell compartment in which cleavage occurs, use was made of inhibitors which block glycoprotein migration at specific points and posttranslational modifications known to occur in specific cell membranes. Cleavage of Fo is inhibited by carbonyl cyanide *m*-chlorophenylhydrazone; thus, cleavage does not occur in the rough endoplasmic reticulum. Monensin blocks the incorporation of Newcastle disease virus glycoproteins into virions and blocks the cleavage of the fusion glycoprotein. However, Fo cannot be radioactively labeled with [^{3}H] fucose, whereas F_1 is readily labeled. These results argue that cleavage occurs in the *trans* Golgi membranes or in a cell compartment occupied by glycoproteins quite soon after their transit through the *trans* Golgi membranes. The implications of the results presented for the transit times of the fusion protein between subcellular organelles are discussed.

Newcastle disease virus (NDV), a paramyxovirus, is an RNA virus composed of a ribonucleoprotein core and a membrane (5). The core (12) contains the genomic RNA as well as three proteins: the nucleocapsid protein (NP), a phosphoprotein (P) (32), and a presumed transcriptase (L) (9, 21). The envelope or membrane, which is derived from the host cell plasma membrane, contains three viral proteins: a nonglycosylated membrane protein (M) and two glycoproteins, the hemagglutinin-neuraminidase (HN) protein and the fusion (F) protein (2, 25).

The fusion glycoprotein mediates membrane fusion between the plasma membranes of two cells, infected and uninfected, or between the membrane of the virus and the membrane of the host cell (1, 17, 18). The fusion protein is synthesized as a precursor (Fo) (26; J. Kaplan and M. A. Bratt, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, V291) and is subsequently cleaved into F_1 and F_2 which remain associated by disulfide bonds (30). The fusion glycoprotein is inactive as a fusogen until cleavage has occurred (18, 28, 29).

The protein responsible for the cleavage of Fo is a host cell enzyme present in some cell types but not in others (25). Although the cleavage of Sendai virus (another paramyxovirus) fusion protein is reported to be external to the cell (13), there are two reports that the cleavage of the NDV fusion glycoprotein occurs intracellularly (19, 31). This finding raises the interesting possibility that internal membranes of infected cells contain an active fusing agent which could potentially disrupt intracellular membrane organization, particularly the ordered lamella of the Golgi membranes. Such a disruption could influence the processing and transport of glycoproteins. Thus, we were interested in determining more precisely the intracellular location of the cleavage of the fusion protein. We present evidence that the cleavage occurs either in *trans* Golgi membranes or just subsequent to the transport of the protein through the Golgi membranes. Thus, most of the Golgi membranes would not be subjected to the fusion activity of the fusion protein. Our results also demonstrate that the fusion protein is transported to the cell surface slowly as is the NDV HN protein (15). However, in contrast to the transit of the HN glycoprotein, slow transit of the fusion protein is due, in part, to rate-limiting steps between the Golgi membranes and the cell surface.

MATERIALS AND METHODS

Cells and viruses. Cells used were chicken embryo (CE) cells prepared as described previously (10). NDV, Australia-Victoria strain, was propagated and purified as described previously (10). Strain Australia-Victoria is a virulent NDV strain that undergoes a productive infection of CE cells to produce infectious virions (1, 2, 10, 21, 28; Kaplan and Bratt, Abstr. Annu. Meet. Am. Soc. Microbiol., 1973).

Infection and radioactive labeling of cells. Monolayers of CE cells (2×10^6 cells) were infected at a multiplicity of 10 PFU per cell. Radioactive labeling was initiated at 5 h postinfection. Labeling with [^{35}S]methionine (100 μCi/ml; 1,450 Ci/mmol; Amersham Corp.) was accomplished in methionine-free minimal essential medium (supplemented with nonessential amino acids and 7% dialyzed fetal calf serum) as described previously (15). For pulse-chase experiments, labeling was for 5 min, followed by the addition of chase medium (minimal essential medium containing 2.5% calf serum, 50 μg of cycloheximide per ml, 2.5% tryptone phosphate broth, penicillin, streptomycin, and fungizone, and 2 mM cold methionine).

Labeling with [^{3}H]fucose (100 μCi/ml; 5 Ci/mM; New England Nuclear Corp.) and [^{3}H]mannose (100 μCi/ml; 27 Ci/mM; New England Nuclear Corp.) was accomplished in glucose-free minimum essential medium containing nonessential amino acids, dialyzed fetal calf serum, and 1 mM pyruvate.

CCCP treatment of cells. Infected CE monolayers were pulse-labeled for 5 min with [^{35}S]methionine as described above and then treated with carbonyl cyanide *m*-chloro-

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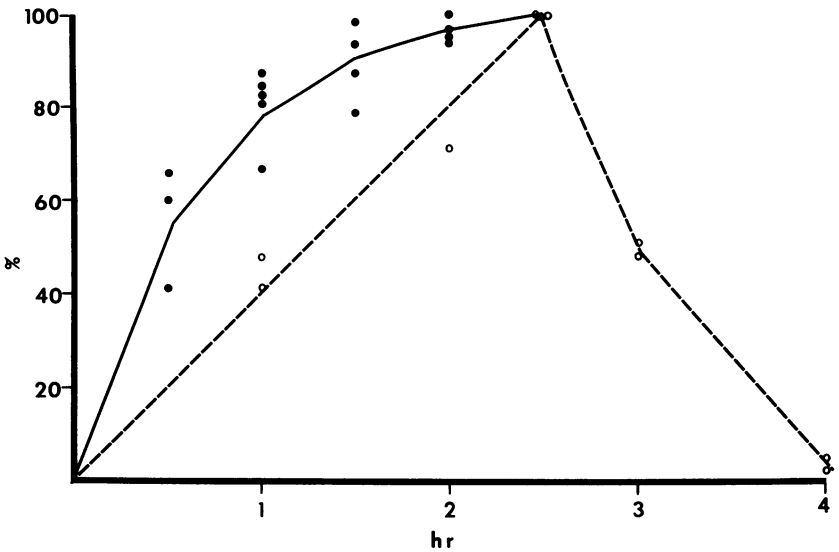

FIG. 1. Comparison of the kinetics of Fo cleavage and appearance of F_1-F_2 at the cell surface. To determine the kinetics of cleavage of Fo, monolayers of infected CE cells were pulse-labeled with [^{35}S]methionine for 5 min and then chased as described in the text. Cells were lysed with 1% Tween–0.5% sodium deoxycholate at various times after the onset of the chase, and proteins present in total cytoplasmic extracts were electrophoresed on 10% polyacrylamide gels. The amount of Fo at the end of the pulse is taken as 100. The percentage of Fo at each time point is subtracted from 100 to plot the percentage of cleavage at each time point. Thus, 100% cleavage represents no detectable Fo in cell extracts. Multiple points at each time represent separate experiments. Results are unchanged when lysis is performed in the presence of inhibitors of proteolytic enzymes such as phenylmethylsulfonyl fluoride, TPCK (L-1-tosylamide 2 phenylethylchloromethyl ketone) and TLCK (1-chloro-3-tosylamido-7-amino L-2-heptanone). The kinetics of appearance of the F_1-F_2 complex at the cell surface is shown by the open symbols. Cell monolayers pulse-labeled or pulse-labeled and then chased were incubated with anti-NDV antibody as described in the text. Cells were lysed, immune complexes were isolated, and proteins present were electrophoresed on 10% polyacrylamide gels in the absence of β-mercaptoethanol. The amount of F_1-F_2 was determined by scanning the resulting autoradiograms with a microdensitometer. The amount of F_1-F_2 at the cell surface was maximal at ca. 2.5 h of chase and was taken as 100%. Multiple points at each time represent the results of separate experiments.

phenylhydrazone (CCCP) as previously described (7, 15). A nonradioactive chase in the presence of CCCP was accomplished as described previously (15).

Monensin treatment of cells. Monensin was added to infected CE monolayers (2×10^6 cells) at various concentrations at 2 h postinfection. Radioactive labeling was accomplished at 5 h postinfection in the presence of monensin as described above.

Detection of cell surface molecules with antibody. After various periods of chase, monolayers were washed in ice-cold phosphate-buffered saline (0.15 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4) and then incubated on ice with 100 μl of undiluted anti-NDV antiserum (heat inactivated at 56°C for 30 min) as described previously (15). This amount of antiserum was determined to be in excess. After 30 min, antiserum was removed, the monolayers were washed extensively, and cells were lysed with RSB (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-hydrochloride [pH 7.4]) containing 1% Tween 40 and 0.5% sodium deoxycholate as described previously (15). Nuclei were removed by centrifugation.

Immune complexes present in cell extracts were precipitated with Immunobeads (Bio-Rad Laboratories) to which goat anti-rabbit antiserum was coupled. Before use, Immunobeads were washed once in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-hydrochloride [pH 7.4]) containing 0.5% Nonidet P-40 and 5 mg of bovine serum albumin per ml and twice in NET buffer–0.5% Nonidet P-40–1 mg of bovine serum albumin per ml. Washed Immunobeads were incubated with cell extracts for 30 min in the presence of 0.4% sodium dodecyl sulfate. Immune complexes were washed four times in NET buffer–0.5% Nonidet P-40 containing 0.4% sodium dodecyl sulfate. Viral proteins were removed from beads in 6 M urea–4% sodium dodecyl sulfate at 100°C.

Polyacrylamide gel electrophoresis. Polypeptides were resolved in 10% polyacrylamide slab gels (14 by 22 by 0.15 cm) prepared and run as previously described (4). The gels were then fixed, dried, and subjected to autoradiography (X-ray film X-Omat AR; Eastman Kodak Co.). The resulting autoradiograms were scanned with a microdensitometer. Gels containing ^{3}H were soaked in Autofluor (National Diagnostics) before drying.

Purification of virions. The virions present in the supernatant of radioactively labeled cell monolayers were purified in 20 to 65% sucrose discontinuous gradients (sucrose solutions were made in distilled water and D_2O) containing standard buffer (0.1 M NaCl, 0.1 M Tris-hydrochloride [pH 7.4], 0.002 M EDTA). The gradient was centrifuged for 2 h in a Beckman SW41 rotor at 24,000 rpm and 4°C.

RESULTS

Intracellular cleavage of Fo. Previous studies of the location of cleavage of the fusion protein (19, 31) used, in one case, cells other than CE cells and, in both cases, strains of NDV other than strain Australia-Victoria. To verify that intracellular cleavage of Fo was also characteristic of NDV strain Australia-Victoria in CE cells, we compared the kinetics of the cleavage of Fo with the kinetics of appearance of the fusion protein at the cell surface.

We previously have devised an assay to detect only cell surface glycoproteins, using antisera directed against NDV

proteins (15). Using this assay, we have previously shown that no uncleaved Fo can be detected at the cell surface (4, 16). This result argues that cleavage occurs intracellularly. If this conclusion is correct, then a comparison of the kinetics of cleavage with the kinetics of appearance of pulse-labeled protein at the cell surface should show a difference in the time course of these two events. And, in fact, the cleavage of Fo occurs before the detection of the fusion protein at the cell surface (Fig. 1). The half time ($t_{1/2}$) for the cleavage of the pulse-labeled fusion protein is ca. 33 min, whereas the $t_{1/2}$ for the appearance of the fusion protein at the cell surface is ca. 75 min. Thus, these results support the idea that cleavage of Fo occurs intracellularly.

Cleavage in the presence of CCCP. CCCP, an inhibitor of oxidative phosphorylation, blocks the migration of pulse-labeled glycoproteins into Golgi membranes (7, 34). After CCCP treatment of NDV-infected, pulse-labeled cells, no radioactively labeled virus particles are released (Fig. 2) and no HN or F glycoprotein can be detected at the cell surface (15). To determine the effect of CCCP on the cleavage of Fo, cells were pulse-labeled with [^{35}S]methionine and then chased with nonradioactive methionine in the presence of the inhibitor. CCCP blocks the cleavage of the fusion protein (Fig. 3). This result argues that if migration of the protein into Golgi membranes is blocked, no cleavage occurs. Thus, cleavage must occur sometime after entry of the protein into Golgi membranes.

Cleavage in the presence of monensin. Monensin is a sodium ionophore which is reported to interfere with the transport of most membrane glycoproteins by preventing their exit from the medial Golgi membranes (8, 33). Thus, the inhibitor is a useful one to locate the intracellular site of cleavage of the NDV fusion protein.

To verify that monensin blocks NDV glycoprotein transport in CE cells, the amount of glycoprotein recovered in virions with increasing concentrations of monensin was monitored. Increasing concentrations of monensin decreased the amount of radioactivity recovered in purified virus, but significant amounts of radioactivity were released into virus particles even at the highest concentrations used (Table 1). However, most of the radioactivity can be accounted for by virus proteins other than glycoproteins. Increasing monensin concentrations preferentially decreased the amount of glycoprotein present in virions (Fig. 4).

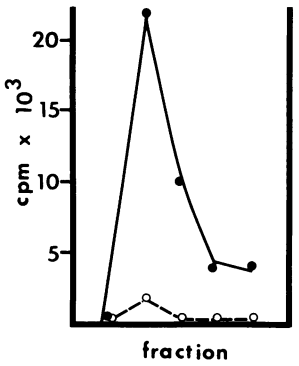

FIG. 2. CCCP blocks the release of virions from infected cells. Infected cell monolayers were radioactively pulse-labeled and then treated with CCCP at 5 h postinfection as described in the text. After 3 h of chase, virions released into the cell supernatant were purified as described in the text. The graph shows trichloracetic acid-precipitable counts across sucrose gradients containing virions and no CCCP (●) or virions plus CCCP (○).

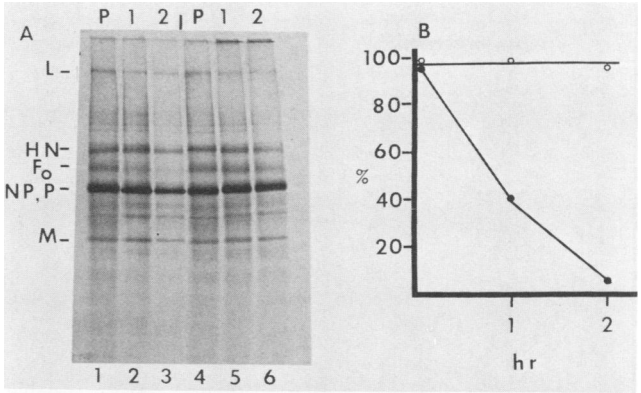

FIG. 3. CCCP blocks cleavage of the Fo glycoprotein. (A) Monolayers of infected CE cells were pulse-labeled with [^{35}S]methionine (P) or pulse-labeled and then chased for 1 or 2 h as described in the text. Cells were lysed, and proteins present in total cytoplasmic extracts were electrophoresed on 10% polyacrylamide gels. Shown are the resulting autoradiographs. Lanes: 1 to 3, untreated cells; 4 to 6; CCCP-treated cells; 1 and 4, pulse-labeled proteins; 2 and 5, 1-h chase; 3 and 6, 2-h chase. (B) The amount of Fo in each extract was quantitated by scanning the autoradiograph shown in panel A with a microdensitometer. Results are presented as the amount of Fo present in the extracts. The amount of Fo after a 5-min pulse-label is taken as 100% (no CCCP, ●; with CCCP, ○).

Virion proteins electrophoresed under nonreducing conditions to resolve both the F_1 and F_2 complex (F_{NR}) as well as any uncleaved Fo (Fig. 4A). With increasing concentrations of monensin, the amount of the fusion protein is reduced to undetectable levels. Figure 4B shows the same virion proteins electrophoresed under reducing conditions. Only Fo which migrates between NP and HN (Fig. 2A) can be resolved under these conditions. F_1 comigrates with NP (16) and cannot be resolved, whereas F_2 is not resolved in this gel system (16). Clearly, the fusion protein detected in virions at low monensin concentrations (Fig. 4A) is primarily in the cleaved form since very little Fo can be detected under reducing conditions. A quantitation of the amount of glycoprotein found in virus at various monensin concentrations is shown in Fig. 5A. The incorporation of the fusion protein is diminished with increasing monensin concentrations. At concentrations of 10^{-5} M monensin, virtually no fusion glycoprotein can be detected in virus particles.

To determine the effect of monensin on cleavage of the fusion protein, cells treated with monensin were pulse-labeled and then chased for 2 h. Control or untreated cells

TABLE 1. Total radioactivity associated with virions released from cells treated with increasing monensin concentrations[a]

Monensin concn (M)	Total virion counts (10^4)
0	2.0 (100%)
1×10^{-7}	1.8 (90%)
1×10^{-6}	1.4 (70%)
5×10^{-6}	1.2 (60%)
1×10^{-5}	0.9 (45%)

[a] Monolayers of infected CE cells were treated with monensin at various concentrations beginning at 2 h postinfection. Radioactive pulse-labeling (5 min) was 5 h postinfection in the presence of monensin. After 5 h of chase, virions were purified from the cell supernatant, and total counts recovered at each monensin concentration are shown.

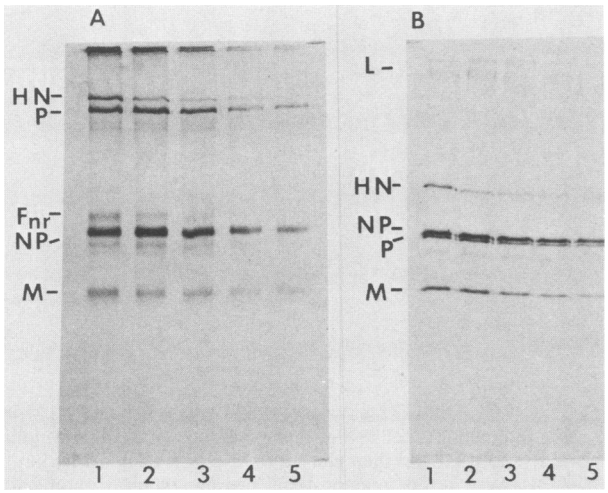

FIG. 4. Proteins in virions released from monensin-treated cells. An autoradiograph of proteins present in virions isolated from the supernatant of cells treated with various concentrations of monensin is shown. (A) Electrophoresis in the absence of β-mercaptoethanol. (F_{nr}, F nonreduced). (B) Electrophoresis in the presence of β-mercaptoethanol. Lanes: 1, control virions; 2, 1×10^{-7} M monensin; 3, 1×10^{-6} M monensin; 4, 5×10^{-6} M monensin; 5, 1×10^{-5} M monensin.

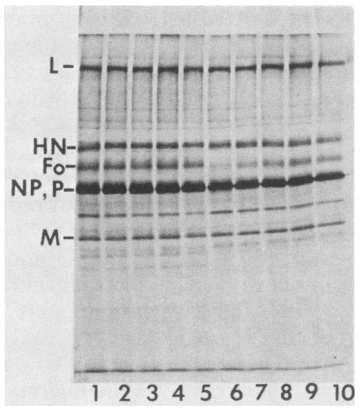

FIG. 6. Monensin blocks cleavage of Fo. Monolayers of infected CE cells treated with various concentrations of monensin beginning at 2 h postinfection were pulse-labeled with [^{35}S]methionine for 5 min at 5 h postinfection. Duplicate monolayers were subjected to a 2-h chase in the presence of monensin. Cells were lysed, and proteins present in total cell extracts were resolved on 10% polyacrylamide gels in the presence of β-mercaptoethanol. Lanes: 1 to 5, pulse-labeled extracts; 6 to 10, 2-h chase extracts; 1 and 6, no monensin; 2 and 7, 10^{-7} M monensin; 3 and 8, 10^{-6} M monensin; 4 and 9, 5×10^{-6} M monensin; 5 and 10; 1×10^{-5} M monensin.

contained little Fo by 2 h of chase (Fig. 6, lane 6). However, increasing monensin concentrations resulted in an increased amount of Fo remaining in the cell extract after 2 h of chase (Fig. 6, lanes 7 to 10). A quantitation of the amount of cleavage at each monensin concentration is shown (Fig. 5B). Clearly, at concentrations of 10^{-6} M monensin cleavage of the fusion protein is virtually eliminated.

Incorporation of [^{3}H]fucose into viral glycoproteins. There are several glycoprotein modifications attributed to enzyme systems of the *trans* Golgi membranes. One of these reactions is the addition of fucose (24). If cleavage of the fusion protein occurs after the addition of fucose, then uncleaved Fo labeled with [^{3}H]fucose should be detected. However, if cleavage occurs before the addition of fucose, then no labeled Fo should be detected. Figure 7 shows the result of labeling infected cells with [^{3}H]fucose.

The HN protein is labeled with fucose (Fig. 7, lane 2). No polypeptide the size of Fo is labeled, but a polypeptide the size of F_1 is labeled. That the F_1-sized polypeptide is F_1 and not NP protein labeled with breakdown products of the [^{3}H]fucose is shown by the fact that no NP-sized material is

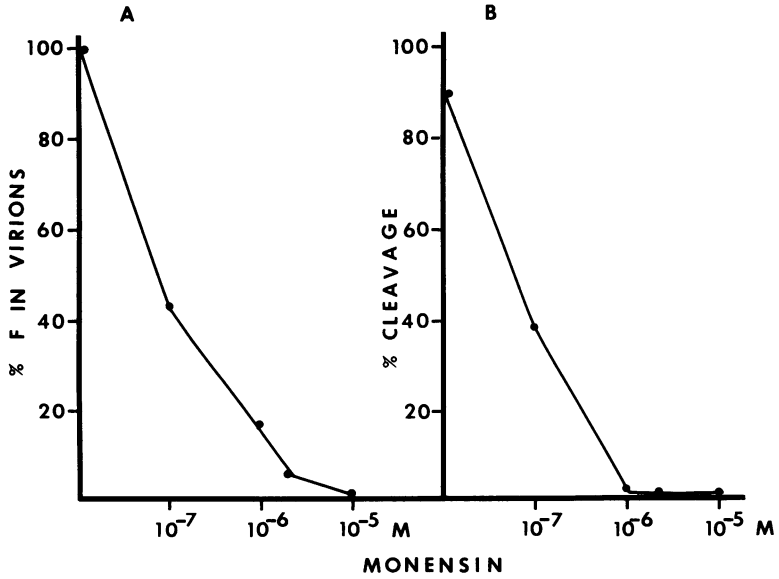

FIG. 5. Quantitation of the amount of fusion protein in virions and the amount of cleavage of Fo in cell extracts with increasing monensin concentrations. The amount of F_1-F_2 in virions with increasing monensin concentrations (A) was determined by scanning autoradiographs shown in Fig. 4 and by setting the amount of F_1-F_2 complex detected in virions released from untreated cells as 100%. The amount of Fo in cell extracts (B) treated with various concentrations of monensin was determined by scanning autoradiographs shown in Fig. 6 with a microdensitometer. The results are expressed as the percentage of cleavage of Fo. The complete absence of Fo is represented as 100%.

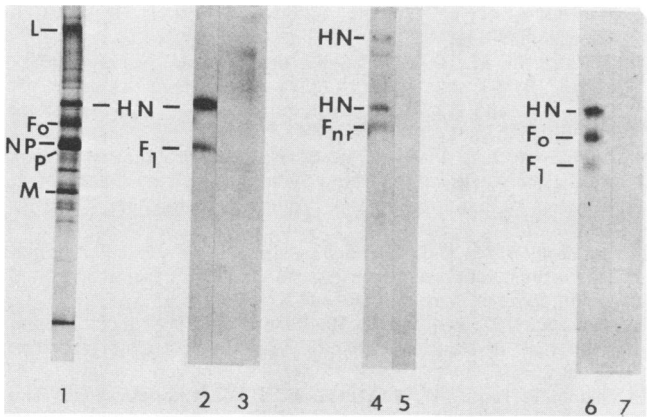

FIG. 7. Radioactive labeling of glycoproteins with [^{3}H]fucose and [^{3}H]mannose. Monolayers of uninfected and infected CE cells were radioactively labeled for 1 h at 5 h postinfection with [^{3}H]fucose or [^{3}H]mannose as described in the text. Infected cells were labeled with [^{35}S]methionine in parallel infections. Proteins present in total cytoplasmic extracts were electrophoresed on 10% polyacrylamide gels in the presence or absence of β-mercaptoethanol. Slab gels contained lanes of [^{35}S]methionine-labeled NDV-infected cell extracts for markers. Lanes: 1, [^{35}S]methionine-labeled cell extracts electrophoresed in the presence of β-mercaptoethanol; 2, [^{3}H]fucose-labeled infected cell extracts plus β-mercaptoethanol; 3, [^{3}H]fucose-labeled uninfected cell extracts plus β-mercaptoethanol; 4, [^{3}H]fucose-labeled infected cell extracts without β-mercaptoethanol; 5, [^{3}H]fucose-labeled uninfected cell extracts without β-mercaptoethanol; 6, [^{3}H]mannose-labeled infected cell extracts plus β-mercaptoethanol; 7, [^{3}H]mannose-labeled uninfected cell extracts plus β-mercaptoethanol. Lanes 1 to 5 are from the same slab gel. Lanes 6 and 7 are from a different gel. The location of the proteins in lanes 6 and 7 was confirmed by electrophoresis of [^{35}S]methionine-labeled viral proteins in adjacent lanes. HN protein exists as both a monomer and a dimer in the absence of β-mercaptoethanol (28). F_{NR}, Fusion protein nonreduced.

seen when the sample is electrophoresed under nonreducing conditions (Fig. 7, lane 4). The F_1-F_2 complex (F_{NR}) comigrates with Fo under these conditions (4, 16, 28). By contrast, Fo can be labeled with [^{3}H]mannose (Fig. 7, lane 6), a core sugar added in the rough endoplasmic reticulum (RER) (24). Thus, cleavage of Fo occurs before or at a time very similar to the addition of fucose.

DISCUSSION

Simple enveloped viruses such as NDV depend upon host cell pathways for the transport and processing of their glycoproteins. These pathways generally involve insertion of the protein into the membranes of the RER, transport to and through the Golgi membranes, and finally transport to and insertion into the cell surface (24). It has been reported that the fusion glycoprotein of NDV is cleaved intracellularly, at some point along the pathway (19, 31). Indeed, we have failed to detect the uncleaved fusion protein on the surfaces of infected cells, using antibody binding to cell surfaces (15) or lactoperoxidase iodination (unpublished data). However, it has been previously noted that the precursor of Sindbis virus E_2 glycoprotein, PE_2, is actually at the plasma membrane but cannot be detected by either lactoperoxidase-mediated iodination or antibody binding (27). Although our antibody does recognize Fo (3), it is possible that the Fo is located at the cell surface in a conformation inaccessible to detection assays. However, our finding that there is a distinct time lag between the appearance of F_1-F_2 at the cell surface and the cleavage of fusion protein argues for an intracellular location of cleavage.

By using inhibitors of glycoprotein transport and taking advantage of the known locations of glycoprotein posttranslational modifications, we have attempted to determine the intracellular location of the fusion protein cleavage. CCCP is an inhibitor which blocks the migration of glycoproteins to the Golgi membranes (7, 34). In the presence of this inhibitor, no fusion protein cleavage occurs, suggesting that the RER is not the site of cleavage.

The *cis* Golgi membranes are also not likely to be the site of cleavage. One function attributed to the *cis* Golgi membranes is the addition of the fatty acid palmitate (11). If cleavage occurs before the addition of palmitate, no labeling of Fo with [^{3}H]palmitate should be detected. However, it previously was reported that both Fo and F_1 can be radioactively labeled with [^{3}H]palmitate (4), suggesting that cleavage occurs after this glycoprotein modification.

Monensin is a sodium ionophore which blocks the migration of most glycoproteins from the medial Golgi membranes of most cell types (33). And indeed, we found that monensin-treated CE cells are unable to incorporate NDV fusion proteins into virions. Monensin also blocks the cleavage of the fusion protein. This result argues that cleavage occurs after the exit of the protein from medial Golgi membranes. However, evidence has been presented which suggests that monensin allows the migration of the glycoprotein of Semliki Forest virus into the *trans* Golgi membranes of CE cells (22) but blocks further transport. Therefore, it is possible that monensin has similar effects on the fusion glycoprotein of NDV. Thus, cleavage may occur in the *trans* Golgi or in a cell compartment beyond the *trans* Golgi.

To pinpoint the cleavage event to the *trans* Golgi membranes or a subsequent cellular compartment, we monitored the addition of fucose, a glycoprotein modification that has been attributed to the *trans* Golgi membranes (24). We asked whether Fo could be labeled with [^{3}H]fucose to determine whether cleavage occurs before or after this modification. We could find little detectable labeling of Fo with [^{3}H]fucose. Therefore, cleavage occurs before the addition of fucose, or alternatively, cleavage occurs just after the addition of fucose, and the pool size of labeled Fo is too small to be detected. This result argues for cleavage in or near the *trans* Golgi membranes.

Another glycoprotein modification attributed to the Golgi membranes is the acquisition of endoglycosidase H resistance (23, 24). However, the fusion protein apparently never becomes resistant to this enzyme (28; A. Semerjian and T. Morrison, unpublished data). Thus, resistance to the enzyme is not a useful tool for the NDV fusion glycoprotein.

Intracellular transport of the fusion protein. We have previously compared the kinetics of the intracellular transport of the vesicular stomatitis virus glycoprotein (G) and the NDV HN glycoprotein (15). We found that these two glycoproteins have widely varying rates of transport to the cell surface. VSV G is transported to plasma membranes with a $t_{1/2}$ of ca. 27 min, whereas the HN glycoprotein reaches the cell surfaces with a $t_{1/2}$ of ca. 78 min. This difference in the transit times resides primarily in the times required for the two proteins to move from the RER to the *trans* Golgi membranes: the HN protein is transported to the *trans* Golgi membranes with a $t_{1/2}$ of ca. 60 min as compared with 13 min characteristic of G. Similar results with other glycoproteins have shown that the transit from the RER to the Golgi membranes accounts for various rates of transit (6, 14, 20, 33).

If our antibody recognizes the fusion protein immediately upon its insertion into the plasma membrane, then our results suggest that similar to the HN protein, the fusion protein is transported to the cell surface slowly with a $t_{1/2}$ of ca. 75 min. We have shown that cleavage of the fusion protein occurs at a location near or in the *trans* Golgi membranes. Thus, the kinetics of cleavage may be used to determine the transit times of the protein into these membranes. By this criterion, the fusion protein is transported to the *trans* Golgi membranes much more quickly than is the HN glycoprotein. The $t_{1/2}$ of cleavage of the fusion protein is ca. 33 min. In addition, our results suggest that the transit time of the fusion protein from the *trans* Golgi membranes to the cell surface ($t_{1/2}$ of 42 min) is much slower than that of the HN glycoprotein ($t_{1/2}$ of 18 min) or G ($t_{1/2}$ of 13 min). These results argue against a nonselective transport system for glycoproteins not only between the RER and the Golgi membranes but also between the Golgi membranes and the cell surface.

Thus in summary, by using inhibitors known to block glycoprotein transport at specific sites and posttranslational modifications known to occur in specific membranes, we have presented results that suggest that cleavage of the Fo glycoprotein occurs in *trans* Golgi membranes. Thus, most Golgi membranes contain only the inactive form of the fusion protein. How, and indeed whether, *trans* Golgi membranes and membranes along the subsequent pathway of glycoproteins escape the activity of the fusion protein remains to be determined. We also have presented results that suggest that the transit times of the fusion protein between subcellular compartments differ from those of the HN glycoprotein.

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